COX-2 Expression and Effects of COX-2 Inhibition in Colorectal Carcinomas and their Liver Metastases

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Abstract. *Nonsteroidal anti-inflammatory drugs are known to reduce the risk and mortality from colorectal carcinoma by inhibiting cyclo-oxygenases (COX). COX-2 expression was investigated immunohistologically in 57 patients with colorectal carcinomas and in the corresponding liver metastases using tissue microarray analysis. Ex vivo COX-2 inhibition with assessment of apoptosis was performed using precision-cut tissue slices of three human liver metastases. Following stimulation with different concentrations of the selective COX-2 inhibitor meloxicam, apoptosis was assessed imunohistochemically after 6 h and 12 h. All primary carcinomas and 56 out of the 57 liver metastases showed various degrees of cytoplasmatic COX-2 expression being with a reduction and in the liver metastases. There was a time- and concentration-dependent change in the number of apoptotic cells in tissue slices, however, this was without statistical significance. COX-2 is constantly involved in the carcinogenesis and metastatic process of colorectal cancer. The antineoplastic effect of COX-2 inhibition may be based on different pathways, including changes in sensivity to apoptosis.*

After the lung and the skeletal system, the liver is the organ most frequently involved in hematogenic metastatic spread of solid tumors. Primary tumors which metastasize into the liver are, in particular, colon, gastric and breast carcinomas, but also melanomas and neuroendocrine tumors, with colorectal carcinomas being the most frequently involved primary site (1). Approximately 15-25% of patients with colorectal carcinoma have metastatic liver disease by the time the primary tumor is diagnosed, and an additional 35-45% of patients will develop hepatic metastases during the course of the disease (2, 3). Prevention and control of hepatic metastasis remain problematic. Hepatic resection is the only curative treatment for liver metastases of colorectal cancer. Unfortunately, most liver metastases are, however, inoperable (4, 5). Adjuvant therapy is necessary to reduce the occurrence of metastatic tumor in the liver.

Increased concentrations of inducible COX-2 have been observed in inflamed and tumor tissue (6, 7). COX-2 effects cell proliferation, tumor growth, apoptosis resistance and immune responsiveness (8). *In vitro* studies have shown that COX-2 overexpression reduces the rate of apoptosis, increases the invasiveness of malignant cells and promotes angiogenesis (9, 10). Several recent reports have suggested an important role of COX-2 in hematogenous metastases of colon cancer to the liver (11-13). A predictive potential, however, was not proven (14). The exact mechanisms still remain enigmatic.

Epidemiological studies have suggested a reduced risk of development of colon cancer overexpressing cyclo-oxygenase-2 (COX-2) in patients with long-term non steroidal drug (NSAID) intake (15-17). A chemotherapeutic effect and a chemoprophylactic potential of NSAIDs have been postulated and investigated *in vitro*, in animal and in clinical studies (9, 13, 18-20). The pathomechanism of this NSAID effect is attributed to arachidonic acid metabolism *via* COX.

Precision cut tissue slices have several advantages in the study of drug-dependent effects. Freshly isolated slices maintain their tissue architecture, cell composition, cell-to-cell-interaction and their original intercellular matrix (21). In addition to pharmacological studies, they can be used as a morphological tool, *e.g.* for the investigation of apoptosis (22).

In this study, we investigated COX-2 expression in colorectal carcinomas and their corresponding liver

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metastases and performed COX-2 inhibition in precision-cut tissue slices (PCTS) of liver metastases using meloxicam to assess the effect on apoptosis.

Materials and Methods

Patient data. In the microarray study, 57 patients with synchronous (42 cases) and metachronous [15 cases with a time gap of 14.6 (1-62) months] liver metastases of colorectal carcinomas were included with the following clinical data: gender: 34 males, 23 females; mean age: 61.3 years (35-83 years); mean diameter of the primary tumor: 4.5 cm; localization: 10 cecum, 13 colon ascendens, 3 colon transversum, 12 colon sigmoideum, 2 colon descendens, 11 rectum, 6 no information; TNM stage: 1 T1, 2 T2, 42 T3, 10 T4; 16 N0; 12 N1, 27 N2, 2 biopsies with no information; grading: 39 G2, 18 G3. The mean diameter of the metastases was 2.4 cm (Table I).

