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ORIGINAL ARTICLE

Transcriptional activation of cyclooxygenase-2 by tumor suppressor p53 requires nuclear factor-kappaB

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Overexpression of cyclooxygenase-2 (Cox-2) is thought to exert antiapoptotic effects in cancer. Here we show that the tumor suppressor p53 upregulated Cox-2 in esophageal and colon cancer cell lines by inducing the binding of nuclear factor-kappaB (NF-kappaB) to its response element in the *COX-2* promoter. Inhibition of NF-kappaB prevented p53 induction of Cox-2 expression. Cooperation between p53 and NF-kappaB was required for activation of *COX-2* promoter in response to daunomycin, a DNA-damaging agent. Pharmacological inhibition of Cox-2 enhanced apoptosis in response to daunomycin, in particular in cells containing active p53. In esophageal cancer, there was a correlation between Cox-2 expression and wild-type *TP53* in Barrett's esophagus (BE) and in adenocarcinoma, but not in squamous cell carcinoma ($P<0.01$). These results suggest that p53 and NF-kappaB cooperate in upregulating Cox-2 expression, promoting cell survival in inflammatory precursor lesions such as BE.
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Introduction

Cyclooxygenase-2 (Cox-2) catalyses the synthesis of prostaglandins, prostacyclins and thromboxanes in inflammatory and transformed cells, and in malignant tissues (Hla *et al*., 1999; Subbaramaiah and Dannenberg, 2003). Expression of Cox-2 is induced by physiological and stress signals including growth factors, cytokines, mediators of inflammation, tumor promoters, oxidizing agents and DNA-damaging agents such as γ-irradiation, UV-rays or anticancer drugs (Das and White, 1997; Rockwell *et al*., 2004; Wu *et al*., 2005). The 5′ flanking region of the *COX-2* gene contains several consensus response elements (REs), four of which play documented roles in Cox-2 expression: c-AMP response element (CRE), nuclear factor interleukin 6, (NF-IL6), E-box and nuclear factor-kappaB (NF-kappaB)-binding motifs (Tanabe and Tohnai, 2002; Zhu *et al*., 2002). Alone or in combination, these REs control *COX-2* expression through multiple signaling cascades, making it an important component in the response to inflammatory and cytotoxic stress.

Enhanced Cox-2 activity and synthesis of prostaglandins (PGs) stimulate proliferation, angiogenesis, invasiveness and inhibit apoptosis (reviewed in Tsuji *et al*., 2001). Pharmacological Cox-2 inhibitors reduce experimental tumor growth (Harris *et al*., 2000; Oshima *et al*., 2001) and the number of intestinal tumors in patients with familial adenomatous polyposis (Steinbach *et al*., 2000). Overexpression of Cox-2 is common in cancers of the digestive tract (esophageal squamous cell and adenocarcinoma, gastric, intestinal and colorectal cancers) (Eberhart *et al*., 1994; Zimmermann *et al*., 1999; Buskens *et al*., 2003; Smith *et al*., 2005; Birkenkamp-Demtroder *et al*., 2005). In esophageal cancers, different patterns of expression are observed in squamous cell carcinomas (SCC) and adenocarcinomas (ADC). Cyclooxygenase-2 expression is low in normal, squamous mucosa, increases in squamous dysplasia but remains low in most SCC (Shamma *et al*., 2000; Yu *et al*., 2003). In contrast, Cox-2 is often expressed in ADC and is already detected in Barrett's mucosa, the precursor lesion in which squamous cells are replaced by intestinal-type cells as the consequence of chronic gastric acid and bile reflux (Kaur and Triadafilopoulos, 2002; Lagorce *et al*., 2003).

Many signals that activate Cox-2 also induce the tumor suppressor p53, a transcription factor that accumulates after post-translation modification in response to DNA damage, inducing antiproliferative responses such as cell cycle arrest, DNA repair or

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apoptosis (Sengupta and Harris, 2005). In 1999, Subbaramaiah *et al.* (1999) reported that expression of p53 at high levels in mouse fibroblasts repressed Cox-2, and proposed that p53 may compete with TATA-binding proteins for binding to the *COX-2* promoter. More recently, Han *et al.* (2002) demonstrated an opposite effect, with namely a physiological, p53-dependent increase in Cox-2 expression in response to DNA damage. Dominant-negative *RAS* or *RAF1* mutants suppressed p53-mediated expression of Cox-2, suggesting an indirect mechanism (Han *et al.*, 2002). However, how p53 activates this mechanism is unknown. In this study, we have investigated the role of NF-kappaB in p53-dependent activation of Cox-2 in response to DNA damage, and we have analysed the correlations between *TP53* gene mutation status and Cox-2 expression in esophageal ADC and SCC.

Results

Upregulation of cycloxygenase-2 expression by p53

To demonstrate that p53 induces Cox-2 expression, increasing amounts of a p53 expression vector (pC53-SN3) were transfected in the colorectal ADC cell line HCT116, together with fixed amounts of *COX-2*-LUC, a reporter construct containing a 1 kb segment of *COX-2* promoter encompassing major functional REs in the 5′ regulatory region of *COX-2* (Gilroy *et al.*, 2001). This fragment contains consensus sites for NF-kappaB, but no identified p53-binding site. A sevenfold increase in luciferase activity was observed with 1 μg of transfected p53 vector (Figure 1a, left panel). Stable expression of the HPV16 E6 viral oncoprotein, which destabilizes and eliminates p53 function, abrogated induction of luciferase activity upon transfection of p53 (Figure 1a, middle panel). Transfection of p53 also increased Cox-2 expression in TE-13, a p53-deficient esophageal cancer cell line (Figure 1a, right panel), as well as in other cancer cell lines with different p53 functional status (HCT15, CACO-2, HT29, Saos-2; data not shown). Next, we generated a stable HCT116 line conditionally expressing p53 in the presence of tetracyclin (HCT116-p53ind). Western blot showed that tetracycline-mediated p53 induction enhanced Cox-2 levels in HCT116-p53ind and that the increase in Cox-2 protein paralleled both p53 accumulation and induction of p21^{waf-1}, the product of a typical p53 target gene (Figure 1b). To further show that p53 could induce Cox-2 enzymatic activity, we used TE-1, a cell line derived from a squamous esophageal carcinoma, containing a temperature-sensitive TP53 mutant (Val to Met at codon 272, V272M). Culture at 32°C (in conditions where V272M preferentially adopts a 'wild-type' conformation) resulted in a strong increase (12-fold) in the level of *COX-2* transcripts (Figure 1c, left and middle panels), correlated with a fourfold increase in prostaglandin E_2 (PGE_2) synthesis (Figure 1c, right panel). Taken together, these data demonstrate that p53 regulates Cox-2 expression in cell lines derived from cancers of upper (TE-1, TE-13) or lower (HCT116, HCT15, CACO-2, HT-29) digestive tract.

