UNIVERSITÀ DEGLI STUDI DI TORINO

Bocca C, Bozzo F, Cannito S, Parola M, Miglietta A
Celecoxib inactivates epithelial-mesenchymal transition stimulated by hypoxia and/or epidermal growth factor in colon cancer cells
MOLECULAR CARCINOGENESIS (2012) 51
DOI: 10.1002/mc.20846

CELECOXIB INACTIVATES EPITHELIAL-MESENCHYMAL TRANSITION STIMULATED BY HYPOXIA AND/OR EPIDERMAL GROWTH FACTOR IN COLON CANCER CELLS

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RUNNING TITLE: CELECOXIB, EGF AND EMT IN NORMOXIC AND HYPOXIC COLON CANCER CELLS

KEYWORDS: HT-29/SW-480 CELLS, CELECOXIB, EMT, HYPOXIA, EGF

ABSTRACT

Celecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor, has been reported to exert chemopreventive and antitumour effects on colon cancer, one of the most common solid epithelial malignancy worldwide. The aim of this study was to elucidate whether celecoxib may be able to affect epithelial-to-mesenchymal transition (EMT), a critical process involved in cancer cell invasiveness and metastasis and then proposed to be relevant for cancer progression.

Human HT-29 colon cancer cells were exposed to carefully controlled hypoxic conditions and/or EGF and then investigated for EMT changes and signal transduction pathways involved by using morphological, molecular and cell biology techniques. Celecoxib inhibited basal and EGF-stimulated proliferation, hypoxia-related HIF-1α recruitment/stabilization as well as hypoxia- and EGF-dependent activation of ERK and PI3K. Interestingly, celecoxib prevented EMT-related changes, as shown by modifications of β-catenin intracellular localization or vimentin and E-cadherin levels, as well as HT-29 invasiveness induced by hypoxia, EGF or hypoxia plus EGF. Finally, experiments performed on SW-480 colon cancer cells (i.e., cells lacking COX-2) exposed to hypoxia, used here as a stimulus able to induce EMT and invasiveness, revealed that in these cells celecoxib was ineffective.

Results of the present study indicate that celecoxib has the potential to negatively affect induction of EMT and increased invasiveness of colon cancer cells as elicited by different signals originating from tumour microenvironment (i.e., hypoxia and EGF). Moreover, these effects are likely be related to the pharmacological inhibitory effect exerted on COX-2 activity.

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-to-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 down-regulation of junctional protein E-cadherin responsible for the loss of cell-cell adhesion, and expression of mesenchymal proteins like vimentin and N-cadherin. Furthermore, cells undergoing EMT express matrix metalloprotease enzymes, the primary mechanism accounting for tumour invasion that allows penetration within the stroma surrounding the original tissue.

Functional changes of EMT take place through complex molecular events involving systems and signalling proteins: central in signalling 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 tumour 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 tumour-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 favour tumour 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, HIF-1 is a transcription factor overexpressed in many solid tumours, whose stabilization and activation correlates with tumour metastasis and poor prognosis. HIF-1 might contribute to tumour progression through the promotion of angiogenesis, the activation/induction of proteolitic enzymes or the activation of genes involved in tumour 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 hypoxia-inducible factor-1 (HIF-1) in colorectal tumour cells (13). COX-2 expression is also known to be up-regulated by specific tumour environmental signals, including EGF and hypoxia (14). Moreover, the COX-2/PGE2 pathway is known to affect signalling 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 nonsteroidal anti-inflammatory drug celecoxib has been shown to reduce cell growth in several tumours, but it has been suggested to also operate through additional COX-2 independent, but still unclear, mechanisms (22).

This study has been designed in order to investigate whether celecoxib may significantly affect EMT induction by either hypoxia and/or EGF in colon cancer cell lines and whether any effect might be related to the classic inhibitory action on COX-2 activity.

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. (MO, USA). 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. (CA, USA). Anti-rabbit Cy3-conjugated secondary antibody was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). The Boyden chambers were from Neuro Probe Inc. (MD, USA).

