SN-38 Overcomes Chemoresistance of Colorectal Cancer Cells Induced by Hypoxia, through HIF1alpha

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Abstract. *Background: Cancer cells can acquire resistance to therapy under hypoxic condition. We aimed to investigate the mechanisms regulating chemoresistance induced by hypoxia. Materials and Methods: Human colorectal cancer cells, HT-29 and SW480, were cultured under hypoxic conditions and the sensitivity to 5-fluorouracil (FU), oxaliplatin, and SN-38 (active metabolite of irinotecan) was tested. The cell cycle was evaluated by flow cytometry after staining of cells with propidium iodide (PI). hypoxia-inducible factor 1α (HIF-1α) expression was evaluated by western blot analysis. Results: Hypoxia induced strong G_0/G_1 cell cycle arrest of cancer cells and abrogated the cytotoxic effects of 5-FU and oxaliplatin, but not that of SN-38. This effect was dependent on the significant inhibition of the accumulation of HIF-1α in cancer cells cultured under hypoxia by SN-38. Neither 5-FU nor oxaliplatin affected HIF-1α expression. Conclusion: SN-38, through inhibition of HIF-1α can overcome chemoresistance under hypoxic conditions of colon cancer cells.*

Most solid tumors develop a hypoxic environment, which significantly affects the biological behavior, response to therapy and prognosis of human cancer (1). Hypoxic cells are more resistant to the inhibitory effect of ionizing radiation and chemotherapy. Such resistance to therapy may be dependent, in part, on poor intratumoral perfusion, which restricts the access of the drugs to the hypoxic areas (2). However, the activation of a family of hypoxia-inducible transcription factors (HIFs), which are important for the regulation of coordinated adaptive responses, and which lead cells to activate survival mechanisms, seems to play the major role.

The HIF-1 heterodimer is composed by two components, HIF-1α and HIF-1β. Whereas the HIF-1β subunit is constitutively expressed in most cells, the HIF-1α subunit has a highly regulated expression. The level of HIF-1α is regulated by the rates of protein synthesis and degradation. The phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) pathways, which are activated *via* O_2-independent mechanisms, are involved in the regulation of HIF-1α protein synthesis, whereas its degradation is regulated *via* proline hydroxylation, through an O_2-dependent mechanism (3).

Various types of human cancer overexpress HIF-1α, which, through the activation of the transcription of a variety of genes, such as vascular endothelial growth factor (VEGF) and matrix metalloproteinase 2 (MMP2), helps tumors to develop metastases. In clinical settings, overexpression of HIF-1α has been reported to be associated with increased chemoresistance, mortality, and metastasis in various types of human cancer, including of the esophagus, bladder, brain, breast, lung, ovary, pancreas, stomach, and colon (4-10).

HIF-1 induces chemoresistance by an antiapoptotic effect (11) and the induction of G_1 cell cycle arrest (12, 13). Various HIF-1 inhibitors have been developed (14-20), but they are not available for clinical use yet. Some other types of clinically available agents, such as ibuprofen, temsirolimus, and cetuximab (21-23), have been shown to inhibit HIF-1 activity. Although they are not selective inhibitors of HIF-1, they may have some beneficial effects on HIF-1α overexpressing tumors.

In this study, we aimed to investigate the effects of the hypoxic environment on the response of colorectal cancer cells to chemotherapy, especially focusing on the mechanisms involving HIF-1α. Since the conventional treatment of colorectal cancer is based on 5-fluorouracil (FU) in combination with either oxaliplatin or the active metabolite of irinotecan, SN-38, these three agents were tested.

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Materials and Methods

Cells and reagents. The human colorectal cancer cell line, HT29, obtained from the Japanese Cancer Research Resource Bank, was used. Cells were cultured in RPMI-1640 medium supplemented with 5% fetal calf serum, 1% antibiotics/antimycotic (complete medium) and incubated in a 5% CO_2 incubator at 37˚C.

5-FU, oxaliplatin, bovine serum albumin, and RPMI-1640 medium were purchased from Sigma-Aldrich (St Louis, MO, USA). Fetal calf sera, and antibiotics/antimycotics were from Gibco BRL (Grand Island, NY, USA). Calcium and magnesium-free phosphate-buffered saline (PBS (–)) was from Wako Pure Chemical Industries, Ltd (Osaka, Japan). SN-38 (active metabolite of irinotecan) was from Toronto Research Chemicals (North York, Ontario, Canada).

