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# Reduced survivin expression and tumor cell survival during chronic hypoxia and further cytotoxic enhancement by the cyclooxygenase-2 inhibitor celecoxib

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**Abstract**

Hypoxia is a characteristic feature of advanced solid tumors and may worsen prognosis. The development of tumor-targeted and hypoxia-inducible gene therapy vectors holds promise to selectively deliver and express suicidal or cytotoxic genes in hypoxic regions of tumors. In this regard, the promoter of the survivin gene, which encodes an anti-apoptotic protein that is strongly expressed in tumor tissue, has received attention because of its supposed inducibility by hypoxia. However, in our present study we demonstrate that treatment of various tumor cell lines with chronic hypoxia or with the hypoxia-mimetic $CoCl_2$ does not result in increased expression of survivin, but rather strongly suppresses this gene's activity. In contrast, expression of glucose-regulated protein 78 (GRP78/Bip) is substantially elevated under chronic hypoxia *in vitro* and in hypoxic areas of tumor tissue *in vivo*. Although tumor cells in general exhibit increased chemoresistance under hypoxic conditions, we found that hypoxic glioblastoma cells are more sensitive to killing by the selective cyclooxygenase-2 (COX-2) inhibitor celecoxib, and this effect is reflected by further decreased expression of survivin. Intriguingly, 2,5-dimethyl-celecoxib (DMC), a close structural analog of celecoxib that lacks the ability to inhibit COX-2, is able to potently mimic the anti-tumor effects of its parent compound, indicating that inhibition of COX-2 is not involved in these processes. Taken together, our results caution against the use of survivin-based promoters to target hypoxic areas of tumors, but favor constructs that include the strongly hypoxia-inducible GRP78 promoter. In addition, our data introduce celecoxib as a drug with increased cytotoxicity against hypoxic tumor cells.

## Introduction

Hypoxia, a unique feature of solid tumors, diminishes therapeutic efficacy and plays a pivotal role in malignant progression. A large number of *in vitro* studies have demonstrated that hypoxic exposure can promote genetic instability and select for tumor cell populations with reduced apoptotic potential, treatment resistance, or enhanced metastatic ability [1, 2]. In addition, hypoxia is a potent signal inducing the expression of a number of genes, many of which contain hypoxia-responsive elements (HREs) in their 5′ promoter or 3′ flanking regions. HREs bind hypoxia-inducible factor (HIF), a key heterodimeric transcription factor that is only active under reduced oxygen tension and is critical to the adaptation of tumor

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cells to hypoxic conditions. Activation of the HRE/HIF axis leads to the upregulation of several factors essential for blood vessel formation and is one of the primary forces driving tumor angiogenesis [3, 4].

Rather than attempting to overcome tumor hypoxia, an alternative new strategy is to take advantage of these tumor-specific conditions and develop gene therapy vectors that display their greatest activity under conditions of chronic hypoxia. The rationale behind this approach envisions that such vectors could deliver their cytotoxic load straight to the most treatment-resistant parts of the tumor and thereby would lead to tumor cell killing directly or would increase the therapeutic ratio of conventional cancer treatments. The promoter regions of most such vectors are being designed by combining one or several HREs with a heterologous minimal promoter fragment derived from a suitable gene [5, 6].

One such heterologous promoter being developed is derived from the gene encoding survivin, which is a member of the inhibitor of apoptosis (IAP) family of proteins that has been implicated in the control of cell division and apoptosis [7]. Survivin's function in mitosis is to preserve the mitotic apparatus and to allow normal mitotic progression, whereas its anti-apoptotic function is executed via its ability to prevent caspase activation. The protein is usually not expressed in differentiated normal adult tissues, but is elevated in the majority of human cancers and appears to be involved in tumor cell resistance to some anti-cancer agents and ionizing radiation (for detailed references, see reviews [8–10]). Due to its prominent activity in many different types of cancer, the survivin promoter has been considered for incorporation into hypoxia-targeted gene therapy vectors [11]. However, as we will show in this study, and contrary to some earlier findings in ischemic tissues [12, 13], survivin expression in tumor cells is severely reduced during chronic hypoxia, and this inhibition can be validated at the level of promoter activity, as well as at the mRNA and protein levels. Thus, our results indicate that the use of the survivin promoter for anti-cancer gene therapy purposes should be approached with caution. As a potentially superior alternative, our study presents the promoter of the glucose regulated protein 78 (Grp78/BiP), a stress-inducible chaperone protein with anti-apoptotic properties that is frequently overexpressed in human tumors [14], and which we find strongly induced by chronic hypoxia in glioblastoma cells *in vitro* and in hypoxic areas of tumor tissues *in vivo*.

Because elevated levels of survivin are associated with increased resistance of tumor cells to conventional therapy [8–10], we also explored whether hypoxia-driven down-regulation of survivin would make tumor cells more sensitive to cytotoxic drug effects. For this purpose, we used celecoxib (trade name Celebrex®), a selective inhibitor of cyclooxygenase-2 (COX-2) that is currently evaluated as a new anti-tumor drug in numerous laboratory studies and clinical trials [15, 16]. Tumor cells are known to mount a defense against hypoxia by increasing the activity of cyclooxygenase-2 (COX-2), which leads to elevated levels of prostaglandin and subsequent stimulation of vascular endothelial growth factor (VEGF), a potent stimulus for new blood vessel growth [17]. The inhibition of this process by celecoxib should be expected to restrain tumor angiogenesis, and several reports indeed support such a view [18–20]. However, in addition to inhibiting COX-2, celecoxib is also known to have COX-2-independent anti-tumor effects (see ref. [21] for a comprehensive review), and because of this, we reasoned that this drug could deliver a one-two punch against tumor growth due to its anti-angiogenic as well as cytotoxic potency.

In the present study, we describe that hypoxic tumor cells have greatly decreased levels of survivin and are significantly more sensitive to killing by celecoxib than normoxic cells with high levels of survivin. This effect can be mimicked by 2,5-dimethyl-celecoxib (DMC), a close structural analog of celecoxib that lacks the ability to inhibit COX-2 [22], indicating that direct cell killing by celecoxib does not involve the inhibition of COX-2. In view of the increased resistance of hypoxic tumor cells to conventional chemotherapeutic drug treatment, which in the past has been well documented in many tumor types and with various anti-cancer drugs [1, 2], our findings presented here suggest that celecoxib might be uniquely useful for inclusion in anti-cancer therapies aimed at the hypoxic regions of tumors. Conceivably, this drug might be able to deliver a one-two knockout punch against hypoxic tumor cells: first, as demonstrated in this present report, celecoxib causes death of hypoxic tumor cells via a COX-2-independent

mechanism; second, as shown in previous studies [18–20], it prevents the rescue of these hypoxic cells via the blockage of new blood vessel growth, which is achieved by its well-established COX-2 inhibitory activity.

