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TGF-β1 mediates the effects of aspirin on colonic tumor cell proliferation and apoptosis

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Abstract. Previous studies have demonstrated that aspirin serves an important role in chemoprevention and the suppression of colorectal cancer (CRC); however, the underlying mechanisms for this inhibition by aspirin remain unclear. Aspirin is capable of promoting apoptosis through prostaglandin-dependent orprostaglandin-independent signaling pathways. In the prostaglandin-dependent pathways, inhibition of cyclooxygenase (COX), particularly COX-2, is the primary mechanism known to be involved in aspirin-induced CRC suppression. Previous studies have implicated prostaglandin-independent signaling pathways and certain associated proteins, including SOX7, in aspirin-induced CRC suppression. In the present study, a newly-characterized association between aspirin, transforming growth factor (TGF)-β1 and CRC inhibition was identified. Specifically, aspirin triggers CRC cell apoptosis by inducing the secretion of TGF-β1, and the increased TGF-β1 then leads to apoptosis and proliferation inhibition in CRC cells.

Introduction

Aspirin, also known as acetylsalicylic acid, is a >100-year-old drug widely used due to its analgesic, antipyretic and anti-inflammatory properties (1). Aspirin is capable of inhibiting the cyclooxygenase (COX) activity of the enzyme prostaglandin G/H-synthase and blocking the biosynthesis of prostaglandins (2). The recognition that oral administration of aspirin may reduce the risk of certain types of cancer makes aspirin a promising candidate for tumor therapy. The high tumor-suppressive efficacy of aspirin treatment observed in colorectal cancer (CRC) has increased the number of studies investigating aspirin-induced CRC suppression.

The pathogenesis of CRC is closely associated with local inflammation in the intestine. Various inflammatory cells or cytokines are responsible for the carcinogenesis of colorectal cells (3). Among them, prostaglandin is well-known for its long history of promoting survival, proliferation and invasion in cancer cells (4). Therefore, the suppression of the production of prostaglandin by inhibiting the activity of COX-2 with non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin becomes a promising choice for CRC suppression. Compared with other types of malignant tumors, CRC is relatively sensitive to aspirin treatment, as revealed by several independent clinical trials (5-7). A number of studies have also demonstrated that COX-2 inhibition is an important strategy for the chemopreventive treatment of colon-associated disorders (8-11), resulting in a lower risk of cancer. The inhibition of COX-2-dependent signaling pathways is one mechanism involved in the good tumor-suppressive efficacy of aspirin. COX-2-independent pathways are also of importance; high concentrations of aspirin may induce apoptosis via 15-lipoxygenase-1 in human HT-29 colonic carcinoma cells (12). Sex-determining region Y-box 7 (SOX7) is upregulated by aspirin and is involved in the aspirin-mediated growth inhibition of human SW480 colonic cancer cells (13). The regulation of SOX7 by aspirin is implemented through the p38/MAPK cascade (13). Although a number of novel associations have been identified, the mechanism underlying the inhibition of CRC by aspirin remains unclear.

Transforming growth factor-β1 (TGF-β1) is a versatile cytokine involved in cell growth, differentiation and immune modulation (14-16). TGF-β1 is a member of the TGF-β superfamily and regulates proliferation and apoptosis in epithelial, endothelial, neuronal and hematopoietic cells (17). TGF-β1 is also capable of inducing apoptosis in a number of malignant tumor cells, contributing to tumor suppression (18). In the present study, the associations between aspirin, TGF-β1 expression and CRC inhibitionwere examined, and a novel aspect of how aspirin increases CRC inhibition and apoptosis was identified.