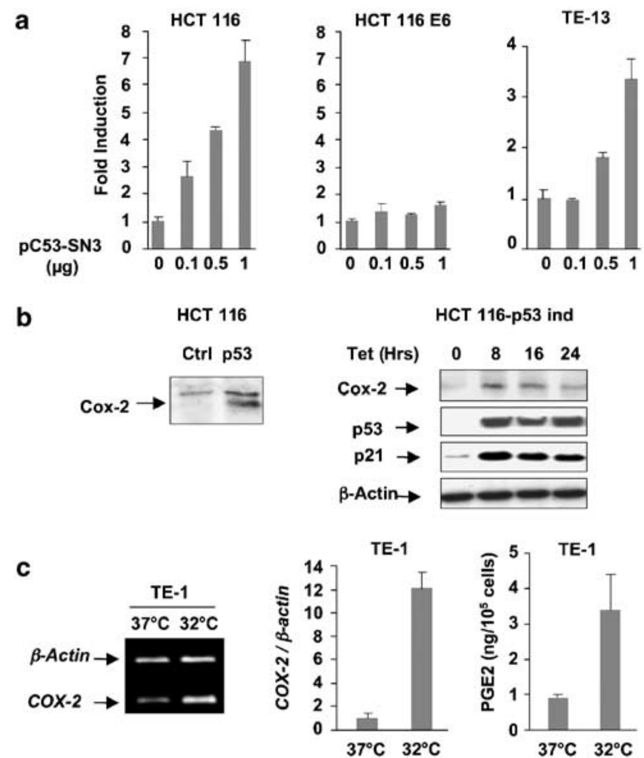
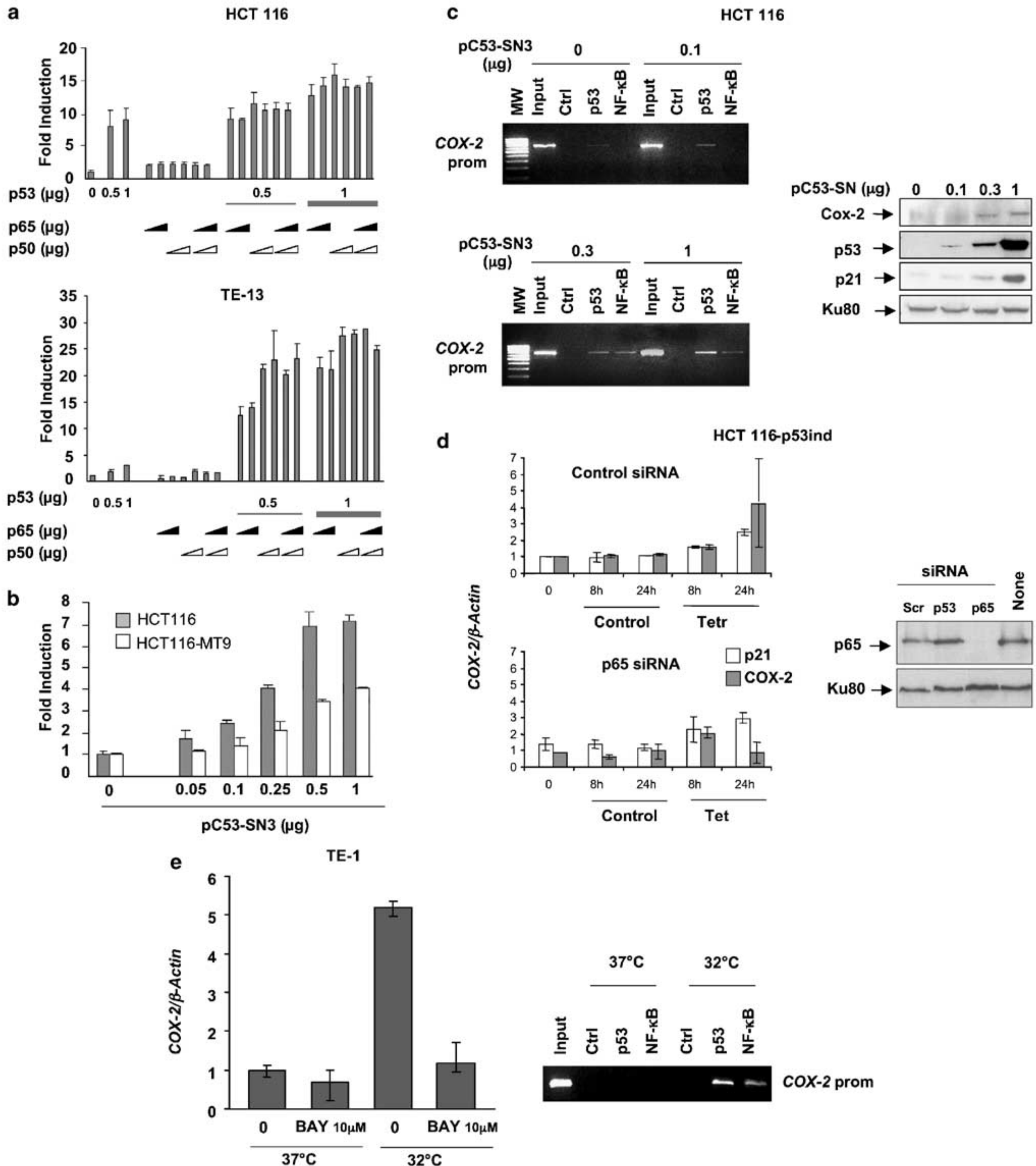


Figure 1 Upregulation of cyclooxygenase-2 (Cox-2) expression by p53. (**a**) p53-dependent induction of a COX-2 reporter construct. Cells were co-transfected with *COX-2*-LUC reporter, increasing amounts of an expression vector for p53 protein (pC53-SN3), and RSV-βGAL reporter as internal transfection standard. LUC activity was normalized to βGAL and expressed as fold induction relative to the activity observed with *COX-2*-LUC reporter alone. (**b**) Increased Cox-2 expression after transfection of p53. Left, HCT116 cells were transfected either with empty vector (pcDNA3, CTRL) or with p53 expression vector (pC53-SN3, p53). Right, a tetracyclin-inducible p53 HCT116 cell line (tet-on) was used (p53-ind). Cells were treated with tetracyclin (1 mg/ml) for 8, 16 and 24 h and levels of p53, Cox-2 and p21^{waf-1} were detected by Western blot using β-actin as a loading control. (**c**) Enhanced COX-2 expression and enzymatic activity in cells expressing a temperature-sensitive p53 mutant. TE-1, a cell line expressing the temperature-sensitive p53 mutant V272M, was used. Left, reverse transcription reverse transcripition–polymerase chain reaction (RT–PCR) detection of *COX-2* mRNA in cells cultured at 37°C (p53 mostly in 'mutant', inactive form) and 32°C (p53 mostly in 'wild-type', active form). Middle, real-time (TaqMan) RT–PCR analysis of *COX-2* expression levels. Right, PGE_2 production at both temperatures.