Exposure of cell monolayer to hypoxia. Cells were exposed to hypoxic conditions at 37˚C within a modular incubator chamber filled with 5% CO_2 and 1% O_2, with the balance made up by the infusion of 94% nitrogen and water vapor (BIOLABO Multigas Incubator, JUJI FIELD, Japan).

Cell growth assessment. In the preliminary experiment, cells were cultured under normoxic conditions for 24 h and subsequently were cultured under normoxic or hypoxic condition for 24, 48, and 72 h. Live cells were counted after staining with trypan blue, and the ratio to the number of the cells cultured for the first 24 h under normoxia was calculated in order to assess cell growth.

Proliferation assay. Cells were plated at 5,000 cells per well in 96-well plates. After culture for 24 h under normoxic conditions (21% O_2, 5% CO_2, 74% N_2), cells were exposed to hypoxic (1% O_2, 5% CO_2, 94% N_2) or normoxic conditions for 24 h. Cells were then treated with relevant doses of 5-FU (0, 2.5, 5, 10, 20, 40 μM), oxaliplatin (0, 2.5, 5, 10, 20, 40 μM), or SN-38 (0, 0.25, 0.5, 1, 2, 4) for 48 h. Subsequently, 1 μl of calcein tetraacetoxymethyl ester (AM) solution was added to each cell. After incubation at 37˚C for 30 min, fluorescence intensity was measured by Tera scan VP (Minerva Tech, Tokyo, Japan). All experiments were performed in triplicate.

Detection of apoptosis by flow cytometry. Cells were prepared and treated as described above, and then stained with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) for 5 min at room temperature, according to the manufacturer's instructions (AnnexinV:FITC Apoptosis Detection Kit; BD Pharmingen, CA, USA). The population of annexinV – PI – viable cells and annexin V + apoptotic cells was evaluated by flow cytometry. Data were collected in a FACS Calibur instrument (Becton-Dickinson, Mountain View, CA, USA) and analyzed using Cell Quest software (Becton-Dickinson).

The experiment was performed three times, and the ratio of apoptotic cells was expressed as mean±SD.

Analysis of the cell cycle by flow cytometry. Cells were prepared and treated as described above, and then the percentage of cells in each phase of the cell cycle was analyzed by flow cytometry, using Cycle TEST PLUS DNA Reagent Kit (BD Pharmingen), which is based on the measurement of the DNA content of nuclei labeled with PI, according to the manufacturer's instructions. Treated cells were trypsynized (250 μl of trypsin buffer) for 10 min at room temperature, and then trypsin inhibitor (200 μl) and RNase buffer were added and allowed to react for 10 min at room temperature. Finally, propidium iodide stain solution (200 μl) was added and incubated for 10 min in the dark, on ice. Samples were immediately analyzed in the flow cytometer (Becton-Dickinson), and the obtained results analyzed by Cell Quest software (Becton-Dickinson). The experiment was performed three times, and the ratio of cells in the G_0/G_1, intra-S, and G_2/M phases were expressed as the mean±SD.

Western blot analysis. Cells were treated with 5-FU (40 μM), oxaliplatin (20 μM), and SN-38 (0.1, 0.2, 0.5 μM) for 24 h under hypoxic conditions. As the control, the cells were incubated under normoxic and hypoxic conditions for 24 h in the presence of the vehicle alone. After washing three times with PBS(–), lysed with 0.5 ml of Tris-saline [50 mM Tris-HCl (pH7.6), 150 mM sodium chloride] containing various protease inhibitors (1 mM EGTA, 0.1 mM diisopropyl fluorophosphates, 0.5 mM phenylmethylsulphonyl fluoride, 1 mg/ml Na-r-tosyl-L-lysine chloromethyl ketone, 1 mg/ml antipain, 0.1 mg/ml pepstain, 1 mg/ml of leupeptin) and 1% Triton-X for 1 h in a cold room and subsequently cells were harvested by scraping the culture dishes with a cell scraper.

After centrifuging at 15,000 rpm for 5 min, the clear supernatant was collected and used for the cell protein extract. Protein concentration was determined using the BCA Protein Assay Kit (Pierce Biomedical Co, Rockford, IL, USA). Cell lysates were separated on 15% and 7.5% Ready Gel J SDS-PAGE (Bio-Rad, Hercules, CA, USA) for Hybond ECL nitrocellulose membrane blotting (Amersham Pharmacia Biotech, Buckinghamshire, UK). The blotted membranes were blocked with 5% skim milk for 30 min and were incubated with each primary antibody overnight at 4˚C. The following primary antibodies were used: anti-HIF1α antibody was from Becton-Dickinson. Anti-β-actin was purchased from Santa-Cruz Biotechnology (Santa Cruz, CA, USA).

