Expression of COX-2, NF-κB-p65, NF-κB-p50 and IKKα in malignant and adjacent normal human colorectal tissue

MP Charalambous¹, T Lightfoot², V Speirs¹, K Horgan³ and NJ Gooderham*⁴
¹Leeds Institute of Molecular Medicine, Wellcome Trust, Brenner Building, St James’s University Hospital, Beckett Street, Leeds LS9 7TK, UK; ²Department of Health Sciences, Alcuin College, University of York, York, UK; ³Department of Surgery, Leeds General Infirmary, Great George Street, Leeds LS1 3EX, UK; ⁴Biomolecular Medicine, Imperial College London, Sir Alexander Fleming Building, London SW7 2AZ, UK

BACKGROUND: Cyclooxygenase-2 (COX-2) is selectively over-expressed in colorectal tumours. The mechanism of COX-2 induction in these tumours is not fully understood, although evidence suggests a possible link between nuclear factor (NF)-κB and COX-2. We hypothesised an association between COX-2 expression and NF-κB-p65, NF-κB-p50 and IκB-kinase-α (IKKα) in both epithelial and stromal cells in human colorectal cancer.

METHODS: Using immunohistochemistry, we measured COX-2, NF-κB-p65, NF-κB-p65 nuclear localisation sequence (NLS), NF-κB-p50, NF-κB-p50 NLS and IKKα protein expression in matched colorectal biopsy samples comprising both non-tumour and adjacent tumour tissue from 32 patients with colorectal cancer.

RESULTS: We have shown that stromal cells of malignant and surrounding normal colorectal tissue express COX-2. In all cell types of malignant tissue, and in vascular endothelial cells (VECs) of neighbouring normal tissue, COX-2 expression was strongly associated with NF-κB-p65 expression (Pearson’s correlation, P = 0.019 for macrophages, P = 0.001 for VECs, P = 0.002 for fibroblasts (malignant tissue), and P = 0.011 for VECs (non-malignant tissue)) but not NF-κB-p50 or IKKα.

CONCLUSIONS: These data suggest that in these cells COX-2 induction may be mediated through activation of the canonical NF-κB pathway. Finally, the lack of association between COX-2, NF-κB-p65 or IKKα in stromal cells with the clinical severity of colorectal cancer as determined by Duke’s stage, suggests that COX-2, NF-κB-p65 and IKKα expression are possibly early post-initiation events, which could be involved in tumour progression.

British Journal of Cancer (2009) 101, 106–115. doi:10.1038/sj.bjc.6605120 www.bjcancer.com

© 2009 Cancer Research UK

Keywords: colorectal cancer; cyclooxygenase-2; fibroblasts; macrophages; nuclear factor-κB; vascular endothelial cells

Adenocarcinoma of the colon and rectum is the second leading cause of death from cancer in the industrialised world (Midgley and Kerr, 1999). The high prevalence of the disease is a driver for understanding the underlying molecular mechanisms of colorectal carcinogenesis.

Several epidemiological studies have reported a 40–50% decrease in the relative risk of colorectal cancer in persons chronically using non-steroidal anti-inflammatory drugs (NSAIDs) (Thun et al., 1991; Marnett, 1992; Giovannucci et al., 1993), indicating that these drugs may have a chemoprotective and possibly chemotherapeutic effect. Data from human studies, have shown that the NSAID, sulindac, can reduce the number and size of polyps in patients with familial adenomatous polyposis (FAP) (Waddell and Loughry, 1983; Giardiello et al., 1993).

Most NSAIDs in current use inhibit the action of cyclooxygenase (COX), a key enzyme in the production of prostaglandins (PGs). Cyclooxygenases are intracellular enzymes that catalyse the conversion of arachidonic acid to PGs and related eicosanoids. Isoforms of the COX-2 gene have been identified, which encode for the constitutively expressed COX-1 and the inducible COX-2. In the last decade, several studies have indicated a link between the expression of COX-2 and the pathogenesis of several types of human cancers, including breast (Brueggemeier et al., 1999; Half et al., 2002), gastric (Ristimäki et al., 1997), lung (Hida et al., 1998) and colorectal adenocarcinomas (Eberhart et al., 1994; Tomozawa et al., 2000).

Although enhanced COX-2 expression in colorectal cancer tissues has been widely observed, the mechanisms that regulate the expression of COX-2 in colorectal tumours are not completely understood. Cyclooxygenase-2 expression can be induced by a variety of stimuli, including oncogenic viruses, growth factors, tumour promoters and cytokines.

Sequence analysis of the 5′-flanking region of the COX-2 gene shows two nuclear factor-κB (NF-κB) sites (Tazawa et al., 1994). In vitro inhibition of this protein has been shown to attenuate COX-2 expression in colorectal cancer cells, indicating that NF-κB may play an important role in COX-2 induction (Plummer et al., 1999; Williams et al., 2003). Earlier, we have shown that upregulation of COX-2 is accompanied by increased expression...
of NF-κB-p65 and 1κB-kinase-alpha (IKKz) in human colorectal cancer epithelial cells (Charalambous et al, 2003).

