Hepatocellular carcinoma cells surviving doxorubicin treatment exhibit increased migratory potential and resistance to doxorubicin re-treatment *in vitro*

SEBASTIAN BUSCHAUER[1], ANDREAS KOCH[1], PHILIPP WIGGERMANN[2],
MARTINA MÜLLER[1] and CLAUS HELLERBRAND[1,3]

Departments of [1]Internal Medicine I and [2]Radiology, University Hospital Regensburg, D-93042 Regensburg;
[3]Institute of Biochemistry, Friedrich-Alexander University Erlangen-Nuremberg, D-91054 Erlangen, Germany

Abstract. Transarterial chemoembolization (TACE) is an established therapeutic approach for the treatment of hepatocellular carcinoma (HCC). Although patients who undergo TACE may have prolonged survival, there are indications that the malignancy of residual HCC tissue can increase subsequent to the procedure. Although hypoxia, which occurs during TACE due to ischemia, is known to contribute to angiogenesis, little is known with regard to the undesirable effects of chemotherapeutic agents on residual HCC cells. Doxorubicin is one of the most commonly used drugs in TACE. The aim of the present study was to analyze alterations in Hep3B and HepG2 human HCC cell lines surviving doxorubicin treatment *in vitro*. Initially, the toxic concentration range was determined, and doxorubicin was subsequently applied in concentrations that killed >80% of the HCC cells. During the first days subsequent to treatment, surviving cells had higher expression levels of the epithelial-mesenchymal transition marker SNAIL, and exhibited increased migratory activity compared with control cells. At 3 weeks after the first doxorubicin treatment, surviving HCC cells tolerated significantly higher doxorubicin concentrations compared with control cells. As a potential explanation for this doxorubicin resistance, significantly increased mRNA expression levels of ATP-binding cassette ABCB1 (multidrug resistance protein 1) and ABCC1 (multidrug resistance-associated protein 1) were observed by reverse transcription-quantitative polymerase chain reaction. In summary, these findings indicate that, following TACE treatment, hypoxia as well as doxorubicin may induce a more

malignant phenotype in surviving HCC cells and decrease susceptibility to further chemotherapeutic treatment.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common type of cancer and the third leading cause of cancer-associated mortality worldwide (1), and the incidence continues to increase in numerous countries (2). In the majority of cases, patients have a background of chronic liver disease leading to liver cirrhosis, which is the main risk factor for the development of HCC (3,4). Currently, surgical resection and liver transplantation are the only curative treatment options (5).

Transarterial chemoembolization (TACE) is a minimally invasive treatment that is frequently used to reduce tumor burden in inoperable situations or as bridging therapy prior to transplantation. Although TACE may permit local tumor control and increase survival time in patients with intermediate HCC (Barcelona Clinic Liver Cancer stage B) (6), there is evidence that TACE enhances angiogenesis in HCC (6,7). While hypoxia, which occurs during TACE due to ischemia, is known to contribute to angiogenesis, little is known about the undesirable effects of chemotherapeutic agents on residual HCC cells subsequent to TACE (8).

The anthracycline doxorubicin is one of the most commonly used drugs in TACE (9). Its main mechanisms of action are intercalation into DNA, inhibition of topoisomerase II and generation of reactive oxygen species (ROS), inducing apoptotic pathways (10,11). While a large proportion of doxorubicin is eliminated from the body unchanged, the main pathway of doxorubicin metabolism is two-electron reduction by cytosolic reductases, of which carbonyl reductase 1 is the most important in the liver (10). However, doxorubicin resistance in HCC cells is predominantly associated with the expression of adenosine triphosphate-binding cassette (ABC) transporters such as ABCB1 (multi-drug resistance gene; MDR1) or ABCC1 (multidrug resistance-associated protein 1; MRP1) (10,12-19).

Previous studies concerning the drug resistance of HCC cells have used doxorubicin-resistant cell lines that were generated through constant exposure to rising levels of

Correspondence to: Professor Claus Hellerbrand, Institute of Biochemistry, Friedrich-Alexander University Erlangen-Nuremberg, Fahrstrasse 17, D-91054 Erlangen, Germany
E-mail: claus.hellerbrand@fau.de

doxorubicin (13,20-22). By contrast, the aim of the present study was to analyze the effects of single-step doxorubicin treatment on surviving HCC cells *in vitro*, mimicking the situation of HCC cells surviving TACE treatment.

