The development of new treatment strategies for oesophageal cancer

Buskens, C.J.

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To develop selective adenoviral vectors for cancer gene therapy, transcriptional targeting

**Inflammation** can be used.

mediated activation

Since cyclooxygenase-2 is compromises tumour frequently upregulated in specificity of
gastrointestinal carcinoma cells, the Cyclooxygenase-2 while undetectable promoter in
in normal cells, adenoviral context

the selectivity of a

COX-2 tumour

specific promoter in

adenoviral context

was analysed in this study.
INTRODUCTION

All clinical cancer gene therapy trials using non-replicating adenoviral vectors (serotype 5) show that this approach is safe but lacks clinical efficacy. A major problem is the limited transduction of tumor cells. Upon injection into a tumor, most studies show that only cells in the proximity of the needle track are transduced. Replicating adenoviral vectors could solve this problem because after transducing an initial small cell population, replication will result in a burst of adenovirus followed by spreading of the viral progeny to surrounding tumor cells. Considering the broad tropism and more specifically the hepatotropism of adenoviral serotype 5 vectors, replication of these adenoviral vectors should be restricted to tumor tissue.

Conditionally replicating adenoviral vectors (CRAds) are a relatively new class of therapeutic vectors that use the normal adenoviral replication cycle to selectively lyse tumor cells, while sparing normal cells. Tumor specific viral replication can be achieved in two ways. One approach is to mutate viral genes essential for viral control of the cell cycle. An example is the ONYX-015 virus, in which tumor specificity is obtained by a deletion in the viral E1B region, restricting viral replication to cells with a defective p53 pathway. However, such mutations seem to result in impaired replication which could reduce the efficacy in patients. A second approach to produce CRAds is to replace replication controlling viral promoters by tumor specific promoters (i.e. transcriptionally targeted CRAds). Such a promoter can be derived from genes that have been found to be upregulated specifically in tumor tissue. Well known examples are the promoter regions of the carcinogenic embryonic antigen (CEA) and that of the alpha-feto-protein (AFP). In addition to a selective ‘tumor on’ phenotype, the candidate promoters should also exhibit a ‘liver off’ phenotype for mitigation of hepatotoxicity.

Recently, promoter fragments of the cyclooxygenase-2 (COX-2) gene have emerged as promising tumor specific elements. COX-2 is the rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins. Under physiological conditions this inducible isoform of the cyclooxygenase family is undetectable in normal tissues, including liver, whereas it is frequently upregulated in pathological conditions especially in gastrointestinal malignancies. Seventy-five to 100% of oesophageal, gastric, colorectal, liver and pancreatic cancers have been reported to show increased expression of COX-2 at the mRNA and the protein level. However, COX-2 is not only expressed in tumors but also in inflamed normal tissue. Since adenoviral vectors have been shown to initiate inflammation by activation of immune cascades, it might be expected that the administration of adenoviral...
vectors could also induce COX-2 expression. Although this induction may involve post-transcriptional mechanisms, detectable expression of COX-2 does indicate that the promoter is active. If adenoviral induction indeed results in increased COX-2 promoter activity, this could compromise the selectivity of CRAds driven by a COX-2 promoter. Inflammation, either directly induced by the adenoviral particles or by cell debris resulting from adenovirus induced cell lysis, could start a positive feedback loop resulting in transcriptional upregulation of COX-2. If COX-2 expression is induced in normal hepatocytes by adenoviral treatment, this could result in viral replication in this organ leading to liver toxicity.

In this study we investigated the therapeutic potential of a COX-2 promoter, in adenoviral context, for the treatment of gastrointestinal tumors. We addressed the possibility of self activation by investigating basal and adenoviral induced COX-2 expression in a panel of gastrointestinal cancer cell lines and freshly isolated primary human hepatocytes. In addition, we determined in vivo COX-2 expression patterns in liver samples of 10 patients who underwent hemi-hepatectomy for primary or metastatic liver cancer, treated with and without preoperative adenoviral cancer gene therapy.

