Hypoxia After Liver Surgery Imposes an Aggressive Cancer Stem Cell Phenotype on Residual Tumor Cells

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Objective: To assess the contribution of hypoxia and bone marrow–derived cells to aggressive outgrowth of micrometastases after liver surgery.

Background: Liver surgery generates a microenvironment that fosters aggressive tumor recurrence. These areas are characterized by chronic hypoxia and influx of bone marrow–derived cells.

Methods: The contribution of hematopoietic cell types was studied in mice lacking specific components of the immune system and in irradiated mice lacking all bone marrow–derived cells. Tumor cells were derived from colorectal cancer patients and from a metastatic tumor cell line. Hypoxia-induced changes in stem cell and differentiation marker expression, clone-forming potential, and metastatic capacity were assessed. The effect of vascular clamping on cancer stem cell (CSC) characteristics was performed in mice bearing patient-derived liver metastases.

Results: Immune cells and bone marrow–derived cells were not required for aggressive outgrowth of micrometastases in livers treated with surgery. Rather, hypoxia was sufficient to promote invasion and accelerate metastatic outgrowth. This was associated with a rapid loss of differentiation markers and increased expression of CSC markers and clone-forming capacity. Likewise, metastases residing in ischemia-reperfusion-injured liver lobes acquired CSC characteristics. Despite their renowned general resistance to chemotherapy, clone-forming CSCs were readily killed by the hypoxia-activated prodrug tirapazamine.

Conclusions: Surgery-generated hypoxia in the liver causes rapid dedifferentiation of tumor cells into immature CSCs with high clone- and metastasis-forming capacity. The results help explain the phenomenon of aggressive local tumor recurrence after liver surgery and offer a potential strategy to kill aggressive CSCs by hypoxia-activated prodrugs.

Keywords: cancer stem cell, colorectal, hypoxia activated prodrugs, hypoxia, inflammation, liver metastases, surgery, tirapazamine

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The fact that metastases frequently arise at sites of injury has been known for over a century. However, the mechanisms that link surgery-induced tissue injury to the stimulation of metastatic growth are still largely obscure. Metastases from colorectal tumors primarily develop in the liver and the lungs as a result of hematogenous spread and subsequent lodging of circulating tumor cells in the microvasculature of these organs. Results from experimental model systems have demonstrated that injury to liver tissue caused by surgical trauma, ischemia-reperfusion, chemotherapy, or irradiation stimulates the outgrowth of liver metastases. Furthermore, surgical resection of liver metastases in colorectal cancer patients results in a significantly better disease control if ischemia-reperfusion injury is limited. Along the same line, it has been argued that perioperative therapy aimed at minimizing the consequences of surgery-induced tissue injury could be beneficial. Partial liver resection and/or local ablation strategies are the only potentially curative treatment modalities in patients with metastatic colorectal cancer. Therefore, it is essential to understand the principles of liver injury-stimulated tumor growth in detail.

Our previous work has demonstrated that micrometastases in the liver adopt a highly aggressive phenotype after liver injury induced by ischemia-reperfusion or local ablation. We found that accelerated tumor growth was primarily observed in tissue areas characterized by hypoxia and inflammation and that this was independent of hypoxia-induced angiogenesis. There is no doubt that both hypoxia and inflammation are protumorigenic conditions (reviewed in references 13–15). Hypoxia-induced factors, cytokines and matrix-remodeling enzymes suppress antitumor immunity. One of the hallmarks of injured tissue is oxygen deprivation (hypoxia), caused by decreased tissue perfusion. Hypoxia-inducible factors (HIFs) promote tissue inflammation by stimulating expression of proinflammatory genes including cyclooxygenase-2, nitric oxide synthase, and various cytokines. Vice versa, expression of HIF1 itself is stimulated by NFκB, a key regulator of inflammatory gene expression. Hypoxia also directly affects tumor cell behavior independently of inflammation by promoting tumor cell dedifferentiation, invasion and metastasis (reviewed in reference 15). The effects of hypoxia on both tumor cells and stromal cells are likely to contribute to hypoxia-associated therapy resistance and aggressive tumor behavior.

Given the intricate relationship between hypoxia and inflammation, it has so far been impossible to identify the major driving force behind aggressive tumor recurrence after liver surgery. In the present report, we have explored the contribution of hypoxia and inflammation to surgery-induced stimulation of tumor growth by using primary patient-derived colonosphere cultures and a mouse model for experimental liver metastasis formation. On the basis of the obtained results, we present a potential therapeutic strategy to limit surgery-induced stimulation of tumor growth.