Requirement of nuclear factor-kappaB for transactivation of cycloxygenase-2 by p53

Nuclear factor kappaB is an important transcriptional regulator of *COX-2* in response to inflammatory signals (Jobin *et al.*, 1998; Konturek *et al.*, 2004). To determine whether NF-kappaB was involved in *COX-2* promoter regulation by p53, HCT116 and TE-13 were transfected with *COX-2*-LUC together with increasing amounts of

expression vectors for NF-kappaB p65 and/or p50, with or without pC53-SN3 expression vector (Figure 2a). Transfection of NF-kappaB subunits alone had only minor effects on luciferase activity in HCT116 cells, whereas co-transfection of NF-kappaB and p53 almost doubled the effect of p53 alone (from 7–8-fold with p53 alone to 14–15-fold with p53 and 1 μg of either p65 or p50 vectors, or both). In TE-13, co-transfection of p53 and NF-kappaB components had overmultiplicative effects (from fourfold and twofold with either p53 or NF-kappaB alone, respectively, to over 25-fold with both factors). Both p50 and p65 showed cooperative effects with p53. Next, we used the HCT116-MT9 cells stably expressing a dominant-negative mutant of I-kappaB-α (Bentires-Alj *et al.*, 1999b, 2001). Transient co-transfection of p53 expression vector and *COX-2*-



LUC reporter showed that luciferase activity was reduced by about 50% in HCT116-MT9 as compared to control cells (Figure 2b), indicating that NF-kappaB was required to mediate at least part of p53 effects on *COX-2* promoter. To verify that NF-kappaB was recruited to *COX-2* promoter during *COX-2* induction by p53, we performed chromatin immunoprecipitation (ChIP) assays in HCT116 cells transfected with increasing amounts of p53 expression vector (Figure 2c). In control cells (transfected with an empty vector), neither binding of NF-kappaB nor p53 was detected. Upon transfection of p53, a dose-dependent increase in the level of p53 bound to the promoter was observed, and was accompanied by an increase in NF-kappaB binding. Interestingly, binding of NF-kappaB was detectable only after transfection of 0.3 and 1 μg of p53 vector, whereas p53 binding was detectable with only 0.1 μg of transfected vector. Furthermore, the proportion of input signal associated with p53 was consistently higher than with NF-kappaB. These results suggest that p53 binding helps the binding of NF-kappaB to the *COX-2* promoter.

To confirm the role of NF-kappaB in the induction of Cox-2 by p53, we inhibited NF-kappaB in HCT116-p53ind cells by using small interfering (si) RNA (Figure 2d). In cells transfected with a control siRNA, induction of p53 with tetracyclin resulted in increased expression of both *WAF-1* and *COX-2* mRNA. In cells transfected with p65 siRNA, the increase in *COX-2* expression, but not in *WAF-1* expression, was inhibited. Finally, we also used BAY117082, a chemical inhibitor of NF-kappaB, in TE-1 cells cultured at 32 or 37°C. This inhibitor induced a drastic reduction in levels of *COX-2* mRNA in cells cultured at 32°C but had no effect at 37°C (Figure 2e). Chromatix immunoprecipitation assays in TE-1 cells at 32°C (active p53) and 37°C (inactive p53) confirmed the presence of both p53 and NF-kappaB onto *COX-2* promoter in cells cultured at 32°C, but not at 37°C. Overall, results in Figure 2 indicate that NF-kappaB is required for activation of *COX-2* promoter by p53. The two factors cooperate for transactivation of *COX-2* promoter, with effects ranging from additive to multiplicative, depending on cellular context.

Effects of mutant p53 on cycloxygenase-2 expression

As the *COX-2* promoter does not contain a canonical p53-binding site, the binding of p53 to the *COX-2* promoter may not obey the same structural constraints than binding to its cognate consensus. As a first step to test this hypothesis, we examined whether mutant p53 may also upregulate Cox-2 expression. HCT116 cells were transfected with three *TP53* mutants, selected as representative of different classes of mutants (Figure 3a). A significant increase in Cox-2 expression was observed with R248Q (DNA contact mutant) and V272M (temperature-sensitive mutant), whereas R175H (conformation mutant) showed only minimal effects. Quantitative analysis of *COX-2*-LUC reporter gene expression confirmed that R248Q and V272M retained partial activity, with *COX-2*-LUC induction levels of about 50% of that of wild-type p53 (Figure 3b, upper panel). However, when transfected into p53-deficient, HCT116-p53−/− cells, these mutants did not show any capacity to increase Cox-2 expression (Figure 3b, lower panel). Similar results were obtained in another p53-deficient cell line, Saos-2 (data not shown). Thus, the effects of mutant p53 on *COX-2* promoter appear to depend on the presence of wild-type p53. Mutant p53 may act by forming complexes with endogenous wild-type p53. Depending on the type of mutant, these complexes may retain a degree of *COX-2* activating capacity. In this respect, it is interesting to note that R175H, the most destructured mutant used here, is the less effective in inducing Cox-2 expression.

p53 is required for cycloxygenase-2 induction by daunomycin, a DNA-damaging agent

We next treated HCT116 cells with daunomycin, a topoisomerase inhibitor that generates strand break damage and induces p53 stabilization and accumulation. Daunomycin induced accumulation of endogenous wild-type p53 and increased Cox-2 protein levels in HCT116, but not in HCT116-E6 cells (Figure 4a). Experiments using the *COX-2*-LUC reporter confirmed that elimination of p53 by E6 almost totally prevented *COX-2* promoter activation in response to daunomycin (Figure 4b). Chromatix immunoprecipitation showed

Figure 2 Requirement of (NF-kappaB) for transactivation of *COX-2* by p53. (**a**) Effect of NF-kappaB protein components on COX-2 promoter activation by p53. Cell lines HCT116 (upper panel) and TE-13 (lower panel) were transfected with *COX-2*-LUC reporter and increasing amounts of p53, p65 and p50 expression vectors alone or in combination, and RSV-βGAL as transfection standard. LUC activity was expressed as fold induction relative to the activity observed with reporter alone, normalized to βGAL. (**b**) A dominant-negative NF-kappaB inhibitor reduces COX-2 promoter activation by p53. HCT116 and HCT116 MT9 (expressing a dominant-negative IkappaBalpha mutant) were transfected with *COX-2* LUC reporter, increasing amounts of p53 expression vector (pc53-SN3) and RSV-βGAL as transfection standard. (**c**) Association of p53 and NF-kappaB with the COX-2 promoter. HCT116 cells were transfected with pC53-SN3 (0.1, 0.3, 1 μg). Left, cross-linked chromatin was immunoprecipitated with anti-p53, anti-NF-kappaB antibodies or control antibody as negative control, and analysed by polymerase chain reaction (PCR) with primers specific for *COX-2* promoter. MW: molecular weight markers. Input: non-precipitated cross-linked chromatin. Right, Western blot analysis showing protein levels of Cox-2, p53 and p21 in nontransfected and transfected HCT116, using Ku80 as loading control. (**d**) Inhibition of NF-kappaB p65 by siRNA prevents p53-dependent induction of COX-2 expression. Left panel: HCT116-p53ind cells were transfected with control or with p65 siRNA, followed by tetracycline treatment. *WAF-1* and *COX-2* mRNA expression was determined by real-time reverse transcription-PCR (RT–PCR) as in Figure 1c. Right panel: Western blot analysis of p65 expression in HCT116 cells transfected with control, p53 and p65 siRNA, or not transfected (none). Ku80: loading control. (**e**) The NF-kappaB inhibitor BAY117082 decreases p53-dependent COX-2 expression. Upper panel: TE-1 cells cultured at 37°C were switched to 32°C, treated with BAY117082 (10 μM) for 12 h, and *COX-2* mRNA expression was determined by real-time RT–PCR as in Figure 1c. Lower panel: cross-linked chromatin was immunoprecipitated with anti-p53, anti-NF-kappaB antibodies or control antibody as negative control, and analysed by PCR as in (**c**).

