Celecoxib Inactivates Epithelial–Mesenchymal Transition Stimulated by Hypoxia and/or Epidermal Growth Factor in Colon Cancer Cells

INTRODUCTION

Colon cancer is the third most common malignancy worldwide, and it stands as the second most common cause of cancer mortality in western countries [1]. Most of these deaths are due to liver metastatic disease, and the prognosis and the overall survival are mainly determined by the progression of the primary carcinoma. An increasingly accepted concept is that colon cancer progression is accompanied by a cellular pathway often referred to as the epithelial–mesenchymal transition (EMT), an important preliminary step in metastasis [2] that allows cells to become motile.

During EMT carcinoma cells become more invasive and aggressive, with loss of epithelial characteristics that cause dissociation from surrounding cells and acquisition of mesenchymal-like properties that allow them to migrate away from the initial neoplastic focus. Thus, the major molecular hallmarks of EMT include downregulation of junctional protein E-cadherin responsible for the loss of cell–cell adhesion, and expression of mesenchy-mal proteins like vimentin and N-cadherin. Furthermore, cells undergoing EMT express matrix metalloprotease enzymes, the primary mechanism accounting for tumor invasion that allows penetration within the stroma surrounding the original tissue.

Functional changes of EMT take place through complex molecular events involving systems and signaling proteins: central in signaling pathways that lead to E-cadherin repression is the activation of the zinc finger factor SNAI1, a strong repressor of E-cadherin transcription, which is overexpressed in colon cancer cells [3].

Another environmental regulator promoting tumor aggressiveness is intratumoral hypoxia, whose importance in development of colon cancer

has been well demonstrated by clinical studies in which hypoxia predicts for poor prognosis. Hypoxia may also influence tumor-associated stromal cells, in a way that may contribute to patient prognosis.

As recently confirmed in human hepatocellular cancer cells hypoxia is able to induce EMT and increased invasiveness in human cancer cells [4,5].

A major mechanism which is likely to link hypoxia to cancer progression is represented by the expression of proteins that favor tumor invasiveness through adaptative mechanisms involving specific hypoxia-inducible transcription factors (HIFs) and resulting in the induction of critical phenotypic and functional cellular changes. Along these lines, hypoxia-inducible factor-1 (HIF-1) is a transcription factor overexpressed in many solid tumors, whose stabilization and activation correlates with tumor metastasis and poor prognosis. HIF-1 might contribute to tumor progression through the promotion of angiogenesis, the activation/induction of proteolytic enzymes or the activation of genes involved in tumor cell invasiveness. HIF-1 can also induce EMT, through the activation of EMT regulators, including SNAI1, although the expression of EMT-related transcription factors may be initiated also following exposure to selected growth factors able to signal through their cognate receptor tyrosine kinases which, in turn, activate MAPK and PI3K pathways, eventually leading to SNAI1/2 upregulation [6,7].

As a matter of fact, epidermal growth factor (EGF) is a potent stimulator of EMT in several cell types and its receptor (EGFR) has been shown to directly interact with β-catenin, leading to its tyrosine phosphorylation and disruption of cadherin-dependent junctions [8]. Endocytosis of E-cadherin results in the release of β-catenin to act on the Wnt pathway, resulting in E-cadherin repression [9,10]. On the other hand, E-cadherin complexes engaged in the intact adherens junction directly inhibit the activity of the EGFR by inhibiting trans-phosphorylation of Tyr845 [9].

An additional critical player in colorectal carcinogenesis is represented by overexpression of inducible cyclooxygenase-2 (COX-2) [11,12], a key enzyme in prostaglandin E2 (PGE2) synthesis which has been identified as a direct target for HIF-1 in colorectal tumor cells [13]. COX-2 expression is also known to be upregulated by specific tumor environmental signals, including EGF and hypoxia [14]. Moreover, the COX-2/PGE2 pathway is known to affect signaling pathways like PI3K/pAkt, ERK/MAPK [15,16], WNT/β-catenin [17], and EGFR [18] which, in turn, are all also able to promote COX-2 expression.

Inappropriate expression of COX-2 has been associated with a poor prognosis, likely through immunosuppression, inhibition of apoptosis and increased metastatic potential of epithelial cells. Indeed, COX-2 overexpression has been linked to the disruption of E-cadherin-mediated cell–cell contacts and in lung cancer cells COX-2 expression results in a significant reduction of E-cadherin, an event which can be rescued by COX-2 inhibition [19]. Conversely, treatment with selective COX-2 inhibitors has been reported to result in a significant decrease in colorectal adenomas in both humans and animals [20,21].

The COX-2-selective non-steroidal anti-inflammatory drug celecoxib has been shown to reduce cell growth in several tumors, but also it has been suggested to operate through additional COX-2 independent, but still unclear mechanisms [22].

MATERIALS AND METHODS

Materials and Reagents

EGF, mouse monoclonal antibodies for β-actin and α-SMA, and anti-goat Cy3-conjugated secondary antibody were purchased from Sigma Chemical Co. (St Luis, MO). Rabbit polyclonal antibodies for EGFR and pAkt1/2/3, mouse monoclonal antibodies for PTEN, COX-2, EGFR, pERK1/2, PI3Kp85α, E-cadherin, β-catenin, vimentin, and HIF1α, and HRP-conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-rabbit Cy3-conjugated secondary antibody was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). The Boyden chambers were from Neuro Probe Inc. (Gaithersburg, MD).

Celecoxib was obtained from Sequoia Research Products Ltd (Pangbourne, UK), solubilized in dimethyl sulfoxide (DMSO), and used at a final concentration of DMSO that never exceeded 0.1%.