MATERIALS AND METHODS

Established human carcinoma cell lines
The oesophageal squamous cell carcinoma cell line TE-2 was kindly provided by Dr. M. Yamamoto (University of Alabama, Birmingham, AL, USA). Human bileduct carcinoma cell lines CCLP-1 and MzChA-1 were obtained from Dr. T. Whiteside (Pittsburgh, PA, USA) and Dr. J.G. Fitz (University of Colorado, Denver, CO, USA) respectively. HT29 and CaCo-2 (colon carcinoma), and OE33 (oesophageal adenocarcinoma) cells were purchased from the European Collection of Cell Cultures (Salisbury, UK). Adenoviral producer cells HEK 293 (human embryonic kidney) were obtained from the American Type Culture Collection (ATCC). All cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum, 300 μg/ml L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37°C in a humidified, 10% CO₂ atmosphere.

Primary hepatocyte cultures
Human hepatocytes were isolated from small liver resection samples (2-5 grams) of patients undergoing partial hepatectomy. Cells were isolated by a standard two-step collagenase perfusion as essentially described by Seglen...
et al., and Ballet et al. After isolation, cells were seeded in primaria plates (Falcon, Becton Dickinson Labware, Milville, NJ, USA) in complete Williams E medium (supplemented with 10% heat-inactivated fetal calf serum, 2μM L-glutamine, 1 mM Dexamethason, 20 μU/ml insulin, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml Fungizon and 1mM ornithin). Hepatocytes were allowed to attach for four hours after which the culture medium was replaced with RocketFuel activated complete CS-C serum free medium (Cell Systems, Kirkland, WA, USA) in order to avoid dedifferentiation and preserve hepatocyte function. Hepatocytes were cultured in a 10% CO2 atmosphere and were allowed to recover for 48 hours before they were used in subsequent experiments. This study was performed in accordance with the guidelines of the local ethics committee.

**Adenoviral constructs**

Recombinant E1-deleted adenoviral vectors expressing the firefly luciferase gene from the human cytomegalovirus (CMV) promoter or one of two control regions of the human COX-2 promoter, COX-2M (-883 to +59 bp) and COX-2L (-1432 to +59 bp), were kind gifts from Dr. I. Dmitriev (University of Alabama, Birmingham, AL, USA).

An E1-deleted adenovirus expressing green fluorescent protein under control of a CMV promoter (AdCMVGFP) was kindly provided by Dr. V. Krasnykh (University of Alabama, Birmingham, AL, USA). Recombinant viral vectors were constructed using the AdEASY system. In short, promoter regions driving the firefly luciferase gene (pGL3 basic vector, Promega, Madison, WI, USA) were cloned into the AdEASY pShuttle vector. The resultant plasmid was recombined with the AdEASY-1 adenoviral backbone in *Escherichia coli* BJ5183. Recombinant plasmids were transfected into HEK 293 cells to generate AdCMVluc, AdCOX-2Mluc, AdCOX-2Lluc and AdCMVGFP. Adenoviral preparations were purified by double cesium chloride (CsCl) density centrifugation, dialyzed against PBS containing 10% glycerol, aliquoted and stored at -80°C until use. All titers were determined on HEK 293 cells and expressed as plaque forming units (PFU)/ml as described previously.

**Adenoviral gene transfer and luciferase assays**

Adenoviral transduction experiments were routinely performed as follows: carcinoma cell lines were seeded in 24 well plates with a density of 2x10^6 cells per well and primary hepatocytes were seeded in primaria 6 well plates with 5x10^5 cells per well. Cell lines were used 24 hours after seeding, and hepatocytes after 48 hours. After rinsing the cells once with PBS, cells were transduced in Dulbecco's Modified Eagle's Medium (DMEM) without serum for 1 hour. To analyse the adenoviral COX-2 induction, cell cultures were incubated for
24 hours with virus either in the presence or absence of 10 ng/ml of the strong COX-2 inducer phorbol 12-myristate 13-acetate (PMA; Sigma Chemical Co., St Louis, MO, USA). Twenty-four hours post transduction cells were harvested for Western blotting (see below) or for luciferase activity assays. Luciferase activity was measured according to manufacturers protocols using the Berthold luminometer (Berthold Detection System, Pforzheim, Germany), the Promega luciferase assay system (Promega, Madison, WI, USA) and the Pierce protein assay (Pierce Biotechnology, Rockford, IL, USA).