## Materials and methods

### *Materials*

Celecoxib is 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide [23]. DMC is a close structural analog, where the 5-aryl moiety has been altered by replacing 4-methylphenyl with 2,5-dimethylphenyl, resulting in 4-[5-(2,5-dimethylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide [24, 25]. Both compounds were synthesized in our laboratory according to previously published procedures; see ref. [23] for celecoxib and ref. [25] for DMC. Each drug was dissolved in DMSO at 100 mM (stock solution) and added to the cell culture medium in a manner that kept the final concentration of solvent (DMSO) below 0.1%. Cobalt chloride ($CoCl_2$) was obtained from Sigma (St. Louis, MO) and dissolved in double-distilled water at 420 mM.

### *Cell lines and culture conditions*

The glioblastoma cell lines U251 and LN229 were kindly provided by Frank B. Furnari and Webster K. Cavenee (Ludwig Institute of Cancer Research, La Jolla, CA); MIA PaCa-2 pancreatic carcinoma cells were provided by Guido Eibl (UCLA, Los Angeles, CA); HeLa cervix carcinoma and U87 glioblastoma cells were obtained from the American Tissue Culture Collection (ATCC; Manassas, VA). All cells were propagated in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin in a humidified incubator at 37 °C and a 5% $CO_2$ atmosphere.

Hypoxic culture conditions were generated by placing the cells in a GasPak EZ Gas Generating Pouch System (BD Biosciences, San Jose, CA). In this system, the amount of oxygen is reduced to 0.7% within 2.5 h, and decreased further to below 0.1% at 24–48 h. In addition to this pouch system, the cells were also cultured in the presence of various concentrations of the hypoxia mimetic agent $CoCl_2$ as an alternative means to simulate hypoxic conditions.

### *Immunoblots and antibodies*

Total cell lysates were prepared by lysis of cells with RIPA buffer [26], and protein concentrations were determined using the bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL). For Western blot analysis, 50 $\mu$g of each sample was processed as described [27]. The primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and were used according to manufacturer's recommendations. The secondary antibodies were coupled to horseradish peroxidase, and were detected by chemiluminescence using the SuperSignal West substrate from Pierce. All immunoblots were repeated at least once to confirm the results.

### *Cell growth and survival assays*

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays were performed in 96-well plates as described in detail elsewhere [28] with the use of $5.0–8.0 \times 10^3$ cells per well. The MTT assay measures the overall metabolic activity of the entire cell culture, which broadly reflects its overall growth and survival in the short-term, i.e., within a few days. Colony formation assays (CFAs) determine the ability of individual cells to survive drug treatment and spawn a colony of clonal descendants. In this assay, the cells were seeded into 6-well plates at 200 cells per well. After complete cell adherence, the cells were exposed to hypoxia and/or drug treatment for 48 h. Thereafter, the drug was removed, fresh growth medium was added, and the cells were kept in normoxic culture undisturbed for 12–14 days, during which time the surviving cells generated a colony of proliferating cells. Colonies were visualized by staining for 4 h with 1% methylene blue (in methanol), and then were counted.

### *Stable transfections and plasmids*

The LN229 glioblastoma cell line was stably co-transfected with individual luciferase reporter plasmids and the pSV2neo plasmid as described

earlier [29]. The survivin reporter plasmid contains 6270 bp of the upstream promoter region of the survivin gene [30] and was kindly provided by the laboratory of Dario Altieri, Yale University (New Haven, CT). CMV-luc is under the control of 880 bp encompassing the promoter of cytomegalovirus (CMV) [31]. Grp78-luc harbors a 3-kb fragment of upstream Grp78 promoter sequences [32] and was kindly provided by Amy S. Lee (USC, Los Angeles, CA).

*In vivo determination of tumor hypoxia*

All animal protocols and experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Southern California (USC). Four- to six-week-old male athymic nu/nu mice (Harlan, Indianapolis, IN) were maintained in a pathogen-free environment at the animal housing facility of USC and were fed commercial laboratory animal diet and water *ad libitum*. For xenograft tumor development, mice anesthetized with a cocktail of Ketamine and Xylazine (10:1, w/w) were injected subcutaneously in the right flank with a 100 $\mu$l suspension of $1.25 \times 10^6$ U251 or $7.5 \times 10^5$ U87 glioblastoma cells. After tumor size had reached approximately 1400 $mm^3$, the animals received an i.p. injection of 75 mg/kg pimonidazole hydrochloride (Chemicon International, Inc., Temecula, CA), a bioreductive chemical probe marker used to assess hypoxia [33]. The animals were sacrificed 90 min later. The tumors were removed, snap-frozen in liquid nitrogen, and stored at −80 °C in Tissue-Tek optimum cutting temperature compound (Miles Inc., Elkhart, IN) until further analysis.

*Tumor tissue processing and immunohistochemistry*

Tumor tissue was cryosectioned (7 $\mu$m) from different representative parts (lateral to hind-leg, medial, and central) and processed for immunohistochemical analysis. Parallel, as well as sequential, double-immunostaining was performed with the use of primary antibodies targeting pimonidazole (Hypoxyprobe-1 mouse monoclonal antibody; Chemicon International), GRP-78 (rabbit polyclonal; Santa Cruz Biotechnology, Inc.), survivin (rabbit polyclonal; Santa Cruz Biotechnology, Inc.), and CD31 (rat anti-mouse CD31; Pharmingen, San Diego, CA). As secondary antibodies, we used biotin-conjugated goat anti-rabbit antibody, Texas Red (TR)-conjugated Avidin, TR-conjugated horse anti-mouse antibody, FITC-conjugated rabbit anti-rat antibodies, and FITC-conjugated goat anti-rabbit antibody (all from Vector Laboratories, Burlingame, CA). All primary antibodies were diluted 1:100 in phosphate buffered solution (PBS) plus 5% v/v species-matched serum of secondary antibodies. Secondary antibodies were diluted 1:100 in PBS.

In the parallel double immunostaining procedure, Hypoxyprobe-1 antibody was mixed with anti-GRP78 or with anti-survivin antibody in 2% goat serum and applied overnight at room temperature. After a brief washing step, TR-conjugated and FITC-conjugated secondary antibodies were applied for 40 min.

In the sequential double immunofluorescence procedure, tumor cryosections were first blocked with 5% goat serum for 25 min. and then incubated with anti-GRP78 antibody or anti-survivin antibody overnight at room temp. After brief intermittent washing steps, biotin-conjugated antibody was applied for 40 min., followed by TR-conjugated Avidin for 15 min. Thereafter, the sections were again incubated with 5% rabbit serum for 20 min to ensure blockage of excess secondary anti-rabbit antibody epitopes. Then, anti-CD31 antibody was applied for 2 h, followed by FITC-conjugated secondary antibody for 40 min.

In all experiments, appropriate controls were incorporated. These included the replacement of the respective primary or secondary antibodies with isotype-matched controls or with species-matched serum, as well as the use of xenograft tumor cryosections from animals that had not received pimonidazole. After completion of incubations with primary and secondary antibodies, the tumor sections were briefly rinsed, mounted with blue-fluorescent DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride) dye mounting media (Vector Laboratories) as nuclear counterstain, covered by glass cover slips, and examined by microscope.

Stained tumor sections were visualized using the Leica DM LB2 universal system microscope (Leica Microsystems Inc., Bannockburn, IL) with the following fluorescence filters: diamidino-2-phenylindole (350 nm excitation) for nuclear counterstain; Texas red (540 nm excitation) for

pimonidazole, GRP-78, and survivin staining; FITC green fluorescence (470 nm excitation) for CD31, GRP-78, and survivin staining. Images were acquired with the Spot RT color camera (Diagnostic Instruments, Sterling Heights, MI) and Spot 4.2 software.