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Materials and methods

Cell culture and treatment. The CT26 mouse coloncarcinoma cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained at 37˚C in a humidified condition of 95% air and 5% CO_2. Cells were cultured in 75-cm^2 flasks or 6-well plates with RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 U/ml streptomycin. Prior to the addition of aspirin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) or LY364947 (Sigma-Aldrich; Merck KGaA), which is an inhibitor of TGF-β receptor (R)-I, cells were allowed to adhere to 6- or 96-well plates for 24 h. For the TGF-β1 treatment experiment, two groups were used: i) 100 ng/ml TGF-β1 (BioLegend, Inc., San Diego, CA, USA); ii) an equal amount of PBS for the control group. For the aspirin treatment group, cells were divided into three groups: i) 3.5 μM aspirin; ii) combination of 3.5 μM aspirin and 1 μMLY364947 (LY364947 was added and after 2 h the culture medium was replaced with an equal volume of RPMI-1640 medium containing 3.5 μM aspirin); iii) an equal amount of dimethyl sulfoxide (DMSO) as the control group. Each group was treated for 24, 36 and 48 h prior to harvesting for additional study.

Cell viability assay. Cell viability was assessed using a MTT Cell Proliferation and Cytotoxicity Assaykit (Beyotime Institute of Biotechnology, Haimen, China). Cells ($1x10^4$) were seeded on 96-well plates and cultured for 24, 36 and 48 h, followed by the addition of MTT solution to the cells for 4 h. Subsequent to the removal of the medium, the remaining MTT formazan crystals were solubilized in DMSO and analyzed at 560 nm using a microplate reader (Benchmark Electronics, Inc., Angleton, TX, USA).

ELISA. CT26 tumor cells were collected and then homogenized in radioimmunoprecipitation assay buffer (0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 150 mM NaCl and 50 mM Tris-HCl), followed by centrifugation at 17,000 x g for 30 min at 4˚C. Adiethylaminobenzaldehyde assay was used to determine the protein concentration of the samples. The prepared samples were stored at -80˚C until used. The levels of TGF-β1 in each sample were assessed using mouse ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA), according to the manufacturer's protocol, and the colorimetric reaction was measured at 450 nm using a microplate reader (Benchmark Electronics, Inc.).

Flow cytometry analysis of apoptosis. CT26 cells were treated, as aforementioned, then harvested and washed in cold PBS prior to staining using an Apoptosis Detection kit (BD Biosciences, San Jose, CA, USA), according to the manufacturer's protocol. Briefly, cells were resuspended to a concentration of $1x10^5$ cells/sample and double-stained with fluorescein isothiocyanate-conjugated Annexin V and propidium iodide. The samples were analyzed by flow cytometry, as previously described (19) (FACSAria™ SORP; BD Biosciences, Erembodegem, Belgium).

Western blot analysis. The cells were harvested, lysed and the total protein was quantified using a Micro Bicinchoninic Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.) according to the protocol of the manufacturer. Total protein (10 μg) from each sample was separated by electrophoresis using 12% SDS-PAGE, transferred onto polyvinylidene fluoride membranes, pre-blocked with 5% skim milk (MerckKGaA) for 90 min at room temperature and then incubated using the primary antibodies (1:1,000), including mouse-anti-β-actin (cat. no., MAB8929), rabbit-anti-B-celllymphoma2 (Bcl-2; cat. no., AF810), rabbit-anti-Bcl-2-associated X protein (Bax; cat. no., AF820), rabbit-anti-Caspase3 (cat. no., AF-605-NA; R&D Systems, Inc.), rabbit-anti-Caspase8 (cat. no., 9429; Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4˚C. The corresponding secondary antibodies [anti-mouse Ig(H+L); cat. no., 0216], [anti-rabbit Ig(H+L) cat. no., A0208; dilution, 1:10,000; Beyotime Institute of Biotechnology, Haimen, China] were applied for 1 h at room temperature β-catenin was used as a loading control. Signals were developed on X-ray films following exposure to electrochemiluminescence advanced luminescence and Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) was used to test the iodine value of the blots.

Statistical analysis. Data are expressed as the mean ± standard deviation. Statistical analyses were performed using SPSS (version 16.0; SPSS Inc., Chicago, IL, USA). Inter-group statistical significance was determined using a Student's unpaired t-test. $P<0.05$ was considered to indicate a statistically significant difference. $^{*}P<0.05$, $^{**}P<0.03$, $^{***}P<0.01$, $^{****}P<0.001$.

