Basic Research Paper

Effects of tamoxifen on apoptosis and matrix metalloproteinase-7 expression in estrogen receptor β-positive colorectal cancer cell line HT-29

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Background and Objective: Studies have shown that estrogen receptor β (ERβ), which is highly expressed in colorectal cancer, correlates to tumor metastasis. Matrix metalloproteinase (MMP)-7 is found overexpressed in colorectal cancer, and plays an important role in tumor invasion and metastasis. This study explored the effect of tamoxifen (TAM) on apoptosis and MMP-7 expression of ERβ-positive human colorectal cancer cell line HT-29.

Methods: After treatment by TAM, the proliferation rate of HT-29 cells was determined by MTT assay, cell apoptosis was evaluated using flow cytometry, expressions of ERβ and MMP-7 in HT-29 cells were measured by western blot. Results: TAM significantly inhibited cell growth of HT-29 in a time (24 h, 48 h, 72 h) and dose (0, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} mol/L) dependent manner. TAM treatment caused significant apoptosis in HT-29 cells [(69.9 ± 4.2)%] at a concentration of 10^{-4} mol/L. Moreover, through binding with ERβ, TAM could downregulate MMP-7 protein expression in HT-29 cells. Conclusion: High concentrations of TAM can inhibit the proliferation rate of ERβ-positive HT-29 cells and effectively downregulate MMP7 expression through binding with ERβ.

Colorectal cancer is a common malignant tumor. Dr. Parkin from the World Health Organization (WHO) reported that in 2002 there are 1,023,000 new cases of colorectal cancer in the world, 529,000 of which lead to death, ranking it third and fourth respectively among all cancers. Currently there are 2.8 million colorectal cancer patients worldwide.1 Back in the early 1960s researchers found that the prognosis for cancer patients of different gender differs even when they suffer from the same type of cancer; tumors in sex hormone targeting organs such as the breast often occur in the gastrointestinal tract concurrently. Therefore, scientists believe that endogenous estrogen may affect the natural progress of certain digestive tract tumors. In 1977, McClendon et al. were the first to prove the existence of estrogen receptor (ER) in colorectal cancer tissues.2 In addition, a domestic group also reported the significantly higher ER expression level in colorectal cancer tissues in comparison to normal tissues.3 ER has two subtypes, namely ERα and ERβ.4 Studies have demonstrated the dominant role of ERβ in colorectal cancers.5 Many studies focusing on ER and cancer metastasis also prove the correlation between the two.6

The role of endocrine therapy in the auxiliary treatment of colorectal cancer is still unclear, given its controversial efficacy for treating colorectal cancer. Marugo et al. confirm that estrogen can promote the growth of ER (+) gastrointestinal tumors.7 Therefore, the objective of this study was to explore whether triphenylethylene non-steroidal anti-estrogen drugs, mainly tamoxifen, could inhibit the proliferation and promote the apoptosis of colon cancer cells. Although there are many reports suggesting the involvement of matrix metalloproteinase (MMP) components in invasive metastasis of colorectal cancers,8,9 whether endocrine therapy alters the function of MMP in colon cancers is still not clear. Therefore, another objective of this study was to investigate whether anti-estrogen receptor drugs can modulate the expression of MMP7 in human colon cancer cells, which should provide a theoretical and experimental basis for the clinical treatment of colon cancer liver metastasis.

Materials and Methods

Major compounds and reagents. Tamoxifen (TAM), 3-(4, 5-dimethylthiazol-2-YL)-2, 5-diphenyl tetrazolium bromide
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[MTT], dimethyl sulfoxide (DMSO) and Annexin-v-FITC apoptosis detection kits (including Annexin-v-FITC, conjugate buffer, propidium iodide) were purchased from Sigma. Mouse anti-human MMP7 monoclonal antibody and rabbit anti-human ERβ polyclonal antibody were purchased from Chemicon. Mouse anti-human ERβ monoclonal antibody was purchased from Abcam and hors eradish peroxidase (HRP) labeled goat anti-mouse IgG antibody was purchased from GE Healthcare. ECL chemiluminescence kit was purchased from Cell Signaling and mouse anti-human β-actin monoclonal antibody was purchased from Thermo companies.

Cell culture. Human highly-metastatic colon cancer cell line HT-29 cell was purchased from the Cell Center of Basic Medical Sciences Institute in Chinese Academy of Science and cultured in DMEM: F12 (1:1) medium containing 10% fetal bovine serum (Gibco) in 37°C, 5% CO2 with saturated humidity.

