Exp Oncol 2008
30, 1, 42–51

Experimental ONCOLOGY

CELECOXIB INHIBITS TUMOR GROWTH AND ANGIOGENESIS IN AN ORTHOTOPIC IMPLANTATION TUMOR MODEL OF HUMAN COLON CANCER

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Aim: **To examine the effect of celecoxib on tumor growth and angiogenesis in an orthotopic implantation tumor model of colon cancer.** ***Methods:*** **Human colorectal adenocarcinoma HT-29 cells were implanted subcutaneously in nude mice. Four groups of animals received different doses of celecoxib after tumor implantation. After 42 days, all animals were evaluated for changes in body weight, the volume and weight of colorectal tumors, and tumor growth inhibition. The content of prostaglandin E_2 (PGE_2) in the tumor tissue homogenate was estimated by radioimmunoassay (RIA). Cyclooxygenase-2 (COX-2) and CD34 expression in tumor tissue was assessed by immunohistochemistry, and the microvessel density (MVD) of tumor tissue was determined. Apoptosis of the tumor cells was detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). The expression of vascular endothelial growth factor (VEGF) mRNA and matrix metalloproteinase-2 (MMP-2) mRNA extracted from the tumor tissue was analyzed by reverse transcriptase polymerase chain reaction (RT-PCR).** ***Results:*** **There was no statistically significant change in the animals' body weight between the treatment groups. However, with increasing doses of celecoxib, the volume and weight of the tumor decreased. The rates of tumor growth inhibition for the L (low), M (medium) and H (high) groups were 25.30%, 38.80%, and 76.92%, respectively, which were significant compared to the C (control) group. There were significant differences in COX-2 expression in the tumor tissue between all groups, except between the L and M groups. Celecoxib exposure also reduced PGE_2 levels in the tumor tissue homogenates. The level of PGE_2 correlated to the weight of tumor ($r = 0.8814$, $P < 0.05$) and to COX-2 expression ($r = 0.8249$, $P < 0.05$). Compared to the control group, the tumor cells from celecoxib-treated mice had a significantly higher apoptotic index. Celecoxib also decreased $CD34^+$ expression in tumors from treated mice. There were significant differences in the MVD between all groups except between groups H and M. Celecoxib significantly reduced the expression of VEGF and MMP-2 mRNA in the group H but not in L and M groups. The MVD in tumor tissue was closely related to the PGE_2 levels, as well as the expression of VEGF and MMP-2 mRNA ($r = 0.9006$, $r = 0.8573$ and $r = 0.6427$, respectively; $P < 0.05$).** ***Conclusions:*** **By inhibiting COX-2, PGE_2 synthesis, and VEGF and MMP-2 mRNA expression in tumor tissue, celecoxib enhances tumor cell apoptosis, thereby inhibiting the growth and angiogenesis of orthotopically implanted tumors in a mouse model of human colorectal cancer.**

Key Words: **colon cancer, orthotopic implantation, cyclooxygenase-2, angiogenesis, prostaglandin E_2.**

According to the recent reports, colorectal cancer is the third most frequently diagnosed cancer in the United States. In 2005, an estimated 105,950 new cases of colon cancer occurred. During the same year, an estimated 55,290 people died from colorectal cancer [1]. In China, the incidence of colon cancer has increased recently, becoming a major threat to the public health. Therefore, improving early detection and treatment, especially the efficacy, to reduce mortality and extend the patients' lives are of the greatest concern.

Cyclooxygenase (COX), a rate-limiting enzyme for the metabolism of arachidonic acid to prostanoids, has two isoforms, constitutive COX-1 and inducible COX-2, which were identified in 1991 [2, 3]. COX-1 is expressed in quiescent and normal cells, while in normal tissue the expression of COX-2 is very low, and, in many cases, undetectable. COX-2 expression is induced by a variety of factors, including interleukin-1 (IL-1), tissue anoxia, ultraviolet ray exposure, epidermal growth factor (EGF), transforming growth factor β (TGF-β), tumor necrosis factor-α (TNF-α) and tumor promoters [2]. Recent researches have demonstrated that COX-2 is overexpressed frequently in various gastrointestinal tract cancers, such as colorectal cancer, esophageal carcinoma, gastric cancer and pancreatic cancer [4].

Epidemiological and clinical observation and laboratory research since 1980s have indicated that non-steroidal anti-inflammatory drugs (NSAIDs) played a crucial role in tumor inhibition both *in vivo* and *in vitro*, particularly in gastrointestinal tract cancers [5]. These researches indicated that COX-2 is maybe a target of NSAIDs.

Since the development of selective COX-2 inhibitors to treat inflammatory diseases, Steinbach et al. [6] first reported in 2000 that in 100 patients with familial adenomatous polyposis, six months of twice-daily treatment with 400 mg of celecoxib, a COX-2 inhibitor, significantly reduced the number of colorectal polyps. More and more studies have demonstrated that non-selective NSAIDs, as well as selective COX-2 inhibitors, can reduce cellular proliferation, induce

Received: January 17, 2008.
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Abbreviations used: AI – apoptotic index; cAMP – cyclic adenosine monophosphate; COX – cyclooxygenase; EP – E-type prostaglandin receptor; ERK – extracellular signal regulated protein kinase; GSK – glycogen synthase kinase; MAPK – mitogen-activated protein kinase; MEK – MAPK/ERK kinase; MMP-2 – matrix metalloproteinase-2; MVD – microvessel density; NSAIDs – non-steroidal anti-inflammatory drugs; PDK1 – 3-phosphoinositide-dependent protein kinase-1; PKA – protein kinase A; PKB – protein kinase B; PGs – prostaglandins; RIA – radioimmunoassay; TIMP – tissue inhibitor of metalloproteinase; TUNEL – terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; TXA_2 – thromboxan A_2; VEGF – vascular endothelial growth factor.

apoptosis, promote immunologic surveillance, and/or reduce neoangiogenesis. These drugs inhibit tumor angiogenesis by both COX-dependent and COX-independent mechanisms [7–10]. However, the exact mechanisms remain poorly understood [11–13].

In this study we established an animal model of orthotopic implantation of colon cancer cells to replicate the clinical conditions of human colon cancer. We then observed the effects of the selective COX-2 inhibitor celecoxib on the tumors to investigate its anticancer mechanism.