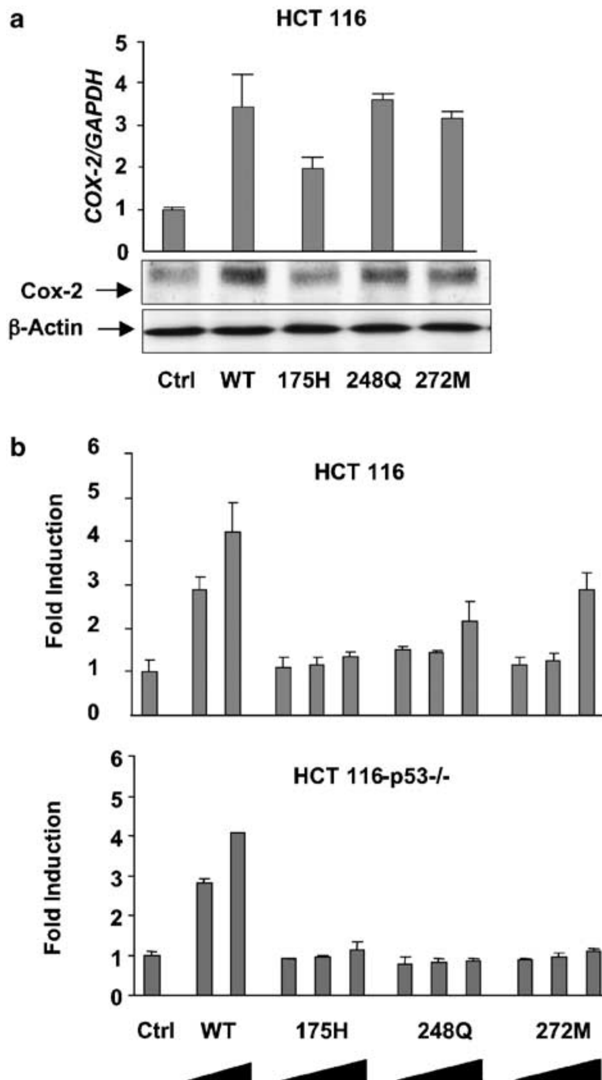


Figure 3 Effect of mutant p53 on cyclooxygenase-2 (Cox-2) expression. (**a**) Induction of Cox-2 expression by mutant p53. HCT116 cells were co-transfected with increasing amounts of wild-type (WT) or mutant p53 expression vectors. Top, *COX-2* mRNA was quantitated by real-time PCR using *GAPDH* as internal control. Bottom, cell extracts were analysed by Western blot with anti-Cox-2 antibody using β-actin antibody as loading control. (**b**) Wild-type p53-dependent effect of mutant p53 on COX-2 promoter activation. HCT116 (upper panel) or HCT116-p53−/− cells were co-transfected with *COX-2*-LUC reporter, control (Ctrl) or increasing amounts of WT or mutant p53 expression vectors (0.1, 0.3, 1 μg), and RSV-βGAL as internal transfection control. LUC activity was expressed as fold induction normalized to βGal.

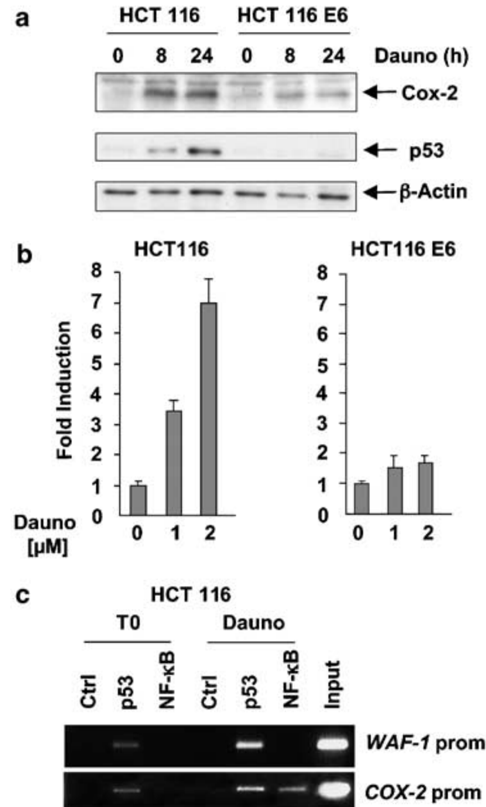


Figure 4 p53-dependent induction of cyclooxygenase-2 (Cox-2) in response to daunomycin. (**a**) p53-dependent increase in Cox-2 protein after treatment with daunomycin. HCT116 and HCT116 E6 cells were treated with daunomycin (2 μM, for indicated times). Cyclooxygenase-2 and p53 proteins were detected by Western blot using β-actin as loading control. (**b**) p53-dependent COX-2 promoter activation in response to daunomycin. HCT116 and HCT116 E6 cells were co-transfected with *COX-2*-LUC reporter and RSV-βGAL as transfection control. Six hours after transfection, cells were exposed to daunomycin (24 h). LUC activity was expressed as fold induction normalized to βGAL. (**c**) Recruitment of p53 and NF-kappaB on COX-2 promoter in response to daunomycin. Cross-linked chromatin from HCT116 cells exposed or not exposed to daunomycin (1 μM, 24 h) was immunoprecipitated with antibodies to p53, NF-kappaB or control antibody as negative control, and analysed by polymerase chain reaction with primers for *WAF-1* and *COX-2* promoter sequences. Input: non-precipitated cross-linked chromatin.

that daumomycin induced both p53 and NF-kappaB to bind to the *COX-2* promoter, whereas only p53 binding was detected on the *WAF-1* promoter (Figure 4c). These results indicate that p53 is required for induction of Cox-2 by daunomycin through the binding of NF-kappaB to the *COX-2* promoter.

