ORIGINAL ARTICLE

Expression of CD40/CD40L in colon cancer, and its effect on proliferation and apoptosis of SW48 colon cancer cells

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Summary

Purpose: To study was to study the expression of CD40/CD40L in colon cancer and investigate the effects of CD40/CD40L overexpression on the proliferation and apoptosis of SW48 colon cancer cell line.

Methods: Immunohistochemistry was used to detect the expression of CD40 protein in colon cancer tissue samples from 70 patients, and 10 adjacent normal tissue samples. A CD40L gene-containing plasmid was transfected into SW48 colon cancer cells using lipofectamine 2000. Cell proliferation was measured by MTT assay. The expression of CD40/CD40L was measured by real-time PCR (RT-PCR). Protein expression of Bcl-2 and Bax was detected by Western blot.

Results: Immunohistochemical analysis showed that CD40 was mainly expressed in the cell membrane and scarcely in the cytoplasm. The expression of CD40 in colorectal cancer tissue was significantly higher than in normal adjacent tissue. RT-PCR showed that CD40 was highly expressed in SW48 cells. The expression of CD40L mRNA was significantly increased in SW40 cells transfected with the CD40L gene-containing plasmid (p<0.01). MTT assay showed that the activity of CD40L transfected cells was significantly inhibited compared with control cells transfected with empty plasmid (p<0.01). Western blot analysis demonstrated significantly decreased Bcl-2 expression, and significantly increased Bax expression in cells transfected with the CD40L gene-containing plasmid compared with the control cells (p<0.01).

Conclusion: In conclusion, CD40 protein expression was significantly higher in colon cancer tissue compared with normal tissue, and the apoptosis of SW48 colon cancer cells can be induced by CD40L gene transfection.

Key words: apoptosis, CD40/CD40L, colon cancer cell SW48, proliferation

Introduction

Colon cancer is a common malignant tumor of the gastrointestinal tract, with rates of morbidity and mortality that are increasing annually. At present, the rate of morbidity of colon cancer ranks third, and the rate of mortality ranks second among all malignant tumors globally [1]. The mortality of colon cancer in China ranks fifth among all malignant tumors, indicating that it severely threatens human health [2]. Surgical resection and adjuvant chemotherapy are two primary methods of treatment for colon cancer [3]. However, surgical resection cannot remove metastasized cancer cells, and adjuvant chemotherapy causes toxicity and side effects, with an insignificant curing effect. With developments of molecular biology, specific molecular targeted therapy has gradually emerged as a new method of tumor treatment [4,5].

As immune co-stimulatory molecules, CD40

and its ligand, CD40L, can complement each other. Previous studies have shown that CD40 and CD40L play pivotal roles in humoral and cellular immunity, and the expression of CD40 and CD40L are closely related to the occurrence and development of various diseases [6]. CD40 was found to be highly expressed in bladder cancer, breast cancer, ovarian cancer, and other tumors [7-9]. CD40L, as the primary ligand of CD40, is mainly expressed on the surface of activated CD4+ T cells. When CD40 binds CD40L, CD40L can activate T lymphocytes and the Fas-mediated apoptotic pathway in tumor cells, thereby enhancing the apoptosis of tumor cells [10]. At present, the effect of CD40/CD40L on tumors has become an area of intense research in the field of tumor pathogenesis.

In this study, immunohistochemistry was used to detect the expression of CD40 in colon cancer tissue. The expression of the CD40 gene in SW48 cells was measured by RT-PCR. To study the function of CD40L, a CD40L gene-containing plasmid was transfected into SW48 cells. The effects of the CD40L gene on the proliferation and apoptosis of SW48 colon cancer cells, and the possible mechanism of apoptosis were analyzed. We believe that our study provides a foundation for targeting CD40/CD40L in the clinical treatment of colon cancer.