Materials and methods

Cells and cell culture. HCC HepG2 (cat. no. HB-8065) and Hep3B (cat. no. HB-8064; American Type Culture Collection, Manassas, VA, USA) cell lines were cultured as described previously (23). Briefly, cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Taufkirchen, Germany) supplemented with penicillin (400 U/ml), streptomycin (50 μg/ml), L-glutamine (300 μg/ml) and 10% fetal calf serum (FCS; Sigma-Aldrich; Merck Millipore, Deisenhofen, Germany) and were passaged at a 1:5 ratio every 3 days. To select cells that survive treatment with a defined doxorubicin dose (1 μM), HepG2 and Hep3B cells were incubated with doxorubicin for 48 h. Subsequently, medium was removed, the cell culture dishes were carefully washed with PBS to remove dead cells, and surviving HCC cells (HCC[surv]) were further cultured in normal, doxorubicin-free medium. Control cells (HCC[ctr]) were continuously cultured in normal medium without doxorubicin. Subsequently, HCC[surv] and HCC[ctr] cells were cultured in parallel and were split when they became confluent. In the two HCC[surv] cell lines this occurred after 1 week. Subsequent to splitting, HCC[surv] cells were further cultured and regularly passaged in parallel with HCC[ctr] cells for another 2 weeks.

Microscope images were captured using an Olympus™ CKX41 microscope (Olympus Corporation, Tokyo Japan) with the ALTRA 20 Soft Imaging System™ and Cell[A] software version 2.6 (Olympus Soft Imaging Solutions GmbH, Münster, Germany). Images were processed using IrfanView™ software version 4.36 (Irfan Skiljan, Jajce, Bosnia).

Analysis of cell viability and proliferation. Cells were seeded in 6-well plates (200,000/well) or 96-well plates (30,000/well), respectively. After 24 h, analysis of lactate dehydrogenase (LDH) secretion into the supernatant (Cytotoxicity Detection Kit PLUS; Roche Diagnostics GmbH, Mannheim, Germany) and a colorimetric XTT assay (Roche Diagnostics GmbH) were used to analyze the viability of HCC cells subsequent to treatment with doxorubicin as described (24). Cell proliferation was assessed using the xCELLigence impedance measurement system (Roche Diagnostics GmbH) according to the manufacturer's protocol.

Analysis of cell migration. The migratory activity of HCC cells was quantified using Cultrex 96-Well Cell Migration assay (Trevigen, Gaithersburg, MD, USA) as described (25). Briefly, HCC cells were seeded into the upper compartment of the provided 96-well micropore plate (10,000 cells/well) in DMEM. The lower compartment was filled with DMEM to study spontaneous cell migration. Subsequent to incubation at 37˚C for 5 h, cell migration was quantified by fluorometry with an EMax Microplate Reader (MWG Biotech, Ebersberg, Germany).

Analysis of mRNA expression. Total cellular RNA was isolated from doxorubicin-treated and control HepG2 and Hep3B cells using the RNeasy Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription was performed as described previously (24). Quantitative polymerase chain reaction was performed using a LightCycler Real-Time PCR System (Roche Diagnostics) (24). In each well, 2 μl of cDNA template was added to 8 μl master mix containing primers and SYBR Green (Bioline GmbH, Luckenwalde, Germany). Melting, annealing and amplification were performed at 95˚C (5 sec), 58˚C (10 sec) and 72˚C (8 sec), respectively and repeated for 45 cycles. ABCB1, ABCC1 and SNAIL mRNA expression were analyzed using QuantiTect Primer assays according to the manufacturer's protocol (Qiagen GmbH, Hilden, Germany). Amplification of cDNA derived from 18S rRNA was used for normalization (24), with the following primer sequences: Forward, 5'-AAACGGCTACCACATCCAAG-3', and reverse, 5'-CCT CCAATGGATCCTCGTTA-3'. Results were evaluated using the $2^{-\Delta\Delta Cq}$ method (26). Analyses were performed in triplicates and experiments were repeated three times.

