Celecoxib inhibits growth of tumors in a syngeneic rat liver metastases model for colorectal cancer

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Abstract

In colorectal cancer, 40% of patients will develop liver metastases. The present study was designed to evaluate the inhibitory effects of the COX-2 inhibitor celecoxib on the growth of colorectal cancer liver metastases in a syngeneic rat model, CC531. The effects of celecoxib on cell viability \textit{in vitro} were evaluated by treatment of CC531 tumor cell cultures with celecoxib. \textit{In vivo}, Wag/Rij rats were inoculated with CC531 tumor cells at two sites in the liver and treated with celecoxib starting one week before, or directly after tumor inoculation. Control rats were inoculated without treatment. Three weeks after tumor inoculation rats were sacrificed. Tumor size, immune cell infiltration and PGE$_2$ and celecoxib levels were determined. CC531 tumors did not show COX-2 expression, tumor growth was significantly inhibited by celecoxib treatment in a dose dependent manner. Immune cell infiltration was decreased after celecoxib treatment, indicating that the immune system was not involved in preventing tumor growth. Celecoxib serum concentration starting at 0.84 μg/ml significantly inhibited the outgrowth of CC531 liver tumors. In contrast, \textit{in vitro} concentrations of celecoxib of at least 12 μg/ml were needed to affect tumor cell viability, suggesting that the effect of celecoxib on tumor growth \textit{in vivo} was not a direct cytotoxic effect.
Introduction

In colon cancer, surgical resection potentially offers cure of the disease. Prognosis is mainly dependent on the occurrence of local or distant metastases, which occur in approximately 40% of the patients. Epidemiological studies have indicated a considerable reduction in risk of occurrence of colorectal carcinoma in patients with reported long-term Non Steroidal Inflammatory Drugs (NSAID) use. In addition to the chemoprophylactic potential, chemotherapeutic effects of NSAIDs have been suggested and evaluated in in vitro, animal and clinical studies. The mechanism by which NSAIDs reduce the risk of colorectal carcinogenesis is generally attributed to the inhibition of the arachidonic acid metabolism via the cyclooxygenase enzymes (COX). COX is a critical step in the synthesis of prostaglandins (PG) that affects cell proliferation, tumor growth and immune responsiveness. Several isoforms of COX exist. The isoform COX-2 is upregulated in many types of malignancies and is responsible for prostaglandin E₂ (PGE₂) production by tumor cells. Several recent reports have suggested that COX-2 expression has an important role in haematogenous metastasis of colorectal carcinomas to the liver, however, the effects of COX-2 inhibition on the growth of established liver metastases remains unknown.

Tumor cells use various strategies to escape host immune surveillance, among others by impairing the effectivity of the host immune response. Overproduction of PG and specifically PGE₂ by tumor cells results in direct down regulation of effector cell cytotoxicity, but also creates an abnormal balance between the T helper (TH)-1 and TH-2 response favouring the TH-2, hereby functionally blunting the host anti-tumor cellular immune response. A recent animal study suggested that the inhibitory effect of COX-2 inhibitors on tumor growth is immunological and is dependent on the presence of B or T lymphocytes. Given the immunomodulating nature of PGE₂ production by tumor cells via COX-2 it has been suggested that COX-2 inhibition can result in an increased anti-tumor immune response by facilitating infiltration.

The aim of the present study was to investigate the effects of the COX-2 inhibitor celecoxib on the growth of established liver metastases by use of the CC531 rat tumor model. In addition we evaluated the effects of celecoxib treatment on prostaglandin production, immune cell infiltration and apoptosis in the liver metastases.

Material and Methods

Animals
Twenty Male Wag/Rij rats weighing approximately 245 g were used (Charles River, Zeist, The Netherlands). All animals were housed in the animal facility of the Leiden University Medical Center. The animals had free access to food and water. The weight of the animals was followed throughout the experiment to monitor their general health state. Principles of laboratory animal care were followed and, according to Dutch law, the Animal Welfare Committee of the Leiden University Medical Center approved the study.
Cell culture and liver metastasis model

The colon adenocarcinoma cell line CC531 (1,2-dimethylhydrazine-induced) which is moderately differentiated and syngeneic to Wag/Rij rats was used for tumor inoculation. Briefly, tumor cells were cultured in RPMI 1640 supplemented with 2 mM L-glutamine (Gibco, Grand Island, NY, USA), 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin sulphate (complete medium). Tumor cells were harvested with a solution of 0.25% (w/v) EDTA and 0.25% (w/v) trypsin in HBSS (Sigma, St. Louis, MO, USA), washed three times in 0.9% (w/v) NaCl solution buffered with 1.4 mM phosphate (PBS) and adjusted to a suspension containing $1 \times 10^6$ viable (trypan blue exclusion test) tumor cells per ml PBS. For local liver tumor induction, $5 \times 10^4$ viable tumor cells (in 50 μl suspension) per site were injected subcapsulary into the upper lobe of the liver at 2 sites.