For preparing precision-cut tissue slices, three resection specimens of liver metastases of colorectal carcinoma were used. The donors were two male and one female, with a mean age of 63 years. The mean diameter of the liver metastases was 5.2 cm.

Tissue microarray. Two separate areas of suitable tissue of the primary carcinoma and the liver metastases were sampled using a 2 mm thick punch needle. The removed tissues were integrated in a prepared receptor block of paraffin. The surface was sealed by superficial heating. Kidney, spleen, pancreas, lymph node and normal colon were used as control tissue.

For immunohistochemistry, 4 μm-thick paraffin sections were cut, deparaffinized in xylene and rehydrated with graded alcohol. Antigen retrieval was performed by microwave pre-treatment (2 × 7 min in citrate buffer, pH 6). After blocking of endogenous peroxidase activity, primary anti-human COX-2 antibody (dilution 1:100; Santa Cruz Biotech, Santa Cruz, CA, USA) was incubated overnight in a moist chamber at 4˚C. Signal detection was performed with EnVision and horsereddish peroxidase monoclonal goat anti-rabbit antibody (DAKO, Glostrup, Denmark) according to the manufacturer's instructions. For counterstaining, Mayers hemalaun was used. Pancreatic islets were used as positive control and appropriate negative controls were stained.

Evaluation was carried out semiquantitatively using the Remmele scoring system (23). Staining intensity was scored in 4 grades (0 negative, 1 weak, 2 moderate, 3 strong) and number of positive cells in 5 categories (0 negative, 1: 1-9%, 2: 10-49%, 3: 50-79%, 4: ≥80%). Multiplication of both values lead to the immunoreactive score (minimal 0; maximal 12).

Precision cut tissue slices. The slices were prepared as described elsewhere (22). In brief, cylindrical tissue cores with a diameter of 0.8 cm were removed from the metastatic tissue and 200-300 μm thick slices were cut using a Brendel Vitron tissue slicer (Vitron, Tuscon, AZ, USA) filled with ice-cold oxygenated physiological Ringer's solution. The slices were incubated in a static incubation system using 34-well plastic tissue culture plates (VWR Darmstadt, Germany) on a rocker platform (Heidolph, Schwabach, Germany) at 37˚C with saturated humidity and 5% CO_2 in 40 ml RPMI medium supplemented with 0.5 ml penicillin/streptomycin, 0.48 ml L-glutamine, 2.4 ml horse serum, 8 ml fetal calf serum and 0.24 ITS supplement (all Sigma, Taufkirchen, Germany).

After an equilibration time of 30 min, a medium change was performed. Following a resting time of 3 h, stimulation with the COX-2 specific inhibitor meloxicam (Mobec; Boehringer Ingelheim, Ingelheim, Germany) started with concentrations of 20 μM, 50 μM and 200 μM. As controls, incubation without meloxicam and incubation with 8 μM DMSO (Sigma) was performed. After 6 h and 12 h, the slices were removed, fixed in 4% neutral buffered formalin overnight and processed routinely for histological and immunohistological investigations.

From the paraffin-embedded tissue, 4 μm-thick slices were cut for conventional histology (H&E staining). Apoptosis was assessed imunohistochemically using M30 antibody (CytoDeath Roche Mannheim, Germany). In brief, after deparaffinization and blocking of endogenous peroxidase, the slices were incubated with the primary antibody M30 overnight at 4˚C in a dilution of 1:50. Detection was performed using monoclonal secondary biotinylated rabbit anti-mouse antibody 1:200 (DAKO) followed by a streptavidin-biotin-complex (Vector, Burlingame CA, USA) and visualized with diaminobenzidine (DAB; DAKO). For evaluation, positive signals in 10 high power fields (HPF) were counted using an ocular grid.

