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The effects of a COX-2 inhibitor meloxicam on squamous cell carcinoma of the esophagus *in vivo*

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Our previous study showed that aspirin induced apoptosis of esophageal cancer cells *in vitro* by inhibiting the pathway of NF-kappaB downstream regulation of cyclooxygenase-2. The purpose of this study was to determine if similar changes occurred *in vivo* in the tumors of patients with SCC of the esophagus who were given a preferential COX-2 inhibitor, meloxicam. Fifty-three patients who had an esophagectomy for SCC were allocated randomly to either a Treatment group ($n = 25$) or a control group ($n = 28$). Patients in the Treatment group were given 7.5 mg/day of meloxicam, for between 10 and 14 days before surgery. Patients in the control group did not take any type of NSAID during this time interval. Samples of the tumor taken from the resected specimens were collected. Proliferation and apoptosis were measured by flow cytometry. The concentration of 6-keto-prostaglandin $F_1\alpha$ in cancer tissue was determined by radio-immuno-assay. Expression of COX-2 mRNA was measured with RT-PCR and COX-2 protein levels with Western blot analysis. Nuclear NF-kappaB and cytoplasmic IkappaB protein levels were determined by electrophoretic mobility shift assay and Western blot, respectively. There were significantly more apoptotic cells in the tumors of patients who were using meloxicam. It also decreased the levels of COX-2 mRNA, COX-2 protein and nuclear NF-kappaB protein and increased the cytoplasmic IkappaB protein in the cancer. We conclude that meloxicam induces apoptosis in SCC of the esophagus *in vivo* by inhibiting the pathway of NF-kappaB downstream regulation of COX-2.

Key words: nonsteroidal anti-inflammatory drugs; meloxicam; squamous cell carcinoma; esophagus; cyclooxygenase; prostaglandin; apoptosis

Esophageal cancer is the seventh most common cancer in the world, and one of the most common causes of cancer death.[1] There are two main forms of the cancer, squamous cell carcinoma (SCC) and adenocarcinoma. In parts of China, the incidence of SCC is among the highest in the world. In the south of Hebei Province its death rate exceeds 100 per 100,000 population/year. Despite surgery and adjuvant chemoradiotherapy, the prognosis for patients with SCC is disappointing, with a 5-year survival rate of less than 30%.[2–5] Thus, new strategies for prevention and treatment are urgently needed.

Epidemiological and experimental studies have shown the potential of nonsteroidal anti-inflammatory drugs (NSAIDs) for the chemoprevention of SCC.[6–9] The effects of NSAIDs are thought to be mediated mainly through the inhibition of the cyclooxygenases (COX), which catalyze the rate limiting step in the formation of prostaglandins from arachidonic acid. There are 2 isoforms of cyclooxygenase, COX-1 and COX-2. COX-1, constitutively expressed at near constant levels and activity in many tissues, has a role in a number of physiological functions. COX-2, normally undetectable in most tissues, is an inducible or early response gene, as well as frequently being over-expressed in tumors, including esophageal SCC and adenocarcinoma.[10–12] The increased concentrations of prostaglandins synthesized by COX-2 can drive tumor growth by promoting angiogenesis, inhibiting apoptosis, stimulating cell proliferation and motility, and modulating inflammatory and immune responses.[13,14]

Our previous study showed that aspirin reduced the proliferation of esophageal cancer cells, and induced apoptosis, in a dose dependent manner *in vitro*. These effects correlated with a reduction in COX-2 expression and prostaglandin synthesis, an increase in IKappaB and a reduction of NF-KappaB and COX-2 mRNA.[15] The purpose of this study was to determine if similar changes occurred *in vivo* in the tumors of patients with SCC of the esophagus who were given a preferential COX-2 inhibitor, meloxicam.

Patients and methods

Patients

Fifty-three patients who had an esophagectomy in the Department of Thoracic Surgery at the Fourth Hospital of Hebei Medical University for SCC were allocated randomly to either a Treatment group ($n = 25$) or control group ($n = 28$). The patient information is given in Table I. There were no significant differences in sex, age, stage of disease or tumor differentiation between the groups. No patients received chemotherapy, radiotherapy or any other cancer related therapies before surgery. The study was approved by the Division of Medical Affairs, the Fourth Hospital, Hebei Medical University, and written consent forms were obtained from all patients.