Cycloxygenase-2 expression protects cells against daunomycin-induced apoptosis

We used a panel of Cox-2 inhibitors to examine the effects of Cox-2 inhibition on proliferation and survival after treatment with daunomycin. First, HCT116 cells were treated with increasing amounts of nimesulid before treatment with daunomycin. In the absence of daunomycin, nimesulid had minor effects on proliferation and survival. However, in the presence of daunomycin, nimesulid strongly reduced proliferation in a dose-dependent manner (Figure 5a, upper left panel). To verify that this effect was due to increased apoptosis, we measured caspase-3 activity in HCT116 treated with nimesulid or with celecoxib and rofecoxib, two inhibitors of the coxib family. Results showed an increase of caspase-3 activity with all three Cox-2 inhibitors in cells treated with daunomycin, but not in untreated cells (Figure 5a, upper right panel). Treatment with nimesulid also induced a dose-dependent increase in Poly (ADP-ribose) polymerase (PARP) cleavage product, an

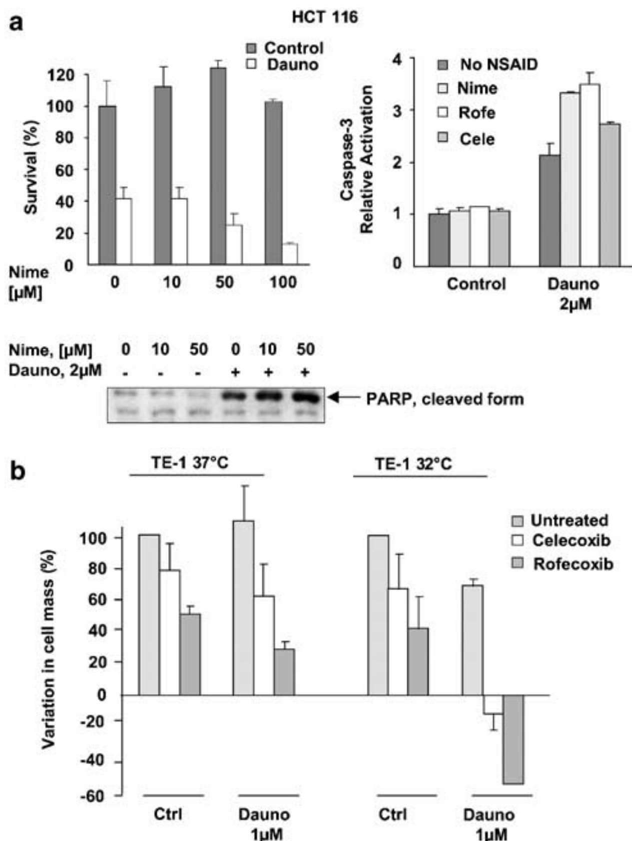


Figure 5 Cyclooxygenase-2 (Cox-2) expression protects cells against daunomycin-induced apoptosis. (**a**) Increased daunomycin-induced apoptosis in HCT116 in the presence of Cox-2 inhibitors. Left, HCT116 cells were treated with the indicated amount of nimesulid before exposure to daunomycin (2 μM, 24 h). Survival, assessed by WST-1 colorimetric assay, was expressed as percentage of the average value observed with untreated cells. Right, HCT116 cells were pre-treated with nimesulid (50 μM), celecoxib (10 μM) or rofecoxib (2 μM) before being exposed or not exposed (Ctrl) to daunomycin treatment (2 μM, 24 h). Cell extracts were incubated with a fluorogenic caspase-3 substrate, and relative caspase activation was expressed by normalizing fluorescence to the level measured in untreated cells. Bottom: Detection of PARP cleavage product in HCT116 treated with nimesulid before exposure to daunomycin (2 μM, 24 h). Cell lysates were analysed by Western blot with anti-cleaved PARP antibody. (**b**) Enhanced proapoptotic effects of Cox-2 inhibitors in TE-1 cells cultured at 32°C. TE-1 cells were grown at 37°C or 32°C, treated with celecoxib (10 μM) or rofecoxib (2 μM) before exposure to daunomycin (1 μM, 24 h). Results were expressed by scoring the percentage of increase (positive values) or decrease (negative values) in global cell mass as measured using the sulforhodamine B assay, with 0 representing no change in global cell mass over the duration of treatment (30 h), positive values representing net cell growth, and negative values indicating a net decrease in cell mass owing to massive cell death.

intracellular marker of caspase-3 activation (Figure 5a, lower panel). Next, we assessed the effects of the two coxibs on the proliferation of TE-1 at 37 and at 32°C (Figure 5b). At 37°C, only rofecoxib had a significant effect on proliferation, with a reduction of 50% in non-treated and 70% in cells treated by daunomycin. The effect of celecoxib was less marked and barely reached statistical significance in cells treated with daunomycin. At 32°C, however, both coxibs induced a clear decrease in cell number owing to massive apoptosis in daunomycin-treated cells. Morphological examination of cells treated by coxibs and daunomycin confirmed the presence of many cells exhibiting fragmented nucleus as well as other typical features of apoptosis (data not shown).

Cycloxygenase-2 expression in relation with TP53 mutation in esophageal cancers

Cycloxygenase-2 expression levels were analysed by immunohistochemistry in two forms of esophageal cancers with known *TP53* mutation status, namely SCC (80 cases) and ADC (61 cases). When available, adjacent, non-cancer mucosa was also examined. A total of 67 mutations were detected (38/80 in SCC, 48%; 29/61 in ADC, 48%; see list of mutations in supplementary data). No expression of Cox-2 was detected in squamous epithelium adjacent to SCC (Figure 6a, panel 1). High Cox-2 expression (high Cox-2 cases, defined as intensity score 2 or 3 in at least 20% of cancer cells) was rare in low- or high-grade dysplasia (Figure 6a, panel 2) and was observed in 11% of SCC (9/80) (Figure 6a, panel 3) In contrast, intestinal (Barrett's) metaplasia, the precursor

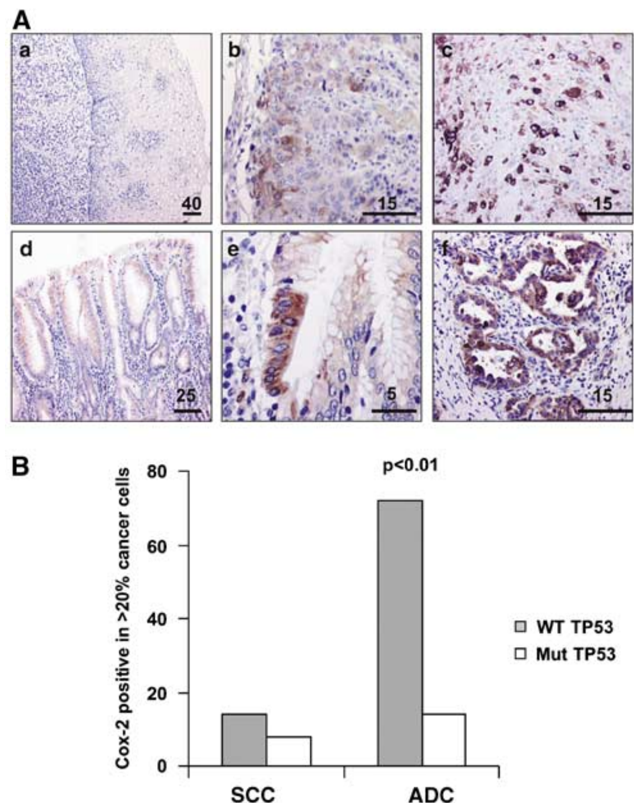


Figure 6 Expression of cyclooxygenase-2 (Cox-2) in esophageal cancers. (**a**) Cyclooxygenase-2 immunostaining in representative sections of normal squamous mucosa (1), squamous dysplasia (2), *in situ* squamous cell carcinoma (SCC) (3), Barrett's mucosa (4, 5) and adenocarcinoma (ADC) (6). Bars: 10 μM. Note the absence of staining in normal squamous mucosa (1) and patchy staining in Barrett's mucosa (5). (**b**) Correlation between Cox-2 immunopositivity in cancer and TP53 mutation status. Histograms show, for SCC and ADC, the proportion of tumors with or without *TP53* mutation that express Cox-2 in over 20% of cancer cells. p: Fisher's exact test for Cox-2 expression in ADC, mutant vs wild-type *TP53*.