Following incubation with the primary antibody, the blots were washed and incubated with 1:10000 dilution of biotinylated anti-mouse IgG (Vector Laboratories, Inc, Burlingame, CA, USA), as appropriate. The immunoreactive bands were visualized by enhanced chemiluminescence (ECL) using the ECL detection system (Amersham Pharmacia Biotech).

Statistical analysis. All of the experiments were repeated at least three times. Statistical significance of the differences was evaluated by the unpaired, two-tailed Student's *t*-test, and an association was considered significant when the significant level of the test was $p<0.05$.

Results

Cell growth inhibition under hypoxic conditions. To evaluate the cell growth ability, HT29 cells were cultured under normoxic and hypoxic conditions, and live cells were counted after staining with trypan blue (Figure 1). After 72 h under normoxia, the HT29 cell growth was 11-fold, whereas under hypoxic condition, it was only two-fold. Similarly, the SW480 cell growth was 16-fold under normoxia, whereas it was only 5-fold under hypoxia. Thus, hypoxia significantly inhibited the cell growth ability of HT29 and SW480 cells.

Under hypoxia, colon cancer cells were resistant to 5-FU and oxaliplatin, but relatively sensitive to SN-38. Treatment of HT29 cells with 5-FU, oxaliplatin, or SN-38 for 48 h, under

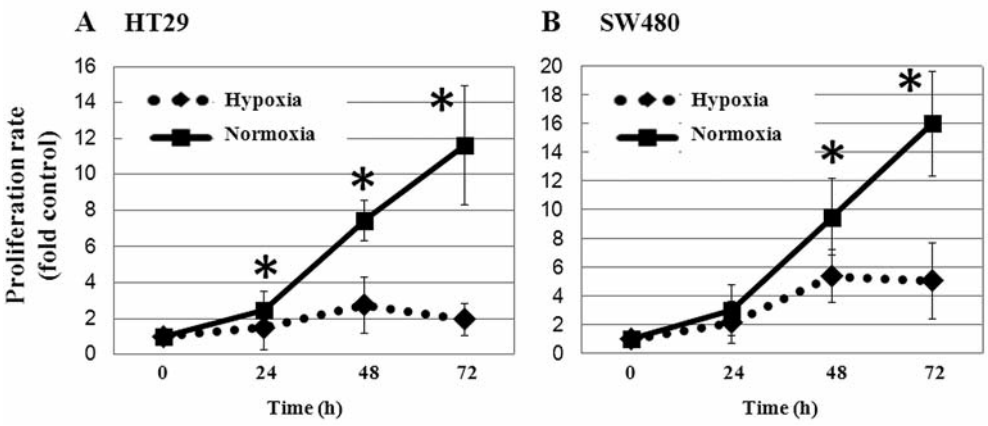

Figure 1. *Cell growth assessment after culture under normoxic and hypoxic conditions. The proliferative activity of HT29 (A) and SW480 (B) cells cultured under hypoxic and normoxic conditions was evaluated for 24, 48, and 72 h. Cell growth was investigated by trypan blue exclusion staining. The proliferation rate was calculated as the ratio to the values obtained with control cells cultured under normoxic or hypoxic conditions for 0 h respectively. Values are given as the mean±SD. The proliferation rates of both HT29 and SW480 cells were significantly lower under hypoxic conditions than under normoxic conditions (*p<0.05).*

Table I. *Cell cycle analysis of cells cultured under hypoxia and normoxia treated with anticancer agents (*p<0.05).*

		Sub G_1	G_0/G_1	S	G_2/M
Control	Normoxia	1.2±0.3	61.3±1.8	19.5±4.9	15.8±4.0
	Hypoxia	10.9±3.3	66.0±3.4	13.7±2.8	9.0±2.6
5-FU	Normoxia	2.4±0.4	26.8±8.2*	63.5±10.8*	6.7±2.3
	Hypoxia	9.5±2.3	68.1±4.3*	19.1±2.4*	2.7±1.3
Oxaliplatin	Normoxia	1.4±0.2	15.4±2.4*	50.6±3.9	31.7±1.4*
	Hypoxia	7.2±1.0	37.3±7.5*	47.7±8.8	7.4±1.0*
SN-38	Normoxia	14.4±6.5	13.3±1.6	58.5±1.6	12.1±3.4
	Hypoxia	14.9±1.3	18.8±5.7	55.5±4.2	8.2±1.9

normoxia, resulted in a dose-dependent inhibition of proliferation ability (Figure 2). On the other hand, when cells were treated with the same doses of 5-FU and oxaliplatin under hypoxic conditions, the inhibitory effects were almost completely abolished. However, the inhibitory effect of SN-38 was clearly observed in the hypoxic environment.

