Prostaglandin E\(_2\) stimulates progression-related gene expression in early colorectal adenoma cells

I Mauritz\(^1\), S Westermayer\(^1\), B Marian*\(^*,1\), N Erlach\(^1\), M Grusch\(^1\) and K Holzmann\(^1\)

1Department of Internal Medicine 1, Institute of Cancer Research, Medical University Vienna, Borschkegasse 8a, A1090 Vienna, Austria

Upregulation of cyclooxygenase-2 (COX-2) and prostaglandin-dependent vascularisation in small adenomatous polyps is an essential part of colon carcinogenesis. To study the underlying cellular mechanisms, LT97 and Caco2 human colorectal tumour cells not expressing endogenous COX-2 were exposed to 1 \(\mu\)M prostaglandin E\(_2\) (PGE\(_2\)) in their medium. At 30 min after addition, expression of c-fos was stimulated 5-fold and 1.3-fold, respectively, depending on the activation of both extracellular signal-regulated kinase and p38. The amount of c-jun in nuclear extracts was increased 20% in LT97 cells. Expression of COX-2 was upregulated 1.7-fold in LT97 cells and 1.5-fold in Caco2 2 h after prostaglandin (PG) addition by a p38-mediated pathway. The known PGE\(_2\) target gene vascular endothelial growth factor (VEGF) was not modulated. Effects of sustained PGE\(_2\) production were studied in VACO235 cells that have high endogenous COX-2 and in LT97 cells infected with an adenovirus expressing COX-2. Prostaglandin E\(_2\) secretion into the medium was 1 – 2 nM and 250 pM, respectively. Expression of both VEGF and c-fos was high in VACO235 cells. In LT97 cells, COX-2 upregulated c-fos expression and c-jun content in nuclear extracts 1.7- and 1.2-fold, respectively, in a PG-dependent way. This shows that exogenous PGE\(_2\) as well as COX-2 overexpression affect signalling and gene expression in a way that enhances tumour progression.

Keywords: colon carcinogenesis; adenomatous polyps; prostaglandins; cyclooxygenase-2; PG-dependent gene expression

Molecular Diagnostics

Upregulation of cyclooxygenase-2 (COX-2) expression in early adenomas is an essential prerequisite of colorectal carcinogenesis. It causes elevated levels of prostaglandins (PGs) in the tissue and PG-dependent neovascularisation (Eberhart et al, 1994). Adenomatous polyps regress under therapy with non-steroidal anti-inflammatory drugs whose main cellular target is COX-2 (DuBois et al, 1996). Mice carrying a hereditary mutation in the APC gene (min-mice or transgenic ΔAPC-mice; (de Wind et al, 1998) develop a multitude of intestinal polyps in a COX-2 and prostaglandin E\(_2\) (PGE\(_2\))-receptor-dependent way (Sonoshita et al, 2001; Seno et al, 2002; Sunayama et al, 2002). Immunohistochemical analysis of tissue sections shows that much of the COX-2 is located in connective tissue but not in the epithelial compartment of the polyps. In the pathology of human colorectal cancer, COX-2 plays a similar role and has become the main target of chemoprevention in high-risk patients (DuBois et al, 1996; Gupta and Dubois, 2001). As in the mouse models, COX-2 is mainly located in the connective tissue of human adenomatous polyps.

This raises the question whether PGs produced in the microenvironment (fibroblasts, endothelial cells or macrophages) can in turn affect early premalignant cells and trigger expression of tumour-associated genes. To answer these questions we have established the human colonic adenoma cell line LT97 from microadenomas of a polyposis patient that do not yet express COX-2 or produce PGE\(_2\) (Richter et al, 2001). Tumour-promoting bile acids have been shown to induce expression of c-fos, COX-2 and vascular endothelial growth factor (VEGF) in these early human colonic adenoma cells (Jurek et al, 2005). The current study analysed the following: (1) the cellular effects of exogenous PGE\(_2\) on the expression of COX-2, c-jun and c-fos in LT97 human colorectal adenoma cells as well as Caco2 colorectal carcinoma cells that also express little COX-2, (2) the consequences of sustained COX-2 expression in VACO235 cells that have a high level of endogenous expression of the enzyme (Willson et al, 1987) and in LT97 cells infected with a COX-2-expressing adenovirus.