Western blot analyses of COX-2 expression
Cells were lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% Tergitol type NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM EDTA, 50 mM Tris/HCl pH 8.0 plus freshly added complete mini protease inhibitor cocktail tablets (Roche Diagnostics GmbH, Mannheim, Germany) and sonicated. Proteins were resuspended in sample loading buffer (125 mM Tris/HCl pH 6.8, 2% SDS, 20% glycerol, 5% β-mercaptoethanol, and 0.015% bromophenol blue) and protein concentration was measured using the Pierce protein assay (Pierce Biotechnology, Rockford, IL, USA). Equivalent samples were separated by 10% SDS-PAGE and transferred to a PVDF membrane (Milipore, Bedford, MA, USA) which was subsequently incubated overnight at 4°C with a monoclonal COX-2 antibody (160112, Cayman Chemical Co., Ann Arbor, MI, USA) in a dilution of 1:1000, and for 1 hour with 1:2000 diluted Horse Radish Peroxidase (HRP) conjugated secondary antibody (DAKO, Duiven, The Netherlands). The bands were visualized, using Lumilight plus substrate (Roche Molecular Biochemicals, Almere, The Netherlands) and chemiluminescence detection (Lumi Imager, Roche, Almere, The Netherlands). Each sample was corrected for loading by normalization with the appropriate β-actin signal. Antibody bands were quantified using Genetools software (Syngene systems, Frederick, MD, USA).

COX-2 immunohistochemistry
To analyse adenoviral COX-2 induction in normal hepatocytes in vivo, liver samples of 10 patients who underwent hemi-hepatectomy for primary or metastatic liver cancer were immunohistochemically stained for COX-2. Five of these patients were treated preoperatively with gene directed enzyme prodrug therapy. The gene therapy treated patients were from the CTL 102/CB 1954 CTC99020 study, in which 1 x 10⁹ or 1x10¹¹ particles of a replication deficient adenovirus (CTL 102) encoding the nitroreductase suicide gene were administered intratumourally. Five days after treatment, the tumour was removed surgically to assess the nitroreductase gene expression
and sections from this material were obtained for this study. The COX-2 immunohistochemical staining procedure was performed as described previously. Briefly, formalin-fixed and paraffin-embedded specimens were sectioned (5 µm), and deparaffinized for antigen retrieval. Immunostaining was performed with the COX-2 specific mouse anti-human monoclonal antibody (160112, Cayman Chemical Co., Ann Arbor, MI, USA) in a dilution of 1:200. An experienced GI-pathologist (GJAO) scored the COX-2 immunoreactivity in both tumour cells and normal hepatocytes of the liver resection specimens.

**RESULTS**

**Endogenous COX-2 expression predicts COX-2 promoter activity in an adenoviral vector**

To assess the transcriptional efficiency of the COX-2 promoter, the expression of endogenous COX-2 protein and the transcriptional activity of two COX-2 promoter fragments (a long 1491 bp fragment designated L and a shorter 942 bp fragment of the same region designated M) were analysed in a panel of gastrointestinal carcinoma cell lines. The cells were transduced with AdCOX-2Mluc and AdCOX-2Lluc, and the COX-2 driven luciferase expression was compared to that of AdCMVluc. The COX-2 promoter driven luciferase expression was correlated to the amount of COX-2 protein present in each cell line as shown in Figure 1. Low COX-2 expressing cell lines like the oesophageal cell line TE-2 and bile duct carcinoma cell line MzChA-1 showed a low COX-2 driven luciferase activity, while high COX-2 expressing cell lines like the cholangiocarcinoma cell line CCLP-1 and oesophageal cell line OE33 showed COX-2 driven luciferase expression levels approaching that of the CMV promoter. Virtually no difference in luciferase activity was observed between the two COX-2 promoter constructs. From these results we conclude that, in the tested panel of gastrointestinal cell lines, the endogenous COX-2 protein expression predicts the activity of the COX-2 promoter in an adenoviral context.

**Induction of COX-2 expression increases COX-2 promoter activity**

To investigate if the promoter region present in the adenoviral vectors is still inducible, PMA was used which is an established pharmacological activator of the COX-2 pathway. Carcinoma cell lines were cultured either in the absence or presence of PMA, and effects on COX-2 protein expression and COX-2 driven luciferase expression were analysed. These experiments show that when COX-2 expression is upregulated, the transcriptional activity of the
Carcinoma cells from a panel of gastrointestinal cell lines were infected with AdCOX-2Mluc and AdCOX-2Lluc (MOI 10). The adenoviral COX-2 promoter driven luciferase expression was correlated to the amount of COX-2 protein levels in all cell lines as demonstrated by immunoblots. Equal loading was confirmed by showing equal β-actin levels.

COX-2L and COX-2M promoters increases accordingly (Figure 2).