Patients in the Treatment group were given 7.5 mg/day of the preferential COX-2 inhibitor meloxicam, marketed as Mobic (Boehringer Ingelheim), from the day of admission to hospital. The length of time between admission and operation varied between patients, depending on the availability of operating theatre time and the time taken to ensure that their nutritional status was at a satisfactory level. Because of this, the number of days that meloxicam was taken ranged from 10 to 14 days within the Treatment group. Patients stopped the drug the day before operation. No side effects which could be attributed to the drug were noted in the Treatment group. Patients in the control group did not take any type of NSAID during this time interval between admission and operation. Samples of the tumor were taken from the resected specimens of each of the patients at esophagectomy, snap frozen in liquid nitrogen, and then stored at −80°C until analysis. Five milliliter of venous blood was collected into the anticoagulant indomethacin-sodium EDTA (the standard anticoagulant used

Abbreviations: AP, apoptotic percentage; COX, cyclooxygenase; EMSA, electrophoretic mobility shift assay; FI, fluorescence index; IOD, integrated optical density; NSAID, nonsteroidal antiinflamatory drugs; RIA, radioimmunoassay; SCC, squamous cell carcinoma.

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TABLE I – CLINICOPATHOLOGIC CHARACTERISTICS OF THE PATIENTS IN THE STUDY OF THE EFFECT OF MELOXICAM, A PREFERENTIAL COX-2 INHIBITOR, ON TUMOR CELLS *IN VIVO*.

	Treatment	Control
N	25	28
Sex (m/f)	19/6	21/7
Age (mean)	58.8	60.1
Age (range)	38–71	35–75
TNM stage		
IIb	20	22
III	5	6

in the hospital). The blood was immediately centrifuged at 3,500 rpm for 15 min at 4°C, and the plasma collected and stored at −20°C.

Methods

Electrophoretic mobility shift assay

An electrophoretic mobility shift assay (EMSA) was used to detect the expression of NF-kappaB in tissues of SCC of the esophagus. A sample of the tissue (15–20 mg) was homogenized in 1 mL of buffer containing 10 mmol/L HEPES, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol (DTT), 0.5mmol/L phenylmethylsulphonylfluoride (PMSF), 0.1% Nonidet P40 (NP-40) and centrifuged at 1,000g for 10 min at 4°C. After the supernatant was removed, nuclear extracts were prepared as described by Liu *et al*.[16]

The protein concentration was determined using the CBB G250 assay kit (Nanjing Jiancheng Biotechnology Research Institute). Double-stranded deoxyoligonucleotides containing the NF-kappaB consensus recognition site (5′-AGT TGA GGG GAC TTT CCC AGG-3′; Promega, USA) were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (Promega) and purified by ethanol precipitation. The nuclear protein (10 μg) was incubated with the radiolabelled probe DNA (3.5 pmol, 10 μCi), with or without a 100- to 500-fold excess of unlabeled probe, in 1 mmol/L $MgCl_2$, 0.5 mmol/L EDTA, 0.5 mmol/L DTT, 50 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 7.5), 0.05 μg poly (dI-dC), and 40 mL/L glycerol to a final volume of 10 μL, for 30 min at room temperature. The DNA-protein complexes were resolved on a 6% nondenaturing polyacrylamide gel which was then dried and visualized by autoradiography at −80°C. The gel was quantified with Gel-Pro analyzer software (Media Cybernetics, USA), and the results expressed as units of integrated optical density (IOD).

Protein extraction and Western blotting

Protein was extracted by homogenizing the esophageal cancer tissues in a lysis buffer containing 100 mmol/L HEPES, 10 mL/L Triton X-100, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 10 mmol/L dithiothreitol (DTT) and 1 mmol/L phenylmethylsulphonylfluoride (PMSF) (Promega, USA) and incubating on ice for 15 min. The lysate was centrifuged at 20,000*g* for 15 min, the supernatant collected, and its protein concentration determined by CBB G250 assay kit (Nanjing Jiancheng Biotechnology Research Institute). Then, 100 μg of protein was separated on a SDS-10% polyacrylamide gel and transferred electrophoretically to a nitrocellulose membrane. After the nonspecific binding sites were blocked with 2% dried skim milk in TBS (120 mM Tris HCl, pH 7.4 and 150 mM NaCl), the membrane was incubated overnight with 2 μg/mL rabbit antibody to COX-2 or I-KappaB (Santa Cruz, USA). After washing with 0.05% Tween 20 in TBS, the membrane was incubated with a horseradish peroxidase-conjugated goat antirabbit IgG secondary antibody for 1 hr at 37°C, washed again, and developed with diaminobenzidine. The immune complex was visualized and quantified with Gel-Pro analyzer software (Media Cybernetics, USA), and the results expressed as units of IOD.