## Results

In order to study molecular events taking place in tumor cells under hypoxic conditions, we either exposed cells to low (~0.1%) oxygen levels *in vitro*, or cultured cells in the presence of $CoCl_2$, a hypoxia mimetic agent [34] that can be applied at different concentrations to simulate a wide range of oxygen tensions. First, two different established glioblastoma cell lines, U251 and LN229, were cultured under hypoxic conditions for various lengths of time, and the expression of survivin protein was analyzed by Western blot analysis. As shown in Figure 1A, the levels of survivin protein were strongly down-regulated by hypoxia, and this effect occurred most prominently between 24 and 48 h. To verify that hypoxic conditions did not just simply shut down all cellular functions, we also determined the expression level of glucose regulated protein 78 (Grp78/BiP), an ER stress protein that is known to be stimulated by chronic hypoxia. We found that Grp78 levels noticeable increased towards the end of the 48 h of hypoxia exposure (Figure 1A), indicating that these cells were still capable of mounting a biological response. Because of an earlier report [35] that hypoxia might lead to increased survivin protein stability in the mitochondria of HeLa cervix carcinoma cells, we included HeLa in our analysis. However, as shown in Figure 1, hypoxia effectively reduced survivin protein levels in these cells as well, with a kinetic that was even faster than what was observed in U251 and LN229 cells. Furthermore, similar to glioblastoma cells, GRP78 was induced by hypoxia in HeLa cells also (Figure 1).

In order to simulate a range of diverse levels of oxygen tension, we next incubated the cells with various concentrations of the hypoxia mimetic $CoCl_2$. As can be seen in Figure 1B, $CoCl_2$ reduced survivin protein levels in a concentration-dependent manner; while 10 $\mu$M of this compound exerted a weak inhibitory effect, higher concentrations of up to 500 $\mu$M led to a further substantial decline in survivin expression. Thus, both conditions, direct reduction of oxygen levels and simulated hypoxia by $CoCl_2$, consistently resulted in greatly reduced levels of survivin protein.

The induction of GRP78 levels in the hypoxic cell culture indicated that these cells were still active and not undergoing massive cell death. To further investigate this aspect, we analyzed the activity of caspase 3, which is the executioner caspase of apoptotic cell death. As shown in Figure 2, chronic hypoxia did not lead to activation of this caspase. Furthermore, there was no cleavage of PARP (poly ADP-ribose polymerase), which is a substrate for activated caspase 3 and an established indicator of caspase activation. In contrast, when cells were treated with staurosporine, a kinase inhibitor and well-known activator of caspase signaling, there was pronounced activation of caspase 3 and massive cleavage of PARP (Figure 2). Together, these results show that hypoxia up to 48 h did not lead to substantial activation of caspase pathways.

We next studied the basis of reduced survivin protein levels by investigating survivin mRNA levels under chronic hypoxia. U251 cells were exposed to hypoxia as above, and RNA was analyzed by semiquantitative RT-PCR. As shown in Figure 3, chronic hypoxia resulted in substantially decreased survivin mRNA levels, which became quite obvious at around 24 h and was even further reduced at 48 h. At the same time, Grp78 mRNA levels gradually increased, further confirming that the down-regulation of survivin was specific and not simply the result of deteriorating cellular functions. In addition, the severe down-regulation of survivin mRNA was fully reversible: when cells kept in hypoxia for 48 h were returned to normoxic culture conditions, survivin mRNA levels were restored to their pre-hypoxia levels within 12 h (Figure 3).

Having established that reduced survivin protein levels were due to a decline in its mRNA, we asked whether survivin promoter activity would be diminished as well. For this purpose, we used LN229 cells that were stably transfected with a luciferase reporter construct under the control of the survivin promoter. For comparison purposes, we used two additional luciferase constructs: one under the control of the Grp78 promoter, and

another under the control of the strong cytomegalovirus (CMV) promoter. All cells were exposed to hypoxia, and luciferase activity was determined at various time points during these culture conditions. As shown in Figure 4, there was an initial, weak (1.8- and 1.6-fold) average increase in luciferase activity from the survivin promoter at 14 and 24 h, respectively. However, this induction was neither statistically significant ($p > 0.05$) nor was it maintained, but was reduced >3-fold during the next 24 h, so that promoter activity at 48 h of hypoxia was less than 50% as compared to normoxia. Thus, chronic hypoxia caused a significant reduction of survivin promoter activity.

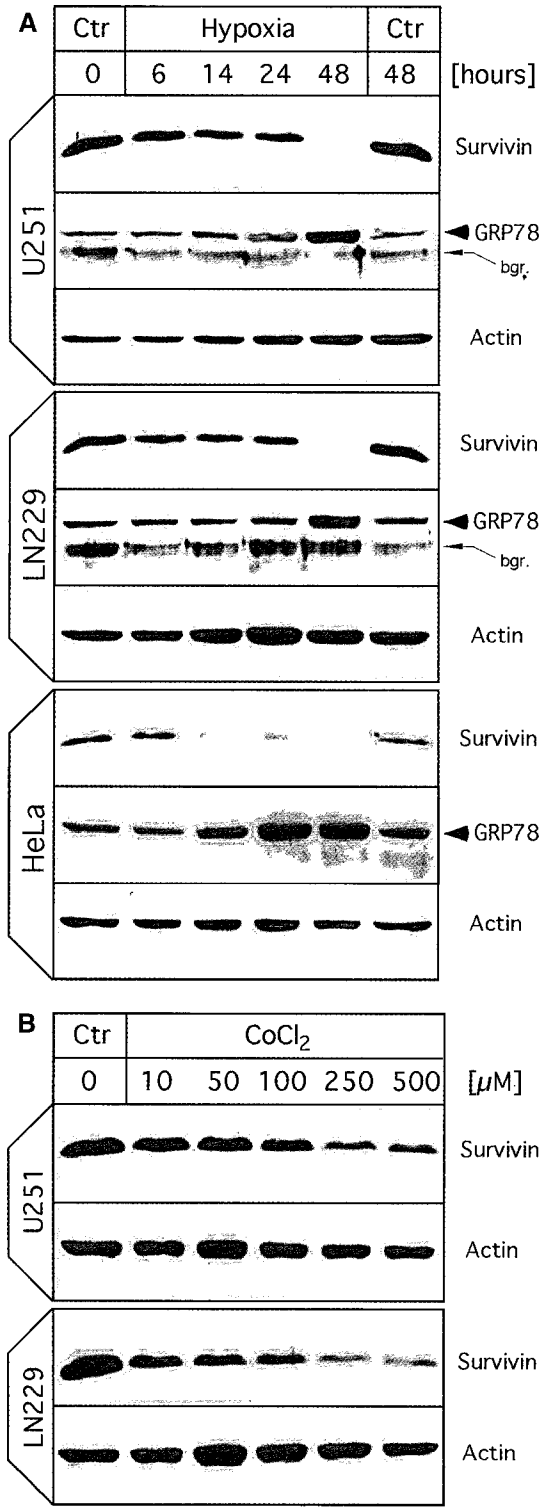
In comparison, the activity of the Grp78 promoter was strongly and continuously stimulated under hypoxia and was elevated 5-, 6-, and 10-fold at 14, 24, and 48 h, respectively (Figure 4). Thus, chronic hypoxia provides a powerful stimulus for Grp78 expression. Of note, hypoxia also induced the activity of the CMV promoter, in particular at the 48-h time point (7.5-fold), which might be relevant in consideration of this promoter's exploration as a hypoxia-targeted gene therapy vector [36]. However, with regards to survivin promoter activity, our results reveal that despite an initial (weak) stimulatory effect, the predominant consequence of chronic hypoxia is repression of this promoter, which is consistent with our data above showing substantial reduction of survivin mRNA and protein levels.