METHODS

Cell Culture

C26 cells were cultured in Dulbecco’s modified Eagle’s medium (ICN Pharmaceuticals, Zoetermeer, The Netherlands) supplemented with 5% fetal calf serum (FCS), 2 mmol/L

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glutamine, 0.1 mg/mL streptomycin, and 100 units/mL penicillin. Human colonosphere cell lines (L145, L146, L167, and L169) were derived from patients harboring colorectal liver metastases and cultured in stem cell medium as described. All cell culture was carried out at 37°C in a 5% CO₂-humidified incubator under normoxic (21% oxygen) or hypoxic (1% oxygen) conditions, the latter using an Invitro2 Hypoxia Workstation (Biotrace International, Spennymoor, UK).

**Clone-Forming Assays**

Cells were preincubated under normoxic or hypoxic conditions for 24 hours. After this 24-hour exposure to normoxia/hypoxia, C26 were seeded as single cells at 1000 cells per 10-cm dish in triplicate under normoxic conditions. Colonies were stained after 10 days with 0.2% crystal violet and counted. Colonospheres were processed as described by Emmink et al. In short, 1000 single cells were plated in Matrigel and colonospheres were counted 3 to 4 weeks after seeding.

The clone-forming potential of cells derived from tumor xenografts was assessed 24 hours after vascular clamping or sham treatment. Tumor-bearing livers were harvested, and tumor tissue was cut into small fragments. Tissue fragments were washed with phosphate buffered saline (PBS) until the supernatant was clear and were incubated in digestion buffer [50 mL Dulbecco’s modified Eagle’s medium, 1.25 mL FCS, 3.5 mg collagenase IX (Sigma, St Louis, MO), 6.2 mg dispase type II (Invitrogen, Carlsbad, CA), 500 μl Primosin] for 60 minutes at 37°C while shaking. Dissociated cells were washed with PBS and were filtered through a 70-μm cell strainer to separate residual tissue fragments from single cells. Cells were plated in Matrigel in a concentration of 5000 cells/well and growth factors B-fibroblast growth factor (10 ng/mL, Abcam, Cambridge, UK) and epidermal growth factor (20 ng/mL, Invitrogen) were freshly added every week. Tumor cells that were not used for the colonosphere forming assays were processed for flow cytometry to determine the percentage human cells using the epithelial marker anti-EpCAM (1/1000, Dako, Glostrup, Denmark). Five weeks after plating, colonospheres were counted and percentage of colonospheres forming efficacy was corrected for the percentage human cells (EpCAM+) in the clamped and sham treated single cell suspension.

To assess the effect of tirapazamine (TPZ) on the number of clone-forming cells colonospheres were exposed to hypoxia or normoxia with or without TPZ (40 μM; kindly provided by Dr Minchinton, BC Cancer Agency, Vancouver, Canada) for 24 hours. After drug-exposure, single cells were washed and plated out in Matrigel (1000 live cells/well) and colonospheres were counted after 3 to 4 weeks.

**Invasion Assay**

Invasion assays were performed as described. After 24 hour incubation under hypoxia or normoxia, 50,000 C26 cells were placed in serum-free conditions as single cells in the upper compartment of BioCoat Matrigel invasion chambers [p No. 354480; Becton Dickinson (BD) Biosciences, Franklin Lakes, NJ]. After another 8 hours of incubation under respectively hypoxia or normoxia transmigrated (toward a 5% serum gradient) cells were counted. Assays were performed in duplicate.

**Western Blotting**

Colonospheres were cultured under normoxic or hypoxic conditions for up to 48 hours. For the TPZ toxicity experiment, colonospheres were cultured for 24 hours under normoxic or hypoxic conditions with or without 40 μM TPZ. Colonospheres were lysed in buffer (containing 20 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 1% NP40, 150 mmol/L sodium chloride, 5 mmol/L magnesium chloride, and 10% glycerol), run out on sodium dodecyl sulphate-containing gels and blotted onto nitrocellulose membranes. After blocking in milk, the blots were probed with antibodies against cytokeratin 20 (CK20, KS20.8; 1:500, M7019; Dako), cleaved caspase-3 (1:5000, ab9665; Cell Signaling, Littleton, MA), or actin (1:20000, NB600-501; Novus Biologicals, Littleton, CO). Horseradish peroxidase-conjugated secondary antibodies were from Dako. Signals were detected by chemiluminescence.