lesion for ADC, frequently displayed patches of highly positive Cox-2 cells in intestinal (Figure 6a, panels 4 and 5), and 44% of ADC (27/61) expressed high levels of Cox-2, detectable in over 50% of cancer cells in 16/27 cases (Figure 6a, panel 6). Quantitative reverse transcription–polymerase chain reaction (RT–PCR) was performed in tumor and adjacent non-involved tissue in six high Cox-2 cases. The average increase in *COX-2* mRNA in tumor tissue was 26.7-fold (range 5–100-fold) (data not shown). In ADC, but not in SCC, there was a significant correlation between high Cox-2 and wild-type *TP53* status. Among cases with wild-type *TP53*, 72% expressed high Cox-2, compared to 14% in cases with mutant *TP53* ($P<0.01$, Fisher's exact test) (Figure 6b). Thus, a correlation between *TP53* status and *COX-2* expression was found in ADC, but not in SCC, suggesting that the participation of p53 in deregulation of *COX-2* expression in cancer is different according to tumor histology and natural history.

Discussion

p53 regulates cycloxygenase-2 expression through nuclear factor-kappaB

The results presented here support the notion that p53 is a physiological regulator of *COX-2* expression in response to the anticancer drug daunomycin, used here as a prototypic DNA-damaging agent. These results also indicate that p53 regulates *COX-2* promoter through an indirect mechanism involving the binding of NF-kappaB to the *COX-2* promoter. This interpretation is supported by the following observations: first, p50 and/or p65 of NF-KappaB cooperates with p53 for activation of a *COX-2* reporter gene, with additive to synergistic effects, depending upon cell line; second, inhibition of NF-kappaB by a dominant-negative I-kappaB-α mutant significantly reduces activation of *COX-2* by p53; third, after transfection of p53, both p53 and NF-kappaB bind to *COX-2* promoter, despite the absence of a canonical p53-binding site; fourth, inhibition of NF-kappaB using a specific siRNA prevented the expression of *COX-2*, but not *WAF-1* RNA after p53 induction; fifth, a chemical inhibitor of NF-kappaB, BAY117082, decreases p53-dependent induction of *COX-2* expression in a cell line expressing a temperature-sensitive p53 mutant. These results are compatible with those of Han *et al*. (2002) showing that p53 upregulates Cox-2 expression by an indirect mechanism (Han *et al*., 2004). However, how p53 and NF-kappaB interact at the molecular level remains to be determined.

Mechanisms of p53–nuclear factor-kappaB cooperation

Previous studies have shown that p53 and NF-kappaB can cooperate in the regulation of gene expression, however, with conflicting results. Webster and Perkins (1999) observed that both p53 and NF-kappaB inhibited each other's ability to stimulate gene expression and that this process was controlled by the relative levels of each factor (Webster and Perkins, 1999). In contrast, Ryan *et al*. (2000) reported that induction of p53 caused activation of NF-kappaB and that this phenomenon correlated with the ability of p53 to induce apoptosis (Ryan *et al*., 2000). We previously showed an additive effect between NF-kappaB subunits and p53 for the transcriptional activation of *TP53* (Benoit *et al*., 2000). Bohuslav *et al*. (2004) described that p53 expression stimulated the S6 ribosomal serine/threonine kinase (RSK1), which in turn phosphorylated and activated the p65/RelA subunit of NF-kappaB (Bohuslav *et al*., 2004). Recently, Corcoran *et al*. (2005) reported that p53 upregulated Cox-2 expression, which in turn physically interacts with p53 and inhibits p53-dependent transcription (Corcoran *et al*., 2005). A mechanism for transcriptional inactivation of p53 by products of Cox-2 activity has been proposed by Swamy *et al*. (2003), who observed that electrophilic PGs caused wild-type p53 accumulation in the cytosol and inhibition of p53-dependent apoptosis (Swamy *et al*., 2003). In HTLV-I Tax-expressing cells, Jeong *et al*. (2005) detected an interaction between p53 and p65/RelA inhibiting p53 transcriptional activity in the absence of NF-kappaB transcriptional activity. Using ChIP assays, they showed that in the presence of Tax, both p53 and NF-kappaB colocalized within the promoter of the p53-responsive gene *MDM2* and repressed its expression. Tax was unable to inhibit p53 in p65−/− mice, indicating that this effect was NF-kappaB dependent. On the other hand, this effect was not seen in the absence of Tax, when both NF-kappaB and p53 were induced (Jeong *et al*., 2005). In the present study, we also describe that p53 and NF-KappaB can colocalize within a promoter and cooperate in the control of its expression. Thus, our results show an intriguing symmetry with those of Jeong *et al*. (2005). These observations suggest that there may be different modes of cooperation between NF-kappaB and p53, in which each factor can recruit the other to promoters that contain a response element for only one of them, resulting in either activation or inhibition of transcription, depending on the promoter and the cellular context. The nature of effects (from repression to activation with additive or multiplicative effects) may depend on how factors synergize or compete in recruiting co-activators such as CBP/p300, in modifying local chromatin complex and in modulating the assembly of transcription initiation complexes. Given the wide and overlapping range of biological activities of p53 and NF-kappaB, cooperation between the two factors may represent a powerful mechanism in the regulation of the fine balance between pro- and anti-apoptotic mechanisms controlled by either factor. This may be of particular relevance in response to DNA damage during chronic inflammation, in conditions where both factors are activated.

Role of p53 in COX-2 activation in response to DNA damage

Many stress stimuli activate both p53 and NF-kappaB, including oxidizing agents such as peroxide and many pleiotropic DNA-damaging stresses such as ionizing or

UV-irradiation (Devary *et al*., 1993; Meyer *et al*., 1993; Li and Karin, 1998), making it difficult to identify the contribution of specific pathways to *COX-2* activation. The novelty of the data presented here resides in the demonstration that NF-kappaB is required for *COX-2* activation by p53 in response to DNA damage by daunomycin, a prototypic DNA-damaging agent primarily activating the p53 pathway. With agents having a wider range of cellular effects, such as hydrogen peroxide, increase in *COX-2* expression was observed in p53-competent as well as in deficient cells (data not shown), reflecting the capacity of this agent to activate NF-kappaB through DNA damage-independent pathways (Meyer *et al*., 1993). We suggest that p53 may provide a plug-in connecting NF-kappaB with DNA damage signaling pathways. Thus, p53 may not be required for activation of Cox-2 expression in response to a number of physiological stimuli. Further studies comparing DNA-damaging and non-damaging signals are required to identify signals that induce p53 to cooperate with NF-kappaB and the nature of post-translational modifications involved.