Hypoxia induced G_0/G_1 phase cell cycle arrest. Culture of the cells under hypoxic conditions resulted in the arrest of the cell cycle at the G_0/G_1 phase (Table I). Treatment of HT29 cells with 5 μM of 5-FU for 48 h under normoxic conditions resulted in S phase cell cycle arrest, whereas the treatment with the same dose of 5-FU under hypoxic conditions resulted in strong arrest at the G_0/G_1 phase (percentage of cells in G_0/G_1 phase: 27% *vs*. 68% for normoxia *vs*. hypoxia, respectively). Furthermore, the treatment of HT29 cells with 5 μM of oxaliplatin for 48 h under normoxic conditions resulted in the arrest of cells at the G_2/M phase, whereas under hypoxic conditions, a strong arrest of cells at the G_0/G_1 phase was observed (percentage of cells in G_0/G_1 phase: 15% *vs*. 37% for normoxia *vs*. hypoxia, respectively). On the other hand, treatment of HT29 with 0.5 μM of SN-38 under normoxic conditions resulted in S phase arrest, with a concomitant increase of the cells in the sub-G_1 phase. Under hypoxic condition, S phase arrest was observed (percentage of cells in G_0/G_1 phase: 13% *vs*. 19% for normoxia *vs*. hypoxia, respectively), also with a concomitant increase in the percentage of cells in the sub-G_1 phase. Under hypoxic conditions, HT29 cells were arrested at the G_0/G_1 phase, which probably contributed to the attenuation of the effect of the chemotherapeutic agents that act through regulation of the cell cycle, including 5-FU and oxaliplatin. Similar results were obtained with another colon cancer cell line, SW480 (data not shown).

Apoptosis induced by 5-FU and oxaliplatin was inhibited under hypoxic conditions. The percentage of apoptotic HT29 cells (annexin V+) was not significantly different between cells cultured under normoxic and hypoxic conditions (8.1% *vs*. 9.4%, normoxia *vs*. hypoxia,

