Expression of macrophage migration inhibitory factor in esophageal squamous cell carcinoma and effects of bile acids and NSAIDs

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The aim of this study was to determine the macrophage migration inhibitory factor (MIF) protein and mRNA expression in esophageal squamous cell carcinoma (ESCC), and the effect of bile acids, aspirin and a selective cyclooxygenase-2 (COX-2) inhibitor, NS398, on MIF expression in ESCC cells in vitro. Specimens from tumors and the adjacent non-cancerous tissues were obtained from 52 ESCC patients. Western blotting was used for the detection of MIF protein expression, and reverse transcription-polymerase chain reaction (RT-PCR) for MIF mRNA expression. Cells of an ESCC cell line, Eca-109, were treated with chenodeoxycholate (CD, 100 mM), glycochenodeoxycholate (GCD, 1 mM), aspirin (1 mM) or NS398 (1 μM). Enzyme-linked immunosorbert assay (ELISA) and RT–PCR were used to detect the expression of the MIF protein and mRNA, respectively, in the supernatant and cultured cells. Western blotting demonstrated that levels of MIF protein were increased in tumors versus non-malignant tissues, with the expression ratio of MIF to β-actin of 0.93 ± 0.21 and 0.57 ± 0.08, respectively (P = 0.012). In vitro, both CD and GCD induced a dramatic increase in MIF protein and mRNA in ESCC cells. On the other hand, aspirin and NS398 significantly decreased MIF protein and mRNA expression, and completely blocked bile acid-induced MIF synthesis in the presence or absence of prostaglandin E₂. In conclusion, MIF expression is increased in ESCC. Whereas bile acids induce MIF expression in ESCC cells, aspirin and NS398 significantly inhibit MIF expression, even in the presence of bile acids, via a COX-independent mechanism.

Introduction

Esophageal cancer is one of the common malignancies, with an increasing incidence and a high mortality in both developed and developing countries (1). Esophageal cancer exists in two main forms with distinct etiological and pathological characteristics, namely esophageal squamous cell carcinoma (ESCC) and adenocarcinoma. Whereas adenocarcinoma is more prevalent in the USA and some other Western countries, ESCC is the predominant type in the other parts of the world, especially in Asia, accounting for > 90% of esophageal cancer worldwide (2,3). However, the underlying mechanisms that determine the biological and molecular behaviors of ESCC have not been fully elucidated, because most current studies are focusing on esophageal adenocarcinoma.

Macrophage migration inhibitory factor (MIF) was originally identified in activated lymphocytes. Macrophages were subsequently found to be an important source (4). MIF inhibits the migration of macrophages and induces a variety of macrophage functions, including adherence and phagocytosis. Moreover, MIF plays a pivotal role in inflammatory and immune diseases (5). In our previous study, MIF was markedly upregulated during acute gastric ulceration in rats, which was associated with accumulation of macrophages (6). Blockade with the neutralizing anti-MIF antibody inhibited macrophage accumulation and MIF up-regulation, indicating an important pathogenic role of MIF in gastric inflammation and ulceration (6). In addition, we observed that Helicobacter pylori infection increased the expression of MIF in gastric epithelial and inflammatory cells (7). Recent studies have revealed that MIF may also play a critical role in the development of cancers as a link between inflammation and tumorigenesis (5,8). MIF is over-expressed in many tumors, including melanoma, neuroblastoma, myelomonocytic leukemia, and cancers of the prostate, breast, lung, colon, liver and stomach (5.8–16). However, the precise role of MIF in carcinogenesis remains unclear.

Studies have shown that reflux of duodenal contents contributes to the development of esophageal cancer, whether adenocarcinoma or ESCC (17–19). Bile acids are likely to be major contributing factors, with previous studies having demonstrated that bile acids cause mucosal injury, induce cyclooxygenase-2 (COX-2) expression, stimulate cell proliferation and promote tumorigenesis (20). On the other hand, a protective role of aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) in the development of both esophageal adenocarcinoma and ESCC has been reported (21).

We hypothesize that MIF expression is increased in ESCC, and that bile acids, aspirin and NSAIDs are involved in the regulation of MIF. Therefore, this study was carried out to determine the expression of MIF protein and mRNA in patients with ESCC, and the effects of conjugated and unconjugated bile acids, aspirin and a selective COX-2 inhibitor on MIF expression in esophageal squamous carcinoma cells in vitro.