MATERIALS AND METHODS

Cell lines and animal model. The human colorectal adenocarcinoma cell line HT-29 was purchased from the Institute of Biochemistry and Cell Biology (Shanghai Institute for Biological Sciences (SIBS), Chinese Academy of the Sciences (CAS), Shanghai, China) and was maintained in McCoy's 5A medium containing 10% fetal bovine serum (GIBCO, USA). Four-week-old female BALB/c nu/nu mice (Shanghai Laboratory Animal Center, CAS) were maintained under specific pathogen-free conditions in a laminar air-flow incubator (25 °C, 40 to 60% humidity, with a 12 h light-dark cycle). To initiate the tumor growth, HT-29 cells in the log phase of growth were implanted subcutaneously in nude mice. After 4 weeks, small pieces of HT-29 tumor tissue (2 mm x 2 mm in diameter) were resected aseptically during the exponential growth phase (4 weeks) and implanted orthotopically on the surface of the cecum of 24 nude mice. The cecum was carefully exteriorized, and the serosa was injured (2 mm x 2 mm in diameter) at the site where the tumor was to be implanted. A tumor piece was then fixed on each injured site of the serosal surface with an 8-0 Vicryl transmural suture. The abdominal wall and skin were closed with 8-0 Vicryl sutures [14].

The present study was approved by the ethics committee of the hospital, and adhered to the tenets of the Declaration of Helsinky.

COX-2 inhibitor treatment. The selective COX-2 inhibitor, celecoxib, was a generous gift of Pharmacia & Upjohn Ltd (Suzhou, China). Postoperatively, all animals were randomly divided into four groups: control (C), and high (H), middle (M), and low (L) doses of celecoxib. Pure water or water containing 1.5, 1.0, or 0.5 mg/L celecoxib was provided daily for these animals to drink freely [15]. The mice were sacrificed at 6 weeks after tumor implantation. After weighing each animal, the tumors were removed and the volume and weight were evaluated. The tumor volume was determined using the formula $V = L \times W^2/2$ where L is length and W is width of colon tumor. The rate of tumor inhibition (TI) was calculated using the formula TI = [(average tumor weight of the control group — average tumor weight of the treatment group)/average tumor weight of the control group] × 100%.

Histopathology. For histological examination, the stomach, intestine, liver and colon of animals were excised and fixed in 10% neutral buffered formalin after the animals were sacrificed at 6 weeks. Paraffin embedded sections (5 μm) were cut and stained with hematoxlin and eosin for histological examination by a pathologist who was unaware of the treatment assignments. If the orthotopically implantated tumors invaded the serosa, muscularis propria, submucosa, or mucosa, or there was pathologic damage to either the stomach or intestine mucosa, or there were liver metastases, the results were observed and recorded.

RIA for PGE_2. After colon tumor tissue samples (50 mg) were washed with 0.9% NS (normal saline), these tissues were homogenized in 1 ml of 0.9% NS. Then these solutions were centrifuged (7500 rpm, 10 min). The supernatant (0.1 ml) was collected and the content of PGE_2 was measured with a PGE_2 RIA kit (a gift from Jiangsu Institute of Hematology, Suzhou, China) according to the manufacturer's instructions. PGE_2 values were expressed as picogram per milliliter in the tumor tissue homogenate samples.

RT-PCR for VEGF and MMP-2 mRNA. Tumor samples were frozen in liquid nitrogen or stored at –80 °C for mRNA analysis. Total cellular RNA was extracted from the frozen tumor samples using TRIzol Reagent (Sigma, MO, USA) according to the manufacturer's protocol. Briefly, 40 mg of tumor tissue was homogenized in 1 ml of TRIzol, and then 0.2 ml of chloroform was added, and the mixture was centrifuged (12 000 rpm, 15 min, 4 °C). The aqueous layer, which contained the RNA, was carefully aspirated and 0.5 ml of isopropanol was added to precipitate the RNA. The resulting solution was centrifuged (12 000 rpm, 15 min, 4 °C) and the pellet was washed with 0.6 ml of 75% ethanol, then with 0.8 ml of 100% ethanol and centrifuged again (12 000 rpm, 15 min, 4 °C). Finally, the pellet containing RNA was dissolved in diethyl pyrocarbonate-treated water (DEPC-H_2O) and was stored frozen at –80 °C until analysis.

The RNA samples were prepared for RT-PCR analysis by first diluting the mixture of total RNA (2 μg) and 2 μl random hexamers (0.1 mmol/L) to 15 μl with DEPC-H_2O and incubated at 70 °C for 5 min. Then, after adding 8 μl of 5 × buffer (Promega co. Madison, WI,USA), 200 units of Moloneymurine leukemia virus reverse transcriptase (Promega), 50 units of RNasin (Promega) and 1.25 μl dNTPs (10 mmol/L), the mixture was diluted to 25 μl in DEPC-H_2O and converted to cDNA by incubation at 37 °C for 1 h and 95 °C for 5 min. Finally, the cDNA solution was stored at -20 °C.

PCR was performed using 5 μl of diluted cDNA in a total volume of 50 μl, including 5 μl 10 × buffer (Promega), 1 μl dNTPs (10 mmol/L), 4 μl $MgC1_2$ (25 mmol/L), 2 μl of sense and antisense primers (10 μmol/L) for VEGF or MMP-2, 2 μl of sense and antisense primers (10 μmol/L) for β-actin, and 2.5 units of Taq Polymerase (Promega). Sequences of the primers for the human VEGF, MMP-2 and β-actin are shown in Table 1. The samples were amplified for 30 cycles of PCR reaction in which predenaturation was done for 5 min at 94 °C, denaturation for 30 s, and extension for 3 min at 72 °C. Annealing time was 30 s; however, the annealing temperature was 60 °C for VEGF transcript and 62 °C for MMP-2. After 30 cycles, the product of the PCR reaction was stored for 7 min at 72 °C and then analyzed.

Amplified cDNAs were separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide. The density of the electrophoretic band for each amplification product was evaluated using the BioCaptMW software. The mRNA values are expressed as relative units calculated according to the following formula: density of the VEGF or MMP-2 amplification product/density of the β-actin amplification product.

Immunohistochemistry for COX-2 protein. Tissue samples were embedded in paraffin, cut into 5 μm sections, deparaffinized, and subjected to microwave irradiation for 15 min at 92 to 98 °C in 0.01 M Citric acid buffer (pH 6.0). Then the slides were immersed in 1% H_2O_2 for 30 min to neutralize endogenous peroxidases. The primary antibody against COX-2 (anti-human COX-2 mouse IgG, Cayman Chemical, USA) was applied to tissue sections at a dilution of 1 : 100 and incubated overnight at 4 °C. The secondary antibody and enzymes were obtained in an Ultravision Detection System kit (Lab Vision, USA). The reaction products were visualized using streptavidin-biotin-peroxidase and 3, 3`-diaminobenzidene chromogen. Finally, the sections were counterstained with hematoxylin, dehydrated, and mounted in diphenylxylene. The expression level of COX-2 was calculated by multiplying a quantitative measure of the extent of COX-2 staining extent by the quantitative measure of the staining intensity. The COX-2 positive staining extent was recorded using a 5-grade system, based on the percentage of tumor cells stained: grade 0 = 0% to 4%; grade 1 = 5% to 24%; grade 2 = 25% to 49%; grade 3 = 50% to 74%; and grade 4 = 75% to 100%. Staining intensity was recorded using 3-grade system: grade 0 = negative; 1 = weakly positive; 2 = positive. All slides were independently evaluated by two blinded observers, both of whom were experienced pathologists.