**Immunohistochemistry**

Nine days after vascular clamping of livers containing L145 tumors, immunohistochemistry was performed on formalin-embedded tissue using antibodies against CK20 (KS20.8, 1:200, M7019, Dako), fatty acid-binding protein 1 (FABP1; 1:500, HPA 028275, Sigma), and Mucin 2 (MUC2; 1:200, 15334; Santa Cruz, Santa Cruz, CA). Slides were incubated overnight with primary antibody at 4°C. For detection goat anti-Ms/Rb/Rt-poly-HRP (Powervision Immunologic, Immunovision Technologies, Brisbane, CA) was used. All slides were developed with diaminobenzidine followed by hematoxylin counterstaining. Pictures were made of the whole tumor lesion and the percentage positive stained tissue per high power field (HPF) was calculated using ImageJ software (National Institutes of Health, Bethesda, MD).

Granulocytes in the transition zone (TZ) after radiofrequency ablation (RFA) procedure were detected by coupling of diazomium salt (pink) to the naphthol-AS-D-chloroacetate esterase created free naphthol compound (characteristic for granulocytes) using standard procedures. Pictures of the TZ were made and LEDER-positive cells were counted per HPF. All quantifications were performed in a blinded fashion.

**Immunofluorescence**

L146 cells were exposed for 24 hours to hypoxia or normoxia before fixation in 3.7% formaldehyde (30 minutes). After fixation, cells were washed with PBS before being permeabilized with ice-cold (−20°C) methanol (10 minutes). Cells were blocked in PBS containing 2% bovine serum albumin and 0.1% Tween (1 hour). Cells were incubated overnight with primary antibody against CK20 (KS20.8, 1:50, M7019; Dako) and stained overnight at 4 degrees with a fluorescence-conjugated IgG (chicken antimouse Alexa fluor 488) mixed with 0.5 μg/mL DAPI. The slides were cover slipped with Vectashield mounting medium. The samples were analyzed using a Zeiss LSM510 META microscope.

**Flow Cytometry**

Aldefluor activity and the expression of the surface marker stem cell antigen 1 (SCA1) were analyzed by fluorescence-activated cell sorting (FACS) using CellQuest software (BD Biosciences). C26 cells and the L145 colonosphere cell line were cultured under normoxia or hypoxia for the indicated time points. For the toxicity experiments, colonosphere line L145 was cultured for 24 hours under normoxic or hypoxic conditions with or without TPZ (20 or 40 μM). Next, cells were trypsinized to obtain single-cell suspensions. Cell doublets and clumps were excluded by prior filtration and by using doublet discrimination gating during FACS analysis. Aldefluor-positive cells were analyzed according to the manufacturer’s protocol by using the ALDH (aldehyde dehydrogenase isofrom 1) substrate BAAA (1 μmol/L per 1×106 cells; StemCell Technologies, Vancouver, Canada). Negative control samples were coincubated with diethylaminobenzaldehyde (50 mM, StemCell Technologies). Percentage of SCA1 expression in C26 cells after 24-hour incubation under normoxia or hypoxia was determined using the antibody against SCA1 (Ly-6A/E(D7), 557403, BD Biosciences). The cell sorting experiments were conducted with DAKO-Cytomation MoFlo High-Speed Sorter.
Quantitative Real Time Polymerase Chain Reaction

RNA was isolated according to the manufacturer’s protocol (RNeasy Mini Kit, Qiagen, Venlo, The Netherlands) from colo-sphere cell lines (L145, L146 and L169) exposed to 48 hours in vitro to normoxia and hypoxia and from the L145 xenografted colo-sphere tumor tissue 24 hours after clamping. c-DNA was synthesized from 250 ng of total RNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Next, cDNA was amplified in an iCycler thermocycler (Bio-Rad) using SYBR Green Supermix (Bio-Rad). mRNA expression levels were quantified using iCycler software (Bio-Rad) and were normalized to B2M. All samples were analyzed in triplicate. Quantitative polymerase chain reaction (qPCR) analysis was performed for the markers hexokinase 2 (HEX2), FABP2, MUC2, sex determining region Y-box 2 (SOX2), octamer-binding transcription factor 4 (OCT4), NANOG, olfactomedin 4 (OLF4M4), and chromo-granin A (CHRA) (all primers were ordered from Sigma).