MATERIALS AND METHODS

Tissue culture and exposure to PG

LT97 colon adenoma cells were established by our laboratory (Richter et al, 2002). They were kept under standard tissue culture conditions by using Ham F-12 medium containing 20% L-15 medium, 2% FCS, 10 \(\mu\)g ml\(^{-1}\) insulin, 2 \(\times\) 10\(^{-10}\) M triiodothyronine, 2 \(\mu\)g ml\(^{-1}\) transferrin, 1 \(\mu\)g ml\(^{-1}\) hydrocortisone, 5 \(\times\) 10\(^{-9}\) M Na-selenite and 30ng ml\(^{-1}\) epidermal growth factor (EGF). Under these conditions, LT97 cells have a doubling time of ~96 h. VACO235 adenoma cells were a gift from Willson et al (1987) and were cultured with minimal essential medium (MEM) supplemented with 2% FCS, 10 \(\mu\)g ml\(^{-1}\) insulin, 2 \(\times\) 10\(^{-10}\) M triiodothyronine, 2 \(\mu\)g ml\(^{-1}\) transferrin, 1 \(\mu\)g ml\(^{-1}\) hydrocortisone, 5 \(\times\) 10\(^{-9}\) M Na-selenite and 30ng ml\(^{-1}\) EGF. Their doubling time was ~60 h. Caco2 colorectal carcinoma cells were obtained from the American Type Culture...
Collection and propagated in MEM containing 10% FCS with a doubling time of about 48 h. HEK293 cells were a gift of Dr M Herlyn (Wistar Institute). They were cultivated in RPMI medium containing 10% FCS.

Prostaglandin E$_2$ or vehicle was added to LT97 cells 96 h, and to Caco2 cells 48 h, after plating. For inhibition of COX-2, SC236 (Searle Skokie, IL, USA) was added concomitantly with the PG in a concentration of 1 $\mu$M that did not affect proliferation of LT97 cells. The kinase inhibitors U0126 and PD169316 were purchased from Calbiochem (San Diego, CA, USA).

**Cell number**

The cell number was determined by neutral red uptake during a period of 2 h from serum-free MEM containing 50 $\mu$g ml$^{-1}$ of the dye, which was taken up into the lysosomes of viable cells. After removal of excess dye by washing with PBS, neutral red taken up into the cells was extracted with 1% acetic acid in 70% ethanol. Absorbance was measured at 562 nm using a plate reader.

**Construction of adenoviral vectors expressing COX-2**

The entire coding region of human COX-2 was obtained by amplification with Pfu DNA polymerase (Stratagene, La Jolla, CA, USA) using primers 5'-TAAGTCGACCGTCTGATGCTG-3' and 5'-GA CTCCTAGACTACAGTTCAGTCGAACG-3' from the cDNA of EST clone C50D10-12Y07 (Invitrogen, CA, USA) from a human cervix carcinoma cell line. The PCR product was first cloned into pCR2.1-TOPO vector (Invitrogen) and further transferred via restriction cloning into the pShuttle-CMV based on the method described by He et al (1998). Briefly, the shuttle vector was linearised with PmeI into the pShuttle-CMV-SPORT6 (Invitrogen) in sense orientation. The recombinant protein from the sense construct was expressed in vitro from the SP6 promoter as a 70 kDa protein using the TNT-SP6 coupled Reticulocyte Lysate System (Promega, Mannheim, Germany). The cDNA fragments were further subcloned with the restriction enzymes HindIII and KpnI into the pShuttle-CMV (Stratagene).

The adenoviral expression vector Ad-COX-2 was created by a double-recombination event in bacteria between cotransformed adenoviral plasmid pAdEasy-1 (Stratagene) and shuttle vector pShuttle-CMV based on the method described by He et al (1998). Briefly, the shuttle vector was linearised with Pmel, gel purified and cotransformed into BJ5183 cells by electroporation. Single clones were selected and confirmed by PacI digestion of plasmid DNA. Plasmids from correct clones were amplified by transformation of XL10 cells (Stratagene) followed by DNA maxiprep (Qiagen, Hilden, Germany). The adenoviral DNA of Ad-COX-2 was linearised with PacI, heat-inactivated, ethanol-precipitated and used for transfection of the packaging cell line HEK293 with Lipofect2000 (Invitrogen). Shuttle vector without any insert was used to construct control virus (Ad-co). Transfected cells were incubated for 7–10 days until cytopathic effect appeared. Primary virus was harvested, amplified and purified using a double CsCl gradient. Virus titer was determined by a standard plaque assay using HEK293 cells.