In an effort to determine whether the above described effects would also take place under *in vivo* conditions, we used a subcutaneous xenograft mouse tumor model and investigated the relationship of tumor hypoxia and expression of survivin and Grp78. Tumor hypoxia was either indirectly inferred from the distribution of blood vessels, or was directly visualized with the use of pimonidazole, a small molecule hypoxia marker that selectively binds to oxygen starved cells [33].

Human glioblastoma cells were implanted into nude mice and were allowed to form sizeable tumors over the course of several weeks. Tumor tissue was then analyzed by double immunofluorescent staining with the use of specific antibodies against survivin and Grp78, in combination with antibodies against platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31), a surface protein specific to endothelial cells and leukocytes [37]. As shown Figure 5, the distribution of blood vessels could easily be defined (green color) throughout the tumor tissue. Tumors also displayed high levels of survivin expression (Figure 5b) and readily detectable Grp78 (Figure 5f) (both indicated by red staining). The overlay of survivin and PECAM-1 stains into the same frame, however, did not reveal a clear correlation of survivin-positive cells with the location of blood vessels, although there were examples of cells with reduced survivin that were located further away from the blood supply (Figure 5c). In comparison, intense Grp78 staining became visible primarily in areas that lacked the green stain indicative of blood vessels, i.e., Grp78 expression tended to be strongest in areas that are presumed hypoxic, based on distance from the blood vessels (Figure 5g). Overall, however, the distribution of survivin and Grp78 was not entirely consistent-perhaps due to the three-dimensional arrangement of blood vessels, which cannot be fully revealed in the two-dimensional sections of this procedure.

To circumvent these limitations, we pursued a more direct approach, where pimonidazole was used to visualize hypoxic regions; this allowed the direct comparison to the simultaneous stain with either survivin or Grp78 antibodies. As shown in Figure 6, various parts of the tumor tissue were positive for pimonidazole (red stain), representing regions of hypoxia. Staining for survivin (Figure 6b, green stain) revealed numerous positive cells that were dispersed throughout the tumor tissue. When both stains were overlayed in the same frame (Figure 6c), there was a tendency of survivin-positive cells to be absent from the most intense hypoxic regions, although, once again, this result was not completely clear-cut. In contrast, when the same analysis was performed for Grp78, there was a strong congruence of elevated Grp78 expression (green stain) with areas of hypoxia (red stain) (Figure 6e–h). Taken together, these results indicate that chronic hypoxia in tumor tissue strongly induces Grp78 expression, whereas at the same time, it appears to have a moderately inhibitory (and certainly no stimulatory) effect on survivin.

Reduction of survivin levels by antisense or siRNA methods has been shown before to cause sensitization of tumor cells to certain chemotherapeutic drugs. On the other hand, there are examples where increased levels of Grp78 appear to provide cytoprotection. Because of this, we




◀*Figure 1.* Hypoxia decreases the amount of survivin protein in various cancer cell lines. (A) U251 and LN229 glioblastoma, as well as HeLa cervix carcinoma, cell lines were cultured under hypoxic conditions for up to 48 h and total cellular lysates were analyzed by Western blot analysis with specific antibodies against survivin or Grp78. Bgr. indicates a non-specific signal that was inconsistently detected. (B) U251 and LN229 cells were treated with increasing concentrations of the hypoxia mimetic $CoCl_2$ for 36 h and analyzed by Western blot analysis for survivin expression. In all cases, the blots were reprobed with an antibody to actin in order to confirm equal loading in all lanes.

wondered how the altered balance of survivin and Grp78 levels during chronic hypoxia would affect chemosensitivity. To answer this question, we used celecoxib, a selective COX-2 inhibitor that is able to induce tumor cell apoptosis *in vitro* and *in vivo* [15, 16]. In addition, we also employed a close structural analog of celecoxib, called 2,5-dimethyl-celecoxib (DMC), which completely lacks the ability to inhibit COX-2 [22]. In a preliminary test, we investigated how these two drugs would affect the basal levels of survivin and Grp78 expression under normoxia in our glioblastoma cell system. As shown in Figure 7, both celecoxib and DMC were able to down-regulate survivin expression, but at the same time cause a substantial increase in Grp78 expression. Because DMC was able to mimic the effects of its parent compound—and in the case of survivin even somewhat more potently—we conclude that the observed drug effects were independent of an involvement of COX-2. Of note, neither celecoxib nor DMC had any effect on the steady state levels of COX-2 protein (Figure 7).

Next we investigated the combination effects of these two drugs together with hypoxia on tumor cell viability. U251 cells were incubated with either drug under normoxic or hypoxic conditions for 48 h, and cell growth and survival was determined with two separate procedures. The first was the conventional MTT assay, which determines the short-term viability (i.e., metabolic activity) of the entire cell population, and the second was the colony formation assay, which reveals the long-term survival of individual cells and their ability to spawn a colony of proliferating cells.

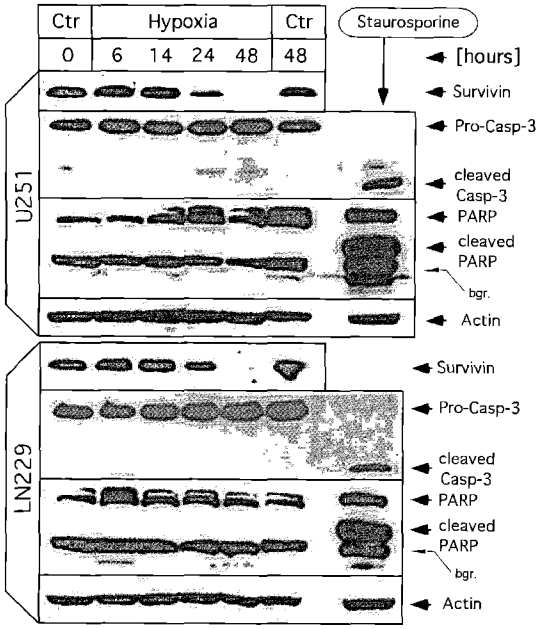



*Figure 2.* Hypoxia does not activate caspase 3. U251 and LN229 cells were cultured under hypoxic conditions for up to 48 h and total cellular lysates were analyzed by Western blot analysis with specific antibodies against survivin, caspase 3, PARP (poly ADP-ribose polymerase; a known substrate for activated caspase 3), and actin (as a loading control). As a positive control for the activation of caspase pathways, cells were also treated with staurosporine, an established inducer of caspase-mediated apoptosis. Note that in all PARP blots, a non-specific background signal was observed (indicated by arrow "bgr."); the specific cleaved PARP fragment was only detected in lysate from staurosporine-treated cells.