Experimental Animal Treatments

All experiments were carried out in accordance with the guidelines of the Animal Welfare Committee of the University Medical Centre Utrecht, The Netherlands. For the experiments using C26 cells, male Balb/c mice, nude mice, severe combined immunodeficiency (SCID) mice, or nonobese diabetic (NOD) SCID mice were purchased from Charles River (10–12 weeks, 20–25 g; Sulzfeld, Germany). For the human L145 colonosphere in vivo experiments CB17/lcr-Prkdc−c-scid−−Crl (F) were purchased (Charles River). Animals were housed under standard laboratory conditions. All surgical procedures were performed under isoflurane inhalation anesthesia. Buprenorphine was administered intramuscularly before surgery to provide sufficient preoperative analgesia.

Tumor induction of C26 cells and RFA were performed as described by Nijkamp et al.3 For the experiments using the colo-sphere cell line L145, cells were injected and grown subcutaneously to a maximum of 1 cm³. Serial transplantation of a little piece of tumor (±10 mm³) was performed into the left liver lob of a new recipient mouse. This tumor was allowed to grow for 8 weeks before hepatic clamping procedure (45 minutes) was performed as described by van der Bilt et al.3 Control mice underwent surgery and liver handling without the clamping procedure itself.

Radiotherapy was used just to gain complete loss of bone marrow function in Balb/c mice. This was given for 1 minute at a dose of 6 gray 2 hours before tumor cell induction. Loss of inflammatory cells was validated by LEDER staining for granulocytes on liver sections and by blood analysis for white blood cells using a Cell-Dyn 1800 (Abbott Diagnostics).

Assessment of Tumor Cell Phenotype After Radiofrequency Ablation

Mice bearing enhanced green fluorescent protein (GFP)–expressing tumor cells were killed 24 hours after RFA. Confocal microscope images were prepared as described by Nijkamp et al.3 In short, livers were excised and placed on a coverslip using immersion oil to improve visualization. Enhanced GFP was excited at 488 nm and length of largest diameter of metastases was measured in both reference zone (RZ) and TZ. The TZ was defined as the area stretching 2 mm outside the necrotic ablated zone. The RZ was defined as the part of the liver unaffected by the RFA treatment (>2 mm from the lesion border).

Tumor Load Analysis After Radiofrequency Ablation

For the tumor load studies after RFA in irradiated mice, animals were terminated 10 days after tumor cell injection (7 days after RFA). For the metastases formation experiments using hypoxia- or normoxia-exposed C26 cells, mice were terminated 7 days after tumor cell injection. Livers were harvested, fixed in 4% buffered formaldehyde and embedded in paraffin for morphometric assessment of tumor growth. Tumor load was scored in the RZ and TZ in the irradiated mice, as described previously.3 For the experiment using hypoxia- or normoxia-exposed tumor cells, the number of metastases and hepatic replacement area were measured in the whole left liver lobe.

Statistics

Data are presented as mean (SEM). All statistical differences between groups were measured by paired (or unpaired when appropriate) Student t tests using GraphPad Prism version 5.0 (Graphpad Software, La Jolla, CA).

RESULTS

Immune Cells Are Dispensable for Injury-Induced Aggressive Tumor Growth in the Liver

We have previously shown that damage to liver tissue induced either by RFA or ischemia-reperfusion injury generates highly localized regions of chronic hypoxia in which aggressive tumor growth is observed.3,5 To study whether immune cells contribute to the aggressive phenotype of tumor cells in postsurgery livers, we made use of irradiation to kill all hematopoietic cells (Fig. 1A). After RFA, neutrophils are the first cells to arrive at the TZ,24 which separates the site of injury from the nonaffected liver. As expected, the recruitment of neutrophils was not observed in irradiated mice (Fig. 1B). Tumor cell invasion into the perinecrotic region can be quantified by making use of GFP-expressing tumor cells and postmortem fluorescence microscopy analysis of the liver. Surprisingly, the absence of neutrophils and other inflammatory cells in the hypoxic TZ had no effect on the highly invasive phenotype that characterizes metastases in this region (Figs. 1C, D). Moreover, irradiation did not affect surgery-stimulated outgrowth of metastases in the TZ (Fig. 1E and reference 3). Similarly, TZ metastases in mice lacking either T lymphocytes (nude), T and B lymphocytes (SCID) or T lymphocytes, B lymphocytes and natural killer cells (NOD/SCID) were all characterized by a highly invasive phenotype (Fig. 1F). From these experiments, we conclude that inflammatory cells are dispensable for establishing an invasive phenotype and accelerated outgrowth of perinecrotic metastases in RFA-injured livers.