Recent studies have shown that tumour stroma also contributes to enhanced COX-2 expression in colorectal cancer. Increased levels of COX-2 have been localised in macrophages (Sheehan et al, 2003; Liu et al, 2005), fibroblasts (Sonoshita et al, 2002; Adegboyega et al, 2004) and vascular endothelial cells (VECs) (Brown and DuBois, 2005), indicating that both host and tumour cells may contribute to the production of PGs within the tumour microenvironment and the subsequent development of cancer growth.

We have therefore studied tissue biopsies obtained from patients with diagnosed primary colorectal carcinoma undergoing surgical treatment for their disease, for differences in expression of COX-2, in epithelial and stromal cells (macrophages, fibroblasts and VECs), in malignant and adjacent normal colorectal tissue, and for alterations in the expression of the upstream intracellular proteins, which seem to be associated to COX-2 expression, namely NF-κB-p65, NF-κB-p50 and IKKz.

MATERIALS AND METHODS

Patients

The study adhered to the tenets of the Declaration of Helsinki. Surgical specimens of primary tumours were obtained with informed consent from 32 patients (21 men and 11 women; aged 44–80 years, mean age 64.0 years ± 1.63 s.e.m.), with histologically verified colorectal cancer, treated at the Department of Surgery, York District Hospital, York, UK. Ethical approval for the study was obtained from the Human Research Ethics Committee at York District Hospital. A total of 17 patients had colon cancer and 15 had rectal cancer. Tumours were classified according to the Duke’s classification (see Table 1). A total of 5 patients had Duke’s A, 12 had Duke’s B and 10 had Duke’s C. The entire study was carried out blind using coded tissue sections.

Expression of COX-2, NF-κB-p65, NF-κB-p50 and IKKz

Table 1 Patient demographic information

| Age | Sex | Tumour site | Duke’s stage | Drug history | Tobacco use | Alcohol |
|-----|-----|-------------|--------------|--------------|-------------|---------|
| 72  | M   | Colon       | B            | None         | No          | 8+      |
| 68  | M   | Colon       | C            | N/A          | No          | N/A     |
| 66  | F   | Colon       | C1           | 5-Fluorouracil, Enalapril | No | 0 |
| 69  | M   | Rectum      | C            | Co-codamol   | No          | 1–7     |
| 49  | F   | Colon       | B            | None         | Yes         | N/A     |
| 63  | F   | Rectum      | N/A          | N/A          | No          | N/A     |
| 52  | M   | Rectum      | C1           | Acelat       | No          | 1–7     |
| 75  | M   | Colon       | C            | N/A          | No          | N/A     |
| 68  | M   | Colon       | B            | Atenolol, Prednisolone, Warfarin, Diltiazem, Isosorbide, Glucolide, Co-danthramer | No | 1–7 |
| 69  | M   | Rectum      | C1           | Atenolol     | No          | 8+      |
| 70  | F   | Rectum      | B            | Lithium, Thyroxine | No | 8+ |
| 72  | M   | Rectum      | B            | Captoril, Naproxen, Allopurinol, Isosorbide, Frusemide, Atenolol, Prochilorperazine | No | 1–7 |
| 78  | M   | Colon       | A            | N/A          | No          | N/A     |
| 56  | M   | Colon       | A            | None         | No          | 8+      |
| 76  | M   | Colon       | A            | None         | No          | 1–7     |
| 44  | M   | Colon       | C            | N/A          | No          | 8+      |
| 58  | F   | Colon       | N/A          | None         | No          | 0       |
| 61  | F   | Rectum      | A            | N/A          | No          | N/A     |
| 66  | F   | Colon       | B            | None         | No          | 0       |
| 54  | M   | Rectum      | C1           | None         | No          | 1–7     |
| 49  | M   | Colon       | B            | None         | No          | 8+      |
| 73  | F   | Colon       | B            | N/A          | Yes         | N/A     |
| 52  | M   | Rectum      | B            | None         | No          | 8+      |
| 68  | M   | Colon       | B            | Salbutamol, Ferrous sulphate | No | 0 |
| 63  | F   | Rectum      | A            | Salbutamol, Beclomethasone, Bendrofluazide | No | 1–7 |
| 56  | M   | Colon       | C            | Losec        | No          | 8+      |
| 68  | M   | Rectum      | B            | Sotalol, Aspirin | No | 8+ |
| 80  | F   | Rectum      | B            | None         | No          | 0       |
| 59  | M   | Rectum      | C1           | None         | No          | 8+      |
| 66  | M   | Rectum      | N/A          | None         | Yes         | 8+      |