Statistical analysis. Values are presented as the mean ± standard error of the mean. Comparison between groups was made using the unpaired Student's *t*-test or two-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference. All calculations were performed using the statistical computer package GraphPad Prism version 6.01 for Windows (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Selection of HCC cells surviving doxorubicin treatment. The present study analyzed the effective dose range of doxorubicin on the HepG2 and Hep3B human HCC cell lines. Analysis of LDH release into the supernatant (Fig. 1A) and XTT activity (Fig. 1B) showed that doxorubicin dose-dependently reduced the viability of HCC cells during the 48 h incubation time. Starting at a dose of 1 μM in HepG2 cells and 0.5 μM in Hep3B cells, doxorubicin caused a significant increase of LDH levels in the supernatant (2.9-fold, P=0.0001 in HepG2; 1.8-fold, P=0.004 in Hep3B). XTT activity was significantly reduced by incubation with 0.125 μM doxorubicin in HepG2 cells (60%; P=0.0075) and Hep3B cells (83%-fold; P=0.0003). The determined toxic dose ranges were comparable to previous *in vitro* studies using the same HCC cell lines (27-32). Phase-contrast microscopy confirmed that, after 48 h incubation with a concentration of 1 μM doxorubicin, 10-20% of HCC cells survived (Fig. 1C). For the next *in vitro* model that was designed to mimic the circumstances of TACE, doxorubicin