Measurement of COX-2 gene expression by reverse transcription PCR

RT-PCR was used to measure COX-2 gene expression in esophageal cancer tissues. Total RNA was isolated using TRIzol reagent following the protocols of the manufacturer (Gibco BRL). An aliquot of this was reverse transcribed into cDNA using an RNA PCR-Kit (Sino-American Biotechnology, Beijing, China). The PCR protocol was an initial 94°C for 5 min, followed by 40 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. Human specific primers were used for COX-2 and beta-actin. The primers used for the COX-2 amplification were 5′-TTC AAA TGA GAT TGT GGG AAA ATT GCT-3′ (forward primer) and 5′-AGA TCA TCT CTG CCT GAG TAT CTT-3′ (reverse primer), giving a 303 bp PCR product. For beta-actin (ACTB), the forward primer was 5′-GTT TGA GAC CTT CAA CAC CCC-3′, and the reverse primer was 5′-GTG GCC ATC TCC TGC TCG AAG TC -3′, giving a 318 bp PCR product. The synthesized PCR products were separated on 1.5% agarose gels and analyzed by Gel-Pro analyzer version 3.1 software. The ratio of COX-2 to beta-actin was used for expressing the relative level of mRNA expression.

Measurement of 6-keto-PGF1α by radio-immuno-assay

The concentration of 6-keto-PGF1α in esophageal cancer tissues and blood from patients, who used meloxicam and controls, was measured with RIA. Venous blood (5 mL) was collected into a syringe containing 0.1 mL indomethacin-Na_2EDTA as anticoagulant, and then centrifuged at 3,500 rpm for 15 min at 4°C. The plasma was collected and stored at −20°C until analysis. Five to ten grams of tissue sample were homogenized in 0.1 mL of 100% ethanol, and homogenization continued after 0.9 mL normal saline was added. The sample was centrifuged at 3,500 rpm for 15 min at 4°C before removal of the supernatant. The precipitate was stored at −20°C until analysis. The concentration of 6-keto-PGF1α in the preparations was measured by radio-immunoassay using a commercially available kit (Beifang Biotechnology, Beijing, China).

Measurement of cell apoptosis and proliferation with flow cytometry

Fresh tissues were fixed in 70% ethanol and stored at 4°C until they were cut into small pieces (about 1 mm^3 in size) with scissors. Cell suspensions were prepared by triturating through a bronze mesh (120 holes/square inch) to remove tissue fragments and cell clusters. The samples were then filtered through a finer sieve (200 holes/square inch). The cell suspensions were centrifuged at 100*g* for 3 min and the cell pellets washed twice with PBS. Finally the pellet was resuspended in PBS to a concentration of 1×10^6 cells/mL. The cells were fixed in 70% ethanol and then stained with 1 mL of propidium iodide (PI), at a final concentration of 50 μg/mL, for 20 min. Flow cytometric analysis was performed on a FACS420 flow cytometer. The apoptotic percentage (AP) was calculated according to the following formula: AP = (the number of apoptotic cells/the number of measured cells) × 100%.

The expression of proliferating cell nuclear antigen (PCNA) in esophageal cancer tissue was measured by flow cytometry. Cell suspension was prepared according to the above procedure. First 100 μL suspensions of the cells were aliquoted into polystyrene tubes and incubated with 100 μL of FITC-conjugated mouse anti-human PCNA antibody (Zhongshan Biotechnology, Beijing, China) diluted 1:100, for 30 min at 37°C. All samples were analyzed using a FACS420 flow cytometer (Becton Dickinson, Sunnyvale, CA). The cells were excited with a single 488 nm argon laser. Fluorescence was detected through a 520 nm band pass (BP) filter. The fluorescence index (FI) was calculated as the ratio of fluorescence intensity of the sample compared to mean fluorescence intensity of normal controls. The mean FI ±2 standard derivations in normal controls was defined as normal expression and