N/A = not available. *Age in years. **Alcohol consumption in units per week (1 unit = half a pint of beer or one glass of wine or one shot of spirits).
tissue was determined using a modified avidin–biotin immunohistochemistry procedure (Goggi et al., 1986). In preliminary experiments, each of the immunohistochemistry assays was optimised using a range of antisera dilutions (1/50–1/5000). For each assay, the negative control antisera (pre-immune sera) were confirmed negative for staining at the dilution optimised for the primary antibody and blocking peptides (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) confirmed specificity. The sections were
deparaffinised and rehydrated through xylene and a series of graded alcohol solutions. Endogenous peroxidase activity was blocked by immersing the sections into a solution of 3% hydrogen peroxide in distilled water for 30 min at room temperature, and then rinsed in cold running tap water for 10 min. Incubating the sections with 5% normal swine serum for 30 min at room temperature reduced non-specific background staining. Sections were then washed twice with phosphate buffered saline (PBS) and 1 ml of either the primary antibody or the normal goat or rabbit IgGs (negative control) was applied to each section, and left at 4°C overnight. The next day, the slides were washed twice with PBS, and then incubated with the secondary antibody solution (Biotinylated swine anti-goat, mouse, rabbit immunoglobulin; 1/150 dilution; 1 ml per section), for 1 h at room temperature. After being washed twice with PBS, they were incubated with the StrepABCComplex solution (Dako Ltd, Ely, UK, 1 ml per section) for 1 h at room temperature, washed twice with PBS and immersed into the substrate (300 ml PBS, 90 μl hydrogen peroxide and 2.5 ml 3,3-diaminobenzidine) for 3 min, and then rinsed with PBS and cold running tap water. Sections were then successively immersed into haematoxylin, acid alcohol and Scott’s tap water to counterstain. Finally, the sections were dehydrated by successive immersion into 70% ethanol, 100% ethanol twice and xylene twice and mounted.

**Immunohistochemical evaluation**

Processed specimens were scored under the light microscope and the intensity and extent of staining with COX-2, NF-κB-p65, NF-κB-p50, NF-κB-p50 NLS and IKKζ antibodies graded blind using coded slides. To assess the grade intensity and distribution of immunoreactivity in the colorectal stromal and epithelial cells, a scoring method, which has been described earlier was used (Yukawa et al, 1994). The distribution was scored according to the number of positive cells; none (not stained), 0; focal (<1/3 of cells stained), 1; multi-focal (1/3–2/3 of cells stained), 2; and diffuse (>2/3 stained), 3. The staining intensity was scored as: none (not stained), 0; mild (between 0 and 2), 1; and strong, 2. The distribution and intensity scored were added to produce the following grades for the staining; 0, negative; 2, weakly positive; and 3, 4 and 5, strongly positive. Sections treated with the normal goat or rabbit IgGs (negative controls) or omitting the primary antibody were devoid of staining. Positive staining controls for COX-2 included sections of kidney, uterus and brain.

**Statistical analysis**

The Wilcoxon’s signed rank test was used to compare the scoring of the respective immunoreactivity for COX-2, NF-κB-p65 and IKKζ between stromal cells of malignant and adjacent normal tissues. The Pearson’s product-moment correlation coefficient test was used to assess the relation between COX-2 expression and NF-κB-p65 and IKKζ, and in addition to assess correlation between COX-2, NF-κB-p65 and IKKζ and the Duke’s stages.

**RESULTS**

**Expression of COX-2 in normal and malignant colorectal tissue**

Tissue sections of malignant and normal bowel from colorectal cancer patients were investigated for COX-2 expression by immunohistochemistry. Approximately, one-third of the patients strongly expressed immunoreactive COX-2 (score ≥3) in stromal cells of both normal and malignant colorectal tissue (Figures 1 and 2A). Only 5 out of 23 patients strongly expressed immunoreactive COX-2 in non-neoplastic epithelial cells. In contrast, there was strong COX-2 expression in malignant epithelial cells in more than half of the patients (17 out of 30 patients). The expression was cytoplasmic. Statistical analysis of matched (normal vs malignant tissue from the same patient) samples showed no significant difference in the respective intensity scores of COX-2 of stromal cells in normal and malignant tissues (Wilcoxon’s signed rank test; n = 23; P = 0.113 for fibroblasts, P = 0.108 for macrophages, and P = 0.066 for VECs). Cyclooxygenase-2 expression was significantly higher in malignant epithelial cells when compared with adjacent normal epithelium (Wilcoxon’s signed rank test; n = 23; P = 0.003).

**Expression of NF-κB-p65 in normal and malignant colorectal tissue**

Tissue sections of normal and malignant bowel from colorectal patients were also investigated for NF-κB-p65 expression. The majority of colorectal cancer patients strongly expressed immunoreactive NF-κB-p65 (score ≥3) in macrophages and VECs of normal colorectal tissue (Figures 1 and 2B). Moreover, about one-third of patients strongly expressed immunoreactive NF-κB-p65 in normal fibroblasts, whereas only 3 out of 24 patients strongly expressed immunoreactive NF-κB-p65 in normal epithelial cells. In cancerous tissue, more than half of the samples showed strong qNF-κB-p65 expression in malignant epithelial cells, whereas only about a quarter to a third of the malignant tissues showed significant NF-κB-p65 expression in stromal cells. In all cell types of both normal and malignant tissue, the NF-κB-p65 staining was both cytoplasmic and nuclear. Cytoplasmic NF-κB-p65 expression represented both inactive protein, which is bound to IκB, as well as...
active NF-κB-p65, which has been phosphorylated and released from IκB, but which has still not translocated into the nucleus. Nuclear NF-κB-p65 expression represented active protein. In order to determine whether any of the cytoplasmic NF-κB-p65 protein was in its active form, we investigated the NF-κB-p65 NLS expression in six patients who expressed significant cytoplasmic NF-κB-p65 (Figure 1). The anti-NF-κB-p65 NLS antibody specifically recognises an epitope overlapping the NLS of the p65 subunit of the NF-κB heterodimer. This epitope is masked by IκB binding, and therefore the anti-NF-κB-p65 NLS antibody selectively binds to IκB free, activated form of NF-κB-p65 (Mitsiades et al, 2006). In all cases, a significant proportion of the cytoplasmic NF-κB-p65 was found to be in its active form. In addition, we also confirmed that nuclear NF-κB-p65 represented active protein. Statistical analysis of matched patient samples showed a significant reduction in the respective intensity scores of NF-κB-p65 in macrophages and VECs of malignant tissues compared with those of normal adjacent colorectal tissue (Wilcoxon’s signed rank test; n = 24; P = 0.027 for macrophages, and P = 0.032 for VECs). No statistical difference was observed in similar analysis of fibroblasts (Wilcoxon’s signed rank test; n = 24; P = 0.109). In contrast, NF-κB-p65 expression was significantly higher in malignant epithelial cells when compared with adjacent normal epithelium (Wilcoxon’s signed rank test; n = 24; P = 0.004).