was used at a concentration of 1 μM, which was in the range of doxorubicin concentrations found in human HCC explants following the administration of TACE (33,34). HCC cells surviving incubation with this doxorubicin dose for 48 h (HCC[surv]) and control cells (HCC[ctr]) were generated as aforementioned.

Analysis of surviving HCC cells in the early phase following doxorubicin treatment. Monitoring of cell growth and morphology with phase-contrast microscopy revealed that HCC[surv] cells developed a spindle-like, outstretched,
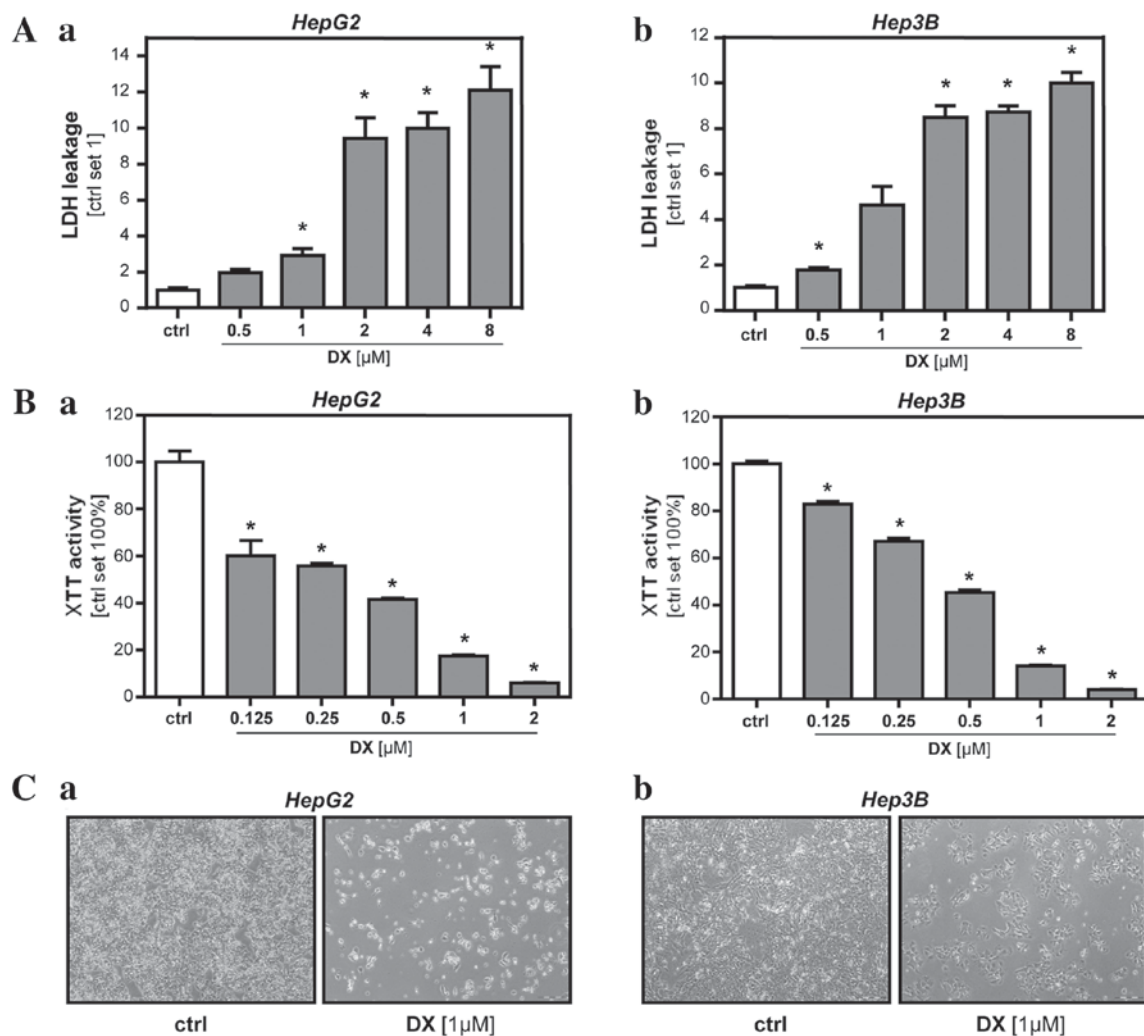
Figure 1. Selection of HCC cells surviving DX treatment. HepG2 and Hep3B human HCC cell lines were treated with the indicated concentrations of DX for 48 h. (A) Analysis of LDH leakage of (a) HepG2 and (b) Hep3B cells into the supernatant. DX caused a dose-dependent increase in LDH levels in the supernatants of the two cell lines. (B) Quantification of XTT activity as a measure of cell viability of (a) HepG2 and (b) Hep3B cells. DX treatment induced a dose-dependent decrease in XTT activity in the two cell lines. (C) Phase-contrast microscopy of ctrl cells and cells treated with 1 μM DX: (a) HepG2 and (b) Hep3B cells (magnification, 40x). In the two cell lines DX treatment caused a marked reduction of cell density, indicative of induced toxicity. *P<0.05 vs. ctrl group. HCC, hepatocellular carcinoma; DX, doxorubicin; LDH, lactate dehydrogenase; ctrl, untreated control.