**Determination of gene expression**

Total RNA was isolated with TRIZOL (Gibco Life Technologies, Paisley, UK) according to the manufacturer’s instructions. RNA (2 $\mu$g) was used to synthesise cDNA by reverse transcription using random hexamer primers and M-MLV reverse transcriptase (Sigma, St Louis, MO, USA).

Genes of interest were amplified from cDNA samples by standard PCR cycles of 1 min denaturation (94°C), 30 s annealing and 1 min synthesis at 72°C. Reaction products were separated on 6% polyacrylamide gels and stained with 0.5 $\mu$g ml$^{-1}$ ethidium-bromide. For quantitation, GelDoc 2000 system and the software Quantity One 4.2.1 (Bio-Rad Laboratories, USA) were used.

Linearity of amplification was determined at the range of 25–35 cycles for target genes and 18–25 cycles for GAPDH to chose the adequate number of cycles for each analysis. For the number of cycles chosen, dose dependency was confirmed by using increasing amounts of cDNA. Primer sequences, annealing temperatures and number of cycles are listed in Table 1.

**Quantification of gene expression by real-time PCR**

c-fos, COX-2 and VEGF were amplified from cDNA samples by real-time PCR using TaqMan assays on an ABI PRISM 7000 Thermocycler (Applied Biosystems, Foster City, CA, USA). After denaturation for 10 min at 95°C, 40 cycles of 15 s denaturation (95°C) and 60 s annealing and synthesis at 60°C were performed using TaqMan Assay-on-Demand kits (Table 2) and TaqMan Universal PCR Master Mix. Quantitative assessment was performed using the $\Delta\Delta$C$_T$ method.

**Western blotting**

Cells were dislodged from the culture plate by using a cell scaper, collected by centrifugation and washed 2 $\times$ with icecold PBS. Cell pellets were homogenised in RIPA-buffer (50 m M Tris/HCl pH 7.4; M$	ext{g}^2+$). Reaction products were separated on 6% polyacrylamide gels and stained with 0.5 $\mu$g ml$^{-1}$ ethidium-bromide. For quantitation, GelDoc 2000 system and the software Quantity One 4.2.1 (Bio-Rad Laboratories, USA) were used.

Linearity of amplification was determined at the range of 25–35 cycles for target genes and 18–25 cycles for GAPDH to chose the adequate number of cycles for each analysis. For the number of cycles chosen, dose dependency was confirmed by using increasing amounts of cDNA. Primer sequences, annealing temperatures and number of cycles are listed in Table 1.

** Quantification of gene expression by real-time PCR**

Genes of interest were amplified from cDNA samples by real-time PCR using TaqMan assays on an ABI PRISM 7000 Thermocycler (Applied Biosystems, Foster City, CA, USA). After denaturation for 10 min at 95°C, 40 cycles of 15 s denaturation (95°C) and 60 s annealing and synthesis at 60°C were performed using TaqMan Assay-on-Demand kits (Table 2) and TaqMan Universal PCR Master Mix. Quantitative assessment was performed using the $\Delta\Delta$C$_T$ method.

**Western blotting**

Cells were dislodged from the culture plate by using a cell scaper, collected by centrifugation and washed 2 $\times$ with icecold PBS. Cell pellets were homogenised in RIPA-buffer (50 m M Tris/HCl pH 7.4; M$	ext{g}^2+$).