Expression of IKKz, and NF-κB-p50 in normal and malignant colorectal tissue

There was little expression of immunoreactive IKKz in either stromal or epithelial cells of normal colorectal tissues, indicating that immunoreactive IKKz protein is not strongly expressed constitutively in these cells (Figure 2C and 3). However, there was a significant increase of IKKz, NF-κB-p50, NF-κB-p65 and COX-2 expression in both stromal and epithelial cells of malignant colorectal tissues. The staining was purely cytoplasmic. Statistical analysis applied to matched patient samples showed a significant increase in the respective intensity scores of IKKz in both stromal and epithelial cells of malignant tissue, compared with those of normal colorectal tissue (Wilcoxon’s signed rank test; n = 18; P = 0.010 for macrophages, P = 0.006 for fibroblasts, P = 0.028 for VECs, and P = 0.005 for epithelial cells). Tissue sections of normal and malignant bowel from colorectal patients were also investigated for NF-κB-p50 expression (Figure 3). A similar expression pattern to that of IKKz was observed for NF-κB-p50 in both normal and malignant tissue. Most of the patients (7 out of 8) did not show any significant NF-κB-p50 expression in either the stromal or epithelial cells of normal tissue. However, there was a significant increase of NF-κB-p50 expression in both stromal and epithelial cells of malignant colorectal tissues. The staining was both cytoplasmic and nuclear. Cytoplasmic NF-κB-p50 expression represented both inactive protein, which is bound to IκB, as well as active NF-κB-p50, which has been phosphorylated and released from IκB, but which has still not translocated into the nucleus. Nuclear NF-κB-p50 expression represented active protein. In order to determine whether any of the cytoplasmic NF-κB-p50 protein was in its active form, we investigated the NF-κB-p50 NLS expression in six patients who expressed significant cytoplasmic NF-κB-p50 (Figure 3). In all cases, only a small proportion of the cytoplasmic NF-κB-p50 was found to be in its active form. As there was available tissue from only eight patients to investigate the expression of this protein, no statistical analysis was performed.

Co-expression of COX-2, NF-κB-p65, NF-κB-p50 and IKKz in normal colorectal tissue

In order to determine whether there was co-expression of COX-2 and NF-κB-p65 or IKKz in normal colorectal tissue, serial sections were examined for expression of the four proteins. In the great majority of patients, strong (≥3) COX-2 expression was accompanied by both cytoplasmic and nuclear NF-κB-p65 expression in VECs (8 out of 9 patients) (Figure 4A). However, only a proportion of patients who expressed strong COX-2 levels in the other cell types within normal tissue, also expressed immunoreactive NF-κB-p65 (4 out of 9 for fibroblasts; 5 out of 9 for macrophages; and 3 out of 5 for epithelial cells) (Figure 4A and B).
In agreement with this histological finding, there was a significant correlation between COX-2 expression and NF-κB-p65 expression in VECs (Pearson’s correlation test, two-tailed, \( P = 0.011, n = 23 \)), but not in the other cell types (\( P = 0.192 \) for macrophages, \( P = 0.171 \) for fibroblasts and \( P = 0.111 \) for epithelial cells) (Figure 4A and B). Cyclooxygenase-2 expression in normal tissue did not correlate with either IKKα expression (Figure 4C and D) (1 out of 10 patients for macrophages; 1 out of 9 for VECs; 0 out of 11 for fibroblasts; and 1 out of 4 for epithelial cells) or NF-κB-p50 expression (1 out of 5 patients for macrophages; 1 out of 4 for VECs; 0 out of 5 for fibroblasts; and 1 out of 3 for epithelial cells). In agreement with this histological finding, there was no correlation between the expression of COX-2 and IKKβ in stromal cells in normal colorectal tissues (Pearson’s correlation test, two-tailed, \( n = 23, P = 0.06 \) for macrophages, \( P = 0.769 \) for VECs, and \( P = 0.222 \) for fibroblasts) (Figure 4C and D).