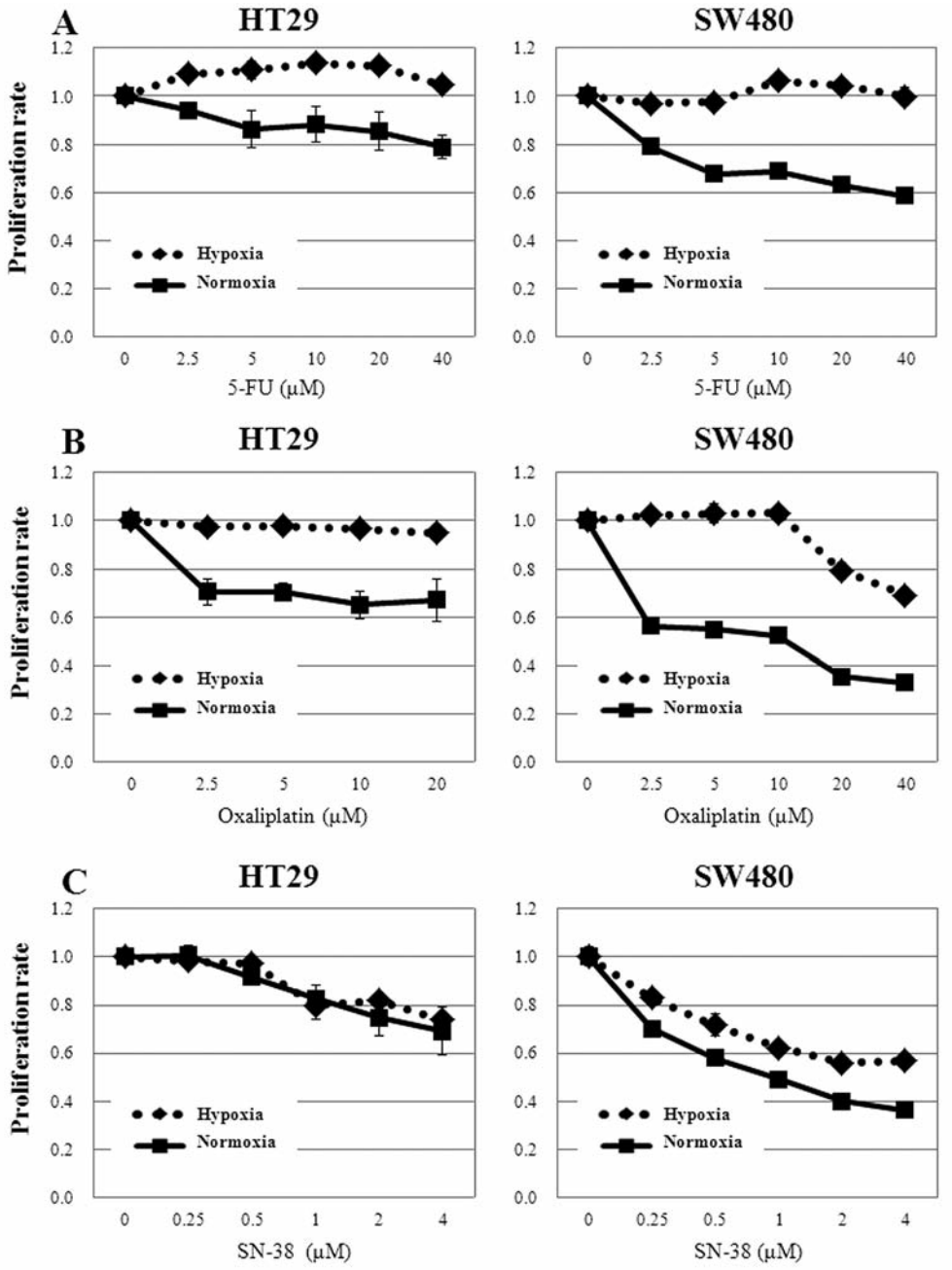

Figure 2. *Effect of anticancer drugs on the proliferative activity of HT29 and SW480 cells. The proliferative activity of HT29 and SW480 cells treated with 5-fluorouracil (5-FU) (A), oxaliplatin (B), and SN-38 (C) for 48 h was assessed by calcein luciferase accumulation. The proliferation rate was calculated as the ratio to the values obtained with control untreated cells cultured under normoxic or hypoxic conditions respectively. Under normoxic conditions, 5-FU, oxaliplatin and SN-38 dose-dependently inhibited the proliferative activity of HT29 and SW480 cells, the effect of each agent being greater in SW480 cells. Under hypoxic conditions, the inhibitory effect of 5-FU and oxaliplatin were attenuated, whereas that of SN-38 was similar to that under normoxic conditions.*

respectively) (Figure 4). Treatment of cells with 5-FU (10 μM) under normoxic conditions resulted in a slightly increased percentage of apoptotic cells (14.6±4.4%). However, when cells were treated with the same dose of 5-FU under hypoxia, the percentage of apoptotic cells did not change (9.6±2.1%). Similarly, apoptosis induced by oxaliplatin (10 μM) was reduced under hypoxia compared with normoxic condition (16.0% *vs.* 10.1%, normoxia *vs.* hypoxia, respectively). On the other hand, when cells were treated with SN-38 (1 μM), there was no difference in the