Statistical analysis. For statistical analysis, SPSS for windows was used (SPSS, Chicago, IL, USA). The Mann-Whitney *U*-test and Student's *t*-test were used for nonparametrical and parametrical data. A *p*-value of <0.05 was considered significant. Statistical analysis was supported by Mr. Hahn of the Department of Medical Statistics, University of Cologne.

The investigations were approved by the Ethical Committee of the University of Cologne. Informed consent was available for investigations with precision-cut tissue slices.

Results

Tissue microarray. All primary carcinomas of the colon and rectum (100%) and 56 out of the 57 liver metastases (98.2%) showed different degrees of cytoplasmatic COX-2 expression (Table I). No nuclear staining was visible (Figure 1).

Four of the primary carcinomas revealed weak staining (Score 1), 27 cases moderate (Score 2) and 26 cases strong staining (score 3). The number of positive tumor cells was distributed as follows: fewer than 10%, 1 case; 10-49%, 5 cases; 50-79%, 6 cases and ≥80%, 45 cases. The was no correlation between the immunoreactive score and the tumor diameter, localization, grading, age, sex or lymph node metastasis.

In the group of the liver metastases, 9 cases showed weak staining (score 1), 42 cases moderate staining (score 2) and 5 cases strong staining (score 3). The percentage of positive cells was as follows: fewer than 10%, 3 cases; 10-49%, 9 cases; 50-79%, 29 cases and ≥80%, 15 cases. There was no correlation to tumor diameter, age or sex.

Comparing primary carcinoma and liver metastases, a weaker expression in the secondary tumors was observed (Figure 2). This difference was significant for the staining intensity ($p>0.05$) as well as for the number of positive cells ($p>0.05$).

Precision-cut tissue slices. There was a time- and concentration-dependent change of apoptosis in the tissue slices (Table II). After 6 h, only a slightly elevated apoptosis

Table I. *Expression of COX-2 in primary colorectal tumors and their corresponding liver metastases*.