**Table 1** Polymerase chain reaction conditions for assessment of gene expression

| Gene    | Primer sequences         | Annealing temperatures | Cycles |
|---------|--------------------------|------------------------|--------|
| GAPDH   | Sense                    | 5'-CGGGAAGCCTTGATCAATGG-3' | 55°C   | 22     |
|         | Antisense                | 5'-GGCAGTGATGCTGAGCTC-3' |        |        |
| c-fos   | Sense                    | 5'-CTCTTCAGAGCAGATGAGATG-3' | 60°C   | 29     |
|         | Antisense                | 5'-GAGTCCAGGATGCCAGCTG-3' |        |        |
| COX-2   | Sense                    | 5'-TTCAATGAGATTGAGAATG-3' | 55°C   | 32     |
|         | Antisense                | 5'-AGATCATCTGCTGACATT-3' |        |        |
| VEGF    | Sense                    | 5'-CTGCTGTTGGGTCATTG-3' | 53°C   | 30     |
|         | Antisense                | 5'-ACACCTGCTGGCTTCTCAG-3' |        |        |
500 mM NaCl, 1% NP-40, 0.5% Na-DOC, 0.1% SDS, 0.05% NaN₃, 20 µl ml⁻¹ Complete Protease Inhibitor (Roche Diagnostics, Vienna, Austria), 500 µM sodium vanadate and 5 mM sodium fluoride). The insoluble fraction was removed by centrifugation for 15 min at 4°C and 14500 r.p.m. A total of 20 µg protein per lane were analysed by electrophoresis on 12 or 15% polyacrylamide gels and transferred to PVDF-membranes. Membranes were probed with antibodies directed against extracellular signal-regulated kinase (ERK) (1:10000; Upstate Biotechnology, Lake Placid, NY, USA) and p-ERK (1:5000; Sigma, St Louis, MO, USA), p38 (1:1000; Santa Cruz, Santa Cruz, CA, USA) and p-p38 (1:1000; Sigma) and COX-2 (1:200; Transduction Laboratories). Secondary horseradish-peroxidase-coupled antibodies were diluted 1:5000 and detected by the Super Signal West Pico Western blotting detection system (Pierce, Rockford, IL, USA).

DNA-binding c-jun in the nucleus

DNA-binding c-jun was determined from nuclear extracts using the Mercury Transfector kit (Clontech, Palo Alto, CA, USA) according to the manufacturer’s instructions. In short, nuclei were isolated from about 1–5 × 10⁷ cells lysed in 10 mM HEPES pH 7.9 containing 1.5 mM MgCl₂, 10 mM KCl, 10 mM DTT and 0.1 mM Complete Protease-Inhibitor (Roche). Nuclei were extracted using 20 mM HEPES pH 7.9 containing 1.5 mM MgCl₂, 0.42 M NaCl, 0.2 mM EDTA, 10 mM DTT, 0.1 µl ml⁻¹ Complete Protease-Inhibitor and 25% (v/v) glycerol. A total of 30 µg extracted protein was bound to 96-well plates coated with c-jun-binding oligonucleotide. After washing the plates and blocking of unspecific binding sites, c-jun was detected using an ELISA detection reaction.

PG production

Cells were left to secrete PGE₂ for 24h after addition of 10 µM arachidonic acid substrate into the medium. Prostaglandin E₂ secreted during this period was determined using an indirect ELISA kit purchased from Caymen Chemicals (Ann Arbor, MI, USA) according to the manufacturer’s instructions.

**Figure 1** Stimulation of adenoma cell growth by PGE₂. A measure of 0.1, 1 and 5 µM PGE₂ was added to the culture medium 2 days after plating and cell number determined 2, 5 and 8 days later.

**Figure 2** Expression of COX-2 and VEGF in LT97 adenoma and Caco2 carcinoma cells. LT97 cells were exposed to 1 µM PGE₂ and transcripts were analysed after 30 min, 2, 4, and 6 h. Expression of COX-2 (A) and VEGF (B) was determined by RT–PCR and PCR products were analysed on 6% polyacrylamide gels. Quantification of gene expression was obtained by real-time PCR and calculated relative to GAPDH. Values given represent mean ± S.E.M from three independent experiments. (C, D) LT97 colorectal adenoma cells; (E, F) Caco2 colorectal carcinoma cells. (C, E) COX-2 and (D, F) VEGF. *Increased above control at \( P < 0.05 \).
RESULTS

Stimulation of cell growth by PGE$_2$

Two days after seeding, increasing concentrations of PGE$_2$ were added to the culture medium of LT97 human colorectal adenoma cells and cell number was determined at days 2, 5 and 8. Growth was stimulated by all PG concentrations, with the optimal concentration at 1 $\mu$M (Figure 1). Consequently, the 1 $\mu$M concentration was used for most experiments elucidating signalling events and gene expression.

Modulation of COX-2 and VEGF gene expression by PGE$_2$

To determine whether exogenous PGs can stimulate COX-2 expression and consequently their own production, LT97 cells as well as Caco2 colorectal carcinoma cells were exposed to 1 $\mu$M PGE$_2$ for up to 6 h and expression of COX-2 was determined by RT–PCR. Expression was elevated 2 h after PGE$_2$ addition (Figure 2A), indicating the possibility that a positive feed-back loop of PG-production is established. Similar but smaller effects were obtained after addition of 5 $\mu$M PGE$_2$ (data not shown). In addition, the expression of VEGF in LT97 cells was analysed from the same RNAs. From five known splice variants, we could detect two main forms – VEGF165 and VEGF121; however, no changes in expression were observed owing to exposure to PGE$_2$ (Figure 2B).