The enhanced chemiluminescence reagent and nitrocellulose membrane (Hybond-C extra) were from Amersham Pharmacia Biotech (Milano, Italy). All other reagents were from Sigma–Aldrich Spa (Milano, Italy).

Cell Line and Culture Conditions

The HT-29 and SW-480 human colon cancer cell lines were obtained from American Type Cell Culture (Manassas, VA). The cell lines were grown and maintained in McCoy's 5A (HT-29) and DMEM (SW-480) medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 μg/ml amphotericin B. Cells were cultured at 37°C in a humidified incubator with 5% CO_2 and 95% air, and regularly examined using inverted microscope.

For treatments, cells were seeded at a density of 3×10^4 cells/cm^2 and cultured in normoxic conditions for 24 h to allow them to adhere to the substratum. The medium was then replaced with new medium supplemented with celecoxib (10 and 50 μM) and/or EGF (100 ng/ml). In experiments designed to evaluate the role of hypoxia, cells were first seeded in normoxic conditions to obtain the desired subconfluence level (65–70%) and then were incubated in strictly controlled hypoxic conditions (3% O_2) for 24 h.

Viability Assay

Cells were seeded in 12-well culture plates and properly treated. Aliquots of cell suspension were incubated with trypan blue solution (0.5% in NaCl) for 5 min to assess cell viability. Finally, cells were transferred to the Bürker chamber and counted by light microscope. Dead cells were defined as those stained with the dye.

Western Blotting

Cells were seeded in 75 cm^2 plates and properly treated. Total extracts, nuclear extracts, and membrane-associated fractions were obtained as previously detailed [23]. Protein contents were measured using a commercially available assay (Protein Assay Kit 2, Biorad, Milano, Italy) with bovine serum albumin as a standard. Extracts were then subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis on 12%, 10%, or 7.5% acrylamide gels. The blots were incubated with desired primary antibody and then incubated with peroxidase-conjugated anti-mouse or anti-rabbit antibodies in Tris-buffered saline-Tween containing 2% (w/v) non-fat dry milk, and developed with the enhanced chemiluminescence reagents. Band intensities were quantified by densitometry and the expression of proteins was reported as a proportion of β-actin protein expression to monitor any discrepancies in gel loading (VersaDoc Imaging System 3000; Biorad).

Fluorescence Microscopy

Cells were seeded on six-well culture plates, allowed to adhere for 24 h, and then treated. After treatment, the cells were fixed and permeabilized with methanol/acetone (1:1). Cells were then incubated with antibodies for E-cadherin, α-SMA, β-catenin, or vimentin, and the immunopositivity was revealed by means of appropriate Cy3-conjugated secondary antibody. Then cells were stained with Hoechst to detect nuclei. After washings with PBS, the slides were mounted with H_2O/glycerol (1:1) and viewed under a fluorescence microscope equipped with a UV light filter (Dialux 20; Leitz, Wetzlar, Germany).

Invasion Assay

Cells were seeded on six-well culture plates, and properly treated. After treatment, cell invasiveness was evaluated with Boyden chambers equipped with 8 μm porosity polyvinylpyrrolidone-free polycarbonate filters coated with 50 μg/ml of Matrigel solution. After a 24 h incubation, the filters were fixed in ice-cold methanol and stained with crystal violet solution. The migrating cells were quantified in 10 randomly selected fields at ×40 magnification in each filter, and the average value (mean ± SD) was defined on three independent filters.

Statistical Analysis

Differences between the means were analyzed for significance using the one-way ANOVA test with Bonferroni post hoc multiple comparisons, used to assess the differences between independent groups. All values are expressed as means ± SD, and differences were considered significant at $P < 0.05$.

RESULTS

Effect of Celecoxib and EGF on HT-29 Cell Proliferation and on EGFR Downstream Signaling Pathways

In a first series of experiments we investigated whether celecoxib was able to interfere with EGF-dependent intracellular signaling pathway. HT-29 cells were cultured for 1 h with EGF (100 ng/ml) and celecoxib, alone or in combination, then rinsed in SFIF and cultured for additional 24, 48, and 72 h in serum-starved medium. Celecoxib was administered at a 50 μM concentration, since we reported previously that this concentration caused a strong (40–60%) reduction of HT-29 cell proliferation, also due to induction of apoptosis [23]. As expected (Figure 1A), EGF led to increased proliferation (significant at 24 and 72 h) whereas celecoxib treatment resulted in a very significant inhibition of basal (i.e., unstimulated) proliferation at any experimental time point, the most effective inhibition being detected at 48 and 72 h (approx. 60% inhibition vs. control values). Celecoxib, although to a lesser extent, also resulted in the inhibition of EGF-stimulated cell growth, with the strongest antiproliferative effect detected at 72 h incubation (approx. 50% inhibition vs. EGF-stimulated proliferation of HT-29 cells).