No significant increase in viral expression was observed with vectors containing the CMV promoter. Particularly strong activation, up to a 23 fold increase in luciferase activity, was seen in the oesophageal carcinoma cell line TE-2 and cholangiocarcinoma cell line Mz-ChA-1 cells, which both have low basal levels of COX-2 expression. These results indicate that inflammation responsive elements are still functional in the COX-2 M and L promoter constructs.

Replcation deficient adenoviral vectors induce COX-2 expression in carcinoma cell lines and primary human hepatocytes

Adenoviral vectors can induce inflammatory responses and since COX-2 expression is affected by inflammation, it was hypothesized that the administration of adenoviral vectors could induce COX-2 expression in cells.
Upregulation of COX-2 expression by treatment of human gastrointestinal carcinoma cell lines with PMA leads to increased transcriptional activity of the COX-2L and COX2M promoters as shown by the increased luciferase levels (MOI 10). No significant increase in viral expression was observed with vectors containing the CMV promoter.

Although this may enhance the efficiency of COX-2 driven adenoviral vectors it could impair their tumour selectivity. To determine if adenovirus indeed can induce COX-2 expression, we transduced TE-2 and CaCo-2 cells with AdCMVluc (MOI 50). COX-2 expression was analysed by Western blot at 12 and 24 hours post-transduction. The results were comparable to those seen after PMA induction. In the TE-2 cell line, which was also very responsive to PMA, strong induction of COX-2 was seen 24 hours after viral transduction (Figure 3). This indicates that in human cancer cells the COX-2 gene can be induced by adenoviral vectors.

Since, one of the main goals of transcriptional targeting is to mitigate liver toxicity, COX-2 induction was also analysed in six primary human hepatocyte isolates. Freshly isolated hepatocytes were cultured and treated with adenoviral vectors (AdCMVluc, moi 50 and AdCMVGFP, moi 10) or with PMA for 24 hours. Cell viability was confirmed by identifying green fluorescent cells with fluorescent microscopy before cells were harvested for Western blot analysis. Clear induction of COX-2 expression by PMA was observed in four
FIGURE 3
Induction of COX-2 expression in gastro-intestinal carcinoma cell lines by adenoviral treatment. After 24 hours, the COX-2 protein is readily induced in the human esophageal carcinoma cell line TE-2, whereas in the human colon carcinoma cells line CaCo-2 the COX-2 induction is limited. Both cell lines were transduced with a non-replicative adenoviral vector (AdCMVluc, moi 50).

out six hepatocyte isolates, and adenoviral COX-2 induction was observed in two out of four hepatocyte isolates (Figure 4A). This COX-2 protein induction was associated with an increase in viral expression (AdCOX-2L, moi 5) (Figure 4B), which was even more pronounced when cell lysates were added to the adenoviral vectors (Figure 4C). Together these data indicate that in normal human hepatocytes COX-2 expression is induced by inflammatory stimuli provided by either PMA or adenoviral vectors.

Induction of COX-2 expression in normal hepatocytes in vivo
Expanding on the in vitro results in primary hepatocytes, COX-2 protein expression was determined in three patients with primary hepatocellular
FIGURE 4
Effect of PMA and adenoviral vectors on COX-2 induction and activation of a COX-2 promoter in eight independent human hepatocyte isolates. Cell viability was confirmed by identifying green fluorescent cells with fluorescent microscopy before cells were harvested (AdCMVGFP, moi 10). A: COX-2 protein induction by PMA (isolate 1-6) or adenoviral vectors (AdCMVluc, moi 50) (isolate 3-6). B: Activation of the COX-2L promoter (AdCOX-2L, moi 5) by PMA (isolate 4-8). C: Activation of the COX-2L promoter (AdCOX-2L, moi 5) by adenoviral vectors with cell lysates (AdNULL-eGFP, moi 100 and 1000).
carcinoma and seven patients with liver metastases from colorectal cancer, who underwent partial hepatectomy. Representative slides of the tumour and surrounding normal liver tissue were stained immunohistochemically for COX-2 expression. COX-2 immunoreactivity was detected in all tumours, of which 8 were classified with moderate to strong staining intensity. In contrast, only weak or no staining was observed in normal hepatocytes, except at sites around inflammation and necrosis (n=4). In tumour bordering non-tumorous tissues, a more intense staining of COX-2 was observed than in non tumour bordering normal liver cells (Figure 5). No identifiable difference in COX-2 expression was observed between the gene therapy treated patients (n=5) and the patients (n=5) who only underwent surgery. These data indicate that COX-2 expression can be induced in normal hepatocytes in vivo.