Assay for microvessel density (MVD). Microvessels in the tumor tissue were immunostained using anti-human CD34 mouse monoclonal antibody and the streptavidin-biotin-peroxidase complex technique (Lab Vision, USA). MVD was evaluated according to the method first described by Weidner et al. [20]. The entire tumor sections was observed under a light microscope (Magnification: 40 x) to find the area that showed the most intense microvessel density, i.e. the highest density of brown stained CD34-positive cells (also referred to as the hot spot). Three different areas within a section were chosen and the stained microvessels were counted under a light microscope at 200x magnification. The average count was considered the MVD for each slide.

Assessing the apoptotic index (AI). Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was performed using a commercial kit (In Situ Cell Detection kit, POD, Roche Diagnostics, Germany) according to the manufacturer's instructions. By this method, the nuclei of apoptotic tumor cells were stained brown. One thousand tumor cells, including the apoptotic tumor cells, were counted for each sample. The apoptotic index (AI) was calculated as follows: AI (%) = (number of apoptotic tumor cells/1000) × 100.

Statistical analysis. The data are expressed as the mean plus or minus the standard deviation (SD). Analyses between multiple groups were determined by ANOVA. Analyses between two groups were determined using the SNK test. The relationships between PGE_2 and the weight of tumor, between MVD and the PGE_2, between COX-2 expression and the PGE_2, between MVD and VEGF mRNA expression and between MVD and MMP-2 mRNA expression were examined by simple linear regression analysis. All statistical analyses were performed using a statistical software package (SPSS, Version 10.0, USA). Statistical significance was defined as *P*-values less than 0.05. All *P*-values were two-sided.

RESULTS

General Observations. None of the nude mice died after implantation and all animals formed colorectal tumor masses before sacrifice. Three animals in group C could touch the mass (about 4 mm in diameter) on the inferior belly in the third week. After all animals were sacrificed, opening the abdominal cavity of each animal revealed masses whose size ranged from 0.50 cm × 0.45 cm to 1.35 cm × 0.95 cm (Fig. 1, *a*). In addition, staining with haematoxlin and eosin confirmed that, in some animals, the tumor cells both the muscularis propria and submucosa (Fig. 1, *b*).

The mean body weight of the control animals was lower than in the treatment groups, but the difference was not statistically significant ($P > 0.05$; Fig. 2). However, the volume and weight of the tumor in group C were significantly higher than in the treatment groups, and were significantly different among all groups ($P < 0.05$; Table 2). Tumor growth was inhibited by 25.30% in the L group, 38.80% in the M group, and 76.92% in the H group, as compared to the control group. No obvious ascites was found in the abdominal cavity of any animal.

Also, no animal showed signs of liver metastasis, hyperemia, edema, erosion, bleeding or ulceration of the stomach and intestine mucosa, confirmed by histopathology.

PGE_2 levels and COX-2 immunohistochemistry. The PGE_2 is one important product of metabolism of arachidonic acid to prostanoids. The COX-2 plays a role in this process. By measuring the PGE_2 levels, we can analyze the activity of COX-2. In our research, the PGE_2 levels in the tumor tissue were higher in group C than that in the treatment groups ($P < 0.05$). With increasing doses of celecoxib, the PGE_2 levels in the treatment groups were reduced ($P < 0.05$; Table 3).

Table 1. RT-PCR primers

	Sense	Antisense	Product size (bp)	Reference
β-actin	ATCTGGCACCACACCTTCTACAATGAGCTGCG	CGTCATACTCCTGTGATCCACATCTGC	838	16
VEGF	GGGCCTCCGAAACCATGAACTT	CGCATCAGGGGCACACAG	259	17
β-actin	GAAACTACCTTCAACTCCATC	CGAGGCCAGGATGGAGCCGCC	219	18
MMP-2	ACCTGGATGCCGTCGTGGAC	TGTGGCAGCACCAGGGCAGC	447	19

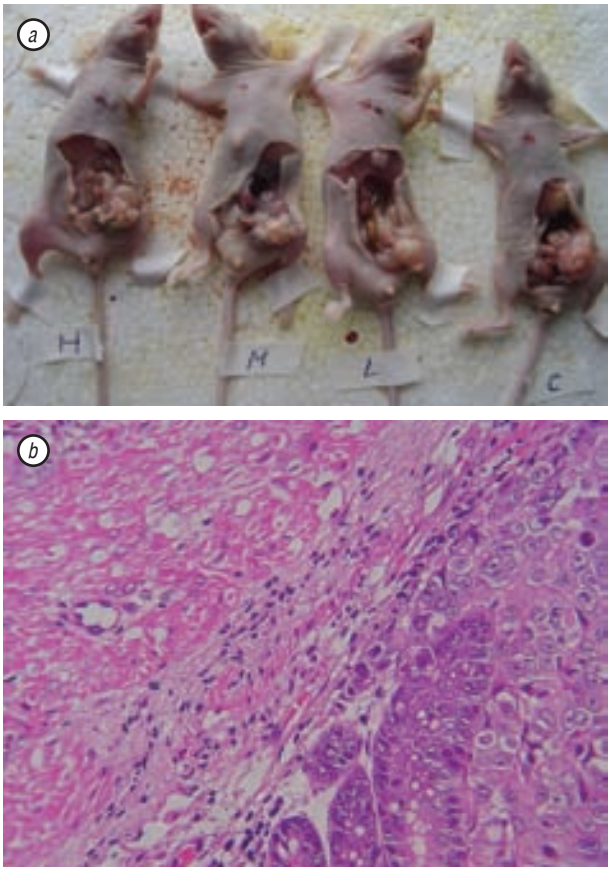

Fig. 1. Macroscopic and microscopic appearance of orthotopically implanted tumors in a mouse model of colon cancer. *a*: Different tumor masses were found in the colon of each animal, and celecoxib obviously inhibited the growth of tumor. *b*: Staining with haematoxlin and eosin showed that the tumor cells invaded muscularis propria and submucosa (Magnification: × 400)