MTT assay to test the inhibitory role of TAM on HT29 cells. Trypsin (0.25%) was used to digest single-layer cultured HT29 cells, and culture medium containing 5% fetal bovine serum was used to make single cell suspension, which was then plated into 96-well plates at 5 x 10^3 cells per well. The volume of each well was 50 μl. After cells were cultured in 37°C, 5% CO2 with saturated humidity for 18 hours, different concentrations of TAM diluted solution were added into each group (0, 1 x 10^-7, 1 x 10^-6, 1 x 10^-5, 1 x 10^-4 mol/L respectively, 50 μl per well). After cells were cultured for 24 hours (group 1–5), 48 hours (group 6–10) and 72 hours (group 10–15), 5 mg/L MTT (10 μl per well) was added and cells were continuously cultured for another four hours, then the supernatant was discarded, 150 μl DMSO was added into each well and shaken for ten minutes to dissolve precipitate. ELISA machine was used to measure the absorbance value (A value) of each well at 490 nm wavelength and the inhibitory rate on cell proliferation was calculated using the following equation: inhibition rate = (1-average A value of experimental group/average A value of control group) x 100%.

Flow cytometry to detect apoptotic cells. 5 x 10^6 HT-29 cells in logarithmic growth phase was plated into 5 x 2.5 cm2 culture flask, cultured for 24 hours, culture medium was discarded, culture medium containing TAM at the concentration of 0, 1 x 10^-7, 1 x 10^-6, 1 x 10^-5 and 1 x 10^-4 mol/L was added, and continued to culture for 24, 48 or 72 hours respectively. Next, trypsin was used to digest and collect all cells, which were centrifuged, washed by PBS twice and re-suspended in 200 μl conjugate buffer. Then 10 μl Annexin V-FITC and 5 μl PI were added, gently mixed and reacted at room temperature in the dark for 15 minutes. Next, another 300 μl conjugate buffer was added and signals were immediately detected on flow cytometer; 1 x 10^4 cells were tested and the light source used was 488 nm Argon laser.

Western blot to test the ERβ and MMP7 protein expression levels in HT29 cells. 5 x 10^6 cells from each group were collected and 80 μl lysis buffer (0.1% SDS, 50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 1 mmol/L EDTA, 100 μg/ml PMSF, 1 μg/ml Aprotinin, 1% NP-40) was added. Cells were centrifuged at 12,000 r/min for five min after 30 min on ice, and total cell proteins were extracted. An equal concentration of proteins was separated by 10% SDS-PAGE electrophoresis and transferred onto nitrocellulose membrane. The membrane was then blocked by TBST (Tween20/TBS) buffer containing 5% nonfat milk at room temperature for three h, and first antibodies (mouse anti-human ERβ monoclonal antibody and mouse anti-human MMP7 monoclonal antibody respectively) were added and incubated at 4°C overnight. After rinsing, another first antibody (mouse anti-human β-actin monoclonal antibody) was added and incubated at room temperature for two hours. After sufficient washing, the membrane was incubated with secondary antibody (HRP labeled goat anti-mouse IgG antibody) for one h, rinsed again and signals were detected with ECL chemiluminescence kit.

Statistical analysis. All quantitative results were shown as mean ± SD, ANOVA was used to determine the significant difference among mean values of multiple samples, and apoptosis percentages were compared by χ2 test. Statistical software SPSS 10.0 was used and p ≤ 0.05 was considered to be statistically significance.

Results

The inhibitory effect of TAM on proliferation of HT-29 cells. After treating HT-29 cells by TAM for 24, 48 and 72 h respectively, results showed that the inhibitory effect of TAM was significantly correlated with drug concentration and treatment duration, with higher inhibitory effects at doses of 1 x 10^-5 and 1 x 10^-4 mol/L than 0.1 x 10^-7 and 1 x 10^-6 mol/L (p < 0.001). The inhibitory effects of TAM treatment for 48 and 72 h were significantly higher than that of 24 h (p < 0.001). No significant difference was detected between 48 h and 72 h treatment group (Fig. 1).