No.	Age (years)	Gender (M=male; F=female)	Primary tumor								Liver metastasis				
			Diameter (cm)	Site	T-Stage	N-Stage	Grading	COX-2 Score			Diameter (cm)	Time (months)	COX-2 Score		
								Intensity	Number	IRS			Intensity	Number	IRS
1	67	F	2.5	Cecum	3	2	3	2	4	8	0.5	0	2	4	8
2	67	M	4.5	Ascendens	3	2	3	2	4	8	0.6	0	2	1	1
3	62	M	4	Sigma	3	0	2	3	4	12	4.5	0	2	3	6
4	43	M	4	Ascendens	3	0	2	3	4	12	1	0	2	3	6
5	48	M	7		3	1	3	2	2	4	1.7	0	1	4	4
6	64	M	3.5	Rectum	3	0	2	3	4	12	1.5	1	2	4	8
7	64	F	4	Rectum	3	0	2	2	4	8	0.8	0	2	3	6
8	63	F	3.8	Ascendens	4	1	3	1	1	1	1.7	3	2	2	4
9	54	F	6.3	Rectum	3	1	3	2	4	8	1.5	47	2	4	8
10	52	M	3.2	Sigma	4	2	2	2	4	8	9.8	1	0	0	0
11	64	M	4	Sigma	3	0	2	2	4	8	1	0	1	2	2
12	65	M		Sigma	4	1	3	2	4	8	1	0	2	3	6
13	53	M	4	Transversum	2	1	2	3	4	12	4.9	24	2	3	6
14	58	M	2.5	Cecum	3	1	2	3	3	9	3.2	0	2	4	8
15	46	F	4	Sigma	4	2	2	3	4	12	1.2	18	2	2	4
16	67	F	5	Cecum	3	2	3	3	4	12			2	4	8
17	49	F	4.7	Descendens	3	1	2	3	4	12	4.5	7	2	3	6
18	66	M	10	Transversum	3	2	3	2	4	8	1.1	0	2	3	6
19	64	F	8	Cecum	3	2	2	2	4	8	0.5	0	2	3	6
20	69	F	5.5		3	1	2	3	4	12	1.2	0	2	3	6
21	87	F	2.2	Sigma	3	2	2	1	4	4	0.3	0	1	4	8
22	63	F	3.2	Sigma	3	1	3	3	4	12	1.2	0	2	3	6
23	72	F	7	Ascendens	3	2	2	2	4	8	7	0	2	3	6
24	59	M	5	Descendens	3	2	2	2	4	8	0.1	0	2	3	6
25	73	F	3	Sigma	4	2	3	2	3	6	1	0	2	3	6
26	80	F	3.5	Ascendens	3	2	3	2	4	8	1.5	0	1	4	4
27	54	M	8.5	Rectum	4	2	2	3	4	12	1.5	0	3	4	12
28	38	M	2.5	Rectum	3	1	2	3	4	12	2.5		2	2	4
29	70	M	3	Rectum	3	2	2	3	4	12	3	0	2	3	6
30	46	F	7.7		3	2	3	2	4	8	3.2	0	1	3	3
31	71	M	5.6	Sigma	4	0	2	3	4	12	3.5	0	3	3	9
32	66	M	4.8	Ascendens	3	2	2	3	4	12	1.8	0	3	4	12
33	49	M	4	Rectum	3	2	3	2	2	4	3	0	1	2	2
34	59	M	5	Rectum	3	2	3	2	3	6	1	0	1	2	2
35	61	M	6.5	Rectum	3	0	2	2	4	8	2	0	2	4	8
36	64	M	8	Transversum	4	0	3	2	4	8	0.7	0	2	4	8
37	49	M	2.7	Cecum	3	2	2	2	3	6	0.3	0	2	3	6
38	83	F	10.5	Cecum	3	0	2	2	2	4	1	0	1	1	1
39	76	F	3.2	Ascendens	3	1	2	1	2	2	1.2	0	2	2	4
41	67	M	4.5	Ascendens	3	2	3	2	4	8	0.6	0	2	1	2
42	43	F	3.5	Ascendens	3	0	2	3	4	12	1	0	2	3	6
43	70	F	2.5	Ascendens	3	1	2	2	4	8	5	1	1	3	3
44	67	F	1.4	Cecum				3	4	12	1.8	0	3	4	12
45	68	M	3.8	Rectum	1		2	3	4	12	1.8	12	2	3	6
46	55	F		Ascendens	4	0	2	2	4	8	8	0	2	3	6
47	35	F	2.2		3	2	3	3	4	12	3.8	0	3	4	12
48	35	F	5	Rectum	4	0	2	2	4	8	0.5	0	2	3	6
49	70	M	3.5	Cecum	3	0	2	3	4	12	1.2	0	2	3	6
50	68	M	1.4	Sigma	3	2	2	3	4	12	0.8	0	2	4	8
51	81	M	3.5	Acendens	3	0	2	3	4	12	4	20	2	3	6
52	68	M	4.3	Acendens	3	2	2	1	2	2	1.9	0	2	3	6
53	77	M	2	Sigma	2	0	2	2	3	6	7	28	2	2	4
54	55	M	5.5	Rectum	3	2	2	2	3	6	2.7	0	2	3	6
55	51	M	6	Sigma	3	0	2	3	4	12	9	2	2	3	6
56	60	M					2	3	4	12	1.8	16	2	2	4
57	58	M					2	3	4	12	0.9	24	2	3	6