**FIGURE 5**
Representative examples of COX-2 immunohistochemistry. A: Strong COX-2 immunoreactivity in tumour cells (40x). B: In tumor bordering non-tumorous tissues, a more intense staining of COX-2 was observed than in non tumor bordering normal liver cells (40x). C: Weak COX-2 immunoreactivity in normal hepatocytes (100x).

**DISCUSSION**

The successful clinical use of adenoviral cancer gene therapy, specifically strategies involving replicating adenoviral vectors, relies on the ability to restrict viral activity to tumour cells. The frequent involvement of the liver in tumour pathology, both as a primary tumour source and major site of metastasis, combined with the natural hepatotropism of adenoviral vectors (serotype 5), makes the need for targeting especially stringent in this organ. An attractive strategy to reduce adenoviral liver toxicity is to use transcriptionally targeted replicating adenoviral vectors. A major obstacle in determining the safety profile of CRAds and their controlling elements is the
lack of adequate animal models. The athymic nude mouse implanted with human tumours is still the only widely available system. However, since the replication of human adenoviral vectors is severely impaired in mouse tissues, these mice models are not suitable to determine 'leakiness' of vector specificity. Therefore normal human material, although difficult to obtain, is still best suited for analysing leakiness of potential tumour specific elements. In this study we have been able to obtain fresh primary normal human hepatocytes, which allowed us to investigate tumour selectivity of the COX-2 promoter in an adenoviral context. High COX-2 expression has been demonstrated in many human gastrointestinal malignancies\textsuperscript{11}, whereas it is not expressed in normal tissue including liver, making the COX-2 promoter a potential tumour targeting element. In recent studies by Yamamoto et al., two regions of the cyclooxygenase-2 promoter have been identified as promising tumour specific transcriptional targeting elements.\textsuperscript{11, 12} In the present study, the activity of the COX-2 promoter was demonstrated to be correlated with the endogenous COX-2 status of various gastrointestinal carcinoma cell lines. Using the strong COX-2 activator PMA\textsuperscript{25}, it was also shown that COX-2 promoter fragments cloned into adenoviral vectors can still be transcriptionally activated, indicating that regions essential for the transcriptional upregulation of COX-2 are present in the used promoter fragments. Previous studies showed that the inducing effects of PMA are mediated by the cyclic AMP response element (CRE, located at -59 bp to -53 bp)\textsuperscript{29} and to a lesser extent the nuclear factor-κB (NF-κB, located at -223 to -214 bp)\textsuperscript{30} of the COX-2 promoter. Furthermore these elements have also been implicated in adenoviral and inflammatory cytokine mediated COX-2 promoter activation.\textsuperscript{31, 32} Both elements are indeed present in the promoter fragments used in this study and may therefore play a role in the activation of the COX-2 promoter. Recently, Hirschowitz et al. showed that adenoviral vectors induce dose-dependent increases in COX-2 protein and Prostaglandin E2 (PGE-2) production in non-small cell lung cancer cell lines and that this increase was independent of the transgene expressed.\textsuperscript{33} In concordance with the results from Hirschowitz et al. we could also induce COX-2 expression by incubating gastrointestinal carcinoma cells with non-replicating adenoviral vectors. More generally, adenovirus induced inflammation has also been described previously. Adenoviral particles, whether replication competent or deficient, are able to induce inflammation upon binding and/or internalization of viral components.\textsuperscript{34, 35} The cell-virus binding, internalization and predominantly adenoviral expression of the E4 coding region, stimulate signal transduction pathways (e.g. mitogen-activated protein kinase (MAPK) and p38/stress-activated protein kinase (p38/SAPK) that activate transcription factors.
These inflammation cascades are not restricted to tumour cells and can also be activated in normal cells. Considering this, we investigated the inducibility of COX-2 in primary human hepatocytes and found that PMA and adenoviral COX-2 induction can also occur in normal human hepatocyte cultures, although it must be noted that COX-2 induction could not be detected in all isolates. Because of these heterogeneous results and the limited availability of primary human hepatocyte isolates, we also analysed COX-2 expression in resection material from ten patients who underwent hemi-hepatectomy for primary or metastatic liver cancer. Five of these patients were preoperatively treated with intratumoural injection of different doses of a non-replicating adenoviral vector. Based on the nitro-reductase expression no spread of the injected virus was seen into the surrounding normal liver tissue, which could explain why