Co-expression of COX-2, NF-κB-p65, NF-κB-p50 and IKKα in malignant colorectal tissue

Serial sections were also examined for co-expression of the four proteins in malignant colorectal tissue (Figure 5). In the majority of patients, COX-2 expression was accompanied by both cytoplasmic and nuclear NF-κB-p65 expression in stromal cells (Figure 4E and F) (11 out of 16 patients for macrophages; and 9 out of 13 for VECs and fibroblasts). A total of 13 out of the 16 patients who expressed COX-2 in malignant epithelial cells, expressed NF-κB-p65 (Figure 4E). In agreement with this histological finding, there was a significant correlation between COX-2 and NF-κB-p65 expression in all cell types (Pearson’s correlation test, two-tailed, \( P = 0.019 \) for macrophages, \( P = 0.001 \) for VECs, \( P = 0.002 \) for fibroblasts and \( P = 0.017 \) for epithelial cells) (Figure 4E and F). Only a proportion of patients who expressed significant COX-2 levels in stromal cells, of malignant colorectal tissues (Pearson’s correlation test, two-tailed, \( n = 3 \) out of 7 patients for macrophages; 4 out of 7 for VECs; and 2 out of 6 for fibroblasts) and NF-κB-p50 levels (3 out of 5 patients for macrophages; 3 out of 5 for VECs; and 2 out of 5 for fibroblasts). Statistically, there was no correlation between COX-2 and IKKα in any of these cells in malignant tissues (Pearson’s correlation test, two-tailed, \( P = 0.322 \) for macrophages, \( P = 0.378 \) for VECs, and \( P = 0.578 \) for fibroblasts) (Figure 4G and H).

Association between COX-2, NF-κB-p65 or IKKα in stromal cells and severity of colorectal cancer

Comparison of the expression of COX-2, NF-κB-p65 and IKKα in stromal cells of both normal and malignant epithelium and severity of colorectal cancer as determined by the Duke’s stage, indicated that protein expression was not correlated with clinical assessment of disease severity (Pearson’s correlation test, two-tailed; \( n = 21 \) and \( P = 0.13 \) for COX-2 in normal macrophages; \( n = 21 \) and \( P = 0.06 \) for COX-2 in normal VECs; \( n = 21 \) and \( P = 0.07 \) for COX-2 in normal fibroblasts; \( n = 23 \) and \( P = 0.16 \) for COX-2 in malignant macrophages; \( n = 23 \) and \( P = 0.20 \) for COX-2 in malignant VECs; \( n = 23 \) and \( P = 0.25 \) for COX-2 in malignant fibroblasts; \( n = 21 \) and \( P = 0.34 \) for NF-κB-p65 in normal macrophages; \( n = 21 \) and \( P = 0.61 \) for NF-κB-p65 in normal VECs; \( n = 21 \) and \( P = 0.47 \) for NF-κB-p65 in normal fibroblasts; \( n = 24 \) and \( P = 0.41 \) for NF-κB-p65 in malignant macrophages; \( n = 24 \) and \( P = 0.46 \) for NF-κB-p65 in malignant VECs; \( n = 24 \) and \( P = 0.22 \) for NF-κB-p65 in malignant fibroblasts; \( n = 15 \) and \( P = 0.07 \) for IKKα in normal macrophages; \( n = 15 \) and \( P = 0.95 \) for IKKα in normal VECs; \( n = 15 \) and \( P = 0.57 \) for IKKα in normal fibroblasts; \( n = 19 \) and \( P = 0.27 \) for IKKα in malignant macrophages; \( n = 19 \) and \( P = 0.51 \) for IKKα in malignant VECs; \( n = 19 \) and \( P = 0.91 \) for IKKα in malignant fibroblasts).

DISCUSSION

We found that both stromal and epithelial cells of malignant colorectal tissue express COX-2, indicating that both could contribute to the production of PGs within the tumour micro-environment. These results are in agreement with earlier studies, that found both stromal and epithelial colorectal cells expressing COX-2 in colorectal adenomas (Aronnvet al, 2003; Pisano et al, 2005; Tatsu et al, 2005) and carcinomas (Battu et al, 1998; Yamashita et al, 2003; Ohta et al, 2006). We have reported earlier (Charalambous et al, 2003), a significant increase of COX-2 expression in malignant colorectal epithelial cells, compared with adjacent normal epithelium. We now report that this difference is not observed in stromal cells. In fact, COX-2 expression was higher in all three stromal cell types in normal colorectal tissue, compared with malignant tissue, although the difference in expression was not statistically significant. These results indicate that in malignant tissue COX-2 expression is predominantly epithelial, whereas in surrounding normal tissue it is predominantly stromal. This latter observation is in agreement with earlier reports suggesting that COX-2 expressed by stromal cells is directly involved in angiogenesis, preparing the surrounding colorectal tissue for local spread of malignant tumour (Williams et al, 2000; Sonoshita et al, 2002; Wendum et al, 2005).

Nuclear factor-κB is an inducible eukaryotic transcription factor, which has a pivotal role in the regulation of the expression of numerous genes involved in immune and inflammatory responses (Sha, 1998; Bowie and O’Neil, 2000). In fact, NF-κB is not a single protein, but a small family of closely related protein dimers, which bind to a common sequence motif known as the κB site (Karim and Lin, 2002). Two regulatory pathways have been described that control the activity of these proteins: the canonical NF-κB pathway, which is normally triggered in response to microbial and viral infections and exposure to pro-inflammatory cytokines; and the alternative pathway, which is triggered by certain members of the tumour necrosis factor (TNF) cytokine family (Karim and Lin, 2002). Nuclear factor-κB-p65 is a member of the canonical pathway, whereas NF-κB-p50 and IKKα are members of the alternative pathway. Once in the nucleus, NF-κB can regulate several genes, including COX-2.