IRS: Immunoreactive score=number × intensity

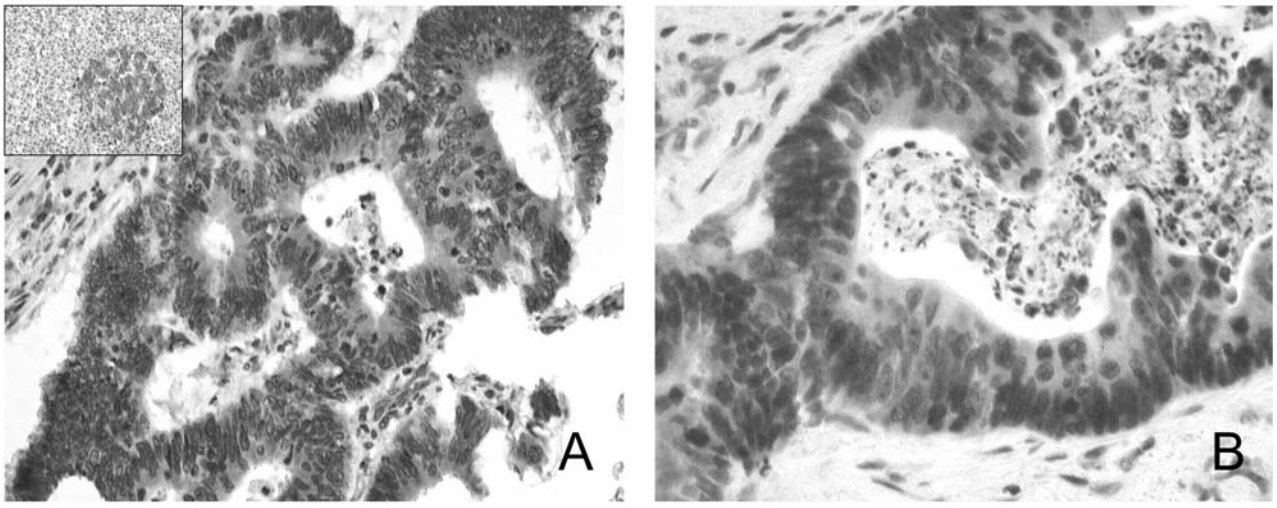

Figure 1. *Immunohistochemical staining of COX-2 in a primary carcinoma of the colon (A) and in the liver metastasis of the same patient (B). Cytoplasmatic staining was seen, which was stronger in the primary tumor compared to the metastasis. Inset: Pancreatic islet as positive control.*

rate was seen in the group stimulated with 20 μM meloxicam. This result was not significant. After 12 h, the slides treated with 20 μM meloxicam showed no increase in apoptosis rate. In the group treated with 50 μM meloxicam, and especially in the group treated with 200 μM, an increase in the number of apoptotic cells was seen with 24.4 apoptotic events/10 HPF (50 μM) and 32.5 apoptotic events/10 HPF (200 μM). No statistical significance, however, was found (Figure 3).

Discussion

Overexpression of COX-2 in colorectal adenomas and carcinomas is described in several studies. Adenomas showed an expression of this enzyme in 40-90% of the cases, whereas carcinomas were positive in over 80% of investigated cases (24-27). In our study, all primary tumors were positive, with different, mostly moderate to high levels of protein expression. This slightly increased level of protein expression in the primary tumor compared to those cited in the literature could be a result of selection of cases with more malignant behaviour and thus an indirect hint of a connection between COX-2 expression and malignancy. In our study, only cases with proven liver metastasis were included to compare expression in both tumor sites. In colorectal cancer, COX-2 expression in the primary tumor failed to show a predictive value for liver metastasis (14), whereas in gastric cancer, such a phenomenon was shown by Yu *et al*. (28).