Results

Aspirin induces the secretion of TGF-β1 by CT26 cells. The levelsof TGF-β1 present in the supernatant fluid of CT26 cells seeded in 6-well plates were determined using an ELISA following aspirin treatment, and the amount of secreted TGF-β1 by a certain number of CT26 cells was calculated: Firstly, the amount of secreted TGF-β1 in $1x10^5$ CT26 cells was tested in each case. Secondly, the number of cells was counted. Then, the average amount of TGF-β1 secreted by $1x10^5$ CT26 cells in each case was calculated using the following formula: Total amount of TGF-β1 in each case/the number of CT26 cells in this case) $x10^5$. The results demonstrated that the levels of TGF-β1 vary based on the duration of treatment. Treatment with aspirin significantly increased TGF-β1 secretion compared with the control ($P=0.0159$ and $P=0.0203$ at 36 and 48 h, respectively; Fig. 1). Treatment of CT26 cells with aspirin for longer time points resulted in increased TGF-β1 secretion. While, one problem should be noted. The secretion of TGF-β1 by CT26 cells is a continuous process and the present study calculated the number of cells following collection of the supernatant fluid of the CT26 cells. The number of cells in control group increased subsequent to culturing for 24, 36 or 48 h, as cellular proliferation had continued, while in the aspirin treatment group, the number of cells increased by a small level, or even decrease, as aspirin inhibits growth of cells (20). Despite this, a statistically significant increase in secreted TGF-β1 in aspirin group was observed.

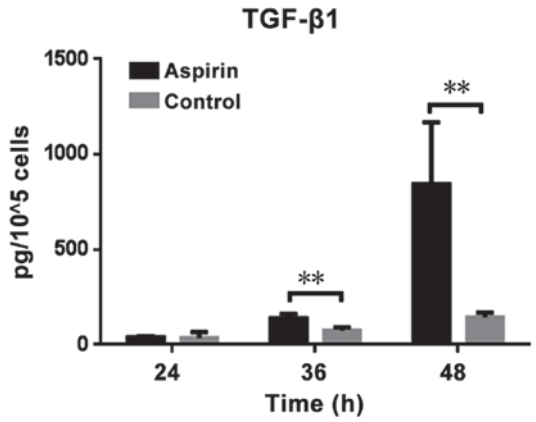

Figure 1. CT26 cells secrete significantly increased levels of TGF-β1 following treatment with aspirin. ELISA was used to determine the levels of TGF-β1 in the supernatant fluid of CT26 cells following treatment with 3.5 μM aspirin for 24,36 and 48 h. **P<0.03. TGF-β1, transforming growth factor b1.

TGF-β1 induces the apoptosis of CT26 cells. An MTT assay demonstrated that TGF-β1 significantly decreases the viability of CT26 cells (P<0.05; Fig. 2A), and induces early and late apoptosis (Fig. 2B and C). Apoptosis serves a key role in cellular development and is a stage-dependent process, including early, intermediate and late-stage apoptotic events. Cells may remain viable if stimulation by aspirin is stopped during early apoptotic events, whereas for late apoptosis defragmentation of DNA is typically observed and this will lead to cell death even if stimulation is halted.

TGF-β1 mediates the effects of aspirin on the viability of CT26 cells. In the present study, an MTT assay was used to examine the effect of aspirin on CT26 cells. The concentration of aspirin used was based on results from a previous study from our group (Wang *et al*, unpublished). A concentration >10 μM was excessive, while no apoptosis or proliferation of CT26 cells was observed when <3 μM aspirin was used(data not shown). Data from the present study demonstrated that treatment with 3 and 3.5 μM aspirin led to a significant decrease in cell viability following 24, 48 and 72 h of treatment compared with the control group (P<0.03, P<0.03 and P<0.01, respectively; Fig. 3A). However, co-treatment witha TGF-βR1 inhibitor, LY364947, resulted in a significant increase incell viability following 48 and 72 h of treatment compared with cells treated with aspirin alone (P<0.05), suggesting that the inhibition caused by aspirin was mediated by TGF-β1. In addition, co-treatment with aspirin and LY364947 significantly decreased the percentage of early and late apoptotic events stimulated by aspirin treatment alone following treatment for 36 h (late apoptotic events) and 48 h (early and late apoptotic events) (P<0.05; Fig. 3B).