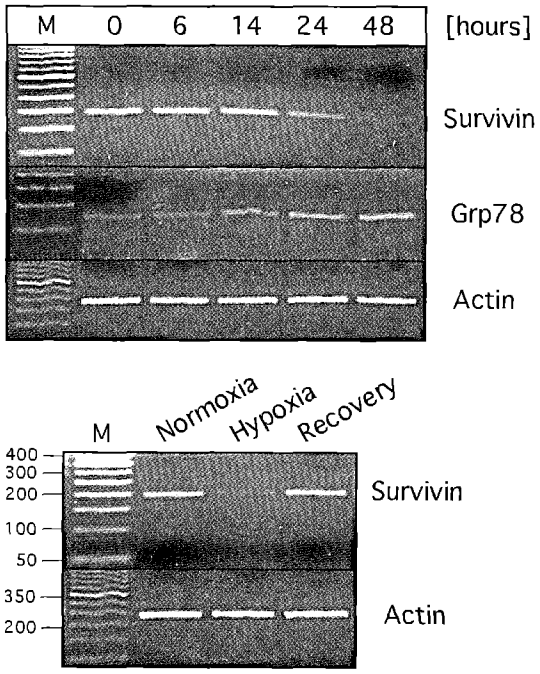



*Figure 3.* Hypoxia decreases survivin mRNA levels, but increases Grp78 mRNA levels. U251 glioblastoma cells were cultured under hypoxic conditions for up to 48 h, and survivin and Grp78 mRNA levels were determined by semiquantitative RT-PCR. In the bottom panel, the cells were kept under normoxic conditions (lane: Normoxia), or were exposed to hypoxia for 48 h (lane: Hypoxia), or were exposed to hypoxia for 48 h and then returned to normoxia for 12 h (lane: Recovery) before analysis by RT-PCR. Actin was used as a control. M: marker lane with 50 bp ladder.

Figure 8A demonstrates that treatment of cells with hypoxia, celecoxib, or DMC lead to 30–40% reduction of metabolic activity, as measured by MTT assays; when hypoxia was combined with either one of the two drugs, viability was further reduced by 20–30%. Similarly, when long-term survival of tumor cells were analyzed (Figure 8B), we found that individual treatment with hypoxia, celecoxib, or DMC reduced the number of surviving cells by 20–45%, and combination treatment of hypoxia with either drug further reduced survival by up to 75%. Thus, it became apparent that celecoxib was able to enhance tumor cell death induced by hypoxia. Intriguingly, DMC faithfully mimicked this effect, indicating that the inhibition of COX-2 was not involved in this cytotoxic process.

Finally, we asked whether the enhancing effect of celecoxib and DMC under hypoxia would be reflected at the level of expression of survivin. To this end, U251 cells were cultured under hypoxic conditions, or with the hypoxia mimetic $CoCl_2$, in the presence or absence of celecoxib or DMC, and survivin protein expression was analyzed by Western blot analysis. As shown in Figure 9, each individual treatment lead to reduced levels of survivin, and the combination of hypoxia or $CoCl_2$ with either of the two drugs enhanced this repressive effect. Taken together, these results show that enhanced tumor cell death in response to drug treatment under chronic hypoxic conditions correlates with further reduced levels of the anti-apoptotic survivin protein.

## Discussion

Novel anti-cancer strategies attempt to take advantage of chronic hypoxic conditions present in advanced tumors by applying gene therapy

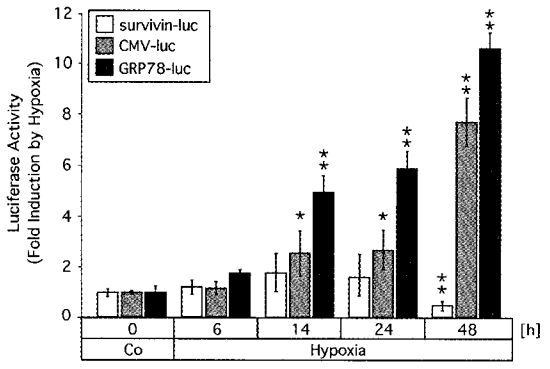



*Figure 4.* Regulation of survivin, Grp78, and CMV promoter activities by hypoxia. LN229 glioblastoma cells, stably transfected with luciferase reporter constructs under the control of the survivin, Grp78, or CMV promoter, were cultured under hypoxic conditions for up to 48 h. Cells were harvested and analyzed for luciferase activity. At each time point, normoxic controls were harvested as well and analyzed in parallel for normalization purposes. Shown is the ratio of luciferase activity between hypoxic and normoxic conditions (fold induction) for each construct (mean ± SE, $n \geq 4$). Statistical analysis was performed and $p$-values were calculated between hypoxia and normoxia values for each time point; one asterisk (*): $p < 0.05$; two asterisks (**): $p < 0.01$. Note that the small increase in average survivin–luciferase activity at 6, 14, and 24 h was not statistically significant.

vectors that display their greatest activity under conditions of chronic hypoxia. In this regard, the promoters of the survivin and the Grp78 gene have attracted some attention, because the activity of either gene generally is found elevated in various types of tumor cells [8–10, 38], and both promoters have been described as being inducible by hypoxia [11, 39]. In our present study, however, we discovered a major discrepancy with previous reports. We found that the expression of survivin is strongly inhibited under chronic hypoxic conditions, and that this effect is obvious at the promoter, mRNA, and protein level. On the other hand, we confirmed that Grp78 expression is highly inducible by hypoxia, and extended these earlier findings by demonstrating that these effects also take place under *in vivo* conditions in tumor tissue. Furthermore, we show that the selective COX-2 inhibitor celecoxib is able to enhance tumor cell death under hypoxic conditions in a COX-2 independent fashion that correlates with further reduced expression of survivin.

What could be the reason for our discrepant finding that survivin is repressed, rather than induced, by hypoxia? To answer this question, it is relevant to distinguish between short-term ($\leq 24$ h) and chronic ($>24$ h) hypoxia. For example, Yang et al. [11] demonstrated 1.5-, 2.3-, and 2.5-fold induction of a survivin–luciferase construct in MCF-7, MDA-MB-231, and MIA PaCa-2 tumor cell lines after 24 h of hypoxia. In comparison, we also obtained this relatively small stimulatory effect (1.8-fold and 1.6-fold) after 14 and 24 h of hypoxia (Figure 4). However, there was much variability, and statistical analysis revealed that this small average induction was not significant ($p > 0.05$), and furthermore did not translate into increased mRNA (Figure 3) or protein levels (Figures 1 and 2). Clearly, after longer exposure to hypoxia, i.e., after 48 h, we found the expression of survivin was greatly diminished at the promoter, mRNA, and protein levels. We believe that longer exposure to hypoxia might be more reflective of the situation in advanced tumors, where deficiencies in the blood supply might generate persistent and chronic conditions of hypoxia. In this regard, we also detected a tendency (but no clear-cut correlation) of survivin-positive cells to be absent from the most hypoxic regions in xenograft tumor tissue *in vivo* (Figures 4 and 5). Therefore, we believe that the survivin promoter is not very well suited for targeting expression constructs to tumor hypoxic regions. In support of our claim, a report by Dohi et al. [35] described that hypoxic exposure of HeLa cervix carcinoma cells *in vitro* led to the stabilization of only mitochondrially located survivin protein, but this effect did not involve the activation of the survivin promoter. We have investigated HeLa cells as well and found a pronounced reduction of overall cellular survivin protein levels during long-term hypoxia (Figure 1A).