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, USA). The cell lines were grown and maintained in McCoy's 5A (HT-29) and DMEM (SW-480) medium supplemented with 10% foetal 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) 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% (wt/vol) 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 6-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, αSMA, 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).

Invasion assay

Cells were seeded on 6-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 ten 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 signalling pathways.

In a first series of experiments we investigated whether celecoxib was able to interfere with EGF-dependent intracellular signalling pathway. HT-29 cells were cultured for 1h 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 over-expressed 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 factor-induced cell proliferation and additional experiments outlined the fact that changes in cell proliferation were correlated with changes in ERK phosphorylation (figure 1C). Pre-treatment 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 (2-fold) 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 hr 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 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 signalling 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 approximatively 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 up-regulated 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 up-regulation 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 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 6 A,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 hrs 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 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 as well as 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 μM or 50 μM concentration, respectively (figure 9B).

DISCUSSION

Celecoxib is currently used as a chemopreventive and therapeutic drug (25,26) and it has also been reported to display anticancer effects both *in vivo* (27) and *in vitro* in experimental models of colon cancer (23,28). However, no studies have addressed whether celecoxib may affect EMT, which is known to be relevant for cancer progression and metastasis formation. EMT is known to be regulated by signals coming from tumour microenvironment, including hypoxic conditions and several growth factors, cytokines and ECM proteins. In addition, tumours themselves can also release growth factors and proteases that are able to modify tumour microenvironment favouring cell motility and invasiveness.

The main message conveyed by the present study is that celecoxib can efficiently inhibit EMT promoted by EGF and hypoxia in colorectal cancer cells which is likely to be, at least in part, dependent on its classic major molecular mechanism leading to COX-2 inhibition. As far as EGF-induced EMT is concerned, celecoxib seems to act by inhibiting EGFR but literature on this matter is controversial. Indeed, literature data suggest the existence of a relationship between COX-2 and EGFR, since COX-2 has been identified as a putative EGF target gene in intestinal epithelial cells, with robust enhancement of prostaglandin production following EGFR activation (29). Several studies emphasize that colon carcinomas bearing increased COX-2-dependent production of prostaglandins are characterized by a very poor prognosis (30,31); moreover, COX-2-derived PGE2 has been reported to transactivate EGFR (11) which overexpression is also known to be associated with poor survival in patients with colorectal cancer. In agreement with these reports, we found that, celecoxib not only decreased EGFR activation/phosphorylation, but also reduced its expression levels, suggesting that the ability of celecoxib to inhibit EGFR downstream signalling pathways may represent a rather complex event. If this hypothesis applies, celecoxib may act similarly to other anticancer agents that have been proposed to exert their antiproliferative effects by promoting degradation or internalization of EGFR (32,33). Since this effect was also reported in COX-2

deficient SW-480 cells, celecoxib effects on EGFR may rely on COX-2 independent, but still unclear, mechanisms of action (22).

EGF signalling is known to affect critical events, including cell differentiation, adhesion, motility and EMT induction, pointing to its unambiguous roles in tumour progression (34,35). Along these lines, MAPK pathway is known to be a major intracellular way to transduce the signals elicited by EGF (36) and indeed here we showed that celecoxib resulted in a significant decrease of EGF-induced activation of ERK and downstream phosphoproteins of the PI3K/Akt pathways in COX-2 positive HT-29 cells (37). Akt plays a central role in EMT and is crucial for transducing extracellular signals such as EGF. It is positively regulated by phosphatidylinositol 3-kinase and inhibited by phosphatase PTEN. In the present study we provided evidence of an increased PTEN expression in HT-29 cells exposed to celecoxib alone which, however, failed to do the same when the cells were exposed to EGF. Deregulation of the PI3K/PTEN/AkT pathway is one of the most common altered pathways in human malignancy and, along these lines, the regulation of AkT may represent an alternative mechanism of action of celecoxib, although at present we can not offer any definitive rationale explanation for this effect on PTEN.