We have shown earlier that upregulation of COX-2 is accompanied by increased expression of NF-κB-p65 and IKKα in malignant colorectal epithelial cells (Charalambous et al, 2003), supporting the proposal that NF-κB is involved in COX-2 induction in these cells. This was in agreement with earlier model systems that showed expression of COX-2 was mediated by NF-κB in human umbilical vein (Jones et al, 1993) and rheumatoid synoviocytes (Crofford et al, 1997). Subsequent in vitro studies provided further evidence in support of this hypothesis (Cherukuri et al, 2005; Duque et al, 2006; Konson et al, 2006). In this study, we have shown that NF-κB-p65 and, to a lesser extent, NF-κB-p50 are not only upregulated in malignant epithelial cells, but they are also significantly activated. Moreover, we have shown that in stromal cells (macrophages, fibroblasts and VECs) of malignant colorectal tissue, as well as in VECs of adjacent normal tissue, COX-2 expression is closely correlated with NF-κB-p65, but not with IKKα or NF-κB-p50. These results indicate that in these cells, COX-2 induction may be mediated primarily through activation of the canonical NF-κB pathway in preference to the alternative pathway. These findings are in agreement with a recent study, which showed that COX-2 expression in colorectal cancer stromal cells, was associated with p-IκB-α, another member of the NF-κB canonical pathway (Vandoros et al, 2006). Interestingly, in normal tissue, COX-2 expression in macrophages and fibroblasts was apparently not associated with either NF-κB-p65, IKKα or NF-κB-p50.

In summary, we have shown that stromal cells of malignant and surrounding normal colorectal tissue express COX-2. In all
cell types of malignant tissue, as well as in VECs of neighbouring normal tissue, COX-2 expression was strongly associated with NF-κB-p65 expression but not IKKα or NF-κB-p50, suggesting that in these cells, COX-2 induction may be mediated primarily through activation of the canonical NF-κB pathway. Finally, the lack of association between COX-2, NF-κB-p65 or IKKα in
Expression of COX-2, NF-κB-p65, NF-κB-p50 and IKKs

MP Charalambous et al

Figure 3 Immunohistochemical localisation of IKKs, NF-κB-p50 and NF-κB-p50 NLS in normal and malignant colorectal tissue from the same patient. The presence of the immunoreactive protein is indicated by brown staining. (A) Human malignant tissue treated with pre-immune sera as primary antibody (negative control for IKKα) (magnification × 10); (B) Human malignant tissue treated with anti-IKKα antibody as primary antibody (positive control) (magnification × 10); (C) Epithelial cells, (D) VECs, (E) macrophages and (F) fibroblasts of normal colorectal tissue treated with anti-IKKα primary antibody (magnification × 20 for epithelial cells, × 10 for VECs and × 80 for other two cell types); (G) Epithelial cells, (H) VECs, (I) macrophages and (J) fibroblasts of malignant colorectal tissue treated with anti-IKKα primary antibody (magnification × 10 for epithelial cells, × 20 for VECs and × 80 for other two cell types); (K) Tissue treated with pre-immune sera as primary antibody (negative control for NF-κB-p50) (magnification × 10); (L) Tissue treated with anti-NF-κB-p50 antibody as primary antibody (positive control) (magnification × 10); (M) Epithelial cells, (N) VECs, (O) macrophages and (P) fibroblasts of normal colorectal tissue treated with anti-NF-κB-p50 primary antibody (magnification × 20 for epithelial cells and VECs, × 40 for other two cell types); (Q) Epithelial cells, (R) VECs, (S) macrophages and (T) fibroblasts of malignant colorectal tissue treated with anti-NF-κB-p50 primary antibody (magnification × 10 for epithelial cells and VECs, × 40 for macrophages and × 50 for fibroblasts); (U) Tissue treated with pre-immune sera as primary antibody (negative control for NF-κB-p50 NLS) (magnification × 10); (V) Tissue treated with anti-NF-κB-p50 NLS antibody as primary antibody (positive control) (magnification × 10); (W) Epithelial cells, (X) VECs, (Y) macrophages and (Z) fibroblasts of normal colorectal tissue treated with anti-NF-κB-p50 NLS primary antibody (magnification × 20 for epithelial cells, × 10 for VECs and × 40 for macrophages and fibroblasts); (AA) Epithelial cells, (AB) VECs, (AC) macrophages and (AD) fibroblasts of malignant colorectal tissue treated with anti-NF-κB-p50 NLS primary antibody (magnification × 20 for epithelial cells and VECs, × 30 for macrophages and × 40 for fibroblasts).

Figure 4 Expression of NF-κB and IKKα compared with COX-2 in normal (A–D) and malignant colonic tissues (E–H). Values are mean ± s.e.m.
stromal cells with the clinical severity of colorectal cancer as determined by the Duke’s stage, suggests that COX-2, NF-κB-p65 and IKKα expression are possibly early post-initiation events, that could be involved in tumour progression.