Methods

Reagents

SW48 colon cancer cells (The Cell Bank of the Chinese Academy of Sciences, Shanghai, China); MTT (Sigma, Saint Louis, MO, USA); Bax, Bcl-2, and GAPDH mouse anti-human primary antibodies, HRP-labeled rabbit anti-mouse secondary antibody (Wuhan Mitaka Biotechnology Co., Ltd., Wuhan, China); DMEM (Gibco, Carlsbad, CA, USA); Trizol kit, reverse transcription kit, RT-PCR kit, Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA); primers, empty plasmid, design and synthesis of CD40L gene-containing recombinant plasmid (TaKaRa, Dalian, China); Mouse anti-human CD40 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA); Immunohistochemical staining kit SP-9001 (Beijing Zhong Shan-Golden Bridge Biological Technology CO.,LTD., Beijing, China).

Patients

From January 2010 to December 2012, 70 patients with colon cancer were selected. A total of 70 colon cancer tissue samples and 10 normal adjacent tissue samples were collected for preparation of paraffin sections. All 70 patients were surgically treated for the first time, and were never treated with chemotherapy. Pathological analysis of tumor tissue samples was performed for diagnosis of colon cancer. Among the 70 patients, there were 36 males and 34 females, with age ranging from 25-78 years (median 45). Sample collection in this study was approved by clinical ethics committee of the First Affiliated Hospital of Soochow University. All patients and their families signed informed consent.

Immunohistochemistry

Paraffin sections were collected and stained using an immunohistochemistry kit (ReyGen, Nanjing, China) Paraffin sections of tumor tissue were deparaffinized, and endogenous peroxidase was inactivated with 3% H_2O_2. Antigen retrieval was then performed, and the sections were blocked with serum. Primary antibody was added followed by incubation at 4°C overnight. After three washes with phosphate buffered saline (PBS) , secondary antibody was added for incubation for 30 min. The slides were washed three times with PBS before the color development step with diaminobenzidine (DAB) The slides were then sealed with gum, and observed under a microscope (TE2000-U, Nikon, Japan) for acquisition of pictures.

Brown particles in the cell membrane or cytoplasm represented a positive signal for CD40 protein staining. The results were classified according to the following scoring criteria: no positive staining or less than 5% stained cells (negative); less than 50% positively stained cells (weak positive); more than 50% positively stained cells (strong positive). The data were statistically analyzed.

Cell culture

SW48 colorectal cancer cells and 786-0 renal cancer cells were cultured in an incubator with Dulbecco's modified eagle medium (DMEM), containing 10% fetal bovine serum at 37°C with 5% CO_2. The medium was changed every 24 hrs until cells reached 80-85% confluence. Once reached 90% confluence, the cells were subcultured following digestion.

RT-PCR

786-0 renal cancer cells were selected as CD40-positive cells. SW48 and 780-0 cells were cultured as described above. After 48 hrs, the cells were collected, and total RNA was extracted with Trizol kit. Only RNA samples with a ratio of absorbance value of A260/A280 between 1.8 and 2.0 were selected for reverse transcription. The expression of CD40 mRNA was measured with an RT-PCR kit, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control. The reaction conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. The primers used are presented in Table 1.

Detection of the effects of CD40L gene transfection on the expression of CD40L mRNA in SW48 cells by RT-PCR

Cells were cultured as described above and trypsinized. After dilution, cell suspensions were seeded in 6-well plates with 10^4 cells per well. A CD40L recombinant plasmid and blank plasmid were transfected into cells in the logarithmic growth phase, according to the manufacturer's instructions. Three groups of cells including the control group (no transfection), empty plas-

Table 1. Primers for RT-PCR

Gene	Primer	Primer sequence
CD40L	Forward	5'-GACGTCAGCATGATAGAAACATACAGCCAACCT-3'
	Reverse	5'-GCCGAATTCTCAGAGTrrGAGTAAGCcAAAAGA-3'
CD40	Forward	5'-CCTTGCGGT-GAAAGCGAA-3'
	Reverse	5'-GCTGGAC-CCGAAGCCCTA-3'
GAPDH	Forward	5'-GCACCGTCAAGGCTGAGAAC-3'
	Reverse	5'-TGGTGAAGACGCCAGTGGA-3'

mid group (transfected with empty plasmid), and experimental group (transfected with CD40L recombinant plasmid) were analyzed. After 48 hrs, cells were harvested and the expression of CD40L mRNA in the different groups of SW48 cells was measured as described above.