Real-time PCR was performed to obtain reliable quantification using cDNAs of the 1 $\mu$M PGE$_2$ groups (Figure 2C and D). The results showed a 50% increase of COX-2 expression (Figure 2C, $p < 0.05$). Vascular endothelial growth factor expression was not increased but rather tendentiously suppressed 4 and 6 h after addition of PGE$_2$ (Figure 2D).

Effects of PGE$_2$ exposure were also measured in Caco2 colorectal carcinoma cells with similar results: expression of COX-2 increased 28% ($p < 0.05$) 2 h after PG addition (Figure 2E), whereas expression of VEGF was variable and not significantly increased (Figure 2F).

Effects on cellular signalling

Expression of c-fos was determined from RNAs isolated after exposure to PGE$_2$, as described above using quantitative real-time PCR. Expression was stimulated about 6-fold 30 min after addition of the PGE$_2$ (1 $\mu$M) after which it rapidly returned to control levels (Figure 3A). In Caco2 cells c-fos induction by the PG was smaller. We did, however, observe significant increases both 30 min and 2 h after addition of PGE$_2$ (Figure 3B).

Induction of c-jun was analysed from nuclear extracts of LT97 adenoma cells using c-jun-binding oligonucleotides and a specific c-jun antibody in an ELISA format. Addition of PGE$_2$ to the cultures stimulated c-jun binding to the promoter sequence by about 20% ($p < 0.05$; Figure 3C).

For analysis of signalling kinases, LT97 cells were homogenised in RIPA buffer after 0.5, 2 and 4 h of exposure to 1 $\mu$M PGE$_2$. Proteins were separated by SDS–PAGE and phosphorylation of ERK and p38 was analysed using phosphorylation-specific antibodies. We observed increased phosphorylation of both signalling kinases (representative Western blot shown in Figure 4A and B). Semi-quantification of band intensities from four independent experiments demonstrates a 2–7-fold increase of ERK phosphorylation from 30 min to 4 h after PGE$_2$ addition (Figure 4A). Phosphorylation of p38 was increased about 2-fold 30 min and 2 h after PGE$_2$ addition. At the 4 h time point, phosphorylation in the control group had reached a level similar to the PGE$_2$ group (Figure 4B).

To investigate whether the activation of ERK was essential for the induction of COX-2 and c-fos expression, the mitogen-activated protein kinase inhibitor U0126 was added to the culture medium together with the PG and COX-2 and c-fos expression assessed by standard RT–PCR. The inhibitor prevented induction of c-fos expression, whereas it did not affect expression of COX-2 (Figure 4C). Addition of PD169316, an inhibitor of p38 signalling, blocked expression of COX-2 as well as c-fos (Figure 4D).

Figure 3 Induction of c-fos and c-jun by PGE$_2$. Expression of c-fos was determined from the same cDNA preparation used in Figure 2 and quantified relative to GAPDH (A) LT97 cells, (B) Caco2 cells. c-jun protein was quantified from nuclear extracts obtained from LT97 cells by using an ELISA format detecting DNA-binding of c-jun (C). Values given represent mean ± s.e.m. from three independent experiments. *Increased above control at $p < 0.05$.
Effects of sustained PGE2 production in adenoma cell cultures

The data described above indicate that PGE2 present in the microenvironment is capable of triggering COX-2 expression and consequently sustained PG production by the adenoma cells themselves that potentially have even more pronounced effects on the expression of tumour progression-associated genes. VACO235 are villous adenoma cells that highly express COX-2 and secrete the expression of tumour progression-associated genes. VACO235 consequently sustained PG production by the adenoma cells microenvironment is capable of triggering COX-2 expression and

To obtain cells with high or low COX-2 expression in the same genetic background, we used an adenoviral vector expressing COX-2 under the control of a CMV promoter. LT97 cells were infected with 10 multiplicity of infection of the virus. At 24 h after infection, COX-2 protein could be detected in the infected cells (Figure 6A) and PG secretion into the medium increased from <15 pm to about 250 pm PGE2 (Figure 6B). Prostaglandin production could be completely inhibited by the specific COX-2 inhibitor SC236 (Figure 6B). Cell growth was not affected by the transfection (data not shown).