Since EGFR is overexpressed in a variety of malignancies including colon carcinoma [24] as well as in HT-29 cells we next evaluated whether celecoxib may affect either EGFR levels or levels of its phosphorylated form (Figure 1B). Celecoxib treatment, irrespective of EGF exposure, resulted in a decrease of EGFR and pEGFR levels after 24 h. ERK activation represents an early step in growth

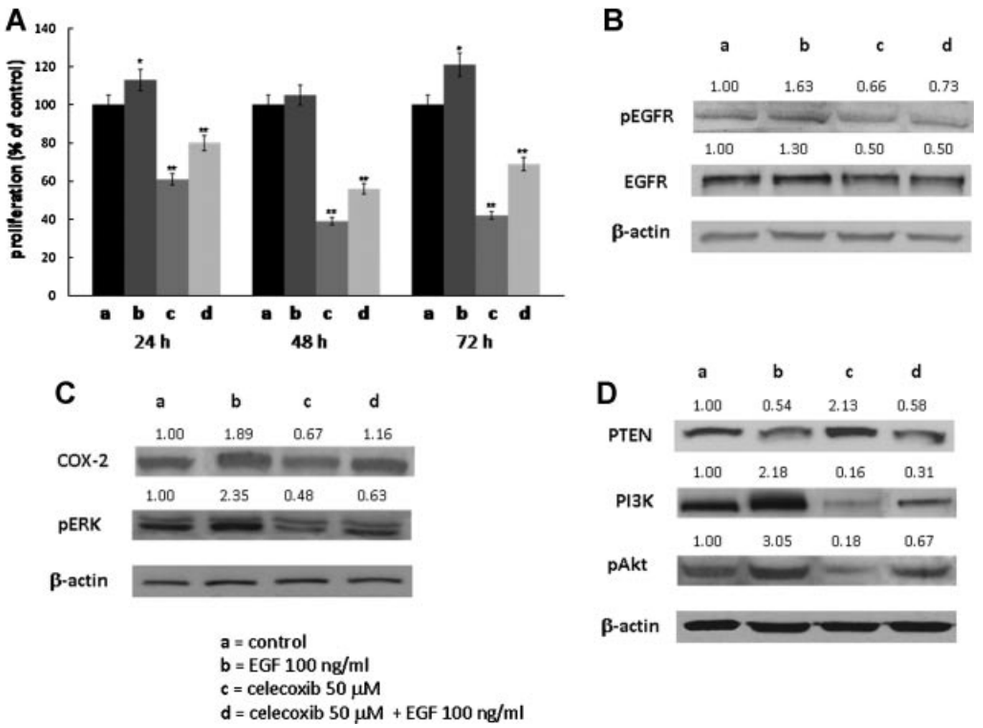

Figure 1. Effect of celecoxib and EGF on HT-29 cell proliferation and on EGFR downstream signaling pathways. The cells were incubated for 1 h in the absence (control) or in the presence of celecoxib (50 μM) and EGF (100 ng/ml), alone or in combination. (A) Viable cell number was then assessed by the trypan blue exclusion test after 24, 48, and 72 h. Results are expressed as percentage versus control (100%) and values represent mean ± SD for three independent experiments, each performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.001$ versus control. (B–D) Whole cell lysates were analyzed by Western blotting with specific antibodies for COX-2, pERK, pEGFR, EGFR, PTEN, PI3K, and pAkt, and then with HRP-conjugated secondary antibody. The blots were normalized with anti-β-actin antibody and quantified as arbitrary values relative to control by densitometry (SD < 10%). The data are representative of three independent experiments.

factor-induced cell proliferation and additional experiments outlined the fact that changes in cell proliferation were correlated with changes in ERK phosphorylation (Figure 1C). Pretreatment with celecoxib resulted in a significant inhibition of pERK levels in control as well as in EGF-stimulated cells. A very similar scenario was also observed for changes in COX-2 levels, as well as for changes in pAkt and PI3K (Figure 1C,D). We next analyzed PTEN activation, since PTEN is known to act as a negative regulator of PI3K and MAPK. As expected, PTEN expression was increased (twofold) by celecoxib, whereas exposure to EGF resulted in a strong reduction of PTEN (approx. 50%) and was not modified by concomitant treatment with celecoxib.

Effect of Celecoxib and EGF on Viability, Expression of HIF-1α, COX-2, pERK, and pAkt in HT-29 Cells Treated With Celecoxib and/or EGF Under Normoxic and Hypoxic Conditions

Transition from the epithelial to the mesenchymal phenotype is believed to represent for cancer cells a major step towards increased malignancy and then a number of experiments have been designed in order to investigate whether celecoxib or hypoxic conditions may affect EMT induction. In these sets of experiments, HT-29 cells were either exposed to normoxic conditions or to carefully controlled hypoxic conditions in the presence or in the absence of EGF and/or celecoxib.

Data obtained at the end of 24-h incubation immediately indicated that, as expected, EGF-stimulated proliferation of HT-29 cells (approx. 30% enhancement, $P < 0.01$), whereas exposure to hypoxic conditions was completely ineffective on proliferation rate irrespective of the specific treatment protocol. More specifically, celecoxib was found to exert a very significant antiproliferative effect at 50 μM on HT-29 cells, in particular on EGF-stimulated ones, whereas the reported inhibitory action of 10 μM celecoxib was definitively less effective (Figure 2A).

HT-29 cells were then evaluated for changes in protein levels of COX-2, pERK, and pAkt as well as