In vivo experimental design

Rats were randomly assigned to one of the following five groups: (1) control group, (2) celecoxib 500 ppm starting at tumor inoculation, (3) celecoxib 1000 ppm starting at tumor inoculation, (4) celecoxib 1500 ppm starting at tumor inoculation (5) celecoxib 1500 ppm starting one week before tumor inoculation (Figure 1). Two tumors were inoculated as described above in the liver at day 0. Tumors were allowed to grow for 21 days after which rats were sacrificed. Abdominal organs were evaluated for signs of toxicity. Liver tumors were separately enucleated from the surrounding liver parenchyma and measured. Blood samples were taken from all rats by aortal puncture at time of sacrifice. Blood samples were allowed to coagulate and were centrifuged for 10 minutes at 13000 rpm (Beckman GS-6R centrifuge, Beckman Coulter, Fullerton, CA, USA); supernatants were collected and stored at -20°C until analysis. The cross sectional tumor area was used for analyses. This was determined using the formula: $L \times W \times 0.25 \times \pi$ in which $L$ is maximum length and $W$ is maximum width of the tumor.

**Figure 1** Design of experiment with celecoxib treatment of CC531 tumors in a rat liver metastases model for colorectal cancer

Groups of 4 male Wag/Rij rats were fed 0, 500, 1000, or 1500 ppm celecoxib starting 7 days before (group 5) or directly after subcapsular tumor cell inoculation in the liver on day 0 (group 1-4). Rats were followed up for 21 days, after which they were sacrificed. After sacrifice, rat serum was collected and rat tumors were enucleated from the liver.
**Medication**
The COX-2 inhibitor celecoxib (SC-58635), obtained as a gift from Pfizer Pharmaceuticals, was incorporated into Altromin 1310 rat breeding diet by Altromin (Altromin Gesellschaft für Tierernährung mbH, Lage, Germany) at various concentrations. Rats were fed this diet according to experimental design as indicated in Figure 1.

**Analysis of celecoxib concentrations in serum**
A high-performance liquid chromatographic (HPLC) method was used and validated for the determination of celecoxib in serum. Ibuprofen was used as an internal standard. Blank serum samples (250 μl) were spiked with celecoxib (range 80ng/ml - 6000 ng/ml) and Ibuprofen (2000 ng/ml) and used as calibrators and quality control samples. The limit of quantification was 100 ng/ml. Within-run and between-run precisions were less than 10 % and average accuracies were between 90 and 110 %. To 250 μl of serum, 50 μl internal standard work solution (10 μg/ml) was added and the sample was mixed thoroughly. To precipitate the proteins, 1 ml of acetonitrile was added and the sample was vortexed again. After centrifugation, the supernatant was transferred to a glass tube and evaporated till dry. The residue was resuspended in 1 ml of the mobile phase and filtered over a 0.45 μm PVDF HPLC-filter (Acrodisc, Waters Corporation) for HPLC injection (40 μl).

Separation was achieved on a Symmetry 300 C18 column (25 cm x 4.6 mm, 5 μm) (Waters, Milford, USA) connected to a Luna C18 guard column (4 mm x 3 mm, 5 μm) (Phenomenex, Torrance, USA). The mobile phase, which was filtered through a 0.20 μm nylon filter before use, consisted of an acetonitrile-water-acetic acid-triethylamine (47 : 53 : 0.1 : 0.03) mixture and was pumped at a flow rate of 1 ml/min. Celecoxib and Ibuprofen were detected by fluorescence detection. Emission and excitation wavelengths of Celecoxib and Ibuprofen were 280/340 and 253/340, respectively.

**Analysis of PGE2 concentrations in liver metastases and serum**
Tumor and serum levels of PGE2 were measured to analyse celecoxib activity in rats fed the control diets or diets supplemented with 500ppm, 1000ppm or 1500ppm. A competitive enzyme immunoassay (R&D Systems Inc., Minneapolis MM 55413, USA) was used for the determination of PGE2 in serum and tumor tissue. The sensitivity of the PGE2 assay was typically higher than 13 pg/ml. Each tissue sample (50-300 mg) was dried for surface moisture and accurately weighed. The sample was then homogenized in 1 ml of distilled water. After centrifugation, the supernatant was treated the same way as serum.