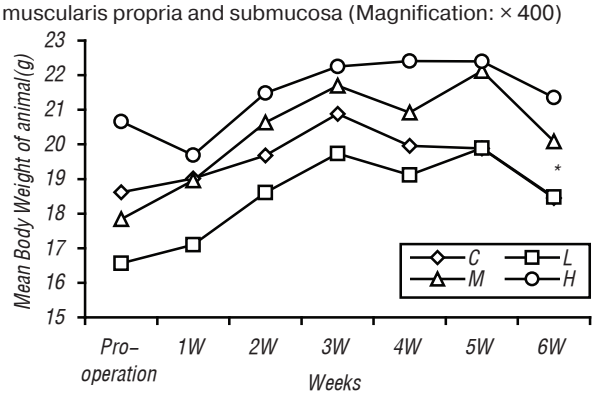

Fig. 2. Celecoxib did not alter the mean body weight of animals in this study. While the mean body weight of group C animals was lower than in the treatment groups, there was no statistically significant difference among all groups (P > 0.05)

Table 2. The volume and weight of colon tumor *in situ*[a]

Group	volume of tumor, cm^3	weight of tumor, g
C	0.53 ± 0.07*	0.59 ± 0.06*
L	0.34 ± 0.10*	0.44 ± 0.08*
M	0.25 ± 0.05*	0.36 ± 0.05*
H	0.06 ± 0.03*	0.14 ± 0.03*

Note: [a]Values represent the mean ± SD; *$P < 0.05$ between all groups.

Table 3. The level of PGE_2 and COX-2 expression in tumor tissue[a]

Group	PGE2 pg/mL	COX-2
C	608.88 ± 76.71*	8.67 ± 1.03**
L	425.27 ± 71.70*	6.83 ± 1.17** b
M	244.77 ± 29.04*	5.50 ± 1.05**b
H	97.92 ± 15.57*	3.83 ± 1.17**

Notes: [a]Values represent the mean ± SD; *$P < 0.05$; [b]There were significant differences in the COX-2 expression of tumor tissue between all groups except between L and M groups; **$P < 0.05$.

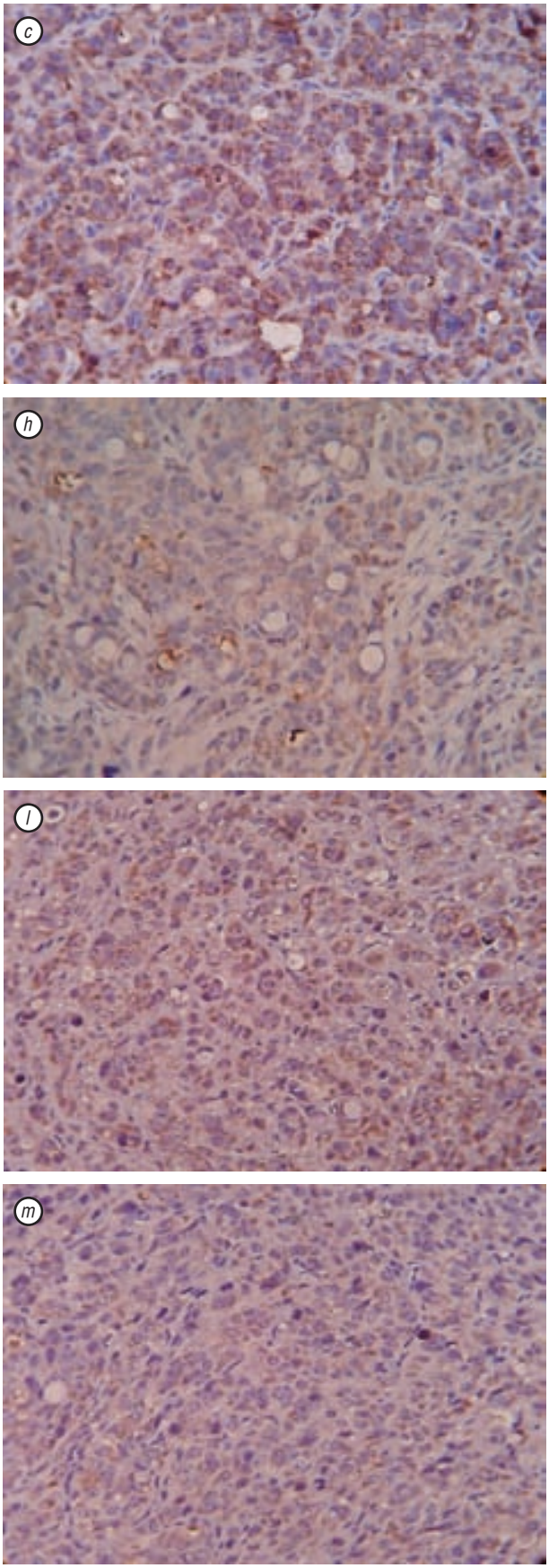

Fig. 3. Expression of COX-2 in tumor tissue as determined by immunostaining. COX-2 expression was detected using the streptavidin-biotin-peroxidase method as described in the Materials and Methods section The extent of COX-2 expression in the treatment groups (group L, M and H) decreased gradually with the increasing dose of celecoxib. (Magnification: X 400)

The expression of COX-2 in the tumor tissue was higher in group C than in the treatment groups ($P < 0.05$). There were significant differences between all groups except the L and M groups in COX-2 expression in tumor tissue ($P < 0.05$), and the expression decreased correspondingly with the increasing dose of celecoxib (Table 3, Fig. 3). In addition, the PGE_2 levels correlated positively with the weight of the tumor (r = 0.8814, $P < 0.05$; Fig. 4, *a*). Between the PGE_2 level and the extent of COX-2 expression significant association was also seen (r = 0.8249, $P < 0.05$; Fig. 4, *b*). By inhibiting COX-2, celecoxib could reduce synthesis of PGE_2 in the tumor tissue with the increasing dose of celecoxib.