We then proceeded to analyse gene expression and signalling kinases in LT97 adenoma cells carrying a COX-2 adenovirus (LT97-COX-2 cells) in the presence and absence of SC236 and consequently, in the absence and presence of PG. Expression of the COX-2 target gene VEGF was highly variable as shown by the large standard deviation (Table 3). Expression of c-fos on the other hand was increased 1.7-fold in a PGE2-dependent manner (Figure 6C). Expression of c-fos in these cells was analysed by RT–PCR and found to be higher than that of LT97 cells (Figure 5).

We then proceeded to analyse gene expression and signalling kinases in LT97 adenoma cells carrying a COX-2 adenovirus (LT97-COX-2 cells) in the presence and absence of SC236 and consequently, in the absence and presence of PG. Expression of the COX-2 target gene VEGF was highly variable as shown by the large standard deviation (Table 3). Expression of c-fos on the other hand was increased 1.7-fold in a PGE2-dependent manner (Figure 6C). Expression of c-fos in these cells was analysed by RT–PCR and found to be higher than that of LT97 cells (Figure 5).

DISCUSSION

Histological examination of polyps in the min-mouse model suggested that COX-2 expression and PG production takes place primarily in the connective tissue. Sonoshita et al (2002) have identified fibroblasts and endothelial cells as the main source, whereas elevated COX-2 protein in the epithelial compartment was not observed. It has to be assumed therefore that at this early stage of tumour development, adenoma cells are subject to elevated levels of prostaglandin produced locally in the tumour microenvironment. This paper has investigated the effects this may have on tumour progression.
As cell line models, we have used LT97 human colorectal adenoma cells and Caco2 colorectal carcinoma cells that express only minimal amounts of COX-2 and produce little PGE2 (Richter et al., 2001). Exposure to PGE2 stimulated growth of the adenoma cells with an optimal concentration of 1 μM as well as in the Caco2 cells (Pai et al., 2002). As compared to the amounts of PG secreted by COX-2-expressing cells into their culture medium, this concentration is very high, which may explain why smaller effects were observed with the highest concentration (5 μM). A similar tendency to inhibit proliferation has been observed in a rat colon tumour model after treatment with 16,16-dimethyl-PGE2. Both in the normal colonic mucosa and in the tumour, a reduction of mitotic index was observed in the highest PG-dose group mediated by an increase of cAMP (Tutton and Barkla, 1980). Prostaglandin E2 effects in min-mice are mediated by the PG receptors EP2 and 4, which signal via cAMP. Therefore, the smaller effects we observed at the 5 μM concentrations in our study may well be caused by the higher production of cAMP. However, it is difficult to assess the local tissue concentration of PGE2 in the in vivo situation so that the conclusions cannot be taken further at this point.

Single doses of PGE2 also induced expression of COX-2 and c-fos and stimulated signalling kinases in a time-dependent way. Maximum effects were observed after 30 min (c-fos, c-jun, signalling kinases) to 2 h (COX-2, signalling kinases). At later time points, the effects diminished as was expected from a single dose of an effector with limited chemical stability. The situation in vivo

Figure 5 Gene expression pattern in adenoma cells. RNA was isolated from VACO235 and LT97 adenoma cell cultures at about 50% confluency. Expression of COX-2, VEGF and c-fos was determined by RT–PCR. Expression of GAPDH was determined for standardisation. Polymerase chain reaction products were analysed on 6% acrylamide gels. The figure shows a composite of two individual analyses each with its separate GAPDH control.

Figure 6 Gene expression in LT97 adenoma cells overexpressing COX-2. LT97 cells were infected with an adenoviral vector expressing COX-2. Control groups were infected with control viruses containing no ectopic gene and cells exposed to the COX-2 inhibitor SC236. (A) Cells were homogenised in RIPA buffer and COX-2 protein analysed by Western blotting. (B) Prostaglandin E2 secretion into the medium was determined by ELISA. (C) RNA was isolated from control transfectants and COX-2-LT97 cells in the presence and absence of the COX-2 inhibitor SC236 and c-fos expression was analysed by standard RT–PCR. Polymerase chain reaction products were separated on 6% acrylamide gels and band intensities were semi-quantified using the Image Quant software. The results represent the mean ± s.e.m. from three independent experiments.

© 2006 Cancer Research UK

British Journal of Cancer (2006) 94(11), 1718–1725
is much better reflected by cultures overexpressing COX-2 so that they produce less PG, but do so continually over time. In our study, this affected gene expression and signalling events in a similar manner but over at least 24 h.