**Immunohistochemical staining of CC531 liver metastases**
Cryostat sections (Cryocut 3000, Leica, Nuss-loch, Germany) 5 μm thick were cut from the tumor tissue that was snap-frozen directly after resection, of the control group (group 1) and the group receiving celecoxib 1500ppm (group 4). Sections were air-dried for at least 16 hr at 60 °C, then fixed in acetone for 10 min and washed twice in PBS. All dilutions of antibodies and conjugates were performed with PBS containing 1% (w/v) bovine serum albumin (BSA, Boehringer, Mannheim, Germany). Immunohistochemistry for detection of tumor cell COX-2 expression was performed as described previously with a polyclonal anti-COX-2 antibody...
MOLECULAR AND BIOLOGICAL INTERACTIONS IN COLORECTAL CANCER

(AXL-210-711, Alexis, San Diego, CA, USA, 1:30022). As negative controls sections were incubated with PBS instead the primary antibody. Immunohistochemical analysis of immune cell infiltration was performed as follows: The tissue sections were incubated for 30 min with a previously determined optimal concentration of protein-A-purified primary antibody. The monoclonal antibody (MAb) 3.2.3 IgG1 (a gift from Dr. W.H. Chambers, University of Pittsburgh Cancer Institute, Pittsburgh, PA) was used for detection of CD161A (NKR-P1A+, Natural Killer cells) cells, the MAb R73, anti-rat T-cell receptor (TCR) (a gift from Dr. Th. Hünig, University of Würzburg, Germany), was used for the detection of T cells. After incubation with the primary antibody, the sections were washed in PBS 3 times for 5 min, followed by two 30-min incubations with horseradish-peroxidase (HRP)-conjugated rabbit anti-mouse Ig (dilution 1:100) and HRP-conjugated swine anti-rabbit Ig (dilution 1:50, both obtained from DAKO, Glostrup, Denmark) and subsequent washes in PBS. Visualization of immune complexes was performed by a 10-min incubation with a 3,3’-diaminobenzidine (DAB) substrate containing 1.8 x 10^{-3} (v/v) H2O2. A polyclonal rabbit anti-laminin antibody (Sigma-Aldrich) was used for the detection of laminin. After 3 wash steps with PBS, the sections were incubated for 30 min with HRP-conjugated swine anti-rabbit Ig (dilution 1:50, DAKO) for the detection of laminin. The immune complexes were visualized by a 12-min incubation step in a buffered TRIS-HCl (pH 7.6) solution containing, per 100 ml, (1) 40 mg 4-chloro-1-naphtol (Merck, Darmstadt, Germany) dissolved in 200 μl dimethylformamide (Baker, Deventer, The Netherlands) and 300 μl ethanol (Merck) and (2) 100 μl of a 30% (v/v) H2O2 solution (Merck). The sections were slightly counterstained using methyl green (Klinipath) and mounted using Kaiser’s glycerine (Merck). Control sections (1 per tumor) were included in which both primary antibodies were omitted21.

Quantification of immunostaining
After immunohistochemical staining slides were directly coded in order to blind the observer for tumor number or treatment group of the tumors. The number of tumor infiltrating R73+ and 323+ cells in tumor epithelium were estimated using a scoring method described by Menon et al23. In brief, an ocular grid, with a total surface area of 38 mm², was used at a 200x magnification to count all leukocytes that were located intraepithelially in 25 different randomly chosen tumor fields of the tissue section. Laminin was used to distinguish between intraepithelially, that is, leukocytes in direct contact with tumor cells, and intrastromally located leukocytes (figure 3A-C). This tumor compartment-specific analysis made it possible to calculate the number of leukocytes per tumor cell area (leukocytes/mm² tumor epithelium). The mean leukocyte infiltration of 25 fields per tumor section was calculated and defined as the intraepithelial leukocyte infiltration. After evaluation, the slides were unblinded for treatment group for further analyses.

Effects of celecoxib on CC531 cell viability in vitro
The cell viability was assessed by the mitochondrial function, measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction activity as previously reported24. Briefly, cells were seeded in a 96-well plate and incubated with increasing concentrations of celecoxib (figure 4). After 72 hours, the cells were incubated with 0.5 mg/ml MTT (Sigma-
Aldrich) for 4 hours at 37°C. Subsequently, 100 μl SDS (10% (v/v) in 0.01 M HCl) was added, after which the absorbance was read at 590 nm, using a microplate reader (Bio-Rad Laboratories, Veenendaal, The Netherlands). Stock solutions of the pure compound celecoxib were made in dimethyl sulphide (DMSO). A final DMSO concentration of 0.1% in medium was used in all in vitro experiments including control experiments.