The expression of a number of apoptosis-associated proteins was analyzed by western blot analysis following treatment with 3.5 μM aspirin and/or 1 μM LY364947. Changes in the expression of caspase 8 P43, caspase 8 P18, caspase 3, Bcl-2 and Bax following treatment with aspirin (data not shown) were significantly rescued when cells were co-treated with aspirin and LY364947 at 24, 36 and 48 h following treatment (Fig. 3C).

Discussion

In the present study, it was demonstrated that aspirin induced the secretion of TGF-β1 by CT26 cells, and that TGF-β1 in turn led to a decrease in viability and increase in the apoptotic rate CT26 cells. To the best of our knowledge, this is a novel mechanism by which aspirin affects CRC suppression. TGF-β1 is one of the mediators of aspirin-induced apoptosis and, through TGF-β1, aspirin treatment resulted in the downregulation of certain proteins known to be involved in apoptosis, including Bcl-2, caspase3 and caspase8 fragments. It has previously been implicated that the downregulation of Bcl-2 family members (21,22), upregulation of the pro-apoptotic factor Bax (23) and the activation of caspase proteases (24) are involved in TGF-β1-induced apoptosis. Bcl-2 and Bax are well known for their roles in apoptosis. Apoptotic caspase proteases are classified into initiators and effectors, according to their point of entry into the apoptotic cascade. Initiator caspases, such as caspase-8, are the first to be activated in a particular cell-death pathway, and they activate effector caspases, such as caspase-3, via the cleavage of linker segments (25,26). Once activated, caspases cleave a variety of important structural proteins, enzymes and regulatory molecules (27), which leads to DNA fragmentation (28-30) and subsequentcell apoptosis. The present study also demonstrated an increase in the levels of caspase-8. Aspirin suppresses tumor cell growth indirectly by inducing the secretion of TGF-β1. This is similar to the results of a previous study that suggested that ethanol exposure increased TGF-β1 signaling through the suppression of Bcl-2 and retinoblastoma proteins, which may have led to apoptotic cell death, including β-endorphin neurons in the arcuate nucleus of the hypothalamus (31).

Previously, aspirin has attracted attention due to its potential benefit in the chemoprevention of CRC (32). Several clinical studies also reported that aspirin may prolong the overall survival of patients with CRC as a fore mentioned (5-7). A systematic review concluded that the regular use of aspirin reduced the risk of CRC and the 20-year risk of mortality due to CRC (33). Another study which combined the analyses of four randomized trials designed for the primary or secondary prevention of cardiovascular events, identified that the use of aspirin at dosages of ≥75 mg daily was associated with a 24% reduction in incidence and a 35% reduction in mortality from colon cancer (34). However, the molecular mechanisms by which aspirin inhibits CRC formation and growth have remained unclear; therefore, the utilization of aspirin in CRC therapy remains a disputed issue.

The best known molecular target of aspirin is COX. Aspirin, but not other NSAIDs, may cause irreversible inactivation of COX isozymes through the acetylation of a specific serine moiety (35); yet there is evidence that, in COX negative SW480 colonic cancer cells, aspirin is also able to inhibit the growth of the cells (36). Another study also identified that aspirin may act through COX-independent mechanisms that result in an increased expression of DNA mismatch repair proteins and the subsequent apoptosis in SW480 cells (37). COX-dependent and COX-independent pathways used by aspirin may work cooperatively. For example, it has been suggested that aspirin may sensitize tumor cells to tumor