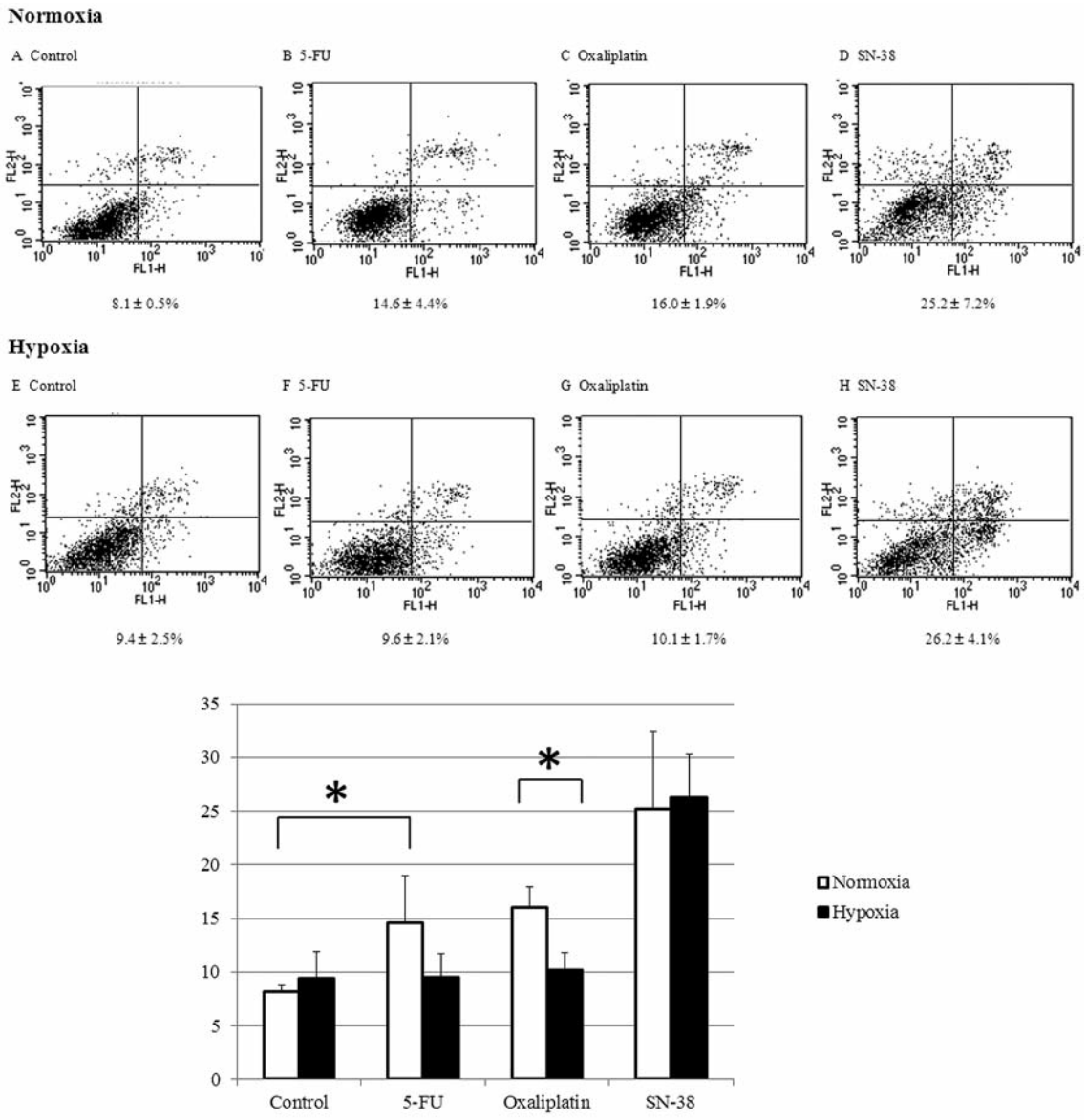

Figure 3. *The change of the proportion of annexin V + apoptotic cells under normoxic and hypoxic conditions after treatment with anticancer agents. The proportion of annexin V + apoptotic cells was evaluated by flow cytometer using annexin V fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining of HT29 cells after treatment with anticancer drug. Treatment with 5-fluorouracil (5-FU) (10 μM) and with oxaliplatin (10 μM) under normoxic conditions resulted in a significant increase in the percentage of annexin V + apoptotic cells. However, under hypoxic conditions, the percentage of annexin V + apoptotic cells was reduced at the same doses of the drugs tested under normoxia. On the other hand, SN-38 (1 μM) induced apoptosis under normoxic conditions, and at a similar rate under hypoxic conditions. Values given are the mean±SD (*p<0.05).*

apoptosis rate between cells under normoxic and hypoxic conditions (25.2% *vs*. 26.2%, normoxia *vs*. hypoxia, respectively). Similar results were obtained with SW480 cells (data not shown).

SN-38 inhibited the expression of HIF-1α in colon cancer cells under hypoxia. HT29 cells were treated with 5-FU (40 μM), oxaliplatin (20 μM), or SN-38 (0.1, 0.25, 0.5 μM) for 24 h under hypoxic and normoxic conditions. Cell lysates were then prepared, and the expression of HIF-1α was investigated by western blot analysis.

Under normoxic conditions, the expression of HIF-1α was negligible, but was clearly observed under hypoxia. The treatment of the cells with 5-FU and oxaliplatin under hypoxia did not affect the expression of HIF-1α (Figure 5). However, the treatment of HT29 cells with SN-38 under hypoxia resulted in an evident decrease of HIF-1α. Similar results were obtained with SW480 cells (data not shown).