Cycloxygenase-2 inhibitors, TP53 mutations and drug-induced apoptosis

The proapoptotic effects of Cox-2 inhibitors are well documented (Sawaoka *et al*., 1998; Jiang *et al*., 2002). In non-small-cell lung cancer cell lines, genetic or pharmacological inhibition of Cox-2 enhances proteosomal degradation of the inhibitor of apoptosis survivin (Beltrami *et al*., 2004; Krysan *et al*., 2004a, b). In turn, depletion of survivin causes arrest of DNA synthesis owing to activation of p53 and $p21^{Waf-1}$ (Beltrami *et al*., 2004; Yang *et al*., 2004). Thus, in tumor cells with wild-type p53, inhibition of Cox-2 may result in increased p53-dependent growth suppression. In agreement with this notion, we found that Cox-2 inhibitors sensitized cultured cells to apoptosis induced by daunomycin, and that this effect was more marked in cells that retained active p53. In particular, the proapoptotic effects of coxibs were greater in TE-1 cells at 32°C (expressing predominantly p53 in an active, wild-type-like form) than at 37°C (expressing predominantly mutant, inactive p53). These observations imply that Cox-2 expression acts as a survival mechanism in cells exposed to forms of damage that activates p53 and may therefore result into apoptosis. In this respect, Cox-2 may have an important contribution in keeping cells alive after DNA damage, tilting the balance towards p53-mediated arrest rather than apoptosis. We believe that chronic inflammation may represent a physio-pathological context in which p53 and NF-kappaB cooperate to activate Cox-2. Inflammatory cells are subjected to a number of signals that activate both factors. In particular, during the inflammatory process, intracellular reactive oxygen and nitrogen species are produced, damaging DNA and inducing p53 activation. In such a context, the cooperation between p53 and NF-kappaB may be a critical factor in determining the fate of cells under inflammatory stress.

Cycloxygenase-2 expression in relation with TP53 status in esophageal cancer

On the basis of the results above, it is predicted that overexpression of Cox-2 in inflammatory pre-cancer and cancer tissues may correlate with wild-type *TP53* status. Despite numerous studies on Cox-2 expression in relation with p53 immunostaining in cancer cells, a correlation between the expression of the two markers has not been demonstrated. Our study in esophageal cancers shed a new light on this question. A correlation between Cox-2 expression and *TP53* mutation status was observed in ADC, but not in SCC. In SCC, Cox-2 expression was not detected in normal mucosa adjacent to cancer but was observed in dysplasias (low- and high-grade) and in a small proportion (11%) of invasive SCC, without correlation with either *TP53* gene status or p53 protein expression. These results are consistent with observations by others, although most series have reported high levels of Cox-2 expression in a larger proportion of SCC (Shamma *et al*., 2000; Kuo *et al*., 2003; Yu *et al*., 2003). Detection of high levels of Cox-2 in dysplasias suggests that overexpression is a marker for intense cell proliferation in early disease (Kuo *et al*., 2003). In contrast, in ADC, Cox-2 expression was frequent in cells of Barrett's esophagus (BE), the precursor lesion, even in the absence of dysplasia, consistent with the inflammatory nature of this lesion. This high Cox-2 pattern was retained in 44% of cancers, irrespective of tumor grade and stage. Tumors with high Cox-2 were significantly more likely to harbor wild-type *TP53* than tumors with low Cox-2. Barrett's esophagus develops as a consequence of exposure of the lower esophagus to gastric reflux stress. Exposure to bile salt and acid upregulates Cox-2 in explants of Barrett's mucosa (Shirvani *et al*., 2000; Kaur and Triadafilopoulos, 2002). Inhibition of Cox-2 by rofecoxib inhibits cell proliferation in BE (Kaur *et al*., 2002). There is also evidence that NF-kappaB activity is significantly increased in BE and is an important contributor to neoplastic progression (Konturek *et al*., 2004). Thus, cooperation between p53 and NF-kappaB increasing Cox-2 expression may represent an adaptative response of cells to stress by reflux that promotes the proliferation and survival of cells with high potential to progress toward ADC.

In conclusion, we describe here a novel mechanism of transcriptional regulation of *COX-2* by p53, in which p53 cooperates with NF-kappaB to activate transcription through the kappaB response element. This mechanism induces anti-apoptotic effects that can be neutralized using therapeutic Cox-2 inhibitors. The cooperation between p53 and NF-kappaB may play a physiological role to neutralize apoptosis in response to DNA damage generated during inflammation. In precursor cancer lesions such as BE, p53-dependent expression of *COX-2* may provide a mechanism for escape from apoptosis and progression toward neoplasia in a highly inflammatory context. A recent study has shown that PGE2 stimulates colon cancer growth by activating a cascade that involves the inactivation and release of glycogen synthase kinase 3β from its complex

with axin, thereby activating β-catenin signaling pathway (Castellone *et al*., 2005). Detailed understanding of these mechanisms will allow to determine in which types of cancer *COX-2* inhibitors may enhance clinical responses to therapeutic drugs.

Materials and methods

Cell lines, transfections and reporter assays

Cell lines TE-1, TE-6 and TE-13 (esophageal SCC) were cultured in RPMI supplemented with 10% fetal calf serum (PAA), penicillin/streptomycin/glutamine (Gibco BRL, Gaithersburg, MD, USA), at 37°C under 10% CO_2. TP53 mutation status and temperature sensitivity of TE-1 has been reported elsewhere (Barnas *et al*., 1997; North *et al*., 2002). HCT116 (colon carcinoma (ATCC CCL247) and HCT116/p53−/− (kindly given by B Vogelstein) were grown in McCoy's 5A modified medium supplemented with 1% L-glutamine, 1% antibiotics and 10% fetal bovine serum (Life Technologies, Cergy Pontoise, France). HCT116 MT9 and HCT116 E6 were grown in the same medium supplemented with G418 (geneticin, 0.5 mg/ml, Roche, Manneheim, Germany) (Bentires-Alj *et al*., 1999a). The following drugs were added in culture medium of exponentially growing cells: BAY 11-7082 (Sigma-Aldrich, St Louis, MO, USA), daunomycin (Cerubidine, Rhone-Poulenc Rorer) and Cox-2 inhibitors nimesulid, celecoxib and rofecoxib (provided by Dr X de Leval, Laboratory of Pharmaceutical Chemistry, University of Liège, Belgium). Transfections were performed with FuGENE (1.5 μl/μg DNA) (Roche, Mannheim, Germany). RNA interference to inhibit NF-kappaB p65 was performed using 10 nM of siRNA from the kit OR-KIT2-SIRA (Eurogentec, Seraing, Belgique) according to the manufacturer's instructions. The sequence OR-0030-neg, from the same supplier, was used as negative control. Transfections were performed using HiPerFect (QiaGen, Venlo, The Netherlands, catalogue number 301705). For Luciferase assays, HCT116 plated at a density of 5×10^5 cells per 35-mm culture dish was transfected with a reporter gene (0.5 μg) containing 5′-flanking sequences (−891 to +9) of human *COX-2* gene constructed into the promotorless luciferase expression vector pGL3 (*COX-2*-Luc, gift from Professor KK Wu, Houston, TX, USA) (Gilroy *et al*., 2001), RSV-βGAL plasmid (0.2 μg) and indicated amounts of expression vectors. Total amount of DNA was kept constant by addition of vector-only pcDNA3 plasmid. After 24 h, luciferase activity was determined using Luciferase Reporter Gene Assay kit and βGalactosidase activity was assessed with Chemoluminescent βGal Reporter Gene Assay Kit (Roche). Luciferase activities were normalized with βGal and expressed as fold induction relative to activity with reporter plasmid alone.