MTT assay

Cell grouping and treatments were the same as described above. After incubation for 48 hrs, cells were washed with PBS, 100 µl of MTT working solution (5 mg/ml) was added to each well, and samples were incubated for 4 hrs. Next, 100 µl of DMSO were added to each well, and the plates were shaken in the dark for 10 min. The absorbance at 570 nm (optical density/OD) was measured using a microplate reader. The relative survival rate was calculated according to the following formula: survival rate (%) = (OD value of experimental group/OD value of control group) × 100%.

Western blot

Cell grouping and treatments were as described above. After incubation for 48 hrs, cells were harvested. Protein was extracted, and protein concentration was determined. Next, 50 µg of protein samples were separated by sodium salt polyacrylamide gel electrophoresis (SDS-PAGE). Protein was transferred to PVDF membranes. The blocking step was performed at room temperature for 1 hr. The primary antibody (1:1000) was added followed by incubation at 4°C overnight. After washing with tris-buffered saline (TTBS), secondary antibody (1:2000) was added followed by incubation at room temperature for 1 hr. After washing with TTBS, color development was performed and pictures were taken.

Statistics

Data are presented as mean±standard deviation. SPSS 17.0 (International Business Machines Corporation, New York, USA) was used for analyses. All data were analyzed using one way ANOVA and $p<0.05$ was considered statistically significant.

Results

Immunohistochemical detection of CD40 in patient tissue samples

As shown in Figure 1, CD40-positive staining was mainly localized in the cell membrane and scarcely in the cytoplasm. Staining was almost absent in the adjacent normal tissue. However, CD40 was found to be highly expressed in colon cancer tissue. CD40 protein was highly expressed in colorectal cancer tissue in 36 (48.5%) of the 70 patients, while low expression or no expression of CD40 was observed in normal colon tissue.
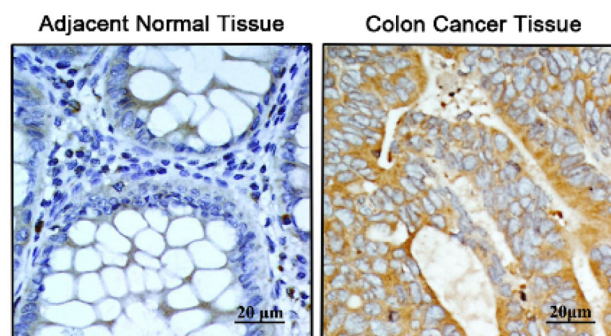
Figure 1. Immunohistochemical detection of CD40 protein in patient tissue samples. CD40 was mainly expressed in colon cancer tissue, and scarcely in adjacent tissues.

The expression of CD40 mRNA in SW48 colon cancer cells

RT-PCR analysis showed that CD40 expression was detected in SW48 colon cancer cells. However, there was no significant difference in the expression of CD40 between SW48 colon cancer cells and 786-0 cells ($p>0.05$) (Figure 2).
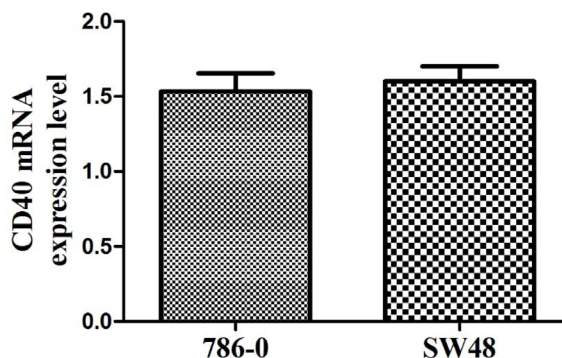

Figure 2. The expression of CD40 mRNA in SW48 colon cancer cells. The expression of CD40 was measured in SW48 colon cancer cells ($p>0.05$).