Exposure of Colorectal Tumor Cells to Hypoxia Promotes Clone Formation, Invasion, and Metastatic Capacity

The finding that inflammatory cells do not play a major role in injury-induced aggressive tumor growth prompted us to test the isolated effect of hypoxia on colorectal cancer cell behavior. To this end, murine colorectal cancer cells and patient-derived “hypoxia” cultures were exposed to hypoxia or normoxia for 24 hours, after which their colony-forming potential was assessed. In all cultures tested, exposure to hypoxia significantly increased the number of cells with colony-forming potential (Fig. 2A). Furthermore, hypoxia also increased the invasive capacity of C26 cells (Fig. 2B). Increased colony-forming potential coupled to increased invasive capacity suggested that a short transient exposure to hypoxia may be sufficient to increase the metastatic capacity of the resulting cultures. To test this, we injected normoxic and hypoxic C26 cells into the spleens of Balb/c mice and analyzed metastatic burden in the liver 2 weeks later. We observed that 5 days of in vitro exposure to hypoxia doubled the metastatic potential of C26 tumor cells (Figs. 2C, E). Moreover, liver metastases formed by hypoxia-exposed C26 tumor cells occupied approximately threefold more liver tissue than those cultured under normoxia (Figs. 2D, E).
FIGURE 1. Immune cells and bone marrow–derived cells do not contribute to the invasive metastasis phenotype and accelerated tumor outgrowth induced by liver surgery. Balb/c mice were subjected to radiation or control treatment directly followed by intrasplenic injection of enhanced GFP-expressing tumor cells. Three days later, liver micrometastases were formed and RFA treatment was performed in the left liver lobe. To assure adequate radiation, white blood cells were measured 24 hours after RFA in the corresponding blood samples (A) and neutrophil influx into the TZ was assessed by LEDER staining on corresponding tissue sections (neutrophils were counted per HPF) (B). Twenty-four hours after RFA treatment, invasion of tumor cells was determined by measuring the longest diameter of metastases in the hypoxic TZ and the normoxic RZ (n = 4–6) (C). Representative pictures are shown (D). Ten days after RFA, tumor load (calculated as hepatic replacement area: HRA) was determined in theTZ and RZ in irradiated mice (n = 11) (E). Invasion of GFP-labeled C26 cells 24 hours after RFA treatment was measured in mice with different immunological backgrounds. Normal Balb/c mice (control), nude mice lacking T lymphocytes, SCID mice lacking T and B lymphocytes and NOD SCID mice lacking T lymphocytes, B lymphocytes and natural killer cells (n = 3) (F). *P < 0.05, Student t test.

Exposure of Colorectal Tumor Cells to Hypoxia Causes Rapid Loss of Differentiation Markers and a Concomitant Increase in Stem Cell Markers

The aforementioned results suggest that hypoxia alone is sufficient to induce a more invasive and aggressive phenotype in colorectal cancer cells. Given that hypoxia can foster the growth of normal stem cells and CSCs and that stem cell signatures are associated with poor survival in colorectal cancer, we analyzed differentiation and stem cell marker expression in colorectal tumor cells exposed to hypoxia or normoxia. Culturing human colonospheres of 4 different patients in hypoxia caused a rapid (24 hours) loss of cytokeratin 20 (CK20), which is a general marker for colorectal tumor cell differentiation (Figs. 3A, B). In addition, hypoxia caused loss of the enterocyte marker FABP2 and of the goblet cell marker Mucin-2, whereas HEX2, a marker for hypoxia, was strongly induced (Fig. 3C). Aldefluor is a fluorescent probe for aldehyde dehydrogenase activity and frequently used to identify normal stem cells and CSCs. Indeed, Aldefluor+ cells express high levels of primitive stem cells markers (Fig. 3F) and recently we have shown that these cells are clonogenic and tumorigenic. Transplantation studies have identified the surface protein SCA1 as a marker for tumor-initiating cells in mice, and it is commonly used to identify murine CSCs. Exposure of C26 cells and human colonospheres to hypoxia caused a dramatic increase in SCA1- and Aldefluor-positive cells, respectively (Figs. 3D, E). Together, the results demonstrate that exposure of human and murine colorectal tumor cells to hypoxia causes a rapid and general loss of differentiation markers and a concomitant increase in stem cell markers and clone-forming capacity.
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FIGURE 2. In vitro exposure of colorectal tumor cells to hypoxia promotes clone formation, invasion, and metastatic capacity. Five different colorectal cancer cell lines were grown under hypoxia or normoxia for 24 hours and their colony-forming potential was assessed in normoxia. All experimenters were performed in triplicate. The mean % of colony-forming cells ± SEM is shown. (A). C26 cells were cultured in transwell migration chambers coated with Matrigel and were exposed to normoxia or hypoxia for 24 hours. Absolute numbers of Matrigel-invaded C26 cells were then quantified in 6 HPFs. Representative HPFs with DAPI-stained migrated cells (white) trough semipermeable membrane (black dots) are shown. The experiment was performed in duplicate (B). C26 cells were exposed to normoxia or hypoxia in vitro for 5 days. After intrasplenic tumor cell injection (n = 4), the livers were harvested 7 days later to assess the number of metastases (C) and the tumor load as hepatic replacement area (% liver tissue occupied by tumor) (D). Representative liver sections are shown (E). * P < 0.05, Student t test.