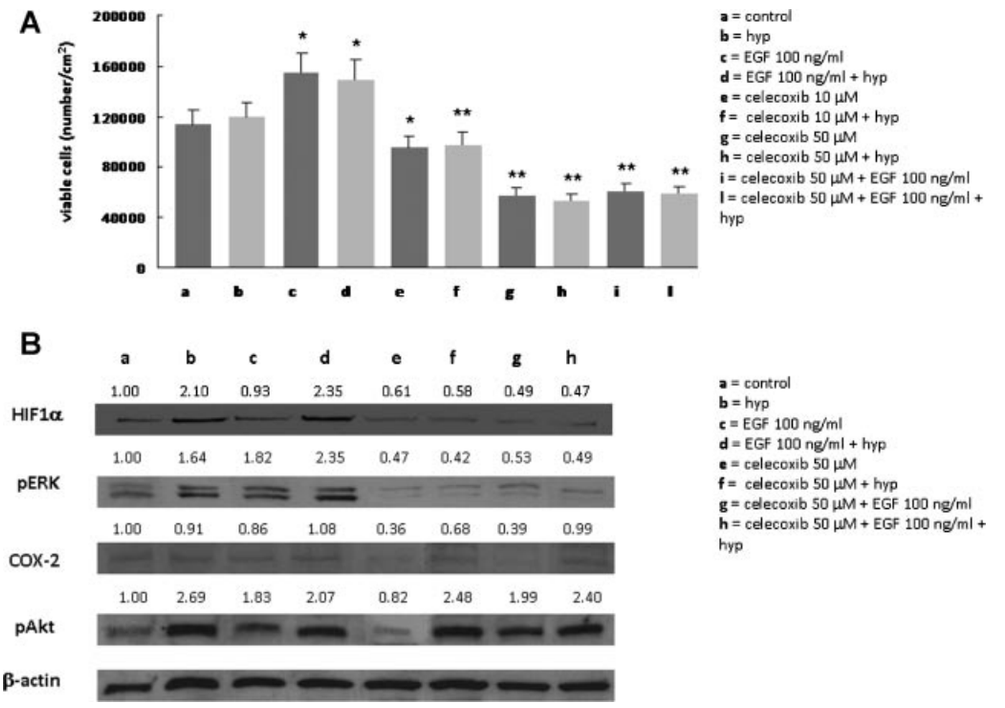

Figure 2. Effect of celecoxib and EGF on HT-29 cell survival and on HIF-1α, pERK, COX-2, and pAkt expression under normoxia and hypoxia. (A) The cells were incubated for 24 h in the absence (control) or in the presence of celecoxib (10 and 50 μM) and/or EGF (100 ng/ml). Viable cell number was assessed by the trypan blue exclusion test, and values represent mean ± SD for three independent experiments, each performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.001$ versus control. (B) The cells were incubated for 24 h in the absence (control) or in the presence of celecoxib (50 μM) and EGF (100 ng/ml), alone or in combination. Whole cell lysates were analyzed by Western blotting with specific antibodies for HIF-1α, pERK, COX-2, and pAkt, and then with HRP-conjugated secondary antibody. The blots were normalized with anti-β-actin antibody and quantified as arbitrary values relative to control by densitometry (SD < 10%). The data are representative of three independent experiments.

for HIF-1α levels (Figure 2B). Exposure to hypoxia resulted in a marked increased expression of HIF-1α, a scenario that was not significantly affected by EGF. Moreover, hypoxia led also to increased phosphorylation of ERK to levels comparable with those elicited by EGF; in particular, under hypoxic conditions EGF treatment induced a further enhancement of pERK levels. Treatment with celecoxib resulted in a very significant (approx. 50%) reduction of pERK and HIF-1α levels in all experimental conditions, suggesting that the drug may affect signaling and transcription factors induced by hypoxia and then likely, responses of target cells. It should be noted that COX-2 expression was unmodified by either hypoxia or EGF but strongly decreased by celecoxib treatment under normoxic conditions. Unexpectedly, celecoxib-induced decrease in COX-2 levels was partially prevented when cells were cultured under hypoxia. By contrast, celecoxib was uneffective on changes of pAkt levels induced by either hypoxia and/or EGF.

Celecoxib Affects Early Changes in EMT Markers Induced by Hypoxia in HT-29 Cells

On the basis of mentioned results, we inferred that in HT-29 cells celecoxib might inhibit hypoxia-induced EMT, a process that requires approximately 72 h to occur [4]. In order to test such an hypothesis we analyzed critical parameters of hypoxia-induced EMT at an early time point (i.e., 24 h). We found that celecoxib was able to fully and significantly counteract early hypoxia-dependent decrease in E-cadherin levels and positive immune staining, which indeed resulted both upregulated by celecoxib as compared to control values (Figure 3A,B).

Whether mesenchymal markers such as vimentin and α-SMA are concerned, celecoxib treatment was found to significantly decrease hypoxia-induced upregulation of vimentin (Figure 4A,B). However, when the action of celecoxib was evaluated in the presence of EGF alone or EGF and hypoxia, celecoxib treatment resulted in a