**Statistical analyses**

Statistical analysis between groups was performed using the Fisher exact test. Correlations between variables were evaluated using Spearman's rank analysis, Mann-Whitney, Kruskal-Wallis, or student's t-test. Values with P<0.05 were considered statistically significant. The Statistical Package for Social Sciences (SPSS) version 12.0 was used for all statistical analyses.

**Results**

**General condition of rats**

The body weights of rats fed the control diet or the experimental diets containing various levels of celecoxib were comparable throughout the study. There was no difference in animal behavior between the treatment groups. Animals experienced a slight weight loss after laparotomy for inoculation of CC531 tumor cells in the liver, but no rats lost more than 5% body weight. The initial tumor induction was successful in all rats and no rats died before the end of the experiment. After sacrifice of the animals no gross intra-abdominal changes were noted that would indicate toxicity.

**Serum celecoxib levels**

To establish if administration of celecoxib to rat diet resulted in adequate levels of celecoxib in rat serum, serum samples were collected after sacrifice. Increased dosage of celecoxib in the rat chow showed a corresponding increase in serum celecoxib levels (figure 2A). Rats in the control group who were fed regular chow had undetectable levels. Steady-state serum levels were as follows: celecoxib 500ppm (group 2): 0.84 ± 0.33 μg/ml, celecoxib 1000ppm (group 3): 1.97 ± 0.77 μg/ml, celecoxib 1500ppm (group 4): 3.10 ± 1.44 μg/ml, celecoxib 1500ppm starting 1 week pre inoculation (group 5): 3.07 ± 0.91 μg/ml (figure 2A). Serum celecoxib levels in the present study were comparable with the 0.1-5.0 μM concentrations in cancer patients treated with celecoxib1,25.

**Effects of celecoxib treatment on liver metastasis growth**

The effects of celecoxib administration on the tumor growth are summarised in figure 2B. Administration of celecoxib resulted in a significant dose dependent reduction of tumor size when compared to the rats that were fed control diet (group 1): Celecoxib 500ppm (group 2): p=0.04, celecoxib 1000ppm (group 3): p=0.02, celecoxib 1500ppm (group 4): p=0.006, celecoxib 1500 ppm starting one week before inoculation (group 5): p=0.007 (figure 2B) (Mann-Whitney). The administration of celecoxib 1 week before tumor cell inoculation did not significantly inhibit tumor growth compared to administration after inoculation (group 4) (p=0.28)
**Figure 2**

Effects of 21 days of celecoxib treatment on CC531 liver metastases and PGE2 serum and tumor level. All treatment groups consisted of 4 rats in each of which 2 tumors were inoculated subcapsularly in the liver. Rats received control diet, or a diet containing: celecoxib 500 ppm, celecoxib 1000 ppm, celecoxib 1500 ppm, starting at tumor inoculation, or celecoxib 1500 ppm, starting 7 days before tumor inoculation. Blood and tumors were obtained from rats after sacrifice. Serum celecoxib levels and serum and tumor PGE2 levels were measured as described in the material and methods section.

Values represent the mean and standard error. 2A: Serum celecoxib concentrations. 2B: Effects of celecoxib on tumor size (cross sectional tumor areas). 2C: PGE2 concentrations in the tumors of the rats. 2D: PGE2 concentrations in sera from the rats.

Effects of celecoxib treatment on serum and tumor PGE2 levels

The effects of celecoxib on tumor and serum PGE2 levels can be seen in figure 2C-D. No significant differences were found in tumor and serum PGE2 levels between the treatment groups (p=0.32 and p=0.51 respectively, Kruskal-Wallis).

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*celecoxib treatment started 7 days before tumor inoculation. In all other groups treatment started at inoculation.*
**Tumor COX-2 expression**

COX-2 expression in CC531 tumors is shown in figure 3: All CC531 tumor cells were negative for COX-2 expression. Surrounding tumor stroma showed light brown immunostaining, this was not affected by celecoxib treatment. Infiltrating macrophages showed to be positive for COX-2 and thus served as internal positive control for the test. All negative controls showed no immunoreactivity.