As described earlier in the literature (29), COX-2 is expressed in nearly all liver metastases of colorectal carcinoma. In our cohort, this enzyme was expressed in

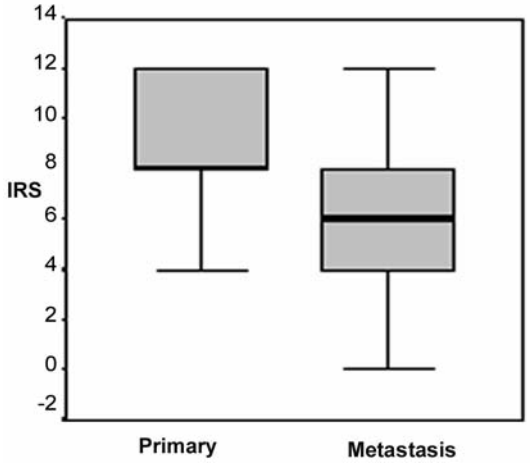

Figure 2. *Comparison of immunoreactive scores (IRS) of the COX-2 expression in primary carcinoma of the colon and in their liver metastases. The COX-2 expression in liver metastases was significantly lower than in the corresponding primaries.*

98.2% of the cases, with no difference between synchronous and metachronous occurrence. The proportion of positive tumor cells and the level of expression, however, were down regulated in comparison to the primary tumor. This is in contrast to the results of other groups. Kobayashi *et al*. performing a similar investigation at the RNA level, found no difference of COX-2 RNA levels between primary tumors with and without liver metastases, nor their liver metastases (30). Investigating tissue of 17 patients using immunoblotting, Chen *et al*. found higher levels of COX-2 expression in the liver metastases (11). The only other

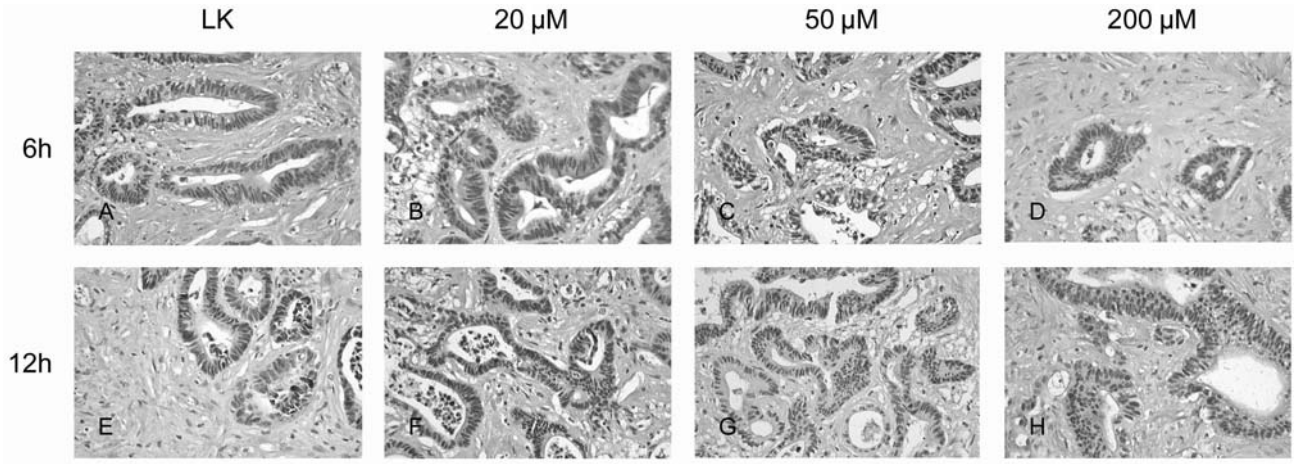

Figure 3. *Immunohistochemical detection of apoptosis in precision-cut tissue slices treated with 20µM, 50 µM and 200 µM meloxicam after 6 h and 12 h. With increasing concentration of meloxicam, a higher proportion of M30 positive cells was seen. LK, Negative control.*

immunohistochemical study comparing COX-2 expression in colorectal cancer and matched liver metastases found scores positively correlated between them (31). These differences and the biological significance of down-regulation at the protein level demonstrated here remain unclear. The reasons could be the result of the tumor itself or of the interaction between the tumor and the liver. Other growth factors or oncoproteins may have taken over the function of COX-2 in the tumor cells. The role of COX-2 could be different in systemic disease with this constant but low level expression. On the other hand, the microenvironment in the liver is different from that in the colon. With good blood supply, a lower level of angiogenic factors seems to be sufficient to maintain tumor growth. This is in concordance with the lower level of microvessel density in liver metastases compared to that in primary tumors (32, 33).