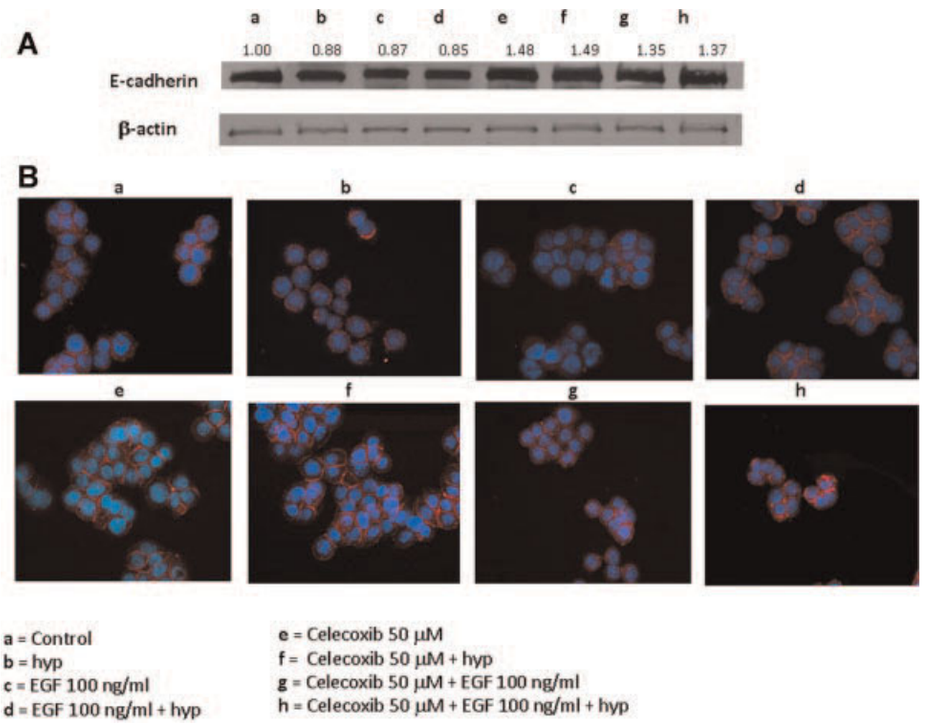

Figure 3. Effects of celecoxib and EGF on E-cadherin expression and its intracellular localization under normoxia and hypoxia in HT-29 cells. HT-29 cells were incubated for 24 h under normoxic and hypoxic conditions in the absence (control) or in the presence of celecoxib (50 μM) and EGF (100 ng/ml), alone or in combination. (A) Whole cell lysates were analyzed by Western blotting with specific antibody for E-cadherin and then with HRP-conjugated secondary antibody. The blots were normalized with anti-β-actin antibody and quantified as arbitrary values relative to control by densitometry (SD < 10%). The data are representative of three independent experiments. (B) The cells were fixed and exposed to anti-E-cadherin primary antibody followed by anti-rabbit Cy3-conjugated secondary antibody. To detect nuclei the cells were stained with Hoechst. The images show the overlaid pictures (400× final magnification) and are representative of three independent experiments.

significant reduction of vimentin levels only, but not of α-SMA, in these experimental conditions (Figure 5A,B).

Celecoxib and EGF Affect β-catenin Intracellular Localization Under Normoxic and Hypoxic Conditions in HT-29 Cells

Since E-cadherin changes and Wnt/β-catenin pathway are known to be strictly connected in EMT induction we next examined whether celecoxib can affect β-catenin intracellular localization.

Treatment of HT-29 cells with celecoxib exposed to either hypoxic conditions alone, EGF (normoxic conditions) or to both EGF and hypoxia resulted in a clear and significant decrease in the nuclear fraction of β-catenin and in a parallel increase of β-catenin bound to the membrane fraction, suggesting that celecoxib may indeed reverse EMT induction (Figure 6A,B).

Celecoxib Inhibits Invasiveness of HT-29 Cells

In order to further investigate the action of celecoxib on EMT-related parameters, we next examined whether the drug was also able to affect invasiveness of HT-29 cancer cells. Results are straightforward in indicating that celecoxib is highly efficient in abolishing either basal invasiveness as well as the one induced by hypoxia alone, EGF alone, or by hypoxia plus EGF (Figure 7).

Effect of Celecoxib on Viability, Expression of E-Cadherin, pERK, and pEGFR in SW-480 Cells Under Normoxic and Hypoxic Conditions

In the experiments depicted in Figure 8, we employed SW-480 colon cancer cells, that we [23] and others (indicated in Ref. [23]) have been reported to be COX-2 deficient; indeed, in our experiments we could not detect any significant level of COX-2 protein in this cell line (data not shown). SW-480 cells were less sensitive to the action of celecoxib, here employed at both 10 and 50 μM concentrations, as evaluated in terms of viable cells detected at the end of 24-h incubation (Figure 8A).

When SW-480 cells were treated or not with celecoxib (50 μM) and exposed to either normoxic and hypoxic conditions we found results that were