Colorectal cancer is characterized by an enormous genetic heterogeneity between individual tumors. Therefore, it is quite possible that Aldefluor (and/or SCA1), while being good CSC markers for the cell lines that were used in the present study, may not be general markers for CSCs in all colorectal tumors.

Vascular Clamping Induces a CSC-Like Phenotype in Resident Liver Metastases

Next, we analyzed whether the in vitro processes described earlier would also occur in liver metastases after tissue injury. To this end, we used patient-derived L145 colonospheres. Implantation of a small piece of tumor tissue into the parenchyma of the left liver lobe resulted in the growth of a single liver tumor in all cases. After 8 weeks, ischemia-reperfusion injury (or sham treatment) was induced. Twenty-four hours after vascular clamping, the livers were isolated and the tumors were processed for analysis of the expression of stem cell and differentiation markers and for colony-forming potential. The stem cell markers SOX2 and OCT4 were elevated in tumors isolated from clamped versus nonclamped liver lobes while the differentiation markers FABP2 and MUC2 were reduced (Fig. 4A). In line with this result, tumor tissue harvested from clamped liver lobes contained significantly more clonogenic tumor cells than tumors isolated from nonclamped lobes (Fig. 4B). Immunohistochemistry of tumor tissue 5 days after vascular clamping also showed a strong decrease in the expression of FABP1 and CK20 in tumors isolated from clamped liver lobes. The differences in Muc2 expression were highly variable (Fig. 4C). These results demonstrate that ischemia-reperfusion injury rapidly imposes an immature, aggressive phenotype on resident tumor tissue.

TPZ as a Potentially Effective Anti-CSC Drug

CSCs are thought to be intrinsically resistant to chemotherapy because of their ability to keep intracellular radical oxygen species low and to high activity of the DNA damage checkpoint and DNA repair enzymes. However, CSCs do not display intrinsic resistance to all (chemotherapeutic) drugs, as they are highly sensitive to killing by Akt inhibitors and by the topoisomerase I inhibitor irinotecan. Interestingly, the hypoxia-activated prodrug TPZ is a topoisomerase II inhibitor, which we have previously shown to be effective in preventing injury-associated aggressive tumor recurrence in the liver.
FIGURE 3. In vitro hypoxia suppresses differentiation and causes increased expression of stem cell markers. The indicated human colonosphere cell lines were exposed to hypoxia for 0, 24, or 48 hours. Lysates were analyzed for the differentiation marker cytokeratin 20 (CK20). Actin served as a control for equal protein loading (A). L146 cells were cultured as above on glass cover slips and immunofluorescence for CK20 was performed. The intensity of CK20 was measured using LSM software. Mean CK20 signal intensity levels were then plotted as means ± SEM. The experiment was performed in triplicate. Representative images are shown (B). Up to 48 hours after exposure to hypoxia, samples of the indicated colonosphere lines were analyzed for RNA levels of the hypoxia marker HEX2 and the differentiation markers FABP2 and MUC2 by qPCR. All experiments were performed in triplicate (C). C26 cells were exposed to normoxia or hypoxia for 24 hours. Cells were then analyzed for SCA1 levels by FACS (D). L145 cells were exposed to either normoxia or hypoxia for 24 hours and Aldefluor activity was measured using FACS analysis. Diethylaminobenzaldehyde was used to identify the negative cell population (E). L145 cells were FACS sorted for Aldefluor<sup>low</sup> and Aldefluor<sup>high</sup> activity and tested for stem cell marker and differentiation marker expression using qPCR (F). All experiments were performed in triplicate at least 2 times. Results are shown as means ± SEM. *P < 0.05, Student t test.