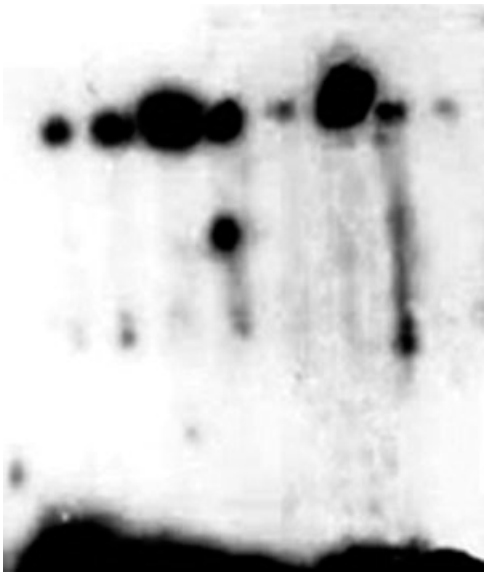

Figure 1 – A gel from a representative EMSA assay for the determination of the activity of NF-κB in tumor tissues resected from patients with SCC of the esophagus. Tissue from a patient in the Treatment group is denoted by T, from a patient in the control group by C.

Figure 2 – A representative Western blot for IκB-α protein levels in resection tissue from SCC of the esophagus. T, Treatment group (tissues from patients who used meloxicam before surgery); C, control group (tissues from patients who did not use meloxicam before surgery).

values higher than the upper limit were regarded as positive for the expression of the antigen.

Statistical analysis

The results are reported as the mean ± standard deviation. Groups were compared by ANOVA test using the SPSS 10.0 software package. Differences were considered to be statistically significant when $p < 0.05$.

Results

The effects of meloxicam on activity of NF-κB in SCC of the esophagus

A gel from a typical EMSA assay is shown in Figure 1. In this figure the activity of NF-KappaB in the lanes 2, 3, 4 and 6, each from a tissue from a Treatment group patient, is greater compared to tissue from a control group patient (lanes 1, 5, 7 and 8). The density of the bands in the EMSA gels was quantitated as IOD units. The mean IOD for the Treatment group (9.8 ± 6.3) was significantly less than that for the control group (37.5 ± 12.4) ($t = 8.992$, $p < 0.0001$).

The effects of meloxicam on the expression of IκB-α protein

One possible reason for a reduction in the level of NF-KappaB in the Treatment group might be an increase in its inhibitor, IKappaB-alpha. Western blotting was used to detect IKappaB-alpha in the tumor tissues resected from patients in the Treatment and the control groups. A gel from a typical Western blot is shown in Figure 2. In this gel, in the control group there was either a weak band (lanes 1 and 5) or no band (lanes 2 and 4), while in the Treatment group the bands were prominent (lanes 3, 6 and 7). The density of the bands in the gels was quantitated as IOD units. The mean IOD for the Treatment group (166.2 ± 55.1) was significantly greater than that for the control group (82.2 ± 24.4) ($t = 7.320$, $p < 0.0001$).

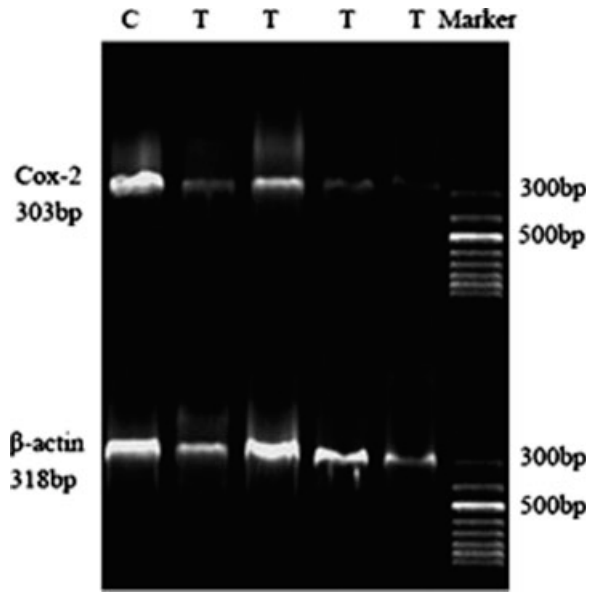

Figure 3 – A representative gel from an RT-PCR assay for COX-2 mRNA in tissue from SCC of the esophagus. The semi-quantitative analysis for COX-2 mRNA was performed by multiplex RT-PCR technique, using beta-actin as the reference standard. T, Treatment group (cancers from patients who used meloxicam before surgery); C, control group (cancers from non-users of meloxicam).