In detail, addition of PGE₂ to tumour cell cultures upregulated expression of COX-2 and consequently stimulated the endogenous production of PG in both LT97 and Caco2 cells. In LT97 cells, this event was preceeded by expression of c-fos and an increase of c-jun in the nucleus and dependent on activation of the p38 signalling kinase.

Of VEGF, a crucial PGE₂ target gene in colonic polyps (Seno et al., 2002), two main splice variants were found representing the main products identified in colorectal tumour cells by Uthoff et al. (2002). Unexpectedly, no stimulation of VEGF expression could be observed in both LT97 and Caco2 cells.

As a model for advanced adenomas VACO235 were used. These cells express high levels of COX-2 and produce as much as 1 – 2 nM PGE₂ within 24 h. Their VEGF expression was higher than that of LT97 cells. As PG production could not be completely blocked by SC236, a highly specific COX-2 inhibitor (Richter et al., 2001), we cannot prove that the effect was PG dependent. Neither could stimulation of VEGF expression be confirmed using LT97 cells overexpressing COX-2 from an adenoviral vector. This may be due to the much smaller amount of PG produced from the transgene in LT97 cultures (250 pM). However, we cannot preclude unrelated causes. Although this seems to contradict the hypothesis that PGs act as an angiogenic switch (Seno et al., 2002; Sunayama et al., 2002), it has to be kept in mind that the adenoma cells are not the only cells capable of response to PGE₂ present in an adenomatous polyp. In polyps in vivo PG can exert paracrine effects on fibroblasts, or endothelial cells, as both cell types have been shown to be responsive in other models (Gupta and Dubois, 2001; Pai et al., 2001; Trompezinski et al., 2001).

The strong expression of COX-2 in VACO235 cells also correlated with higher expression of c-fos. In this case, upregulation was confirmed by use of LT97 cells overexpressing COX-2. Inhibition by 1 μM SC236 demonstrated that the effect was PG dependent. In addition, c-jun protein in the nucleus was increased in a PGE₂-dependent manner. Induction of c-fos has also been achieved by tumour-promoting but not by non-promoting bile acids (Jurek et al., 2005). Stimulation of c-fos and c-jun together indicates activation of AP1 which is frequently observed following exposure to tumour-promoting compounds in the colon as well as the skin (Qiao et al., 2000; Angel et al., 2001; Bachelor and Bowden, 2004) and is regarded as a therapeutic target (Suto et al., 2004). Expression of c-fos was dependent on the activation of both ERK and p38 in cells exposed to PGE₂. This indicates that more than one stimulus is necessary for induction of c-fos, which was also shown after exposure to bile acids (Qiao et al., 2000). In our study, both kinases were also activated in COX-2-overexpressing cells, but p38 activation could not be shown to be PG dependent.

In summary, we provide evidence that both PGE₂ from the microenvironment and upregulation of COX-2 affect signalling and gene expression in human colorectal adenoma cells in a way that enhances tumour progression by both autocrine and paracrine mechanisms.

### Table 3 Relative VEGF expression in COX-2-LT97 cells

| Experimental group   | VEGF (% of uninfected control) |
|---------------------|--------------------------------|
| Ad-co               | 112.3 ± 20.7                   |
| Ad-co + SC236       | 107.6 ± 5.4                    |
| Ad-COX-2            | 236.7 ± 48.7                   |
| Ad-COX-2 + SC236    | 333.0 ± 155.7                  |

VEGF = vascular endothelial growth factor; COX-2 = cyclooxygenase-2.

---

**Figure 7** Activation of signalling kinases in COX-2-overexpressing cells. Activation of ERK and p38 was analysed from protein lysates from control cells as well as COX-2-LT97 cells in the presence and absence of SC236. Band intensities were semi-quantified using the Image Quant software. The results represent the mean ± s.e.m. from three independent experiments. *Larger than control at \( P < 0.05 \).
ACKNOWLEDGEMENTS
We thank Meenhard Herlyn (Wistar Institute, Philadelphia, PA) for his support in constructing the adenoviral vector and to Doris Mejri for excellent technical assistance. This work was supported by grants of the Herzfelder’sche Familienstiftung to KH and BM and by the 3R programme of the Austrian Ministry for Education, Science and Culture to BM (GZ70.082/2-Pr/4/2002).