Therefore, we tested whether TPZ was able to kill hypoxia-exposed CSCs. We found that TPZ readily killed colonosphere cultures from 3 different patients in a hypoxia-dependent manner (Fig. 5A) and completely abolished clone-forming capacity of hypoxia-exposed colonospheres (Fig. 5B). Strikingly, TPZ treatment completely prevented the hypoxia-induced increase in Aldefluor<sup>+</sup> CSCs in the tumor cell population surviving treatment (Fig. 5C).

DISCUSSION

Previously, we have shown that the HSP90-blocker 17-DMAG, a potent destabilizer of HIF1, could prevent hypoxia-associated aggressive tumor growth surrounding RFA-generated lesions. In the present report, we provide evidence that tumor cell exposure to hypoxia is sufficient to drive aggressive local outgrowth of liver tumors without the need for infiltrating inflammatory cells or BMDPCs. Exposure of patient-derived “colonosphere” cultures to hypoxia caused a rapid loss of differentiation markers and imposed an invasive CSC phenotype. These effects were also observed in mice harboring human xenotransplant in the liver after ischemia-reperfusion injury. We propose that the hypoxia-induced phenotypic change to a more primitive CSC-like state contributes to the observed aggressive postsurgery phenotype of residual micrometastases. Given the enormous genetic heterogeneity that characterizes colorectal cancer, further work is needed to establish the generality of these findings in a large panel of human colorectal tumors and in patients undergoing partial liver resection. Nonetheless, given that hypoxia-induced increase in cancer stemness was observed in all cell lines analyzed, it may be regarded as a relatively common phenomenon. In line with this, colorectal cancer patients whose tumors are characterized by a primitive stem cell–like expression pattern are at high risk of developing metastases and tumor recurrence.

Local recurrence is a major concern after liver surgery. Local recurrence rates of up to 60% have been reported after RFA procedures. A key issue is that more than 50% of resected liver tumors harbor microsatellite lesions, which are undetectable by...
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FIGURE 4. Ischemia-reperfusion in mouse livers bearing human tumor xenografts causes loss of tumor cell differentiation and increased colony-forming potential. Small pieces of subcutaneous L145 human tumor xenografts were implanted into the left liver lobes of immunodeficient mice. Eight weeks after implantation, the left liver lobes were subjected to an ischemia (45 minutes) reperfusion (24 hours) procedure. The livers were harvested and tumor tissue was analyzed for stem cell marker and differentiation marker expression using qPCR (A). Alternatively, the tumor tissue was processed to single cells. The number of colony-forming units was assessed by plating single cells in Matrigel and analyzing the number of colonies 5 weeks later (B). Nine days after the clamping procedure, tumor-containing livers were analyzed by immunohistochemistry for the differentiation markers FABP1 and CK20 (C). All experimenters were performed in triplicate. *P < 0.05, Student t test.

conventional imaging. Moreover, clinically inconspicuous rest liver frequently (up to 70%) contains micrometastases. When left behind after surgery, such micrometastases could benefit from the hypoxic protumorigenic microenvironment and undergo the transition into a CSC-like state. It is important to note that the “CSC state” is not a stable feature of a select group of cells but one that may be obtained or lost over time, depending on cues from the microenvironment. Our results suggest that postsurgical hypoxia is one such dominant cue.

Although there is no doubt that inflammatory cells and BMD-PCs can promote tumor formation, these cells were not required for the changes in tumor behavior after liver surgery (this report). Apparently, the mechanisms that link chronic inflammation to tumor development are distinct from those linking postsurgical hypoxia to an aggressive tumor phenotype. Nevertheless, hepatitis is an independent predictor for local recurrence after RFA treatment of hepatocellular carcinoma (HCC). Whether this is due to inflammation-associated hypoxia or to the inflammatory cells themselves is not known.