However, in SW-480 COX-2-negative cells this effect was not observed (data not shown), suggesting an involvement of COX-2 in this pathway. Moreover, it is well known that the state of phosphorylation of ERK and Akt is the result of a balance between the action of upstream kinases and phosphatases, which dephosphorylate and inactivate protein kinases. Along these lines, in HT-29 cells downregulation of the EGF pathway may also be related to an increased expression of PTEN, the negative regulator of PI3K and MAPK activity. In the present study we provided evidence suggesting increased PTEN expression in the presence of celecoxib which, however, failed to do the same in HT-29 cells exposed to EGF. Although these data may suggest that celecoxib-related changes in PTEN expression may contribute to the impairment of EGFR downstream signalling, we can not offer any rationale explanation for this effect on PTEN.

Intratumoral hypoxia is another environmental condition able to induce EMT and increased invasiveness in human cancer cells (4). Hypoxia may also affect tumour-associated stromal cells, and its relevance in the development of colon cancer has been outlined by studies in which hypoxia predicts for poor prognosis (5).

However, a direct mechanistic link between hypoxia, EMT and celecoxib action in colon cancer cells has never been described. To our knowledge, data in this study provide the first evidence on the putative role of celecoxib as negative regulator of hypoxia-induced EMT, with a mechanism that, based on results obtained for SW-480 cells, seems to mostly depend on COX-2inhibition. This statement is also suggested by results related to cell viability which indicate that the consistent

inhibitory action of 50 μM concentration on HT-29 cells may be partly due to toxic effects, whereas the action of 10 μM concentration, which is less effective on viability, is likely to depend mainly on the inhibitory effect exerted by celecoxib on COX-2. On the other hand, in SW-480 COX-2-negative cells the inhibitory effect of both the concentrations of celecoxib on viability is not remarkable, confirming COX-2 inhibition as the main mechanism of action for this drug.

In this connection, it is well known that hypoxia is likely to contribute to cancer progression through adaptative mechanisms involving recruitment/stabilization of hypoxia-inducible transcription factors (HIFs). HIF-1 is overexpressed in many solid tumours and has been proposed to up-regulate genes involved in cell invasiveness and angiogenesis, with an overall scenario in which HIF-1 expression correlates with an increased risk to develop metastasis and a poor prognosis.

Although it is well known that under hypoxic conditions HIF-1α accumulation occurs because of the shutdown of the proteasomal degradation system in the absence of oxygen, it has also been reported that HIF-1α levels may be enhanced in tumor cells as a consequence of the action of several growth factors, including EGF. Indeed, under normoxic conditions, it has been shown that EGFR signalling activates the PI3K/Akt pathway, subsequently increasing HIF-1α levels (38,39).

Although PI3K has many potential downstream targets, we focused our attention on Akt because of several reports outlining the importance of this kinase, which is frequently constitutively active in cancer cells, for its functional role in aggressive, therapy-resistant malignancies (40). Although several drugs are currently investigated for their ability to inhibit Akt signalling, in our hands celecoxib was unlikely to inhibit HIF-1α accumulation by exerting a negative effect on Akt activity. As a matter of fact, although celecoxib treatment resulted in down-regulation of HIF-1α in HT-29 cells under normoxic conditions and this was associated with a slight inhibition of Akt phosphorylation, the drug was unable to prevent Akt activation induced by hypoxia or EGF. This may suggest that, at least under hypoxic conditions, celecoxib may interfere with HIF-1α translation and/or degradation, rather than affect the PI3K/Akt pathway. Moreover, according to previous results published by some of us (4), activation/phosphorylation of Akt under conditions of hypoxia is likely to depend mainly on mitochondrial release of reactive oxygen species. By contrast, EGF is able to activate Akt as a consequence of interaction with its cognate receptor and down-stream activation of the related signaling pathway.