Materials and methods

Patients

Fifty-two ESCC patients [39 males and 13 females, with a mean age of 58.9 years (range, 34–81)] who underwent surgical treatment without preoperative therapy in the Department of Surgery, Beijing Friendship Hospital, The Capital University of Medical Sciences, between January 1999 and December 2002,
were included in the study. Surgical treatment consisted of resection of the esophagus with lymph node dissection. Specimens from tumors and from adjacent normal-appearing tissues (at least 5 cm away from the tumor) were obtained from each patient, and immediately frozen in liquid nitrogen.

Cell culture and treatment with chenodeoxycholate, glycochenodeoxycholate, aspirin and NS398 in the presence or absence of prostaglandin \( E_2 \)

An ESCC cell line, Eca-109 (22; kindly provided by Prof. G.R. Yang, Henan Institute of Medical Sciences, Henan Medical University, Zhengzhou, China) was grown in DMEM containing 10% fetal calf serum (FCS) with 100 U/ml penicillin and 100 \( \mu \)g/ml streptomycin in a 5% \( CO_2 \) atmosphere at 37 °C. Bile acids, chenodeoxycholate (CD, 100 \( \mu \)M, Sigma Chemical, St Louis, MO) or glycochenodeoxycholate (GCD, 1 mM, Sigma), aspirin (1 mM, Sigma) or a selective COX-2 inhibitor, NS398 (1 \( \mu \)M, Biomol, Plymouth Meeting, PA), were added to medium containing 1% FCS for 24 h, in the presence or absence of prostaglandin \( E_2 \) (2.8 \( \mu \)M). The concentrations for the bile acids were selected according to a previous study (20), and also from pilot experiments where a wide range of concentrations were evaluated. Concentrations chosen were those that induced MIF protein expression by ~2-fold. There was no obvious toxicity from these substances, as assessed by measuring the cell number, trypan blue exclusion and the release of lactate dehydrogenase (data not shown).

Western blot

Tissue was frozen and thawed in ice-cold RIPA buffer (PBS with 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS) containing protease inhibitor mixture, with 3 ml RIPA buffer per gram of tissue. The tissue was homogenized, kept on ice for 30 min and centrifuged twice at 10,000 r.p.m. for 10 min at 4 °C. The supernatant was collected and protein content in the supernatant was measured using a bicinchoninic acid reagent (Pierce, Rockford, IL). Protein (25–30 mg) was electrophoresed using a 10% denaturing SDS gel, and transferred onto an Immobilon-P membrane (Millipore, Bedford, MA). Blots were incubated with an anti-MIF monoclonal antibody (R&D Systems, Minneapolis, MN), followed by a horseradish peroxidase-conjugated anti-IgG antibody (Santa Cruz, CA). The immunoblot bands were developed and visualized by enhanced chemiluminescence (Amersham, Piscataway, NJ) and autoradiography. The intensity of the bands was quantified by densitometry, with \( \beta \)-actin monoclonal antibody (Santa Cruz) as a control.

Semi-quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated from frozen tissues as well as cultured cells using the RNeasy Mini Kit (QIAGEN, Valencia, CA). Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed using oligo (dT) as the random primer for reverse transcription and specific MIF primers and \( \beta \)-actin primers for PCR. Briefly, total RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (QIAGEN) at a concentration of 2 U/mg for 2 h at 42 °C. PCR was carried out using QuantiTECTTM SYBR PCR Kit (QIAGEN). The following primers were used to amplify a 185-bp fragment of MIF: sense primer, 5′-GTT CCT CTC GA CCA GCA GC-3′; antisense primer, 5′-GCA GCT TGC TGT AGG AGC GGT TCT G-3′. Primers for the amplification of human \( \beta \)-actin mRNA were as follows: sense primer, 5′-ATG GAA TTC CCG TGG AAC AGG AAG GAA AGT AGC AG-3′; antisense primer, 5′-CGT CAT ACG CCT GCT TGC TGA TCC ACA TCA GC-3′. The amplification profile for MIF was 28 cycles of 45-s denaturation at 95 °C and 1-min annealing, 1-min extension at 63 °C, followed by extension for 5 min at 63 °C. For \( \beta \)-actin, the amplification profile was 35 cycles of 1-min denaturation at 94 °C and 1-min annealing at 56 °C and 1-min extension at 72 °C, followed by extension for 5 min at 72 °C. Afterwars, the PCR products were resolved on a 1.7% agarose gel, stained with ethidium bromide, and analyzed by densitometry. Data are expressed using an MIF expression ratio, which was defined as the MIF band area intensity divided by the \( \beta \)-actin band area intensity.