Figure 4 – Western blot for COX-2 expression in SCC of the esophagus. T, Treatment group (tumor tissue from patients who took meloxicam before surgery); C, control group (tumor tissue from patients who took no NSAIDs before surgery).

The effects of meloxicam on the expression of COX-2 mRNA

A gel of the amplified products from a representative RT-PCR is shown in Figure 3. The lower are the housekeeping beta-actin bands, used as a common reference for comparing the results from the different tissues, and the upper are the COX-2 bands. The left column is from a patient in the control group, the next 4 columns to the right were from 4 patients in the Treatment group. There is a prominent COX-2 band for the tissues from the control group, while tissues from the Treatment group show weak COX-2 bands.

The optical density of each of the bands was quantitated and the ratio of IOD of the COX-2 band to the IOD of the beta-actin band was calculated. The mean ratio for the Treatment group (0.60 ± 0.15) was significantly lower than the median for the control group (0.89 ± 0.07) ($t = 7.837$, $p < 0.0001$), indicating that the COX-2 gene expression was less in the tumor of patients in the Treatment group than in the control group.

The effects of meloxicam on the expression of COX-2 protein

Weak expression of COX-2 was seen in 16 tumors and no expression in 9 tumors from patients in the Treatment group. In a representative gel shown in Figure 4 there is weak expression (lanes 1, 5 and 6) or no expression (lane 2) in tissue from 4 patients in the Treatment group (T), and strong expression in

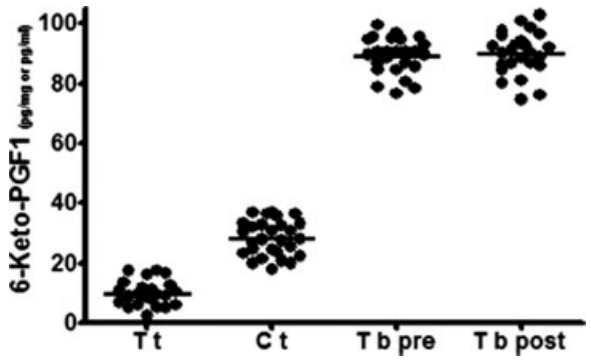

FIGURE 5 – The concentration of 6-Keto-PGF1α in resected tumor issue from the Treatment group (Tt), or the control group (Ct) ($t = 13.195$, $p < 0.001$), and the concentration in the plasma before (Tbpre) and after taking meloxicam (Tbpost) in the Treatment group ($t = 0.366$, $p = 0.716$). The horizontal bars represent the medians of the groups.

tissue from 2 patients (lanes 3 and 4) in the control group (C). The amount of COX-2 measured as IOD in the Treatment group (7.5 ± 4.6) was significantly less than that in the control group (28.0 ± 10.5) ($t = 8.99$, $p < 0.0001$).

The effect of meloxicam on prostaglandin concentration in plasma and in esophageal cancer tissue

To determine if the reduction in COX-2 expression resulted in a reduction in prostaglandin, the concentration of 6-keto-prostaglandin F1α (6-Keto-PGF1α) was measured in the plasmas and the resected tumor tissues. This prostaglandin was measured as a representative prostaglandin as it was the one for which the necessary reagents, in particular the specific antibody, were readily available.

The results in Figure 5 show that the concentration of 6-keto-PGF1α was significantly lower in the tumor taken from patients in the Treatment group (9.9 ± 4.1 pg/mg) compared to that from the control group (28.3 ± 5.8 pg/mg) ($t = 13.195$, $p < 0.001$). This reduction in concentration in the Treatment group was due in the main to the inhibition of COX-2 in the tumor by the drug, as the concentration of 6-Keto-PGF1α did not differ between plasma samples taken after treatment compared to samples taken before treatment (89.1 ± 6.1 *vs*. 89.8 ± 7.2 pg/mL) ($t = 0.366$, $p = 0.716$).