mesenchymal shape within the first 6 days after treatment with doxorubicin (Fig. 2A). By contrast, HepG2ctr and Hep3Bctr did not change their characteristic, cubic and compact cell form during the whole observation period. Additionally, expression of the epithelial-mesenchymal transition (EMT) marker SNAIL was 1.9-fold (P=0.03) increased in HepG2surv compared to HepG2ctr cells (Fig. 2B). Also in Hep3Bsurv SNAIL expression was 5.2-fold (P=0.0002) higher compared with Hep3Bctr cells (Fig. 2B). Functional analysis revealed similar rates of proliferation of HCCsurv and HCCctr cells (data not shown). However, HCCsurv cells exhibited significantly increased migration in Boyden chamber assays compared to HCCctr cells (Fig. 2C). Migration ability in HepG2surv was 2.4-fold increased (P=0.001) compared with HepG2ctr. Hep3Bsurv exhibited a 3.3-fold increase (P=0.009) in migratory potential compared with Hep3Bctr.

Analysis of surviving HCC cells 3 weeks after doxorubicin treatment. After ~1 week, HCCsurv cells became confluent and required splitting. Subsequently, HCCsurv cells were further

cultured in parallel with HCCctr cells for another 2 weeks. During that time, the HCCsurv cells reverted to their original shape. The spindle-like, outstretched cell form disappeared and the HepG2surv and Hep3Bsurv no longer differed from their respective control cells (Fig. 3A). SNAIL expression and migratory potential were similar in HCCsurv and HCCctr cells (data not shown). However, 3 weeks following doxorubicin treatment, HCCsurv cells exhibited significantly higher expression levels of MDR1 (ABCB1) and MRP1 (ABCC1) compared to HCCctr cells (Fig. 3B). ABCB1 expression was 1.7-fold increased in HepG2surv (P=0.029) and 3.4-fold in Hep3Bsurv (P=0.002) compared with their respective control cells. ABCC1 expression was increased 2.1-fold in HepG2surv (P=0.016) and 1.4-fold in Hep3Bsurv (P=0.09) cells compared with their respective control cells. Consistently, HCCsurv cells tolerated significantly increased doxorubicin concentrations compared with HCCctr cells (Fig. 3C). Although XTT-activity was reduced to 33% in HepG2ctr treated with 0.5 μM doxorubicin, HepG2surv exhibited an XTT-activity of 74% (P=0.0001) upon incubation with the same doxorubicin dose. Similarly,
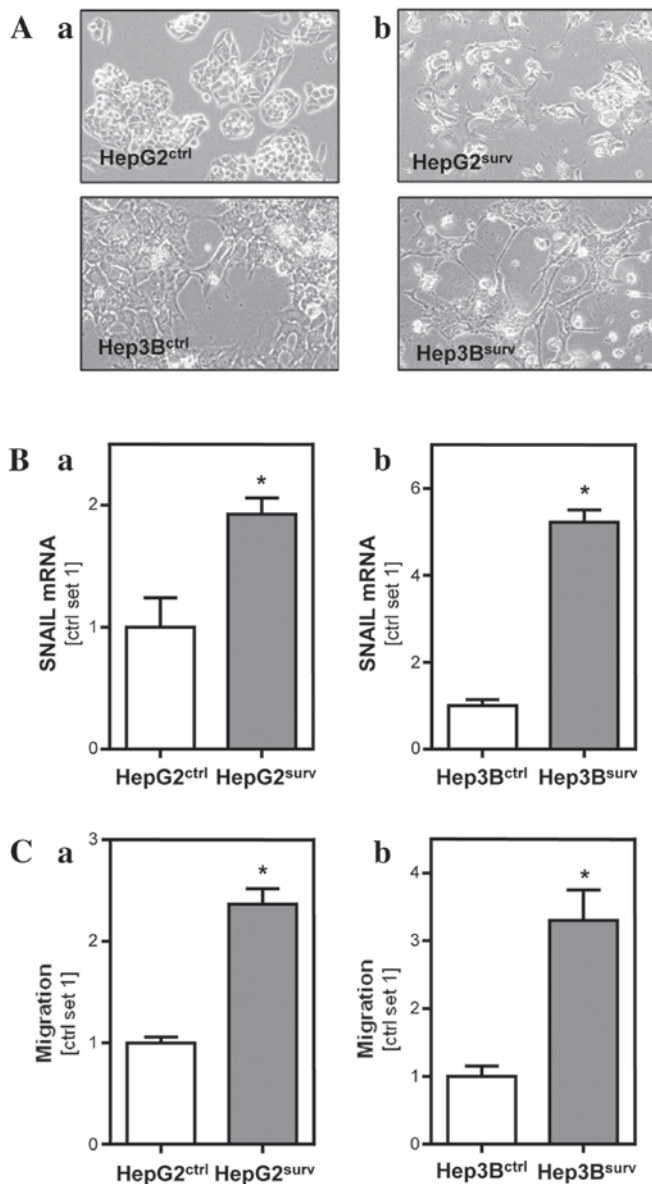
Figure 2. Analysis of surviving HepG2 and Hep3B HCC cells in the early phase subsequent to doxorubicin treatment. (A) Phase-contrast microscopy of (a) untreated control cells (HepG2ctrl and Hep3Bctrl) and (b) cells surviving 1 week after doxorubicin treatment (HepG2surv and Hep3Bsurv; magnification, 100x). The two HCCsurv cell lines exhibited spindle-like, outstretched cell forms, whereas HepG2surv cells retained cubic, compact forms. (B) Analysis of SNAIL mRNA levels by reverse transcription-quantitative polymerase chain reaction in (a) HepG2 and (b) Hep3B cells. HCCsurv cells exhibited significantly higher SNAIL expression than HCCctr cells. (C) Analysis of migratory potential by Boyden Chamber assays in (a) HepG2 and (b) Hep3B cells. HepG2surv cells exhibited significantly higher migratory activity than HepG2ctr cells. *P<0.05 vs. ctrl group. surv, surviving cells; ctrl, untreated control.

impairment of XTT-activity in response to 0.5 μM doxorubicin in Hep3Bsurv cells (64%) was significantly lowered (P=0.0006) compared with the reduction of XTT-activity (34%) in Hep3Bctr cells.