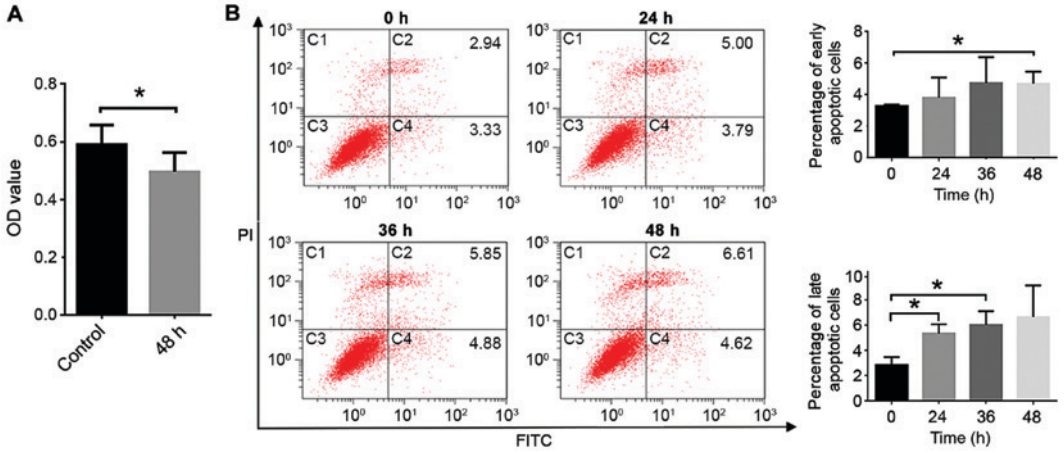

Figure 2. TGF-β1 promotes apoptosis of CT26 cells. (A) MTT assay and (B) flow cytometry results following treatment of CT26 cells with 100 ng/ml TGF-β1 for the indicated time points. *P<0.05. TGF-β1, transforming growth factor b1; OD, optical density; FITC, fluorescein isothiocyanate; PI, propidium iodide.