Western blotting

Cells were lysed in RIPA-like buffer (250 mM NaCl, 50 mM Tris-HCl pH 7.4, 0.1% sodium dodecyl sulfate (SDS), 2 mM Dithiothreitol, 0.5% NP40) containing protease inhibitors (complete™, Roche). After clearing by centrifugation, an equal amount of proteins were resolved onto 10% SDS–polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose, probed with appropriate antibodies and revealed with enhanced chemiluminescence (ECL, Amersham, UK). The following antibodies were used: p53 (DO7, Dako, Glostrup, Denmark, 1/1000), Cox-2 (C20, Santa Cruz, CA, USA, 1/1000), $p21^{Waf-1}$ (C19, Santa Cruz, 1/1000), cleaved PARP (BD Pharmingen, San Diego, CA, USA, 1/1000), anti-β-actin (C4, Santa Cruz, 1/10 000).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assays were performed as described by Strano *et al*. (2002). After cross-linking with formaldehyde (1% final concentration) for 10 min and neutralization in 125 mM glycine pH 2.5, cells were lysed for 15 min at 4°C in SDS-lysis buffer (1% SDS, 10 mM ethylene diamin tetraacetic acid (EDTA), 50 mM Tris-HCl) containing protease inhibitors. Lysates were sonicated to generate 200–1000 bp DNA fragments, cleared by centrifugation and diluted 10-fold with immunoprecipitation buffer (16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl, 1.2 mM EDTA, 0.01% SDS, 1.1% Triton X-100). After preclearing with salmon sperm DNA/protein G agarose beads (Upstate, Charlottesville, VA, UK), lysates were incubated overnight with anti-p53 (CM-1, Novocastra, Newcastle, UK), anti-NF-kappaB p65 (sc-109, Santa Cruz) polyclonal antibodies or an irrelevant antibody (labeled as Control, Ctrl, in relevant figures) as negative control. Immunoprecipitated complexes were collected with salmon sperm DNA/protein G agarose beads, washed, and eluted with 1% SDS, 0.1 M $NaHCO_3$. Cross-linking of protein–DNA complexes was reversed at 65°C for 4 h, DNA was digested with 10 mg/ml of proteinase K for 1 h at 45°C, and recovered by phenol/chloroform extraction and ethanol precipitation. Detection of promoters was performed with the following primers: *COX-2:* F 5′-CTGTTGAAAGCAACTTA GCT-3′, R 5′-AGACTGAAAACCAAGCCCAT-3′, *p21WAF1:* F 5′-CATTGTTCCCQGCACTTCCTCTC-3′, R 5′-AGAAAG CCAATCAGAGCCACAG-3′.

Cell proliferation, prostagladin E_2 activity and caspase assays

Cells were plated in 96-well plates (5000 cells/well) and incubated with daunomycin and/or Cox-2 inhibitors for 24 h. Cell viability was assessed by WST-1 colorimetric test (Roche) and cell numbers were determined using the sulforhodamine B (SRB) colorimetric assay (Skehan *et al*., 1990). For PGE_2 assay, TE-1 cells were plated in six-well plates at a density of 10^6 cells/well, grown for 24 h at indicated temperature, and PGE_2 in culture medium was measured by enzyme immunoassay (R&D systems, Abingdon, UK). Levels of PGE_2 were normalized according to the number of cells at the time of the medium collection. The results were expressed in nanograms of PGE_2 per 10^5 cells. Caspase-3 activation test was performed using the caspase-3 fluorometric assay kit (Alexis Biochemicals, Lausen, Switzerland). In brief, HCT116 cells pre-treated or not pre-treated with Cox-2 inhibitors were exposed to daunomycin. Cell lysates were incubated with a caspase-3 fluorogenic substrate and the fluorescence of generated cleaved product was measured with a spectrofluorometer (460 nm).

Semiquantitative and real-time reverse transcription-polymerase chain reaction

Total RNA isolated from cell lines or frozen tissues using Trizol (Gibco-BRL, Gaithersburg, MD, USA) was reverse transcribed with SuperScript™ Reverse Transcriptase (Invitrogen, Cergy Pontoise, France). *COX-2* cDNA (F 5′-GTTT GCATTCTTTGCCCAGC-3′ and R 5′-CAGGCACCAGAC CAAAGACC-3′) was co-amplified with *β-actin* (F 5′-GACAC CACTGGAGGGTGACT-3′ and R 5′-CCACATGGTCTTC CTCTGCT-3′) for semiquantitative analysis. A PCR amplification cycle included denaturation at 94°C for 30 s, annealing at 59°C for 30 s and extension at 72°C for 5 min. Real-time PCR was performed with ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Courtaboeuf, France).

Relative quantification was performed in a multiplex reaction using *β-actin* as endogenous control. The primers and fluorogenic probes for PCR reactions were the following: *COX-2*: F 5′-GCCCTTCCTCCTGTGCC-3′, R 5′-AATCAG GAAGCTGCTTTTTAC-3′/5′ FAM-ATGATTGCCCGACT CCCTTGGGGTGT-BHQ1 3′ and *β-actin*: F 5′-CTGGCA CCCAGCACAATG-3′, R 5′-GCCGATCCACACGGAGTA CT-3′/5′ TET-TCAAGATCATTGCTCCTCCTGAGCGC-BHQ1 3′. Each sample was analysed in quadruplicate. Relative quantification was performed using the comparative threshold cycle (C_T) method as described in the User Bulletin, ABI PRISM 7900 HT Sequence Detection System.

Tumor tissues, cyclooxygenase-2 immunohistochemistry and TP53 mutations

A consecutive series of 141 patients with esophageal cancer were recruited at Edouard Herriot Hospital, Lyon (France) between 1995 and 2002, including 80 SCC and 61 ADC with pathological evidence of Barrett's metaplasia (Siewert and Stein, 1998). All tumors were locally invasive, T3 or T4 carcinoma in patients who did not receive chemo- or radiotherapy before resection or biopsy. Tissues were formalin fixed and paraffin embedded using standard methods. For immunohistochemistry, rehydrated tissue sections were exposed overnight at 4°C to the anti-Cox-2 polyclonal antibody C-20 (1/1000, Santa Cruz) and fixed antibodies were detected using biotinylated anti-rabbit IgG (1/200, Vectasin Elite-ADC kit, Vector Laboratories Inc., Burlingame, CA, USA) followed by streptavidin–peroxidase and diaminobenzidine-based detection (Vector Laboratories). Tumors were scored by evaluating the percentage of tumor cells that stained for Cox-2 and were considered as positive when containing over 20% of stained tumor cells. TP53 mutation detection (exons 4–9) was performed by temporal temperature gradient electrophoresis (TTGE) using the DCode system (BioRad, Marnes La Coquette, France) using primers and conditions described elsewhere (Sepehr *et al*., 2001) and DNA segments that generated abnormal TTGE profiles were further analysed by sequencing on ABI Prism 3100 automated sequencer. All mutations were confirmed by a second, independent sequence analysis using the same primers.

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