in the gene therapy group no additional COX-2 induction was observed. Comparable to the in vitro hepatocyte cultures, COX-2 expression was heterogeneous in liver in vivo. Still, clear COX-2 expression could be observed in normal hepatocytes, specifically in those surrounding the tumour cells. The presence of COX-2 expression in normal hepatocytes demonstrates that other factors can also disrupt COX-2 tumour specificity in vivo. Activation of MAPK/ERK and p38/SAPK pathways can increase the quantity and activation state of nuclear transcription factors leading to pro-inflammatory cytokine gene expression, especially in tissue macrophages. These various cytokines (e.g. TNF-α, IL-1 and IL-8) can rapidly induce the COX-2 protein level and enzyme activity via activation of the nuclear factor-κB (NF-κB) transcription factor. A publication of Reid et al. demonstrated a systemic upregulation of COX-2 inducing cytokines after hepatic arterial infusion of a replication-selective oncolytic adenovirus in patients with liver metastasis of gastrointestinal malignancies. Together these data suggest that the COX-2 promoter activation can be more pronounced in vivo than demonstrated in the in vitro experiments due to additional cytokine production and activation of immune cells. The results of this study seem to contradict the outcome of a previous study conducted by Yamamoto et al. regarding the COX-2L and COX-2M promoter elements. Yamamoto et al. administered COX-2 promoter controlled CRAds systemically in non-tumour bearing mice and histopathological analysis did not reveal findings of toxicity in major organs. However, they did observe increased numbers of acute inflammatory cells in the liver parenchyma. Considering the impaired replication of human adenoviral vectors in mouse tissues, the presence of inflammation in the liver complements our data and also suggests that the tumour specificity of COX-2 promoter elements can be compromised when replicating adenoviral vectors are used to treat primary and secondary liver tumours.
One could easily imagine the loss of COX-2 tumour specificity when, after initial tumor specific replication, considerable amounts of adenoviral particles are released from the tumour to adjacent normal cells, inducing inflammation and subsequent activation of COX-2 in normal cells. In fact, in their study Yamamoto et al detected E1A mRNA in the livers of treated animals indicating that the COX-2 promoter is active albeit on a very low level. Since low levels of E1A are already sufficient for adenoviral replication, even this low expression could result in efficient replication and cell lysis in a permissive tissue such as human liver. Furthermore adenovirus induced cell lysis does not only result in the release of viral particles but also exposes surrounding cells to cellular debris. Since cell debris is also able to induce pro-inflammatory cytokines, extensive release of both adenoviral particles and cellular debris is expected to have enhanced effects on COX-2 induction in vivo. This cascade would further compromise the clinical use of COX-2 transcriptionally targeted CRAds. Therefore, our results indicate that in its present form the COX-2 promoter may not be suitable for controlling the replication of a CRAd. Still, in light of the promising results in intestinal cancers, we think that modifications of the COX-2 promoter to enhance tumour specificity may result in an attractive tumour specific vector.

In this context, it would be interesting to see how deletions or mutations in the CRE and NF-κB domains that reduce the inflammation sensitivity, would influence the tumour specificity of the COX-2 promoter. A complementary approach could be to reduce COX-2 activating activity via mutations in the E4 region of adenoviral vectors, although it should be considered that such mutations can have severe implications for the oncolytic activity of the virus (e.g. complete deletion of the E4 region renders the virus replication incompetent). In addition, adapting the viral genome does not affect cell debris induced inflammation. Other interesting possibilities are to combine the currently used COX-2 promoter fragments with additional tumour specific promoters in dual promoter activated CRAds and/or combine the use of the COX-2 promoter with transductional targeting.

In conclusion, this study demonstrates that awareness of adenoviral vector effects on infected tumour cells and especially normal human (liver) cells is relevant and should be acknowledged in the development of adenoviral cancer gene therapy. Although the tumour specificity of the COX-2 promoter still warrants further research, our data suggest that the tumour-specificity of COX-2 promoter elements can be compromised when incorporated into adenoviral vectors systems due to possible self-activation of these vectors. Therefore, the currently used native COX-2 promoter regions seem unsuitable as tumour specific regulators in replicating adenoviral vector systems.
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