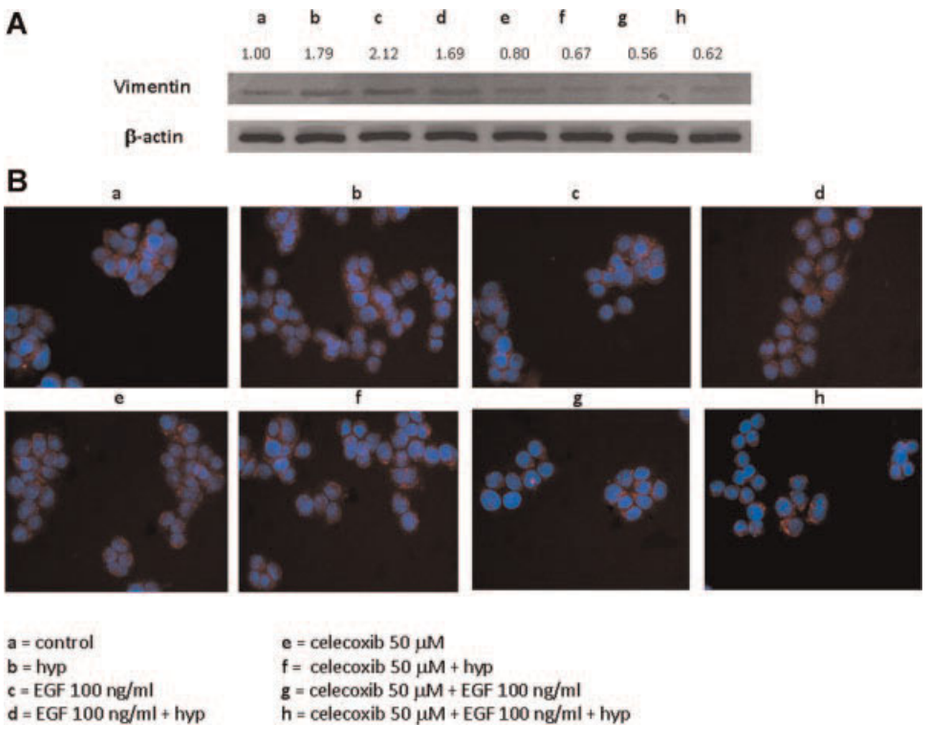

Figure 4. Effects of celecoxib and/or EGF on vimentin expression and its intracellular localization under normoxia and hypoxia in HT-29 cells. HT-29 cells were incubated for 24 h under normoxic or hypoxic conditions in the absence (control) or in the presence of celecoxib (50 μM) and EGF (100 ng/ml), alone or in combination. (A) Whole cell lysates were analyzed by Western blotting with specific antibody for vimentin and then with HRP-conjugated secondary antibody. The blots were normalized with anti-β-actin antibody and quantified as arbitrary values relative to control by densitometry (SD $<$ 10%). The data are representative of three independent experiments. (B) The cells were fixed and exposed to anti-vimentin primary antibody followed by anti-rabbit Cy3-conjugated secondary antibody. To detect nuclei the cells were stained with Hoechst. The images show the overlaid pictures (400$\times$ final magnification) and are representative of three independent experiments.

somewhat different from those observed for HT-29 cells. In particular, E-cadherin levels that were reduced under hypoxic conditions also in this cell line, were not reverted by celecoxib. On the other hand, pERK levels increased slightly under hypoxia, irrespective on the treatment with celecoxib (Figure 8B).

Celecoxib Inhibits Hypoxia-Induced Invasiveness of Both HT-29 and SW-480 Cells

Since previous results were consistent with a significant effect exerted by celecoxib on critical parameters of EMT, we next performed in both HT-29 and SW-480 cells a comparative analysis of the drug action on hypoxia-induced invasiveness, chosen as a model condition for its ''in vivo'' putative relevance. Results are straightforward in indicating that in HT-29 cells both 10 and 50 μM celecoxib concentrations were highly effective (Figure 9A) in inhibiting either basal or hypoxia-induced invasiveness. On the other hand, celecoxib displayed no or modest inhibitory effect on basal and hypoxia-induced invasiveness of SW-480 cells when used at either 10 or 50 μM concentration, respectively (Figure 9B).

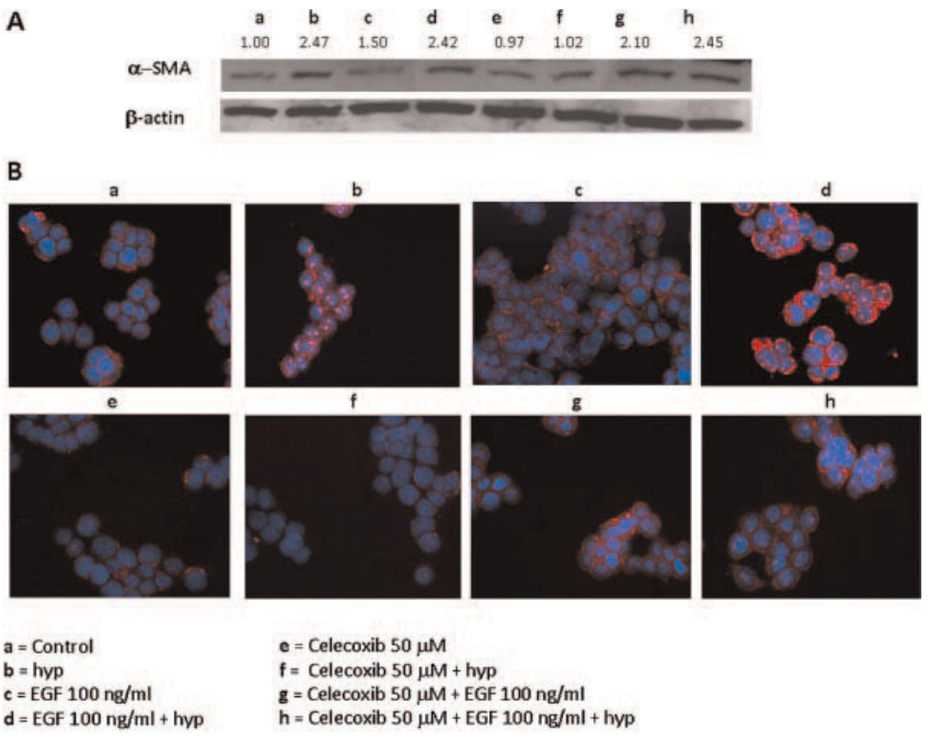

Figure 5. Effects of celecoxib and/or EGF on α-SMA expression and its intracellular localization under normoxia and hypoxia in HT-29 cells. HT-29 cells were incubated for 24 h under normoxic and hypoxic conditions in the absence (control) or in the presence of celecoxib (50 μM) and EGF (100 ng/ml), alone or in combination. (A) Whole cell lysates were analyzed by Western blotting with specific antibody for α-SMA and then with HRP-conjugated secondary antibody. The blots were normalized with anti-β-actin antibody and quantified as arbitrary values relative to control by densitometry (SD $<$ 10%). The data are representative of three independent experiments. (B) The cells were fixed and exposed to anti-α-SMA primary antibody followed by anti-rabbit Cy3-conjugated secondary antibody. To detect nuclei the cells were stained with Hoechst. The images show the overlaid pictures (400× final magnification) and are representative of three independent experiments.