A well-established mechanism to boost promoter activity under hypoxic conditions is the insertion of a hypoxia-inducible element (HRE), which is an enhancer sequence present in several robustly hypoxia-inducible genes, such as the vascular endothelial growth factor (VEGF) gene or the erythropoietin (EPO) gene [3, 6]. Single or multiple copies of the HRE have been inserted into various heterologous promoters in order to successfully achieve increased stimulation by hypoxia. In the case of survivin, it was shown that the insertion of six tandem VEGF-HRE copies increased the inducibility by hypoxia between 1.6-

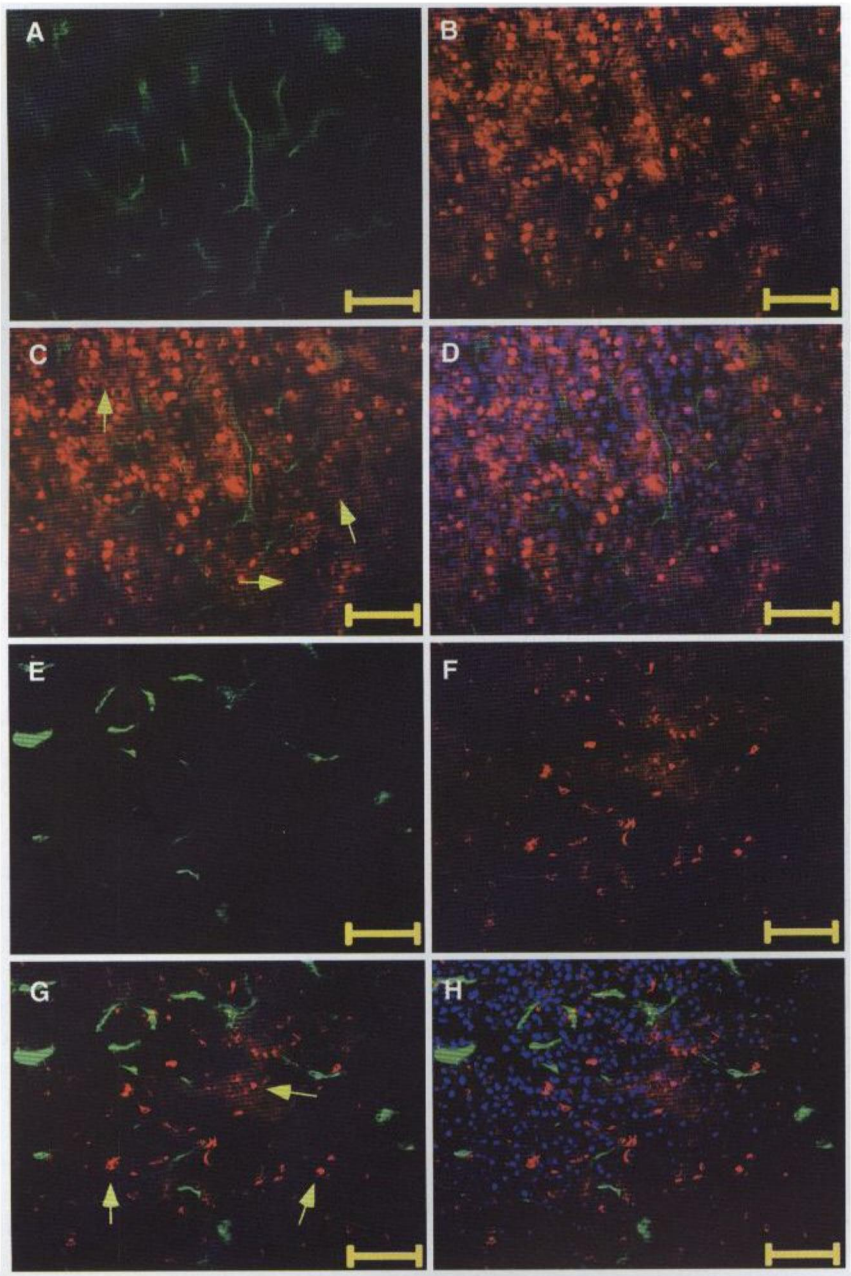



*Figure 5.* Localization of tumor blood vessels in relation to cellular expression of survivin and Grp78 proteins. Subcutaneous tumors grown from U87 glioblastoma cells were analyzed for distribution of blood vessels and expression of survivin and Grp78 by sequential double immunohistochemical staining. Blood vessels were visualized with PECAM-1 antibodies (green stain), whereas survivin and Grp78 were detected with their respective antibodies and are indicated by red stain. DAPI nuclear counterstain is shown in blue. (a) Distribution of blood vessels; (b) expression of survivin (note prominent nuclear stain); (c) overlay of a + b (arrows indicate examples of low survivin expression distant from blood cells); (d) addition of nuclear counterstain to c (pink color reflects red survivin stain superimposed on blue nuclear DNA stain. The bottom panels, e–h, are similar to the above, except that red stain represents Grp78 protein instead of survivin (arrows in g indicate examples of high Grp78 expression distant from blood vessels). Scale bar (yellow): 100 $\mu$m. In all cases, representative sections are shown.

and 4.7-fold, depending on the cell type [11]. Although this increase appears promising, it was not investigated whether this boost would be maintained for longer time periods, i.e., under chronic hypoxia. In general, based on our results, it might be quite informative and indeed critical to