ACKNOWLEDGEMENTS

This study was supported by Grants from the United Kingdom Food Standards Agency, the AG Leventis Foundation, Paris and Imperial College, London.

REFERENCES

Adegboyega PA, Ololade O, Saada J, Mifflin R, Di Mari JF, Powell DW (2004) Subepithelial myofibroblasts express cyclooxygenase-2 in colorectal tubular adenomas. *Clin Cancer Res* 10: 5870–5879

Arnoletti JP, Upson J, Babb JS, Bellacosa A, Watson JC (2005) Differential stromal and epithelial localization of cyclooxygenase-2 (COX-2) during colorectal tumorigenesis. *Exp Clin Cancer Res* 24: 279–287

Battu S, Chable-Rabinovitch H, Rigaud M, Beneytout JL (1998) Cyclooxygenase-2 expression in human adenocarcinoma cell line HT29 cl.19A. *Anticancer Res* 18: 2397–2403

Bowie AG, O’Neill LA (2000) Vitamin C inhibits NF-kappa B activation by TNF via the activation of p38 mitogen-activated protein kinase. *J Immunol* 165: 7180–7188

Brown JR, DuBois RN (2005) COX-2: a molecular target for colorectal cancer prevention. *J Clin Oncol* 23: 2840–2855

Bruggemeier RW, Quinn AL, Parrett ML, Joarder FS, Harris RE, Robertson Bowie AG, O’Neill LA (2000) Vitamin C inhibits NF-kappa B activation by TNF via the activation of p38 mitogen-activated protein kinase. *J Immunol* 165: 7180–7188

Charalambous MP, Maihofner C, Bhambra U, Lightfoot T, Gooderham NJ, Brueggemeier RW, Quinn AL, Joarder FS, Harris RE, Robertson Bowie AG, O’Neill LA (2000) Vitamin C inhibits NF-kappa B activation by TNF via the activation of p38 mitogen-activated protein kinase. *J Immunol* 165: 7180–7188

Konson A, Mahajna JA, Danon A, Rimon G, Agbaria R (2006) The involvement of nuclear factor-kappa B in cyclooxygenase-2 over-expression in murine colon cancer cells transduced with herpes simplex virus thymidine kinase gene. *Cancer Gene Ther* 13: 1093–1104

Liu ES, Shin VY, Ye YN, Luo JC, Wu WK, Cho CH (2005) Cyclooxygenase-2 regulates cyclooxygenase-2 expression through nuclear factor-kappa B in human colorectal cancer epithelial cells. *Br J Cancer* 88: 1598–1604

Cherukuri DP, Goulet AC, Inoue H, Nelson MA (2005) Selenomethionine regulates cyclooxygenase-2 (COX-2) expression through nuclear factor-kappa B (NF-kappaB) in colon cancer cells. *Cancer Biol Ther* 4: 175–180

Cofford LJ, Tan B, McCarthy CJ, Hla T (1997) Involvement of nuclear factor kappa B in the regulation of cyclooxygenase-2 expression by interleukin-1 in rheumatoid synoviocytes. *Arthritis Rheum* 40: 226–236

Duque J, Diaz-Munoz MD, Fresno M, Ilizaga MA (2006) Up-regulation of cyclooxygenase-2 by interleukin-1beta in colon carcinoma cells. *Cell Signal* 18: 1267–1269

Eberhart CE, Coffey RJ, Radhika A, Giardelio FM, Ferrenbach S, DuBois RN (1994) Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* 107: 1183–1188

Giardelio FM, Hamilton SR, Krush AJ, Piantadosi S, Hyldin LM, Celano P, Booker SV, Robinson CR, Offerhaus GJ (1993) Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. *N Engl J Med* 328: 1313–1316

Giovannucci E, Egan KM, Hunter DJ, Stampfer MJ, Colditz GA, Willett WC, Speizer FE (1995) Aspirin and the risk of colorectal cancer in women. *N Engl J Med* 333: 609–614

Goggi G, Dell’Orto P, Viale G (1986) *Immunohistochemistry, Modern Methods and Applications* pp 54–70. Butterworth-Heinemann: London

Half E, Tang XM, Gwyn K, Sahin A, Water K, Sinicrope FA (2002) Cyclooxygenase-2 expression in human breast cancers and adjacent ductal carcinoma in situ. *Cancer Res* 62: 1676–1681

Hida T, Yatabe Y, Achika H, Muramatsu H, Kozaki K, Nakamura S, Ogawa M, Mitsudomi T, Sugitani T, Takahashi T (1998) Increased expression of cyclooxygenase 2 occurs frequently in human lung cancers, specifically in adenocarcinomas. *Cancer Res* 58: 3761–3764

Jones DA, Carlton DP, McIntyre TM, Zimmerman GA, Prescott SM (1993) Molecular cloning of human prostaglandin endoperoxide synthase type II and demonstration of expression in response to cytokines. *J Biol Chem* 268: 9049–9054

Karin M, Lin A (2002) NF-kappaB at the crossroads of life and death. *Nat Immunol* 3: 221–227