Discussion

The aim of the present study was to analyze human HCC cells surviving doxorubicin treatment *in vitro*, in an experimental setting resembling the circumstances of HCC cells surviving doxorubicin application during TACE. For this, two different human HCC cell lines were incubated with doxorubicin at a concentration that killed >80% of the tumor cells within 48 h. The applied concentration of doxorubicin was in the range of tissue drug concentrations found in experimental TACE models *in vivo*, as well as in HCC explants of patients after the administration of TACE (33,34). After 2 days, cell culture of surviving HCC cells was continued without doxorubicin exposure to mimic the situation of a single doxorubicin dose application during TACE.

Applying these experimental conditions, the present study observed an increased expression of the EMT marker SNAIL and morphological changes to a mesenchymal cell shape in HCC cells surviving doxorubicin exposure. Additionally, doxorubicin-surviving HCC cells exhibited increased migratory activity. Expression of SNAIL has been found to positively correlate with poor clinical outcomes in different types of cancer, including HCC (35). Furthermore, several studies indicate that EMT is a crucial event in HCC progression, being associated with tumor cell invasion and metastasis (36). Accordingly, a previous study reported that the incidences of poorly differentiated histology and intrahepatic metastases are significantly increased in post-TACE HCC tissues compared with in HCC tissues of patients who have not undergone TACE treatment (37). Furthermore, Zen *et al* (38) found a combined hepato-cholangiocellular phenotype was more frequently detected in HCC tissues after TACE compared to untreated HCC. In the context of these previous studies and the present *in vitro* data, one may hypothesize that doxorubicin application during TACE promotes a more malignant phenotype in surviving HCC cells. Currently, the present study can only speculate why the alterations in cell morphology, SNAIL expression and migratory activity in doxorubicin-surviving HCC cells regressed with prolonged cell culture. It may indeed have been an intermediate effect, or trypsinization and splitting of the cells may have triggered this reversion.

However, for up to 3 weeks after a single doxorubicin application, surviving HCC cells were significantly less susceptible to retreatment with doxorubicin. As a potential explanation for this increased chemotherapy resistance, significantly increased expression levels of MDR1 (ABCB1) and MRP1 (ABCC1) were found; these genes are known to contribute to multidrug resistance in HCC (12-15,17,18,20). MRP1, which is overexpressed in HCC (39), performs an important role in the intrinsic multidrug resistance of HCC and is also associated with an aggressive tumor phenotype and has been suggested to indicate a progenitor cell origin (18).