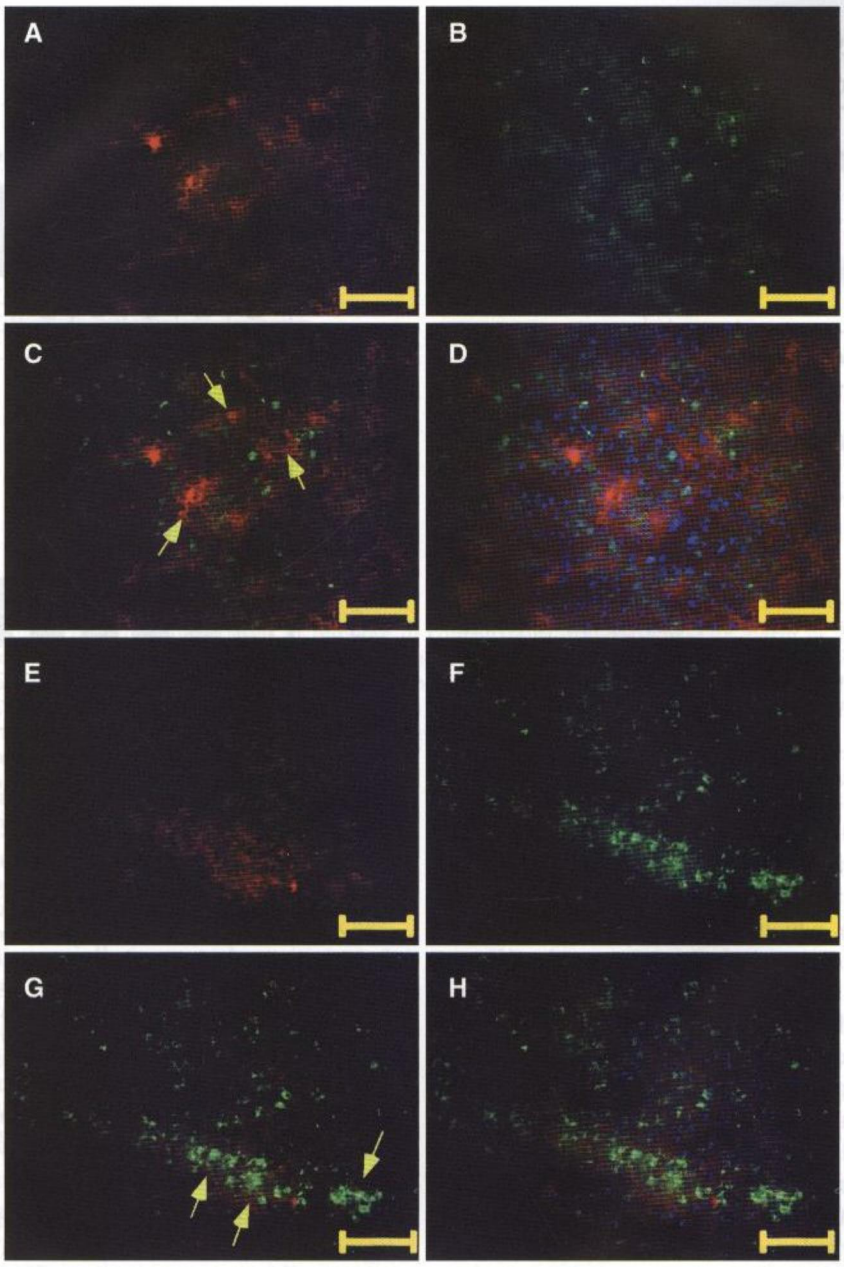



*Figure 6.* Localization of hypoxic regions in relation to cellular expression of survivin and Grp78 proteins in tumor tissue. Subcutaneous tumors grown from U251 glioblastoma cells were analyzed for the presence of hypoxic regions and the expression of survivin and Grp78 by parallel double immunohistochemical staining. Hypoxic regions were visualized with anti-pimonidazole antibodies (red stain), whereas survivin and Grp78 were detected with their respective antibodies and are indicated by green stain. DAPI nuclear counterstain is shown in blue. (a) Distribution of hypoxic regions; (b) expression of survivin; (c) overlay of a + b (arrows indicate examples of low survivin expression in hypoxic areas); (d) addition of nuclear counterstain to c (brownish color reflects green survivin stain superimposed on blue nuclear DNA stain). The bottom panels, e–h, are similar to the above, except that green stain represents Grp78 protein instead of survivin (arrows in g indicate examples of high Grp78 expression in hypoxic areas). Scale bar (yellow): 100 $\mu$m. In all cases, representative sections are shown.

apply extended periods of hypoxia, perhaps in combination with *in vivo* studies that include the hypoxia marker pimonidazole, when hypoxia-targeted gene therapy vectors are being tested.

The magnitude of hypoxia-inducibility of various promoter constructs reported in the literature varies greatly, from $<2$-fold to $>500$-fold (for a recent review, see [6]). Besides variations in basal

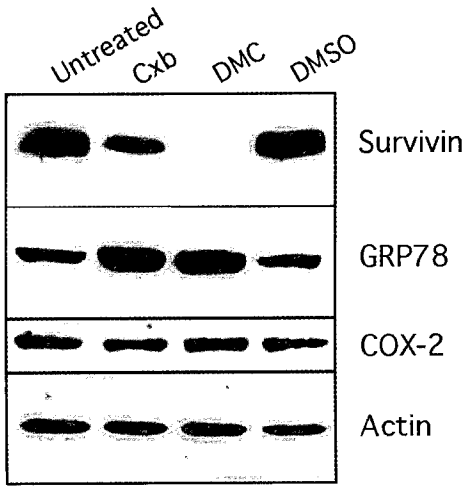



*Figure 7.* Down-regulation of survivin and increase in Grp78 protein by celecoxib and DMC. U251 glioblastoma cells were treated with 50 $\mu$M celecoxib (Cxb) or DMC for 48 h. As controls, the cells were either left untreated or received solvent (DMSO) alone. Western blot analysis was performed with antibodies specific to survivin and Grp78. In addition, we established that neither celecoxib nor DMC were able to change the expression levels of COX-2 protein (inhibition of COX-2 by celecoxib takes place at the post-translational, i.e., enzyme activity, level). Actin was used as a loading control. Similar results were also obtained with LN229 and other glioblastoma cell lines.

promoter activity and the number of HREs present in a given construct, a major determinant is oxygen tension, which is heterogeneous not only in human tumor tissue, but also in the experimental conditions applied. In general, promoter activity of hypoxia-inducible genes increases as oxygen tension diminishes, and greatest stimulation is achieved at 0.1% oxygen and below [6]. In our experimental *in vitro* system, oxygen levels were reduced to approximately 0.1%, which is a value that has been measured in human tumors [40, 41]. Under these conditions, survivin expression is greatly reduced between 24 and 48 h, whereas at the same time the expression of the Grp78 promoter is strongly (10-fold) induced (Figure 4). Hypoxia-inducibility of Grp78, which lacks a consensus HRE sequence, has been reported before [39, 42, 43], and we extend these earlier studies by demonstrating that Grp78 mRNA expression *in vitro* is maximally stimulated by long-term (>24 h) hypoxia (Figure 3), which constitutes a most beneficial feature for the specific targeting of this promoter to hypoxic tumor tissue *in vivo*. This view is further supported by our observation that Grp78 is very prominently