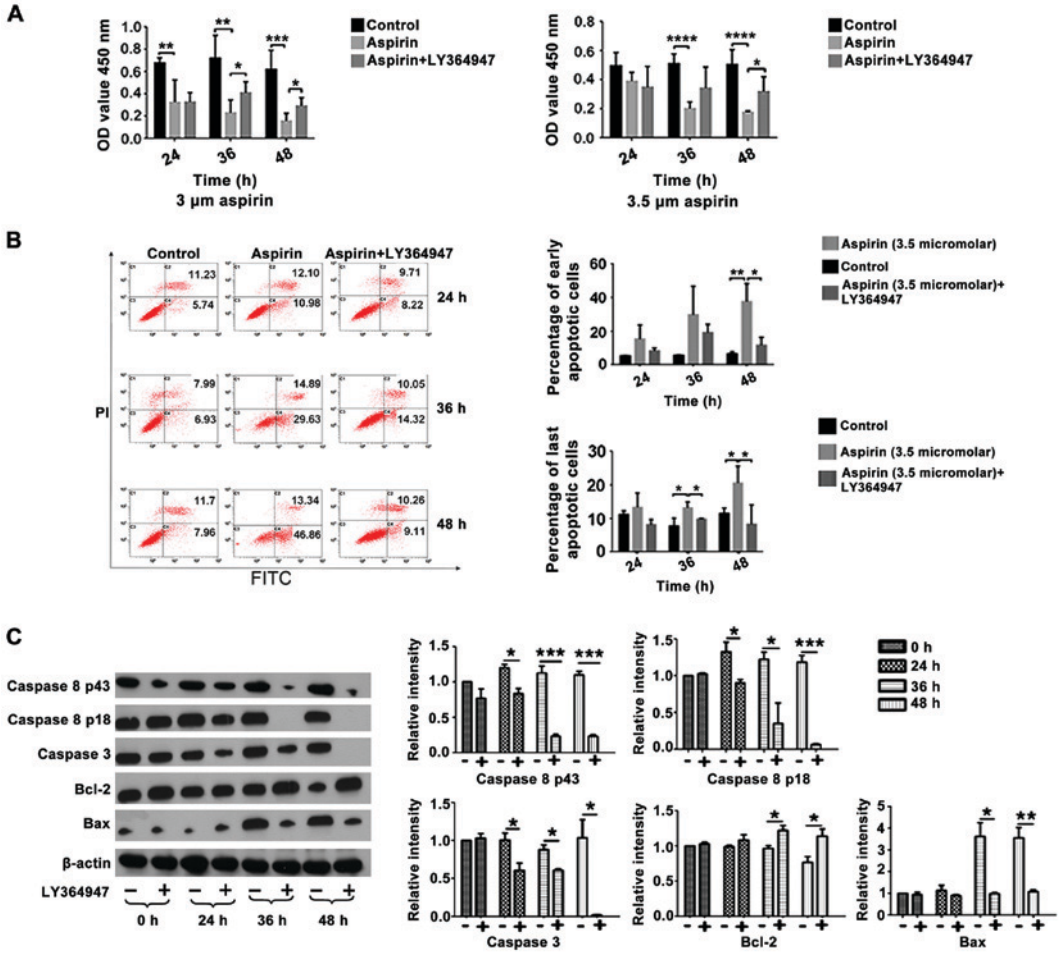

Figure 3. LY364947 partially rescues the effect of aspirin on CT26 cell viability. (A) Viability of CT26 cells following treatment with 3 or 3.5 μM aspirin and/or 1 μM LY364947. (B) Flow cytometry results and percentage of early and late apoptotic cells following treatment with 3.5 μM aspirin and/or 1 μM LY364947 for the indicated time points. (C) Representative western blot analysis and quantification of protein levels of caspase 8, caspase 3, Bcl-2 and Bax following treatment with 3.5 μM aspirin and/or 1 μM LY364947 for the indicated time points. The control group was 0 h, which received no treatment. *P<0.05, **P<0.03, ***P<0.01, ****P<0.001. FITC, fluorescein isothiocyanate; PI, propidium iodide; OD, optical density; Bcl-2, B-cell lymphoma 2; Bax, Bcl-associated X protein.

necrosis factor-related apoptosis-inducing ligand, and may act synergistically with the inhibition of COX-2-dependent prostaglandin formation (38,39). The two pathways work together and result in the apoptosis of tumor cells.

The life span of cancer cells within a living system is significantly affected by the rate of apoptosis (40), which has been recognized as an important physiological event in the pharmacology of anticancer agents (41,42). The regulation

of apoptosis has become an area of extensive analysis in cancer studies (43). Aspirin may affect apoptosis through prostaglandin-dependent or prostaglandin-independent pathways (44). In prostaglandin-dependent pathways, the inhibition of COX, particularly COX-2, is the primary mechan is minvolved in CRC suppression by aspirin (8-11). COX-2 is undetectable in the normal epithelium, but is detectable in >80% of patients with CRC and is associated with larger tumor size and deeper tissue invasion (45). However, not all human colonic cancer cells express COX-2 and produce prostaglandins (46,47). Several studies have demonstrated that high aspirin dosages may also affect apoptosis and cell proliferation in CRC via COX-2 independent pathways (37,48,49). These mechanisms include: i) Wnt/β-catenin pathway inhibition (50); ii) interleukin-6/signal transducer and activator of transcription 3 signaling pathway (51); iii) the downregulation of special protein(Sp)1, Sp3, Sp4 and numerous Sp-regulated gene products (52); iv) the regulation of various targets, including 15-lipoxygenase-1 (53) or the pro-apoptoticproteinprotease-activated receptor 4 (54); v) activation of p38/mitogen-activated protein kinase (MAPK) (55), caspases (56) or the ceramide pathway (57); vi) the release of mitochondrial cytochrome *c* (58); vii) the induction of autophagy in colorectal cancer cells (59); viii) inhibiting the activation of nuclear factor-κB (60), which has been implicated in cellular adhesion (61), and the promotion of metastasis (62).