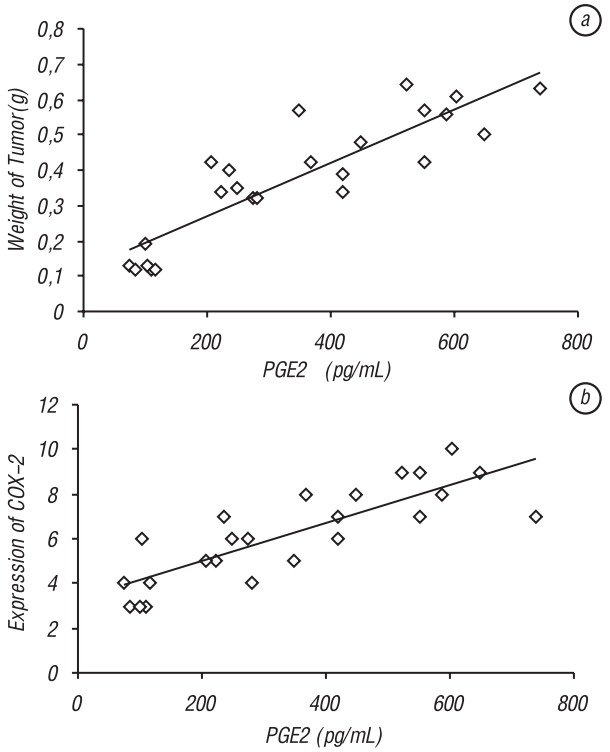

Fig. 4. Correlation between PGE2 and either the weight of the tumor (*a*) or the expression of COX-2 (*b*). The correlation coefficient between PGE2 and the weight of tumor was 0.8814 ($P < 0.05$), whereas the correlation coefficient between PGE2 and expression of COX-2 was 0.8249 ($P < 0.05$)

Apoptosis in the tumor cells. Apoptosis of the tumor cells was detected by TUNEL assay to determine the apoptotic index (AI) in the different groups. Our experiment showed that the AI was significantly lower in group C (2.77 ± 0.70) than in the treatment groups ($P < 0.05$) (Fig. 5). As the dose of celecoxib increased, so did the AI, from 5.90 (± 0.65) in the L group, to 7.47 (± 0.96) in the M group and 9.27 (± 0.97) in the H group. These differences were statistically significant ($P < 0.05$). The results revealed that celecoxib could dose-dependently enhance tumor cell apoptosis.

Microvessel density (MVD). The MVD is the important biomarker for angiogenesis of tumor. Our experiment investigated the anti- angiogenesis of celecoxib by measuring the MVD in the tumor tissue. The MVD in group C (30.50 ± 4.60) was significantly higher than in the treatment groups ($P < 0.05$). Celecoxib obviously decreased the MVD of groups L (24.33 ± 3.78), M (13.17 ± 3.19), and H (9.00 ± 3.58). There were significant differences in

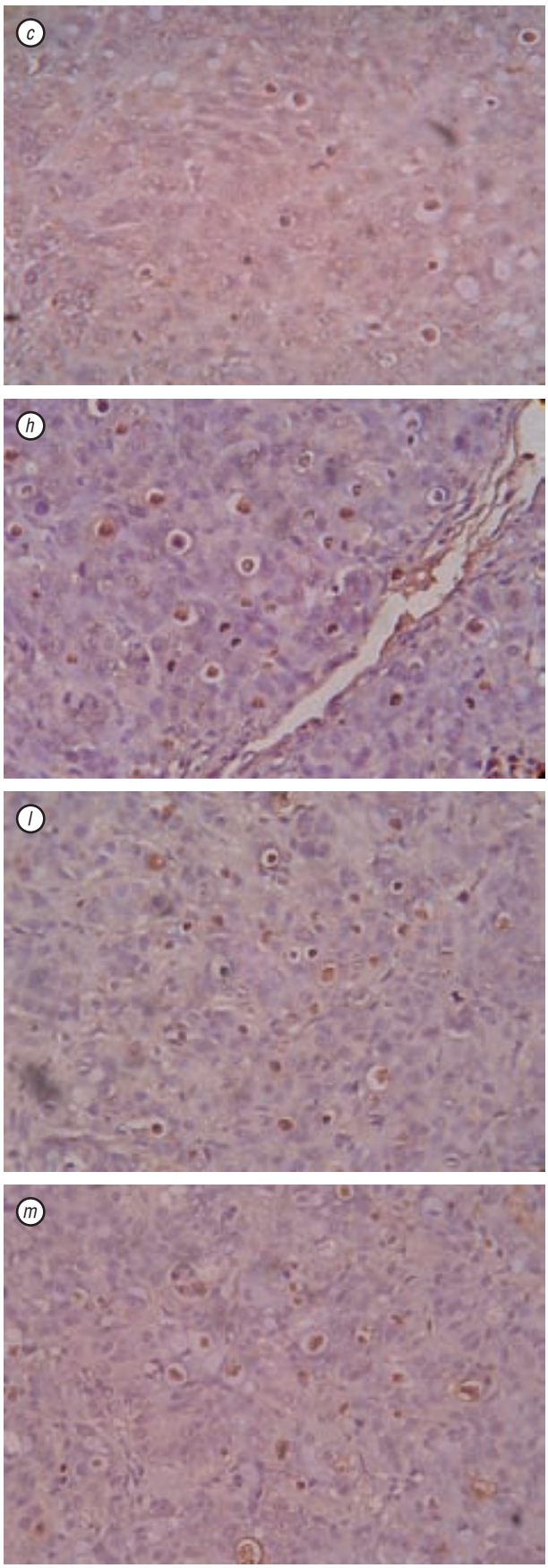

Fig. 5. Detection of apoptotic HT-29 cells by the TUNEL assay. The apoptosis of the tumor cells was detected using the TUNEL method as described in the Materials and Methods section. TUNEL-positive cells (apoptotic cells) are stained brown (Magnification: × 400). There were more apoptotic cells in the samples from celecoxib-treated mice than in the samples from the control group