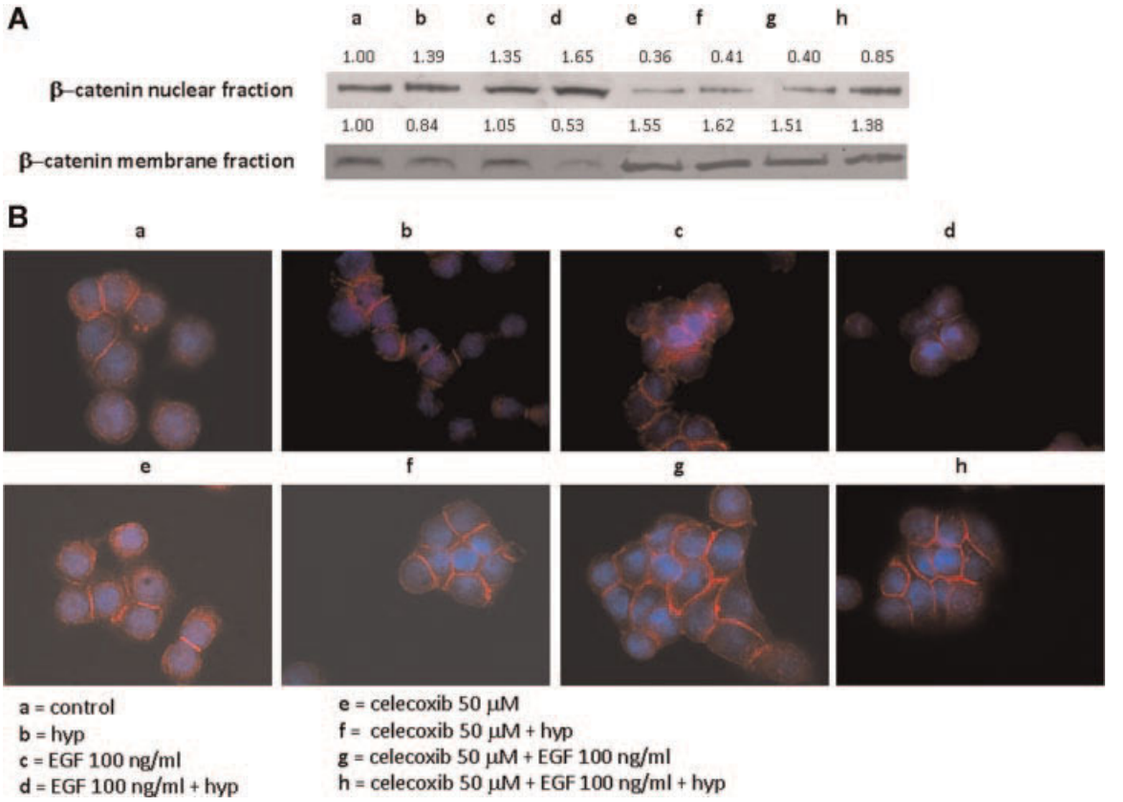

Figure 6. Effects of celecoxib and EGF on β-catenin expression and its intracellular localization under normoxia and hypoxia in HT-29 cells. HT-29 cells were incubated for 24 h under normoxic or hypoxic conditions in the absence (control) or in the presence of celecoxib (50 μM) and EGF (100 ng/ml), alone or in combination. (A) Nuclear and membrane-associated fractions were routinely stained using Ponceau Red dye and analyzed by Western blotting with specific antibody for β-catenin and then with HRP-conjugated secondary antibody. The blots were quantified as arbitrary values relative to control by densitometry (SD < 10%) and are representative of three independent experiments. (B) The cells were fixed and exposed to primary antibody for β-catenin followed by anti-mouse Cy3-conjugated secondary antibody. To detect nuclei the cells were stained with Hoechst. The images show the overlaid pictures (400× final magnification) and are representative of three independent experiments.

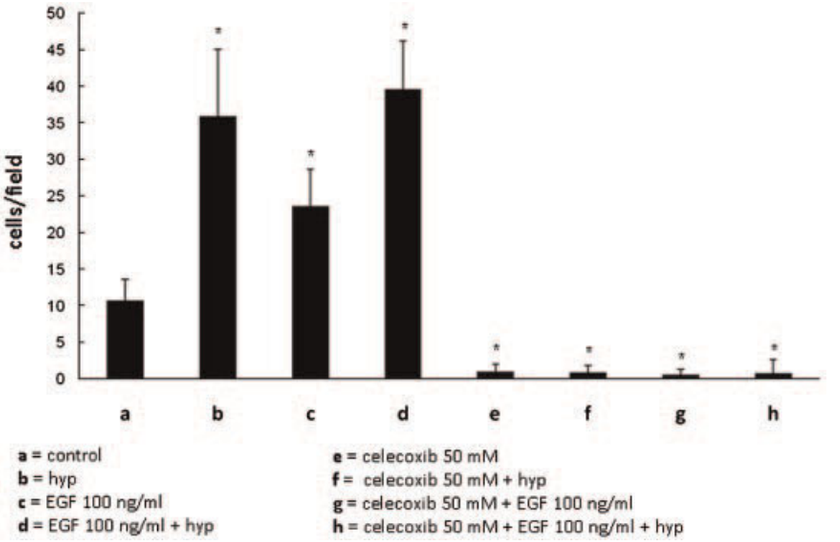

Figure 7. Effects of celecoxib and EGF on cell invasiveness under normoxia and hypoxia in HT-29 cells. HT-29 cells were incubated for 24 h under normoxic and hypoxic conditions in the absence (control) or in the presence of celecoxib (50 μM) and EGF (100 ng/ml), alone or in combination. Matrigel invasion was evaluated with a Boyden chamber assay; each filter was examined with a Zeiss microscope and the number of cells that penetrated through the matrigel was counted. Data are expressed as number of cells per high-power field and represent the mean of three independent experiments, each performed in triplicate (bars, SD). $^{*}P < 0.001$ significant difference between treated groups and the control.