The effects of meloxicam on proliferation and apoptosis in squamous cell carcinoma of the esophagus

The results in Table II show that there was no difference in FI value of PCNA between the tumors from patients who were on meloxicam and those who were not. Typical histograms of cell preparations from tumors from a patient in each of the Treatment and the control group are shown in Figure 6. The results in Table II show that there were significantly more apoptotic cells in the tumors of patients who were using meloxicam (mean 6.2%) compared to those who were not using meloxicam (mean 1.2%) ($p = 0.0005$).

TABLE II – THE FI VALUE OF PCNA AND APOPTOTIC RATE IN SCC OF THE ESOPHAGUS BY FCM

	FI of PCNA	AR (%)
Treatment ($N = 15$)	1.23 ± 0.1	6.2 ± 2.5
Control ($N = 20$)	1.32 ± 0.1	1.2 ± 1.1
P	0.16	0.0005

Discussion

Overexpression of COX-2 has been observed in many human malignancies including esophageal cancer.[17–19] For example, Zimmermann detected COX-2 expression in 91% of esophageal SCC and in 78% of esophageal adenocarcinomas by immunohistochemistry.[11] Our previous study also found enhanced expression of COX-2 in esophageal cancers compared with normal esophageal squamous epithelium.[20] Sharma reported that the expression of COX-2 protein was increasingly upregulated from low grade to high grade dysplasia compared with normal esophageal epithelium in the esophagus, and also increased in SCC.[21] This suggests that COX-2 might be involved in the progression from dysplasia to SCC. Similar results were found by Morris in the Barrett's metaplasia-dysplasia-adenocarcinoma sequence with an increase in COX-2 expression in the progression from low grade to high grade dysplasia.[22] France suggested that COX-2 protein expression might be a better prognostic indicator in esophageal adenocarcinoma than traditional histopathological staging.[12] Overexpression of COX-2 *in vitro* has been shown to have a number of cellular effects including increasing proliferation, reducing apoptosis,[23] promoting angiogenesis,[24] decreasing E-cadherin expression,[25] and increasing invasive/metastatic potential.[13] The cyclooxygenase enzymes catalyze the rate-limiting step for prostaglandin synthesis, although it must be noted that the downstream prostaglandin metabolites might differ markedly between cell types and under different conditions. COX-2 has been shown to increase prostaglandin synthesis in many cancer cells, including esophageal,[11] colon,[18] lung[26] and prostate.[23] Prostaglandins have been reported to promote cell proliferation, inhibit the immune response to malignant cells, inhibit apoptosis[19] and regulate angiogenesis.[24]

Our previous study showed aspirin dose- and time-dependently inhibited cell growth and induced apoptosis in esophageal SCC TE-13 cells,[15] which was consistent with other reports. Li et al. noted that aspirin-induced apoptosis in both esophageal squamous cell carcinoma and adenocarcinoma cell lines,[8] and the treatment of OSC-2 esophageal cancer cell line with the preferential COX-2 inhibitors, NS-398 and flosulide, suppressed proliferation and also induced apoptosis. We also demonstrated that aspirin inhibited COX expression, inhibited the proliferation of, and induced apoptosis in, the cultured esophageal SCC cell line TE-13. These changes correlated with a reduction in COX-2 mRNA and protein expression, prostaglandin synthesis and an inhibition of NF-KappaB nuclear translocation and an increase in cytoplasmic IkappaB.[15] These results supported the conclusion that aspirin induced apoptosis in TE13 cells by inhibiting the pathway of NF-kappaB regulation of COX-2.[15] This study was designed to determine if a NSAID, meloxicam, had the same effect on esophageal tumor cells *in vivo* as aspirin had on the esophageal SCC cell line TE-13 *in vitro*. Meloxicam, a preferential COX-2 inhibitor that has a higher degree of COX-1 inhibition than selective COX-2 inhibitors such as celecoxib, was selected for use in this study because of its more widespread availability at the time in China. Although it does inhibit thromboxane A, it is also reported not to interfere with platelet function at the dosage of 7.5 mg/day.[27]

In the present study there was a reduction in the expression of COX-2 protein in the resected esophageal SCC tissue from the Treatment group. This was mirrored by a decrease in the concentration of prostaglandin in the tissue. The concentration of prostaglandin in the plasma did not differ between the two groups as would be anticipated. The plasma pool of prostaglandin reflects the production bodywide by the constitutive COX-1, which is not inhibited by meloxicam. There was reduction in the expression of COX-2 mRNA in the tumor tissue, which paralleled a reduction in NF-KappaB expression and an increase in IKappaB expression. In tissues from the Treatment group there were more apoptotic cells than in the control tissues.