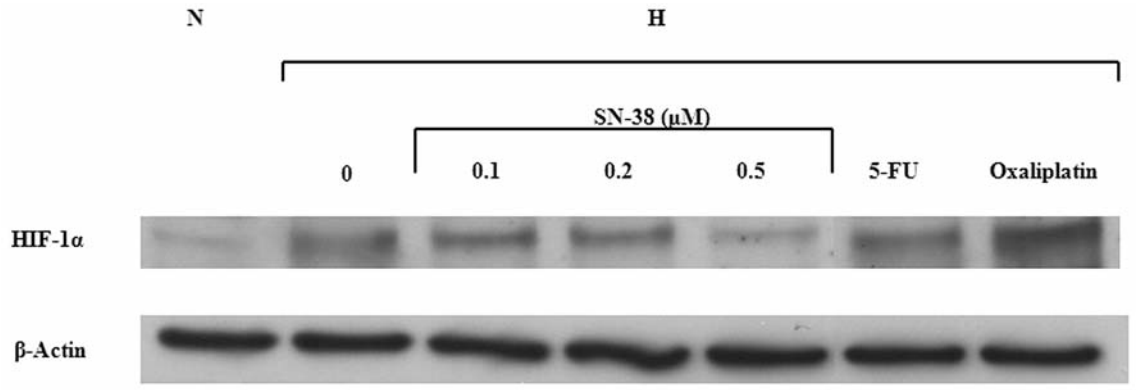

Figure 4. *Western blot analysis of hypoxia-inducible factor 1α (HIF-1α) expression with anticancer drug. The expression of HIF-1α was detected by western blot in HT29 cells cultured under normoxic and hypoxic conditions and treated with anticancer agents for 24 h. HIF-1α expression was very weak in cells cultured under normoxia, but under hypoxic conditions, significant accumulation of HIF-1α was observed. Treatment with 5-fluorouracil (5-FU) (40 μM) and oxaliplatin (20 μM) under hypoxic conditions for 24 h did not affect HIF-1α expression, but treatment with SN-38 inhibited HIF-1α in a dose-dependent manner.*

Discussion

Fluorouracil and folinic acid with either oxaliplatin (FOLFOX) or irinotecan (FOLFIRI) are widely used as first-line and second-line chemotherapy for metastatic colorectal cancer. However, the results still remain poor in advanced cases, especially those with metastatic lesions, often dependent on the acquisition of resistance to therapy. The hypoxic environment in the central area of the growing tumor, dependent on the insufficient neovascularization, seems to be in part responsible for this phenomenon. In fact, hypoxic cells are known to be more resistant to ionizing radiation and chemotherapy (24).

In the present study, we aimed to investigate the effect of the hypoxic environment on the chemosensitivity of colorectal cancer cells to chemotherapy and the mechanisms underlying it. We demonstrate that the HT29 colorectal cancer cell line, under hypoxic conditions, is more resistant to 5-FU and oxaliplatin compared to under the normoxic conditions. Both 5-FU and oxaliplatin dose-dependently inhibited the proliferative activity of HT29 cells under normoxia, but under hypoxia, the inhibitory effects of both agents were significantly abrogated. Chemoresistance to 5-FU and oxaliplatin was partly dependent on the arrest of the cell cycle at the G_0/G_1 phase, induced by hypoxia, which seemed to prevent these agents from fully exerting their effects on the cell cycle. SN-38 treatment also dose-dependently inhibited the proliferative activity of HT29 cells under normoxia, however, different from 5-FU and oxaliplatin, the inhibitory effect of SN-38 was not abrogated by hypoxia. Treatment with SN-38 under normoxia resulted in the accumulation of the cells at the S phase, as well as an increase of cells in the sub-G_1 phase, *i.e.* apoptotic cells. Although less evident than under normoxia, under hypoxia, the accumulation of cells in the S phase and an increase of cells in the sub-G_1 phase were observed. Similar results were obtained with another colorectal cancer cell line, SW480 (data not shown). 5-FU and SN-38 have been reported to exert their functions through induction of S phase cell cycle arrest (25-27), whereas oxaliplatin exerts its function through the accumulation of the cells in the G_2/M phase (28). Since hypoxia led cells to be arrested at the G_0/G_1 phase, with consequent inhibition of the cell growth, the inhibitory effects of 5-FU and oxaliplatin could not be fully exerted under hypoxia. Analysis of apoptosis revealed that treatment with 5-FU under normoxia resulted in an increased rate of apoptosis, which was inhibited by hypoxia. Similarly, the apoptosis induced by oxaliplatin under normoxia was inhibited under hypoxia. Under normoxia, SN-38 treatment also resulted in increased apoptosis, but it was not substantially affected by hypoxia. Thus, the hypoxic environment significantly abrogated the inhibitory effects of 5-FU and oxaliplatin, but not of SN-38, on human colon cancer cells, dependent on the cell cycle arrest at G_0/G_1 phase and the inhibition of apoptosis.