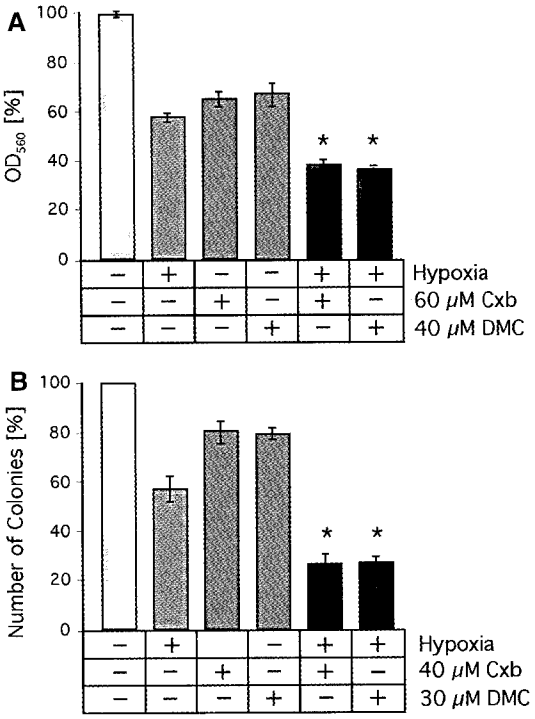



*Figure 8.* Further reduced survival by combination of hypoxia with celecoxib or DMC. U251 glioblastoma cells were treated with hypoxia, celecoxib, or DMC either alone or in combination for 48 h as indicated. (A) MTT assays were performed immediately at the end of the 48-hour incubation period. The OD value (reflective of metabolic activity, which indicates overall cell growth and survival) from untreated/control cells was set at 100%. Each bar represents the mean ($\pm$SE, $n$ = 8). (B) Colony formation assays were performed, and the number of surviving cells that were able to spawn a colony was determined two weeks after exposure of cells to hypoxia and drugs. Shown is the mean ($\pm$SE) from triplicate experiments. These experiments were also repeated with LN229 cells and yielded essentially the same outcome. Asterisks indicate statistically significant ($p < 0.01$) differences between combination treatments and individual drug treatment.

induced in the most hypoxic regions of tumor tissue *in vivo* (Figures 4 and 5). Thus, for purposes of cancer therapy, it would seem that Grp78 might be the superior choice among these two promoters, and two previous studies [44, 45] have indeed demonstrated the anti-tumor efficacy of this promoter when directing the expression of the herpes simplex virus thymidine kinase (HSV-tk) suicide gene in xenograft tumor models.

The down-regulation of survivin expression under chronic hypoxic conditions points to a further critical issue, namely the chemosensitivity of hypoxic cancer cells. In general, hypoxic cells are more resistant to conventional chemotherapeutic

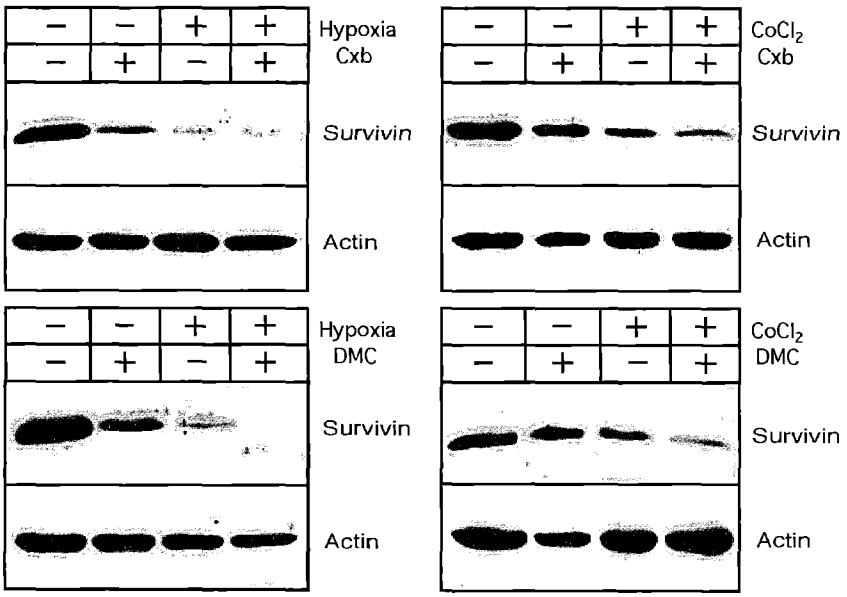



*Figure 9.* Further reduced survivin protein levels in response to combination of hypoxia with celecoxib or DMC. Glioblastoma cells were exposed to hypoxia (left panels) or the hypoxia mimetic $CoCl_2$ (right panels) in the presence or absence of celecoxib or DMC for 36 h. Total cellular lysates were analyzed for expression levels of survivin protein (or actin as a loading control) by Western blot analysis with specific antibodies. U251 and LN229 cells were used for all experiments and yielded similar results. Representative results are shown for U251 on the left and for LN229 on the right.

drugs, which constitutes a major problem during chemotherapy [2, 46]. On the other hand, it has been demonstrated in several experimental systems that the down-regulation of survivin expression, for example by antisense or siRNA approaches [47], results in substantially increased sensitivity of such tumor cells to killing by chemotherapeutic drugs (for examples, see [48–53]). Therefore, one wonders why hypoxic cells with reduced survivin levels are not becoming more chemosensitive. One of the reasons for this might be found among some of the other hypoxia-stimulated effects [1, 2], such as variations in the activity of DNA repair enzymes, cell cycle regulatory components, metallothioneins, or increased Grp78 levels. Grp78 is a component of the ER stress response and functions to protect cells from various types of stress that impinge on ER function [14]. Intriguingly, however, it has been demonstrated that elevated levels of this protein support cellular survival during chronic hypoxia [54] and exert protective function against certain chemotherapeutic drugs [55, 56]. Thus, in this context, one could speculate that the protective function of elevated Grp78 might perhaps outweigh the sensitizing effect of reduced survivin levels. While our study did not address this particular hypothesis, we did investigate the sensitivity of hypoxic tumor cells to celecoxib, a selective COX-2 inhibitor that appears to hold promise for the treatment and prevention of colorectal cancer and possibly for other cancers as well.

We chose celecoxib because the chemical inhibition of COX-2 might be beneficial in view of the proclivity of hypoxic tumor cells to mount a defense against hypoxia by increasing the activity of COX-2. Elevated COX-2 activity leads to higher levels of prostaglandin and subsequent stimulation of vascular endothelial growth factor (VEGF), which is a potent stimulus for new blood vessel growth [17, 18]. However, besides acting via COX-2, celecoxib is also known to have COX-2-independent anti-tumor effects [21], and thus might not require COX-2 to inhibit hypoxic tumor cell growth. As we show in this study, celecoxib significantly reduces the survival of cells under chronic hypoxia (Figure 8). The underlying mechanism of this cytotoxic effect appears to be independent of COX-2, because the celecoxib derivative DMC, which entirely lacks COX-2 inhibitory ability [22], potently mimics this effect; furthermore, COX-2 independency is also consistent with numerous other examples demonstrating that tumor cell killing by celecoxib and

DMC can take place without the involvement of COX-2 [24, 28, 29, 57–60]. In this context, it is noteworthy that recent reports indicated that the ER stress response pathway is involved in mediating apoptosis during treatment of cells with certain NSAIDs, including celecoxib [61, 62]. It is therefore conceivable that the ER stress response might participate in drug-induced cell death after chronic hypoxia as well, and that this effect perhaps might add to and exacerbate ER stress induced by hypoxia, thereby overwhelming the protective function of this mechanism. The potential involvement of ER stress pathways would suffice to explain the observed induction of Grp78 by hypoxia, and maybe also the reduction of survivin expression. Because the typical events during ER stress combine an inhibitory effect on general translation with the selective up-regulation of ER stress genes (such as increased translation of GRP78) [63], it is entirely possible that survivin may have fallen victim to the general inhibition of translation that was initiated by hypoxia-induced ER stress. However, further studies are needed to confirm this conjectural scenario.

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