REFERENCES
Angel P, Szabowski A, Schorpp-Kistner M (2001) Function and regulation of AP-1 subunits in skin physiology and pathology. Oncogene 20: 2413 – 2423
Bachelor MA, Bowden GT (2004) UVA-mediated activation of signaling pathways involved in skin tumor promotion and progression. Semin Cancer Biol 14: 131 – 138
de Wind N, Dekker M, van Rossum A, van der Valk M, te Riele H (1998) Mouse models for hereditary nonpolyposis colorectal cancer. Cancer Res 58: 248 – 255
DuBois RN, Giardiello FM, Smalley WE (1996) Nonsteroidal anti-inflammatory drugs, eicosanoids, and colorectal cancer prevention. Gastroenterol Clin North Am 25: 773 – 791
Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN (1994) Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology 107: 1183 – 1188
Gupta RA, Dubois RN (2001) Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. Nat Rev Cancer 1: 11 – 21
He TC, Zhou S, Costa LY, Kinzler KW, Vogelstein B (1998) A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci 95: 2509 – 2514
Jurek D, Fleckl E, Marian B (2005) Bile acid induced gene expression in LT97 colonic adenoma cells. Food Chem Toxicol 43: 87 – 93
Pai R, Soreghan B, Szabo IL, Pavelka M, Baatar D, Tarnawski AS (2002) Prostaglandin E2 transactivates EGFR receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. Nature Medicine 8: 289 – 293
Pai R, Szabo IL, Soreghan BA, Atay S, Kawanaka H, Tarnawski AS (2001) PGE(2) stimulates VEGF expression in endothelial cells via ERK2/JNK1 signaling pathways. Biochem Biophys Res Commun 286: 923 – 928
Qiao D, Chen W, Stratagoules ED, Martinez JD (2000) Bile acid-induced activation of activator protein-1 requires both extracellular signal-regulated kinase and protein kinase C signaling. J Biol Chem 275: 15090 – 15098
Richter M, Jurek D, Wrba F, Kaserer K, Wurzer G, Karner Hanusch J, Marian B (2002) Cells obtained from colorectal microadenomas mirror early premalignant growth patterns in vitro. Eur J Cancer 38: 1937 – 1945
Richter M, Weiss M, Weinberger I, Furstenberger G, Marian B (2001) Growth inhibition and induction of apoptosis in colorectal tumor cells by cyclooxygenase inhibitors. Carcinogenesis 22: 17 – 25
Seno H, Oshima M, Ishikawa TO, Oshima H, Takaku K, Chiba T, Narumiya S, Taketo MM (2002) Cyclooxygenase 2- and prostaglandin E(2) receptor EP(2)-dependent angiogenesis in Apc(Delta716) mouse intestinal polyps. Cancer Res 62: 506 – 511
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
Sonoshita M, Takaku K, Sasaki N, Sugimoto Y, Ushikubi F, Narumiya S, Oshima M, Taketo MM (2001) Acceleration of intestinal polyposis through prostaglandin receptor EP2 in Apc(Delta 716) knockout mice. Nat Med 7: 1048 – 1051
Sunayama K-I, Konno H, Nakamura T, Kashiwabara H, Shoji T, Tsuneyoshi T, Nakamura S (2002) The role of cyclooxygenase-2 (COX-2) in two different morphological stages of intestinal polyps in APC(Delta 474) knockout mice. Carcinogenesis 23: 1351 – 1359
Suto R, Tominaga K, Mizuguchi H, Sasaki E, Higuchi K, Kim S, Iwao H, Arakawa T (2004) Dominant-negative mutant of c-Jun gene transfer: a novel therapeutic strategy for colorectal cancer. Gene Therapy 11: 187 – 193
Trompezinski S, Pernet I, Schmitt D, Vlac J (2001) UV radiation and prostaglandin E2 up-regulate vascular endothelial growth factor (VEGF) in cultured human fibroblasts. Inflamm Res 50: 422 – 427
Tutton PJ, Barkla DH (1980) Influence of prostaglandin analogues on epithelial cell proliferation and xenograft growth. Br J Cancer 41: 47 – 51
Ushoff SM, Duchrow M, Schmidt MH, Broll R, Bruch HP, Strik MW, Galandiuk S (2002) VEGF isoforms and mutations in human colorectal cancer. Int J Cancer 101: 32 – 36
Willson JK, Bittner GN, Oberley TD, Meisner LF, Weese JL (1987) Cell culture of human colon adenomas and carcinomas. Cancer Res 47: 2704 – 2713

© 2006 Cancer Research UK
British Journal of Cancer (2006) 94(11), 1718 – 1725