Konson A, Mahajna JA, Danon A, Rimon G, Agbaria R (2006) The involvement of nuclear factor-kappa B in cyclooxygenase-2 over-expression in murine colon cancer cells transduced with herpes simplex virus thymidine kinase gene. *Cancer Gene Ther* 13: 1093–1104

Marnett LJ (1992) Aspirin and the potential role of prostaglandins in colon cancer. *Cancer Res* 52: 5575–5589

Midgley R, Kerr D (1999) Colorectal cancer. *Lancet* 353: 391–399

Mitsiades CS, McMillin D, Kotoula V, Poulaki V, McMullan C, Negri J, Fanourakis G, Tseleni-Balafouta S, Ain KB, Mitsiades N (2006) Antitumour effects of the proteasome inhibitor Bortezomib in medullary thyroid carcinoma cell lines. *Clin Cancer Res* 12: 18: 226–236

Ohta T, Takahashi M, Ochiai A (2006) Increased protein expression of both NF-κB-p65 and IKKα is accompanied by increased expression of nuclear factor-kappa B and I kappa B kinase-alpha in human colorectal cancer epithelial cells. *Br J Cancer* 88: 1598–1604

Plummer SM, Holloway KA, Manson MM, Munks RJ, Kaptein A, Farrow S, Howells L (1999) Inhibition of cyclooxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-kappaB activation via the NIK/IKK signalling complex. *Oncogene* 18: 6013–6020
Expression of COX-2, NF-κB-p65, NF-κB-p50 and IKKα

MP Charalambous et al

is activated and correlates positively with COX-2 expression in stromal myofibroblasts surrounding colon adenocarcinomas. J Cancer Res Clin Oncol 132: 76 – 84

Waddell WR, Loughry RW (1983) Sulindac for polyposis of the colon. J Surg Oncol 24: 83 – 87

Wendum D, Comperat E, Boëlle PY, Parc R, Masliah J, Trugnan G, Fléjou JF (2005) Cytoplasmic phospholipase A2 alpha overexpression in stromal cells is correlated with angiogenesis in human colorectal cancer. Mod Pathol 18: 212 – 220

Williams CS, Tsuji M, Reese J, Dey SK, DuBois RN (2000) Host cyclooxygenase-2 modulates carcinoma growth. J Clin Invest 105: 1589 – 1594

Williams JL, Nath N, Chen J, Hundley TR, Gao J, Kopelovich L, Kashfi K, Rigas B (2003) Growth inhibition of human colon cancer cells by nitric oxide (NO)-donating aspirin is associated with cyclooxygenase-2 induction and beta-catenin/T-cell factor signaling, nuclear factor-kappaB, and NO synthase 2 inhibition: implications for chemoprevention. Cancer Res 63: 7613 – 7618

Yamashita K, Arimura T, Shimizu H, Takahashi H, Endo T, Imai K, Yamano H (2003) Increased cyclooxygenase-2 expression in large flat colorectal tumors (laterally spreading tumors). J Gastroenterol 38: 69 – 73

Yukawa M, Fujimori T, Maeda S, Tabuchi M, Nagasako K (1994) Comparative clinicopathological and immunohistochemical study of ras and p53 in flat and polypoid type colorectal tumours. Gut 35: 1258 – 1261

Ristimäki A, Honkanen N, Jänkkälä H, Sipponen P, Härkönen M (1997) Expression of cyclooxygenase-2 in human gastric carcinoma. Cancer Res 57: 1276 – 1280

Sha WC (1998) Regulation of immune responses by NF-kappa B/Rel transcription factor. J Exp Med 187: 143 – 146

Sheehan KM, Sabah M, Cummins RJ, O'Grady A, Murray FE, Leader MB, Kay EW (2003) Cyclooxygenase-2 expression in stromal tumors of the gastrointestinal tract. Hum Pathol 34: 1242 – 1246

Sonoshita M, Takaku K, Oshima M, Sugihara K, Taketo MM (2002) Cyclooxygenase-2 expression in fibroblasts and endothelial cells of intestinal polyps. Cancer Res 62: 6846 – 6849

Tatsu K, Hayashi S, Shimada I, Matsui K (2005) Cyclooxygenase-2 in sporadic colorectal polyps: immunohistochemical study and its importance in the early stages of colorectal tumorogenesis. Pathol Res Pract 201: 427 – 433

Tazawa R, Xu XM, Wu KK, Wang LH (1994) Characterization of the genomic structure, chromosomal location and promoter of human prostaglandin H synthase-2 gene. Biochem Biophys Res Commun 203: 190 – 199

Thun MJ, Namboodiri MM, Heath Jr CW (1991) Aspirin use and reduced risk of fatal colon cancer. N Engl J Med 325: 1593 – 1596

Tomozawa S, Tsuno NH, Sunami E, Hatano K, Kitayama J, Osada T, Saito S, Tsuruo T, Shibata Y, Nagawa H (2000) Cyclooxygenase-2 overexpression correlates with tumour recurrence, especially haematogenous metastasis, of colorectal cancer. Br J Cancer 83: 324 – 328

Vandoros GP, Konstantinopoulos PA, Sotiropoulou-Bonikou G, Kominea A, Papachristou GI, Karamouzis MV, Gkermpsi M, Varaklis I, Papavassiliou AG (2006) PPAR-gamma is expressed and NF-kB pathway...