Our results using human colorectal tumor cell lines freshly isolated from liver metastases in a mouse model demonstrate that liver surgery promotes the CSC phenotype and causes accelerated tumor outgrowth. These results warrant clinical studies in which the phenotype and growth behavior of liver metastases is analyzed before and after the first surgical procedure. Interestingly, a recent study investigated the growth speed of 60 HCC tumors before and after transarterial chemoembolization. This procedure induces high levels of hypoxia due to the embolization spheres. After transarterial chemoembolization, the mean tumor doubling time was significantly shorter, suggesting hypoxia-associated acceleration of tumor growth. This is in line with the results that we have obtained in the model of surgery-stimulated hypoxia-associated outgrowth of colorectal liver tumors. A clinical imaging study should address the relationship between liver surgery and the rate of local and distant recurrent tumor growth in patients with metastatic colorectal cancer. On the basis of the results so far, we suggest that minimizing surgery-induced hypoxia may help prevent aggressive local tumor recurrence.

Hypoxia not only causes changes in tumor phenotype, it is also strongly associated with poor response to radiotherapy and chemotherapy. This may be due to the lack of oxygen itself (reactive oxygen species cannot be formed in the absence of oxygen), but it may also be directly related to the hypoxia-induced CSC phenotype, as CSC are generally considered to be relatively chemoresistant. Overcoming hypoxia-associated resistance of tumor cells to chemotherapy is one of the holy grails in oncology. Approaches include the pharmacological targeting of HIFs and the use of hypoxia-activated prodrugs such as TPZ. Previously, we have shown that this drug is effective in overcoming hypoxia-driven aggressive outgrowth of C26 liver metastases after RFA. In the present report, we show that TPZ has the remarkable capacity to lower the CSC fraction in drug-treated cell populations and kill all clone-forming capacity, while treatment with most standard chemotherapeutics (such as, oxaliplatin and 5FU) increases the CSC fraction. TPZ inhibits topoisomerase II. Recently, we found that irinotecan,
FIGURE 5. The hypoxia-activated prodrug TPZ kills colorectal CSCs. The indicated colonosphere cell lines were cultured with or without TPZ (40 \( \mu \)M) for 24 hours in hypoxia (H) or normoxia (N). Cells were lysed and analyzed for the processing of caspase-3 as an indicator of tumor cell apoptosis. Actin was used as a loading control (A). Cells were grown in hypoxia for 24 hours in the presence or absence of TPZ (40 \( \mu \)M). Single cell suspensions were prepared and the colony-forming potential was assessed by plating 1000 live cells in Matrigel. The number of colony forming units was assessed 3 to 4 weeks later. Representative pictures are shown (B). The effect of TPZ treatment on the number of Aldefluor+ cells population was assessed by FACS. L145 cells were cultured in normoxia or hypoxia for 24 hours with or without TPZ (20 \( \mu \)M or 40 \( \mu \)M). Cells were then processed for FACS analysis of the % Aldefluor-positive cells as in Figure 4 (C). All experimenters were performed in triplicate. *\( P < 0.05 \), Student t test.

a topoisomerase I inhibitor, is also effective in eliminating the CSC population.22 It follows that CSCs may not be selectively resistant to the type of DNA damage induced by topoisomerase inhibition. An alternative explanation could be that TPZ inhibits expression of HIF1.63 This could affect cellular differentiation, invasion, and metastasis formation.15 However, in the colorectal tumor cells studied here, apoptosis was the primary outcome after hypoxic exposure to TPZ, and this completely prevented the hypoxia-induced increase in CSC numbers. This suggests that the cytotoxic effects rather than the effects on HIF1 may be dominant. In any case, this study together with our prior work22 strongly suggests that topoisomerase inhibition may be a good strategy to kill colorectal CSCs.

How may these results be implicated in the treatment of liver tumors? Surgical procedures, which generate hypoxia in the liver, include RFA and embolization techniques. The clinical value of embolization spheres loaded with doxorubicin has recently been established and may become standard treatment in HCC (reviewed in64). We propose that the unique capacity of TPZ to kill colorectal CSCs may be exploited in the treatment of unresectable metastases. These drugs could be included in embolization beads, ensuring local delivery in areas of hypoxia, or could be administered as adjuvant therapy after RFA. In addition, by locally killing the CSC fraction, these drugs could prevent the initiation of new metastatic clones. The combination of surgical and/or radiological approaches with CSC-targeting chemotherapeutics such as TPZ should reduce intrahepatic recurrence rates in metastatic colorectal cancer.

CONCLUSION

Surgery-generated hypoxia in the liver causes rapid de-differentiation of tumor cells into immature CSCs with high clone-and metastasis-forming capacity. The results presented in this report help explain the phenomenon of aggressive local tumor recurrence following liver surgery and offer a potential strategy to kill aggressive CSCs by hypoxia-activated pro-drugs such as TPZ.

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