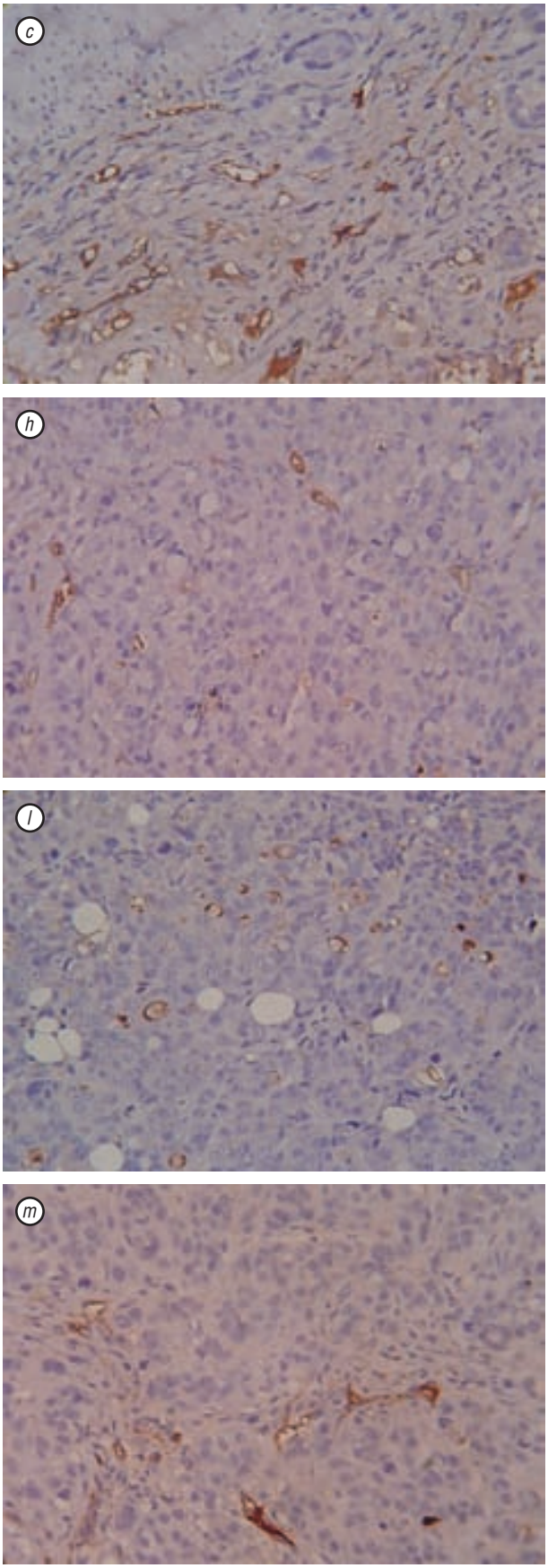

Fig. 6. Detection of microvessels in tumor tissue by immunostaining for CD34. CD34 expression was detected using the streptavidin-biotin-peroxidase method as described in the Materials and Methods section. The microvessels were more numerous in the control group than in the treatment groups (Magnification: × 200)

the MVD ($P < 0.05$) between all groups except between groups H and M (Fig. 6). A significant correlation was found between MVD and PGE_2 levels in the tumor ($r = 0.9006$, $P < 0.05$; Fig. 7). The results showed that celecoxib could inhibit the angiogenesis of tumor and PGE_2 could have a significant correlation with angiogenesis.

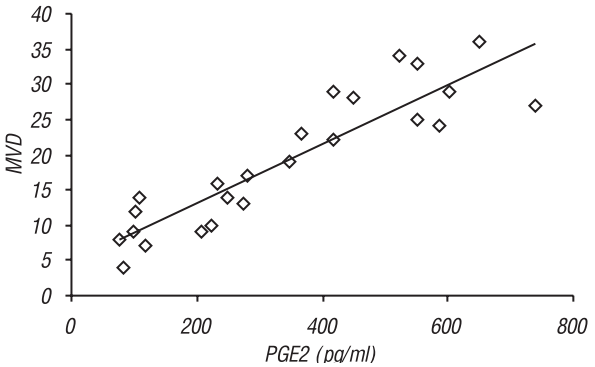

Fig. 7. Correlation between PGE2 and MVD in tumors from celecoxib-treated mice. The correlation coefficient between PGE2 and MVD was 0.9006 ($P < 0.05$)

VEGF and MMP-2 mRNA expression. VEGF and MMP-2 play an important role in the angiogenesis of tumor. Additionally MMP-2 might correlate to invasion and metastasis of tumor. Our experiment showed that celecoxib significantly reduced the expression of VEGF and MMP-2 mRNA in the treatment groups compared with to the control group ($P < 0.05$). There were significant differences in the expression of VEGF and MMP-2 mRNA ($P < 0.05$) between all groups except groups L and M (Table 4, Fig. 8 and 9).

Table 4. Expression levels of VEGF mRNA and MMP-2 mRNA in tumor tissue[a]

Group	VEGF/β-actin	MMP-2/β-actin
C	0.66 ± 0.10*	0.59 ± 0.14**
L	0.49 ± 0.06*[b]	0.42 ± 0.04**[c]
M	0.43 ± 0.08*[b]	0.34 ± 0.06**[c]
H	0.23 ± 0.06*	0.22 ± 0.08**

Notes: [a]Values represent the mean ± SD; [b]There were significant differences in the expression levels of VEGF mRNA between all groups except between group L and M; *$P < 0.05$; [c]There were significant differences in the expression levels of MMP-2 mRNA between all groups except between groups L and M; **$P < 0.05$.

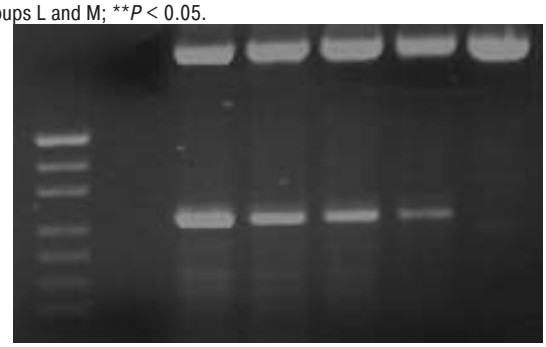

Fig. 8. Expression of VEGF mRNA in tumor tissue as determined by RT-PCR.

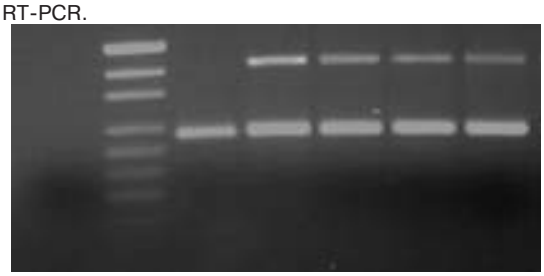

Fig. 9. Expression of MMP-2 mRNA in tumor tissue as determined by RT-PCR

In addition, the MVD of tumor tissue significantly correlated to the expression of VEGF and MMP-2 mRNA in the tumor tissue (r = 0.8573, r = 0.6427, respectively; P < 0.05; Fig. 10, A and B). By inhibiting VEGF and MMP-2 mRNA expression in tumor tissue, celecoxib inhibited the angiogenesis of colorectal cancer