To gain insight into the functional role of COX-2 in liver metastases of colorectal carcinomas, we used the *in vivo*-like experimental model of precision-cut tissue slices. Liver metastases were treated with different concentrations of meloxicam, a selective COX-2 inhibitor. The effect was studied by assessing the apoptotic rate. COX-2 has a proven antiapoptotic function. Overexpression of COX-2 is correlated with inhibition of cytochrome *c* release and increase of bcl 2. Thus, mitochondrial-induced apoptosis is reduced (34). Tang and co-workers were also able to show an inhibition of death receptor-induced apoptosis by COX-2 (35). Selective inhibitors of COX-2 have been demonstrated to induce apoptosis in several tumor cell lines, including those of colon, gastric and prostate cancer (36-39). Our results support this. Precision-cut tissue slices have a system-inherent spontaneous apoptosis rate. Compared to this spontaneous apoptosis rate, an increase of cell death was observed here after COX-2 inhibition. In spite of this trend, these results failed to reach statistical significance in this study. For the lack of statistical significance, methodological reasons cannot be completely excluded. Only three samples of human liver metastases were investigated with the innovative technique of precision-cut tissue slices. On the other hand, there may be biological reasons. In a clinical study by Fenwick and co-workers treating 23 patients with a selective COX-2 inhibitor prior to resection of liver metastases from colorectal carcinoma, no difference was found in the apoptotic index (40). They did however, find a reduced microvessel density in the treated population. Thus, the antineoplastic effect of COX-2 inhibition is possibly based on an additional antiangiogenetic effect (30, 41). This is supported by suppression of the vascular endothelial-like growth factor (VEGF) receptor by COX-2 inhibitors in cell lines of colorectal carcinoma (42), as well as positive correlation between COX-2 and VEGF in tissue samples of primary and metastatic human colorectal cancer (31). An inhibitory effect of celecoxib, another COX-2 inhibitor, on tumor growth in a syngeneic rat liver metastasis model for colorectal cancer by creating an unfavourable environment for tumor growth was postulated (13). An

Table II. *Rate of apoptosis in tissue slices after treatment with Meloxicam (apoptotic events/10HPF).*

	6 h	12 h
Negative control	14.4 (±4.4)	16.0 (±2.7)
Meloxicam 20 µM	19.0 (±9.2)	16.8 (±5.4)
Meloxicam 50 µM	16.7 (±6.0)	24.4 (±3.4)
Meloxicam 200 µM	12.0 (±3.8)	32.5 (±7.0)
DMSO	15.0 (±6.7)	15.0 (±5.2)

inhibitory effect of COX-2 inhibition on the invasion of a tumor cell line in an *in vitro* model was shown as another pathway (10). Reduction of matrix metalloproteinase-9 has also been discussed as one possible mechanism of this effect (43). Precision-cut tissue slices, however, cannot be used to investigate such long-term effects or a possible anti-invasive potential.

In summary, all primary colorectal carcinomas investigated here and nearly all liver metastases showed COX-2 expression, with down-regulation of positive tumor cells and the level of expression in metastastic tissue. Using the *in vivo*-like experimental model of precision-cut tissue slices, a trend for decreased cell death was observed after COX-2 inhibition. The antineoplastic effect of COX-2 inhibition seems to be based on antiapoptotic effects, but additional effect such as antiangiogenic action should also be considered. These mechanisms of COX-2 inhibition can contribute to the protective effects of NSAIDs.

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