**Effects of celecoxib treatment on infiltration of tumors by R73+ and 323+ cells**

Previously, it was established that intraepithelial immune cells that are in direct contact with tumor target cells affect tumor growth. Therefore, infiltration of intraepithelial immune cells in the tumor was evaluated. Figure 4A-D shows the effects of celecoxib treatment on the intraepithelial infiltration of R73+ (TCR-positive cells, T cells) and 323+ (CD161A-positive cells, Natural Killer cells) cells. Intraepithelial infiltration of R73+ and 323+ cells was significantly diminished in the 1500ppm celecoxib group (group 4) compared to the control group (group 1) (p=0.01 and p=0.02 respectively). Infiltration with R73+ cells was positively correlated with 323+ cell infiltration (p=0.03, Spearmans’ rank analysis).

**Effects of Celecoxib administration on tumor cell viability in vitro**

In the present study we observed that concentrations of 0.84 μg/ml were sufficient to reduce tumor growth. When CC531 cells were exposed to celecoxib concentrations equal to *in vivo* concentrations after 72 hours, no effect on cell viability was observed. In vitro, treatment with concentrations of at least 12 μg/ml or higher were needed to inhibit cell growth.
viability $86.7 \pm 11.5$, p=0.10) and exposure to 2.4 $\mu$g/ml celecoxib resulted in a significant inhibition of cell viability as compared to the control group (mean % cell viability $43.0 \pm 3.7$, p<0.0001, one sample t-test).

A representative staining for T cell and NK cell infiltration of tumor sections from celecoxib-treated (1500ppm) and -untreated rats 21 days after tumor inoculation. Sections were double-stained with laminin and R73 (anti-TCR, 1:100, Fig. 4A, 4C) or 323 (anti-CD161A, 1:50, Fig. 4B, 4D) antibodies respectively. R73+ and 323+ cells were stained brown, as revealed by immunohistochemistry (see material and methods). The matrix protein laminin was stained blue, blank spaces represent tumor nodules, delineated by a laminin-containing basal-membrane-like structure. The majority of R73+ and 323+ cells were localized in the tumor stroma, few positive cells were found in the tumor nodules. (200x magnification)
**Discussion**

The current study demonstrates that treatment of rats with levels of celecoxib equal to therapeutic levels in humans, showed an inhibitory effect on the growth of liver metastases, even in a situation of low COX-2 activity. Recent RNA expression array data from a study by Germann et al. demonstrated that the CC531 cell line shows low COX-2 RNA expression. The low COX-2 expression was confirmed in our study as tumor epithelium was negative for COX-2 immunostaining. In addition, PGE$_2$ serum and tumor levels were not affected by celecoxib treatment. Furthermore, the level of PGE$_2$, assumed to reflect COX-2 activity, was very low as compared to a similar study using MC-26 cell line, that showed a 2000-fold higher PGE$_2$ production in untreated COX-2 positive tumors.

In our model, increasing levels of celecoxib were associated with a corresponding decrease in tumor size. Celecoxib is known to have direct cytotoxic effect on tumor cells as well as indirect effects, in which the immune system and angiogenesis is involved. Treatment of CC531 cells *in vitro* for 36 hours with concentrations of up to 12 μg/ml (32μM) did not have any significant effect on cell viability while *in vivo* already 0.84 μg/ml significantly inhibited tumor growth, suggesting no direct effect of celecoxib on tumor cell viability *in vivo*. These observations are supported by a study by Williams et al. Celecoxib may create an unfavourable host environment for tumor growth. Several environmental interactions that determine tumor growth have been described to be affected by celecoxib treatment, including the immune system.

In vitro production of PGE$_2$ by COX-2 prevents activation of natural killer cells and T-cell mediated anti-tumor response, impairs the function of DC's and suppresses lymphocyte proliferation. The before mentioned studies suggest that these effects can be reversed by selective COX-2 inhibition. A recent study indicates that, in addition to enhancement of lymphocyte accumulation in tumors by COX-2 inhibition, the anti-tumor effects of COX-2 inhibition are immunological and depend on the presence of lymphocytes in the tumor. In the current study we quantified the immune cell infiltration: Surprisingly, we found a significant decrease in T-cell and NK-cell infiltration in tumors receiving celecoxib treatment, showing that the effect of celecoxib on tumor growth in our model can not be attributed to immune effector cells. A decrease in infiltration after treatment with NSAIDs or COX-2 inhibitors has been described in inflammatory processes as inflammatory bowel disease and rheumatoid arthritis and indicates that the effects of COX-2 inhibition on tumor growth is not mediated through an increased anti-tumor immune response.

Most studies evaluating the immunological effects of COX-2 inhibition in tumor growth were performed with COX-2 overexpressing tumors. However it is estimated that 25-30% of human colorectal cancer does not express the COX-2 enzyme. The results from the current study indicate that effects of COX-2 inhibitors on tumors with low COX-2 activity are still significant, but independent of immune effector mechanisms.
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