Coming back to EGF-induced EMT, in order to preserve cellular shape and polarity, intracellular domains of cadherins are connected to the actin cytoskeleton through α-catenin and β-catenin, with downregulation of E-cadherin in cancer cells being a critical disruptor of epithelial homeostasis, favouring cell invasiveness (41). Accordingly, EGF has been found to promote disassembly of cell-

cell junctions and downregulation of the E-cadherin cell-cell adhesion molecules (42), a key event for EMT in tumour progression. Loss of E-cadherin expression is considered as a hallmark of EMT and is mainly due to an upregulation of transcription factors that directly repress E-cadherin expression (42). On the other hand, E-cadherin complexes engaged in the intact adherens junction can directly inhibit the activity of the EGFR by inhibiting transphosphorylation of Tyr845 (9). Our results indicate that exposure of untreated or EGF-treated HT-29 cells to hypoxia resulted in a significant reduction of E-cadherin levels, with celecoxib being able to substantially abrogate hypoxia-induced E-cadherin downregulation. On the other hand, celecoxib was unable to counteract hypoxia-induced E-cadherin down regulation in SW-480 cells, suggesting that the effect of the drug on hypoxia-induced down-regulation of E-cadherin in HT-29 cells may be mediated mostly through inhibition of COX-2. During EMT, the decrease in epithelial traits is accompanied by a parallel increase in other mesenchymal markers, including α-SMA and fibronectin (43). Furthermore, a "cadherin switch" has been described, whereby E-cadherin loss is accompanied by gain of N-cadherin (44) or vimentin and indeed it has been reported that vimentin expression is, in colon and other cancers, correlated closely with increased invasiveness and poor prognosis (45). In the present study we provide evidence that celecoxib can induce a decrease in vimentin overexpression in HT-29 cells undergoing EMT following exposure to EGF and/or hypoxia.

In addition, endocytosis of E-cadherin is known to result in a mobilization of β-catenin that can then act on the Wnt pathway, resulting in further E-cadherin repression (9). It has been reported that in tumour cells β-catenin is aberrantly activated and acts as a tumour-promoting factor, and HIF-1α has been proposed to inactivate β-catenin by sequestering it under hypoxia (46). Furthermore, many cancer cells express both HIF-1α and HIF-2α in response to hypoxia and it has been recently proposed that HIF-1α/HIF-2α balance may contribute to cell growth regulation when hypoxia and Wnt stimulation coexist (47). We found that celecoxib reduced β-catenin nuclear accumulation and increased membrane-bound β-catenin, independently on hypoxic conditions. These results suggest that in addition to its role in COX-2 inactivation, celecoxib may contribute to inhibit cancer cell growth by modulating β-catenin signalling.

Furthermore, hypoxia has been described to induce migration and invasiveness in different cell lines (48) and indeed invasiveness of both HT-29 and SW-480 cells increased under hypoxia. Under both normoxic and hypoxic experimental conditions celecoxib significantly inhibited invasiveness at both 10 and 50 μM concentrations only in COX-2 positive HT-29 cells but, of relevance, not in COX-2 deficient SW-480 cells treated with the 10 μM concentration. According to viability data, the modest effect of 50 μM celecoxib on SW-480 hypoxia-induced invasiveness may be partly related to some cytotoxicity.

The overall final message from this study indicates then that celecoxib can act as a drug able to prevent EMT and invasiveness of colon cancer cells as stimulated by either EGF or by exposure to hypoxia. Moreover, as indicated by the comparison of data on hypoxia-related changes obtained in HT-29 versus SW-480 cells, these inhibitory actions on EMT and invasiveness are likely to be at least in part dependent on the usual inhibitory mechanism on COX-2 activity.

Acknowledgements

The financial support was received from Regione Piemonte and University of Torino.

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FIGURE LEGENDS

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Fig. 1. Effect of celecoxib and EGF on HT-29 cell proliferation and on EGFR downstream signalling 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. * $p<0.05$, ** $p<0.001$ *vs* 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.

Fig. 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. * $p<0.05$, ** $p<0.001$ *vs* 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.

Fig. 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 24h 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.

Fig. 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× final magnification) and are representative of three independent experiments.

Fig. 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.

Fig. 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.

Fig. 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.

Fig. 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 *vs* 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.

Fig. 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.