Hypoxia, which also occurs after TACE through ischemia, is known to induce EMT and to enhance migration and therapy resistance in HCC cells (40,41). The findings of the present study suggest that the chemotherapeutic agent doxorubicin may also cause unfavorable alterations in surviving HCC cells. These findings are of importance for the understanding of HCC recurrence observed subsequent to TACE. Future studies are required to analyze whether maintaining doxorubicin levels for a prolonged period, such as with doxorubicin-eluting beads, or switching to other anticancer agents may omit certain pathological alterations found in the present *in vitro* model.
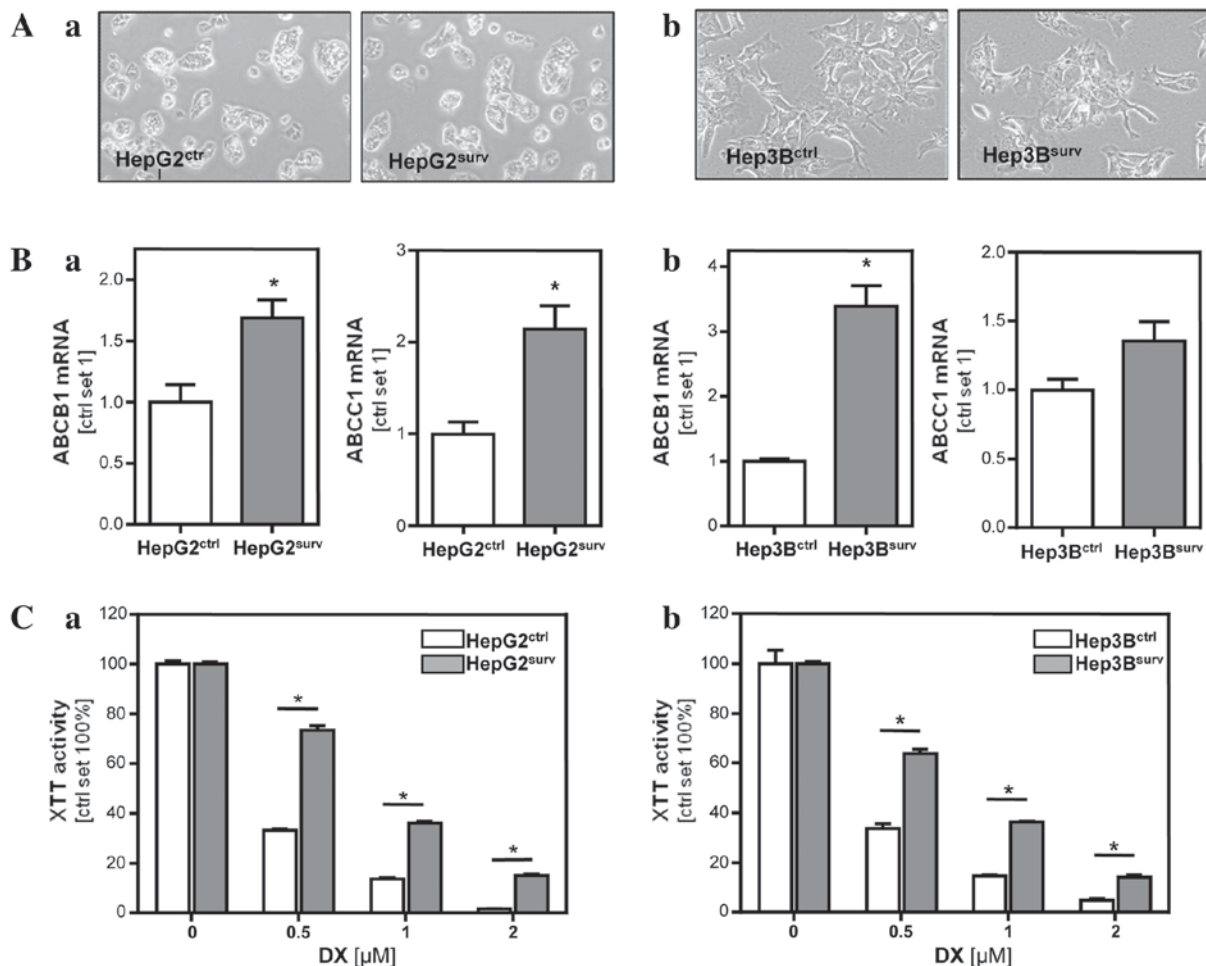
Figure 3. Analysis of surviving HCC cells 3 weeks after doxorubicin treatment. (A) Phase-contrast microscopy of untreated control cells [(a) HepG2[ctrl] and (b) Hep3B[ctrl]] and cells surviving 3 weeks after DX treatment [(a) HepG2[surv] and (b) Hep3B[surv]] (magnification, 100x). The cell morphology of HCC[surv] and HCC[ctrl] cells did not significantly differ. (B) ABCB1 and ABCC1 mRNA expression analyzed by reverse transcription-quantitative polymerase chain reaction in (a) HepG2 and (b) Hep3B cells. ABCB1 expression was significantly higher in HCC[surv] compared with HCC[ctrl] cells. In addition, ABCC1 expression levels were significantly higher in HepG2[surv] cells compared with HepG2[ctr] cells, whereas differences did not reach the level of significance in Hep3B[surv] vs. Hep3B[ctrl] cells. (C) XTT activity of untreated control cells and DX-surviving cells following re-incubation with the indicated concentrations of DX for 48 h: (a) HepG2 and (b) Hep3B. HCC[surv] cells revealed significantly higher XTT activity than HCC[ctr] cells in response to treatment with the same doxorubicin concentrations, as an indication that HCC[surv] cells tolerated significantly higher doxorubicin concentrations compared with HCC[ctr] cells. *P<0.05 vs. ctrl group. DX, doxorubicin; surv, surviving cells; ctrl, untreated control.

Furthermore, it must be investigated whether such altered therapeutic strategies may improve the outcome of HCC patients following TACE treatment, and this *in vitro* model may be used for preclinical analyses addressing these questions.

Acknowledgements

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