Next, in order to investigate the mechanisms underlying these phenomena, we focused on HIF, which has been reported to be involved in the acquisition of resistance to therapy, including radio- and chemotherapy. Various mechanisms have been implicated in the chemoresistance induced by HIF-1, including the induction of an antiapoptotic mechanism (11) and the arrest of the cells at the G_0/G_1 phase (12, 13, 29). Our present data revealed the accumulation of HIF-1α in cells cultured under hypoxia, which was not affected by treatment with 5-FU and oxaliplatin. Interestingly, however, the treatment of these cells with SN-38 resulted in a dose-dependent inhibition of the accumulation of HIF-1α. Cells accumulating HIF-1α are less sensitive to pro-apoptotic signals (30, 31), which is partly explained by the inhibition of B-cell lymphoma

2 homology domain 3 interacting domain death agonist (BID), B-cell lymphoma 2 associated death protein (BAD), and B-cell lymphoma 2 associated X protein (BAX) (11). This is compatible with our findings that treatment with 5-FU and oxaliplatin, which did not affect the accumulation of HIF-1α, resulted in the inhibition of the pro-apoptotic effect compared to cells cultured under normoxia, but not for those cells treated with SN-38, which inhibited the accumulation of HIF-1α. The apoptosis rate was similar between cells treated with SN-38 under normoxia and those under hypoxia. The G_0/G_1 cell cycle arrest induced by hypoxia has also been reported to be dependent on HIF-1α, which inhibits cyclin D1 and induces P21 and P27 (29, 32, 33). Thus, it is suggestive that SN-38 attenuates the antiapoptotic effect of hypoxia *via* an HIF-1α dependent pathway.

Topoisomerase I inhibitors, such as topotecan and SN-38, have been reported to inhibit HIF-1α expression in malignant glioma cells (34-36). SN-38 has been shown to inhibit VEGF expression through inhibition of HIF-1α in glioma cells and, as a consequence, to inhibit endothelial proliferation and tube formation (35). In colorectal cancer, the combination therapy of rapamycin, which is known to target HIF-1α, and irinotecan, which is able to inhibit the accumulation of HIF-1α, has been shown to induce massive death of colon cancer cells under hypoxic, but not normoxic, conditions *in vitro*, and a dramatic reduction of tumor volume *in vivo* (37). Therefore, HIF-1α, a key transcription factor with a pivotal role in tumor cell metabolism, is a promising target in the treatment of colorectal cancer. But the inhibitory effect of irinotecan itself on the accumulation of HIF-1 α has been shown to be limited (37).

In our study, SN-38, a derivative of irinotecan, was found to be able to inhibit HIF-1α accumulation, even at very low doses (IC-10 under normoxia). The inhibitory effect of SN-38 on HIF-1α was stronger than that of irinotecan. This may result from the stronger antitumor activity of SN-38 compared to irinotecan (more than 30 times) (38, 39). In addition to the inhibitory effect on HIF-1α, SN-38 has antiangiogenic effects through inhibition of VEGF production and a direct effect on endothelial cells. Thus, SN-38 seems to exert at least triple effects on solid tumors: a direct cytotoxic effect on cancer cells, an inhibitory effect on HIF-1α, and an antiangiogenic effect.

We conclude that SN-38 is an anticancer agent with the ability to inhibit the accumulation of HIF-1α in colorectal cancer cells and, consequently, to actively kill these cells even under hypoxic conditions. Oxaliplatin, similar to 5-FU, was less effective against hypoxic colorectal cancer cells.

In colorectal cancer, HIF-1α overexpression is associated with mortality and metastasis (6-10). Thus, targeting HIF-1α would be a very promising strategy for the treatment of colorectal cancer, especially poorly vascularized tumors and those overexpressing HIF-1α. In these kind of tumors, FOLFIRI therapy could be more effective than FOLFOX, and these features should be evaluated and considered when choosing the ideal therapeutic protocol.

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