TAM-induced apoptosis in HT-29 cells. This study detected TAM-induced apoptosis in HT-29 colon cancer cells by flow cytometry. The apoptosis rate after 4 h of TAM treatment at the concentrations of 0, 1 x 10^-7, 1 x 10^-6, 1 x 10^-5, 1 x 10^-4 mol/L were 2.7 ± 1.1%, 2.9 ± 1.3%, 2.9 ± 1.2%, 3.1 ± 1.2% and 69.9 ± 4.2% respectively. Sub-G1 peak (apoptosis peak) was obvious after treatment with 1 x 10^-4 mol/L TAM for 48 h, and the apoptosis rate compared to the control group showed significant difference (69.9

Figure 1. Cytotoxicities of TAM on HT29 cells. Values are presented as mean ± SD of three independent experiments. *(A)* p < 0.001 compared to 48 h and 72 h groups.
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Effects of TAM on protein expressions of ERβ and MMP7 in HT-29 cells. Western blot showed that HT-29 cells had marked expression of ERβ and MMP7 proteins, which had molecular weights of 57 kDa and 30 kDa, respectively. After cells were treated with different concentrations of TAM for 24 h, 48 h or 72 h, the protein expression levels of ERβ and MMP7 were significantly reduced, and this inhibitory effect was strongly correlated with drug concentration and treatment duration (Fig. 3).

Discussion

Currently, people name the ER discovered in 1986 as ERα and another ER subtype, identified in 1977, as ERβ. Our results showed that the expression rate of ERβ was 90.4% in 620 cases of colon cancer tissues. In addition, Dr. De-Sen Wan et al.10 report an ERα positive rate of 37% in 73 colorectal cancer patients admitted to our hospital from 1987 to 1993, which supports the hypothesis that ERβ is the main ER subtype expressed in colon cancer. In this study we selected human highly-metastatic colon cancer cell line HT-29, which exclusively expressed ERβ, as our experimental model.11 After treatment with 1 x 10^-4 mol/L TAM for 48 hours, cell growth was significantly inhibited and apoptosis appeared.
Meanwhile, ERβ expression was also significantly reduced in TAM-treated HT29 cells. TAM has dual biological roles given its anti-estrogen effect and weak estrogen activity, while its dominant role is the anti-estrogen effect through binding with estrogen receptors on target cells. The complex will enter the cell nucleus and bind DNA. Although it has certain biological activities similar to estrogen, its competition with estrogen receptors will block the biological effects of intrinsic estrogen.12

Currently, many studies have proven that ERβ plays an important role in tumor growth and metastasis,13,15 but the mechanism remains unclear. A retrospective study by Dr. De-Sen Wan et al.10 shows that the five-year survival rate of patients treated with post-operative TAM and that of untreated patients, is 66.7% and 72.5% respectively, though the difference is not statistically significant. In contrast, the remote metastasis rate in TAM group is much less than that of the untreated group (3.0%: 20.0%, p < 0.05). However, the above study uses ERT-positive as the criteria for patient selection. Therefore, in further clinical studies, we still need to thoroughly explore the relationship between ERβ and endocrine therapy in colorectal cancer, and compare the clinical efficacy of endocrine therapy based on both ER subtypes.

MMP-7 (Matrix metalloproteinase) is the smallest known member of the MMPs family. Initially, the cDNA of MMP-7 is cloned from human cancer cDNA library, and its protein product is first detected by Miyazaki et al.16 in culture medium of human rectal cancer cell line. Because that MMP-7 has strong extracellular matrix (ECM) degradation activity, many studies detect MMP-7 overexpression in metastases of malignant colorectal cancer and colorectal cancer liver metastasis.17 In our experiment, the MMP7 protein expression in HT-29 cells reduced at different degrees after treatment with TAM. Therefore, we believe that MMP-7 may become a new target for endocrine therapy of colon cancer and requires further in-depth study. Research demonstrates that TAM can not only competitively bind to estrogen receptor to form anti-estrogen receptor complex, thereby reducing the vitality of cancer cells and inhibiting tumor growth, but can also induce apoptosis in cancer cells through a variety of non-estrogen receptor-dependent mechanisms such as protein kinase C, calmodulin, transforming growth factor β, c-myc, MAPK, tumor suppressor factor maspin, oxidative stress, mitochondrial membrane permeability transition and ceramide production, etc.18,19

In summary, high concentrations of TAM could inhibit the proliferation rate of ERβ-positive colon cancer cells in vitro, and effectively reduce the expression of MMP7 protein, which is involved in cell invasion and metastasis. Thereby we could conclude that the anti-estrogen endocrine therapy for colon cancer not only inhibited tumor growth, but also precipitated in suppressing tumor metastasis. In order to further support the above conclusion, animal experiments and prospective multicenter randomized controlled trials still need to be performed.

References