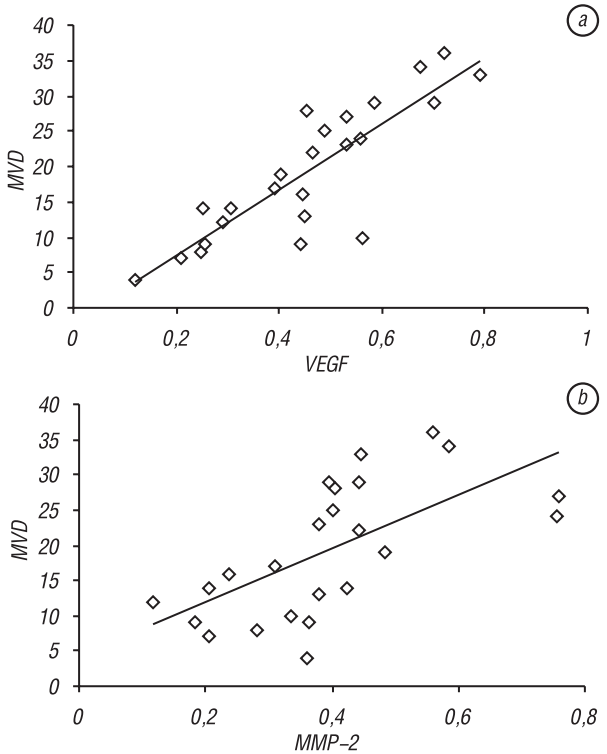

Fig. 10. Correlation between MVD and the expression of VEGF (*a*) and MMP-2 mRNA (*b*) in tumor tissue. The correlation coefficient between MVD and the expression of VEGF mRNA was 0.8573 ($P < 0.05$), whereas the coefficient between MVD and expression of MMP-2 mRNA was 0.6427 ($P < 0.05$)

DISCUSSION

In the past 20 years, epidemiological, molecular and clinical studies have proved that COX-2 plays an increasingly important role in the occurrence and development of colon cancer by various mechanisms [5, 12, 13]. Since selective COX-2 inhibitors (e.g. celecoxib) have lower toxicity and side effects compared to the traditional NSAIDs (e.g. aspirin, sulindac, piroxicam, ibuprofen, ibuprofen, and indomethacin), we have investigated the effect of celecoxib on the growth and angiogenesis of orthotopically implanted tumors in a mouse model of human colon cancer [21–24].

Our first priority was to establish a suitable preclinical animal model. According to the 'seed and soil' theory of S. Paget, the animal model of orthotopic implantation of colon cancer is the most suitable to study the anti-cancer mechanism of celecoxib, because this model more accurately replicates the clinical condition of human colon cancer, thereby more accurately reflecting the efficacy of celecoxib against colon carcinoma. In this study, none of the nude mice died and all animals formed an *in situ* mass consistent with colorectal tumor. Though liver or lymphoid node metastasis and ascites were not found because of the relatively short observation time and the biological character of HT-29 cells, this model still contributes to the study of the local biology behavior and pathophysiological changes of colon cancer.

This study showed that both the volume and weight of the tumor can be reduced significantly by celecoxib treatment. This effect was dose-dependent, as demonstrated by the rate of tumor growth inhibition. Moreover, this effect can be seen even at the lowest dose of celecoxib (0.5 mg/L).

There are many possible anticancer mechanisms of celecoxib. The most obvious is the inhibition of COX-2 activity [2]. By inhibiting the enzyme activity of COX-2, celecoxib reduces the synthesis of PGE_2. PGE_2 might enhance the proliferation and invasive potential of colon carcinoma cells by activating major intracellular signal transduction pathways [25], such as cAMP/PAK/EP [26], c-Met-R/β-catenin [27], Raf/MEK/ERK [28], among others. In addition, recent reports show that PGE_2 stimulates angiogenesis, which provides oxygen and nutrients essential for tumor growing [29]. The positive correlation in this study between PGE_2 levels and the weight of the tumor shows that, by inhibiting the activity of COX-2, celecoxib reduced the synthesis of PGE_2 and the growth of tumor is suppressed.

Celecoxib might exert its effect by reducing the expression of COX-2. In this study, the expression of COX-2 is lower in the treatment groups than in the control group, resulting in the inhibition of the synthesis of PGE_2. COX-2 expression could be suppressed by a few possible mechanisms. Tjandrawnata et al. [30] found that PGE_2 can up-regulate the expression of COX-2mRNA. When PGE_2 levels are reduced, COX-2 expression decreases, which further suppresses the synthesis of PGE_2. Alternatively, the findings of Chun et al. [31] suggest that celecoxib can down-regulate COX-2 expression by blocking activation of p38 mitogen-activated protein (MAP) kinase and the transcription factor AP-1. Finally, Shishodia et al. [32] have reported that celecoxib inhibits NF-κB activation through inhibition of IKK and Akt activation, leading to down-regulation of synthesis of COX-2. Thereby, not only by directly inhibiting the activity of COX-2, but by down-regulating the synthesis of COX-2, celecoxib takes effect.

In this study, the TUNEL assay was used to detect apoptosis, demonstrating that more HT-29 cells underwent apoptosis in the treatment groups than in the control group. These data also show that celecoxib enhanced apoptosis of colon cancer cells in a dose-dependent manner. Recent reports have implicated many different mechanisms in the induction of apoptosis in tumor cells by celecoxib. First, PGE_2 can inhibit apoptosis by inducing expression of the Bcl-2 proto-oncogene. Also, PGE_2 and other prostaglandins often elevate intracellular cAMP concentrations, which can suppress apoptosis. COX-2 inhibitors may prevent colon cancer by interfering with both or either of the above processes [25].

Alternatively, Arico et al. [33] have reported that celecoxib induces apoptosis through the major anti-apoptotic PDK1/Akt/PKB signal transduction pathway.

Another possible mechanism, reported by Wu et al. [34], is activation of caspase-9 and caspase-3, and release of cytochrome C in a Bcl-2-independent manner. Grosch et al. [35] indicated that celecoxib can inhibit the transition from the G0/G1 to the S phase of the cell cycle in colon cancer cells in a concentration-dependent manner, and induce apoptosis in HT-29 cells. Moreover, this study showed that the effects of celecoxib were independent of the COX-2 status of the cells, strongly suggesting that the anticancer activity of celecoxib is independent of its ability to inhibit COX-2. In other studies, the mechanisms by which selective COX-2 inhibitors induce apoptosis in cancer cells also was found to be COX-2 independent, by affecting such pathways as the 15-lipoxygenase-1 [36], PPAR-δ [2, 37] and NF-κB [38] pathways, for example. So, celecoxib might induce the apoptosis of colon cancer cells by dependent and independent pathways.

Angiogenesis plays a critical role in providing oxygen and nutrients essential for tumor growth. Antiangiogenic therapy has great potential to become the new method for conquering cancer in the future [39]. In recent studies, there is evidence that COX-2 may indirectly induce angiogenesis *in vitro* by increasing the production of angiogenic factors, such as VEGF. In these studies, selective COX-2 inhibitors were shown to be antiangiogenic [7].