Enzyme-linked immunosorbent assay

MIF in the supernatants was measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instruction (R&D Systems). Briefly, 100 \( \mu \)l of capture anti-human MIF antibody (2 \( \mu \)g/ml) in phosphate-buffered saline (PBS, pH 7.4) was transferred to each well of an ELISA plate, and blocked with 1% BSA. 100 \( \mu \)l of each sample, or recombinant human MIF (R&D Systems) were added, in triplicate, into corresponding wells for 2 h at room temperature. Biotinylated anti-human MIF antibody (0.2 \( \mu \)g/ml, R&D Systems) was added and incubated for 2 h at room temperature. After plates were washed three times, 100 \( \mu \)l streptavidin-HRP (R&D System) at a dilution of 1 in 200 was added to each well. Following washing for a further four times, 100 \( \mu \)l of a 1:1 mixture of reagents A (\( H_2 O_2 \)) and B (tetramethylbenzidine) was added. Finally, the reaction was stopped by adding 50 \( \mu \)l \( H_2 SO_4 \). Absorbance was read using a microtiter plate reader (NovaPath™, Bio-Rad, Hercules, CA) at 450 nm.

Statistical methods

Data obtained from the study were expressed as the mean ± SD. Statistical analysis was performed using SPSS software (version 10.0, SPSS, Chicago, IL). Independent-sample t-test and the Mann-Whitney U-test, where appropriate, were used to determine the differences between numeric variables. The P values calculated were two-tailed. The alpha level of significance was set at \( P < 0.05 \).

Results

Expression of MIF protein in tumor and the corresponding non-malignant tissues

Western blotting of ESCC tumor and the corresponding non-malignant specimens demonstrated the expected band for MIF protein with a molecular weight of 12.5 kDa. In 50 of 52 cases (96.15%), the expression levels of MIF protein were higher in tumors than in the corresponding non-malignant tissues (Figure 1). Overall, the mean expression ratio of MIF over \( \beta \)-actin was 0.93 ± 0.21 for tumor samples and 0.57 ± 0.08 for the corresponding non-malignant tissues (\( n = 52, P = 0.012 \)).

Expression of MIF mRNA in tumor and the corresponding non-malignant tissues

Further, RT-PCR analysis showed that MIF mRNA was expressed both in the tumor and in the corresponding non-malignant tissues, but the expression levels were higher in the former than in the latter for 48 of 52 cases (92.31%) (Figure 2). The mean ratio of the MIF mRNA band area intensity divided by the \( \beta \)-actin band area intensity was 1.33 for tumor tissue and 0.71 for corresponding non-malignant tissue.
by the $\beta$-actin mRNA band area intensity was 0.82/0.12 for tumors and 0.34/0.05 for the corresponding non-malignant tissue ($n = 52, P = 0.011$).

Effect of CD, GCD, aspirin and NS398 on MIF protein and mRNA expression in esophageal squamous carcinoma cells in vitro (Figure 3). On the other hand, aspirin and NS398 significantly decreased the MIF protein and mRNA expression. Moreover, aspirin and NS398 completely blocked bile acid-mediated induction of MIF synthesis at both protein and mRNA levels (Figures 3 and 4). However, the inhibitory effect of aspirin and NS398 was not reversed by addition of prostaglandin E$_2$, and prostaglandin E$_2$ did not have any impact on MIF expression (Figure 3).

Discussion

In the present study, the expression of MIF protein and mRNA was significantly increased in ESCC, compared with the corresponding non-malignant tissues, suggesting that MIF is involved in the pathogenesis of ESCC. Further investigation is required to elucidate the underlying mechanisms, although promotion of cell proliferation, tumor angiogenesis and metastasis has been implicated (9,13,23–25).