In addition, aspirin may be administered for cancer treatment due to its indirect function in reducing drug resistance or enhancing the antitumor effects of other drugs (63). Furthermore, aspirin may suppress the pro-invasion and pro-metastasis effects of sorafenib in hepatocellular carcinoma (HCC) through the upregulation of oxidoreductase HTATIP2, which is potentially mediated by the inhibition of COX2 expression, and it may improve the efficacy of sorafenib (63). An additional study revealed that aspirin enhanced doxorubicin-induced apoptosis and reduced tumor growth in human HCC cells *in vitro* and *in vivo* (64).

TGF-β1is a versatile cytokine that is involved in cell-cycle control, the regulation of early development, differentiation, extracellular matrix formation, hematopoiesis, angiogenesis, chemotaxis and immune functions (15). Proliferation of a number of epithelial cell types may be inhibited by TGF-β1, including intestinal epithelial cells, whereas the growth of mesenchymal cells is stimulated (65). TGF-β1 is also an inhibitor of tumor growth that is capable of inducing apoptosis in various types of cancer cells; however, the reaction of diverse malignant cells to TGF-β1 is different: During the process of cancer development, certain transformed cells become partly or completely resistant to TGF-β1 growth inhibition (12), resulting in the uncontrolled proliferation of the cells (52). However, specific cancer cells remain sensitive to TGF-β1, which may lead to proliferation inhibition and the induction of apoptosis in these cells (16). A number of transgenic mouse studies have also provided evidence for the hypothesis that one of the roles of the TGF-β1 signaling pathway is to provide protection against malignant transformation (17,18). Transgenic mice that produce a constitutively active form of TGF-β1 are resistant to DMBA-induced mammary tumor formation (17). Patients with breast cancer and high expression levels of TGF-β1 appear to exhibit longer disease-free intervals and a significantly improved probability of survival (66).

In summary, the present study has suggested a novel pathway for aspirin-induced CRC cell apoptosis. More specifically, aspirin induces CRC cell apoptosis by elevating the secretion of TGF-β1 by those cells, and the increased levels of TGF-β1 in turn lead to apoptosis and proliferation inhibition in the CRC cells. There are certain limitations associated with the present study. Firstly, concentrations of 3 and 3.5 μM aspirin were used to treat the CT26 cells, as lower doses of aspirin were unable to induce apoptosis and proliferation inhibition following treatment for 48 h; this concentration of aspirin induced a secretion of ~1,000 pg TGF-β1 by $1x10^5$CT26 cells. However, 100 ng/ml TGF-β1, a20-fold lower concentration compared with the amount induced by aspirin, was used to examine whether TGF-β1 is capable of inducing apoptosis and proliferation inhibition in CT26 cells. It was not possible to test higher concentrations of TGF-β1 induced by aspirin due to the high cost of commercial TGF-β1 protein. However, if a significantly lower dose of TGF-β1 was able to induce apoptosis and proliferation inhibition in CT26 cells, it is reasonable to predict that higher concentrations would elicita similar effect. Alterations in the expression levels of various apoptotic proteins in CT26 cells following treatment with 3.5 μM aspirin were observed, but these changes did not appear in the cells following treatment with 100 ng/ml TGF-β1. Whether 100 ng/ml TGF-β1 is able to induce apoptosis in CT26 cells without affecting the caspase proteins remains to be determined. Furthermore, caspase-independent pathways, such as the AIF-endonuclease G-Omi/HtrA pathway, may also induce apoptosis in these cells (67). Whether this pathway was involved at the relatively low concentrations of TGF-β1-induced apoptosis in the present study, and whether higher and lower concentrations of TGF-β1may induce apoptosis via alternate pathways remains to be elucidated, and additional studies are required. Secondly, the present study only focused on a small number of apoptotic proteins, including the caspase 8 fragments p43 and p18, caspase3, Bcl-2 and Bax. An association has previously been demonstrated between TGF-β1 and the well-known extracellular signal-regulated kinase (ERK)1/2-MAPK signaling pathway (68). Whether aspirin exhibits any significant effects on this pathway has yet to be determined and additional studies are required.

Acknowledgements

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