Our study also showed that celecoxib suppressed angiogenesis in the treatment groups. The MVD of tumors from mice treated with celecoxib was lower than in the control group. Additionally, celecoxib significantly decreased the expression of VEGF and MMP-2 mRNA in the treatment groups compared with the control group. Both VEGF and MMP-2 expression are closely related to angiogenesis. VEGF has been described as the fundamental and strongest regulator of angiogenesis and vascular permeability [7]. Celecoxib suppresses the expression of VEGF by inhibiting the synthesis of PGE_2 and the activation of the EPR/cAMP/PKA signal transduction pathway by which the expression of VEGF was upregulated [7, 40]. This pathway is the primary mechanism by which selective COX-2 inhibitors prevent angiogenesis. In our study positive correlations between MVD and PGE_2 levels and VEGF mRNA were the best evidence of celecoxib inhibiting this signal pathway.

MMP-2 plays an important role in matrix degradation, an important process in tumor angiogenesis. Moreover, the digestion of vascular basement membranes and an invasive/angiogenic phenotype has been closely associated with the overexpression of MMP-2. When the basement membranes are degraded, growth factors are released which can promote tumor angiogenesis [41]. Recently Takahashi et al. [42] reported that overexpression of COX-2 in tumor cells up-regulated the expression of MMP-2, an effect which is blocked by selective COX-2 inhibitors. The results of our study also showed that celecoxib suppressed the expression of MMP-2. Moreover, the expression of MMP-2 was positively related to the MVD. This is another probable mechanism of antiangiogenesis by a selective COX-2 inhibitor. There are two potential mechanisms by selective COX-2 inhibitors suppress the expression of MMP-2. The first is by suppressing transcription of MMP-2 [43], which is probably a COX-2-independent effect. The second was described by Attiga et al. [44], that selective COX-2 inhibitors suppress the synthesis of MMP-2 by decreasing the COX-2 metabolites which stimulate the synthesis of MMP-2. There are other potential mechanisms by which selective COX-2 inhibitors inhibit angiogensis, such as inhibiting TXA_2, alpha V beta 3 integrin, MARK/ERK2 and Akt/GSK signal pathway.

In summary, this study tested the hypothesis that the selective COX-2 inhibitor celecoxib can inhibit the growth and angiogenesis of orthotopically implanted tumors in a mouse model of human colon cancer by suppressing COX-2, the synthesis of PGE_2 and the expression of VEGF mRNA and MMP-2 mRNA. We did not find any pathologic damage of gastrointestinal tract in any animals, suggesting that celecoxib may have minimal side effects, as least in the gastrointestinal tract, in a clinical setting. The further development of safer selective COX-2 inhibitors could be a promising approach for the treatment of colon cancer. However, celecoxib alone did not induce complete tumor regression in our study. Accordingly, a careful study of combination therapy with chemotherapeutic agents, or radiotherapy, could lead to more successful and promising treatment for colon cancer [45].

ACKNOWLEDGMENTS

We express our gratitude to Wenhong Shen (Jiangsu Institute of Hematology, the First Affiliated Hospital of Soochow University, Jiangsu, China) for her assistance in the RIA assay and Yuhai Chai (Department of Pathology, Soochow University, Jiangsu, China) for his assistance in IHC assay. We highly appreciate Dr. Jiannong Chen for PCR assay. This work was supported by grants from the 135 Medical Talent Project of Jiangsu Province (No.37RC2002037) and the Important Research Topic Foundation of Health Department of Jiangsu Province of P.R. China (K2000507), China.

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ЦЕЛЕКОКСИБ ИНГИБИРУЕТ РОСТ ОПУХОЛИ И АНГИОГЕНЕЗ ПРИ ОРТОТОПИЧЕСКОЙ ИМПЛАНТАЦИИ РАКА ТОЛСТОЙ КИШКИ ЧЕЛОВЕКА

Цель: **изучить влияние целекоксиба на рост опухоли и ангиогенез в модели ортопической имплантации опухоли толстой кишки человека.** ***Методы:*** **клетки колоректальной аденокарциномы человека HT-29 подкожно имплантировали бестимусным мышам. После имплантации опухоли четыре группы животных получали разные дозы целекоксиба. Через 42 дня изучали изменения веса животных, объем опухолей, эффект ингибирования роста опухоли. С помощью радиоиммунного анализа (RIA) в гомогенате опухолей определяли содержание простогландина E_2 (PGE_2). В опухолевой ткани иммуногистохимическим методом выявляли экспрессию циклооксигеназы-2 (COX-2) и CD34 и оценивали плотность микрососудов (MVD). Апоптотические клетки выявляли методом TUNEL. Экспрессия мРНК фактора роста эндотелия сосудов (VEGF) и металлопротеиназы-2 (MMP-2) в опухолях проанализирована методом обратной транскриптазной реакции (RT-PCR).** ***Результаты:*** **статистически достоверных различий в весе животных между разными группами обнаружено не было. Вто же время, с увеличением дозы целекоксиба объем и вес опухоли уменьшался. По сравнению с контрольной группой (C), рост опухоли статистически достоверно ингибировался в L (низкая доза), M (средняя доза) и H (высокая доза) группах животных на 25,30%, 38,80% и 76,92% соответственно. Были обнаружены значительные отличия в экспрессии COX-2 в опухолевых тканях между всеми группами животных, кроме групп L и M. Было показано целекоксиб-зависимое уменьшение уровня PGE_2 в гомогенатах опухолей. Уровень PGE_2 коррелировал с весом опухоли ($r = 0,8814$, $P < 0,05$) и экспрессией COX-2 ($r = 0,8249$, $P < 0,05$). По сравнению с контрольной группой опухолевые клетки мышей, получавших целекоксиб, имели значительно более высокий апоптотический индекс. Целекоксиб также снижал экспрессию $CD34^+$ на поверхности опухолевых клеток. Были обнаружены статистически достоверные различия в MVD между всеми исследованными группами, кроме H и M. Целекоксиб способствовал уменьшению экспрессии мРНК VEGF и MMP-2 в группе H, но не в группах L и M. MVD в опухолевой ткани кореллировал с уровнем PGE_2, а также с экспрессией мРНК VEGF и MMP-2 ($r = 0,9006$, $r = 0,8573$ и $r = 0,6427$ соответственно; $P < 0,05$).** ***Выводы:*** **целекоксиб способствует апоптозу опухолевых клеток, ингибирует рост опухоли и ангиогенез при ортотопической имплантации мышам колоректальной аденокарциномы человека. Такой эффект целекоксиба связан с угнетением синтеза COX-2, PGE_2 и экспрессии мРНК VEGF и MMP-2 в опухолевой ткани.**

Ключевые слова: **рак толстой кишки, ортотопическая имплантация, циклооксигеназа-2, ангиогенез, простогландин E_2.**