Duodenogastroesophageal reflux, exposing esophageal tissue to acid, pepsin, bile and pancreatic juices play a significant role in the development of Barrett’s metaplasia and esophageal carcinoma, and refluxed duodenal contents per se cause esophageal carcinoma in rats (17–19). Bile acids within the duodenal contents are likely to be major contributing factors in tumorigenesis through mucosal injury and stimulation of cell proliferation (20,26,27). In explants of Barrett’s esophagus ex vivo and in esophageal adenocarcinoma cells, bile acids up-regulate COX-2 expression and prostaglandin E (PGE) release, and increase cell proliferation (20,28–30). In cultured esophageal squamous carcinoma cells, bile acids also significantly increase COX-2 expression and PGE release, with the unconjugated bile acid, CD, being more potent than the conjugated bile acid, GCD (20). In the present study, both bile acids significantly stimulated MIF expression at both protein and mRNA levels in cultured esophageal squamous carcinoma cells, suggesting that bile acids promote esophageal squamous carcinoma cells, at least in part, through an MIF-mediated pathway (i.e. involving MIF-mediated cell proliferation and angiogenesis).

Aspirin/NSAIDs are protective against both esophageal adenocarcinoma and ESCC, probably through inhibition of COX activity, with subsequent stimulation of apoptosis and suppression of cell growth (21,31–38). In addition, aspirin/NSAIDs may also reduce the incidence of esophageal cancers by decreasing esophageal inflammation, which is a risk factor for both esophageal adenocarcinoma and ESCC (39,40). In the present study, we observed that both aspirin and the COX-2 inhibitor, NS398, decreased MIF expression in the esophageal squamous carcinoma cells. Moreover, the effect of aspirin/NSAIDs on MIF expression was not influenced by addition of bile acids, with stimulatory effect of bile acids on MIF expression.

![Fig. 3. Effect of CD, GCD, aspirin and NS398, with and without prostaglandin E$_2$, on MIF protein expression in ESCCs in vitro. The bar represents the SD. $^*P < 0.001$, compared with the control.](https://academic.oup.com/carcin/article-abstract/26/1/11/2476004)

![Fig. 4. Effect of CD, GCD, aspirin and NS398 on MIF mRNA expression in esophageal cancer cells in vitro as demonstrated by RT–PCR analysis.](https://academic.oup.com/carcin/article-abstract/26/1/11/2476004)
expression being completely blocked by aspirin/NSAIDs. These findings further support the notion that aspirin and NSAIDs play a protective role in the pathogenesis of esophageal cancer including ESCC, and suggest that regulation of MIF expression by aspirin and the COX-2 inhibitor, contribute to this protective role. A few studies have looked at the association between MIF and COX-2 (41,42). One such study reported that recombinant human MIF up-regulated COX-2 mRNA, and anti-MIF monoclonal antibody significantly reduced COX activity and mRNA expression (42). It has been shown that MIF inhibits p53 activity, and importantly, that this regulation coincides with the induction of arachidonic acid metabolism and COX-2 expression (43,44). The latter supported our initial hypothesis that inhibition of MIF-induced COX-2 activity by aspirin/NSAIDs might contribute to the protective role of these drugs in the development of ESCC. However, our further experiment indicates that aspirin/NSAIDs modulate MIF expression by a COX-independent mechanism, because addition of prostaglandin E2 in the culture medium did not reverse the inhibitory effect of aspirin/NSAIDs.

Further studies are required to determine the exact mechanisms by which aspirin/NSAIDs inhibit bile acid-induced MIF expression. It has been demonstrated that bile acids activate protein kinase K and the transcription factors AP-1 and NF-kB, induce IL-8 expression and up-regulate some oncoproteins such as c-myc in esophageal adenocarcinoma cells (45–48). Therefore, it would be helpful to determine whether bile acids exert similar effects in esophageal squamous carcinoma cells and whether aspirin/NSAIDs inhibit bile acid-induced MIF expression by antagonizing one or more of these pathways. In addition, it is generally accepted that cigarette smoking and alcohol intake are closely associated with the development of ESCC, an association that may account for 90% of ESCC cases in the developed world (49). There is evidence that one of the molecular targets for cigarette smoking is the p53 gene, and damage to this gene is known to depend on the number of cigarettes smoked per day (50). It is possible that cigarette smoking exerts its effect on p53 through an MIF-mediated pathway, but this hypothesis needs to be tested further.

In conclusion, MIF expression is increased in ESCC. Whereas bile acids induce MIF expression in esophageal squamous carcinoma cells, aspirin and NS398, a COX-2 inhibitor, significantly inhibit MIF expression even in the presence of bile acids, in a COX-independent mechanism.

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References


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