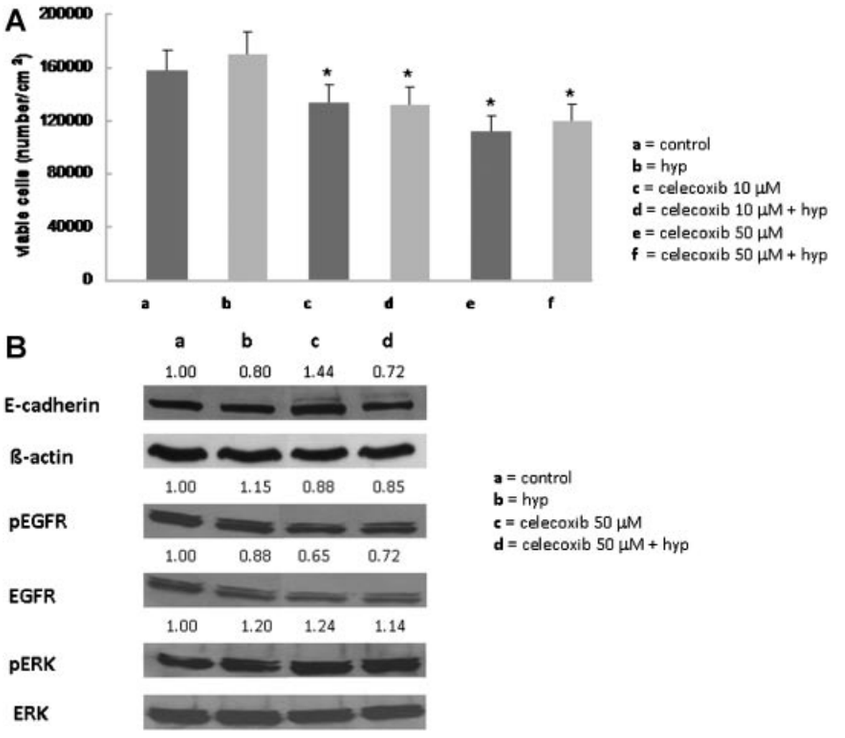

Figure 8. Effect of celecoxib on SW-480 cell viability and on E-cadherin, pEGFR, EGFR, and pERK expression under normoxia and hypoxia. (A) The cells were incubated for 24 h in the absence (control) or in the presence of celecoxib (10 and 50 μM). Viable cell number was assessed by the trypan blue exclusion test, and values represent mean ± SD for three independent experiments, each performed in triplicate. $^{*}P < 0.05$ versus control. (B) The cells were incubated for 24 h in the absence (control) or in the presence of celecoxib (50 μM). Whole cell lysates were analyzed by Western blotting with specific antibodies for E-cadherin, pEGFR, EGFR, and pERK and then with HRP-conjugated secondary antibody. The blots were normalized with anti-β-actin or ERK1/2 antibody, and quantified as arbitrary values relative to control by densitometry (SD < 10%). The data are representative of three independent experiments.

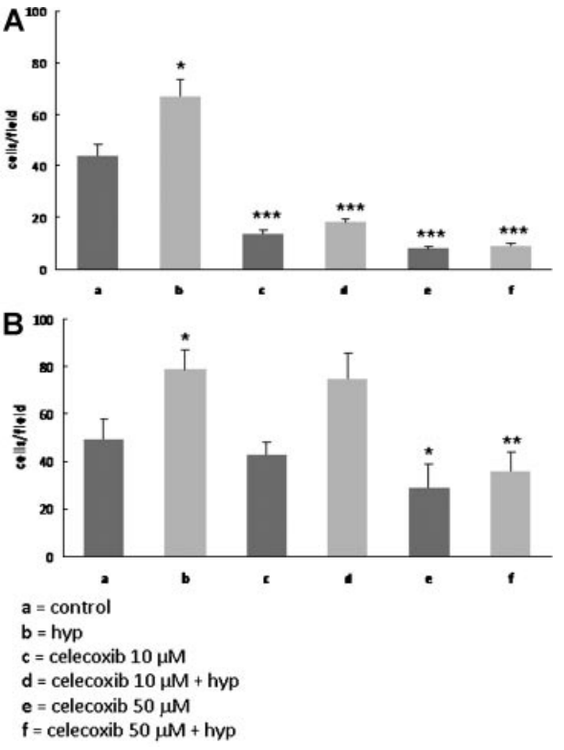

Figure 9. Comparative effects of celecoxib on HT-29 and SW-480 cell invasiveness under normoxia and hypoxia. HT-29 (A) and SW-480 (B) cells were incubated for 24 h under normoxic and hypoxic conditions in the absence (control) or in the presence of celecoxib (10 and 50 μM). Matrigel invasion was evaluated with a Boyden chamber assay; each filter was examined with a Zeiss microscope and the number of cells that penetrated through the matrigel was counted. Data are expressed as number of cells per high-power field and represent the mean of three independent experiments, each performed in triplicate (bars, SD). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ significant difference between treated groups and the control.