This is one of the few *in vivo* studies to examine mechanisms by which NSAIDs affect cancer tissues in general or esophageal SCC tissue specifically. Yin *et al*.[28] demonstrated that aspirin and sodium salicylate specifically inhibited IKK-beta activity *in vitro*

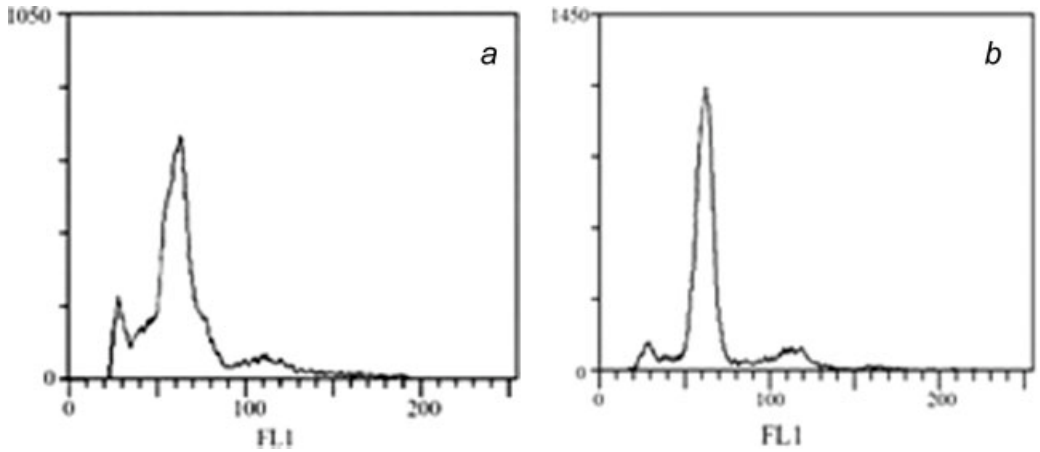

FIGURE 6 – Representative FACS histograms of cells prepared from esophageal SCC tumor tissue of a patient in the Treatment (*a*) and the control (*b*) groups. The apoptotic rate in (*a*) is 10.6%, in (*b*) 5.5%.

and *in vivo*, thereby preventing activation of NF-kappaB responsive genes. Sclabas *et al.*[29] reported that aspirin, given before or at the time of transplantation into mice of a human pancreatic carcinoma cell line, repressed tumor formation in mice, possibly through inhibition of NF-kappaB activation. Futakuchi *et al.*,[30] based on results from an *in vivo* metastasis model in rats, suggested that aspirin has the potential to inhibit lung metastasis from chemically induced hepatocarcinoma cells by inhibiting NF-kappaB and its downstream genes. There are several clinical studies, however, which show no benefit in esophageal cancer from NSAID use. Heath reported that the long term administration of celecoxib in patients with Barrett's esophagus with dysplasia for 48 weeks did not appear to prevent progression to cancer.[31] Limburg reported results from a randomized controlled trial of selenomethionine and/or celecoxib for 10 months in residents from Linxian, China, with histologically confirmed mild or moderate dysplasia on trial entry. Selenomethionine had a protective effect for a subset of patients with mild esophageal squamous dysplasia patients, but there was no association between celecoxib and changes in the grade of dysplasia, nor synergy with the selenomethionine.[32]

The results in the present study extend these reports by examining the effect of meloxicam on human primary tumors. They are consistent with meloxicam inducing apoptosis in esophageal SCC tissue *in vivo* through the mechanisms of inhibiting the pathway of NF-kappaB regulation of COX-2, which is similar to those by which aspirin affected esophageal SCC TE-13 cells *in vitro*[15]. Together, the *in vitro* and *in vivo* results support further investigation of the potential for aspirin or NSAIDs in esophageal SCC prevention or treatment.

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