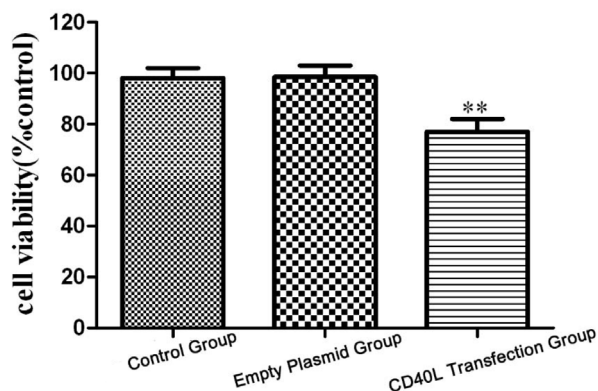*The effect of CD40L gene transfection on CD40L mRNA level in SW48 colon cancer cells*

The level of CD40L mRNA in cells transfected with the CD40L gene was significantly higher than in the control and empty plasmid groups (p<0.01), indicating that the CD40L gene was successfully transfected in SW48 cells. However, there was no significant difference in the level of CD40L mRNA between the empty plasmid group and control group (p>0.05) (Figure 3).
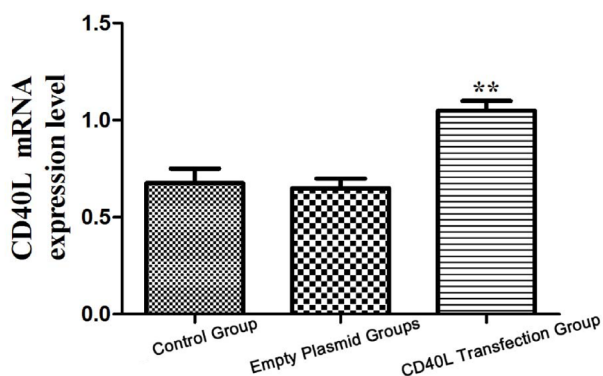
Figure 3. The effect of CD40L gene transfection on the level of CD40L mRNA in SW48 colon cancer cells. The level of CD40L mRNA in cells transfected with the CD40L gene was significantly higher than in the control and empty plasmid groups. **p<0.01.

*The effect of CD40L gene transfection on the proliferation of SW48 cells*

Compared with the control and empty plasmid groups, the cell proliferation rate of the experimental group was significantly reduced (p<0.01), indicating that the CD40/CD40L signaling pathway was activated by CD40L gene transfection, which in turn inhibited cell proliferation (Figure 4).

Figure 4. The effect of CD40L gene transfection on the proliferation of SW48 cells. Compared with the control and empty plasmid groups, the cell proliferation rate of the experimental group was significantly reduced. **p<0.01.

The effects of CD40L gene expression on Bax and Bcl-2 protein levels

After 48 hrs of incubation, the expression of Bax protein was significantly increased, and the expression of Bcl-2 protein was significantly decreased (p<0.01) in the experimental group compared with the control and empty plasmid groups (Figure 5). These results indicated that CD40L gene expression resulted in activation of CD40/CD40L signaling, which in turn promoted the expression of Bax, and inhibited the expression of Bcl-2.
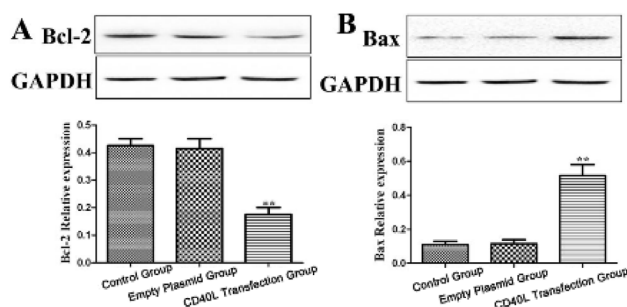
Figure 5. The effects of CD40L gene expression on Bax and Bcl-2 protein levels measured by Western blot. **A:** The protein expression of Bcl-2. **B:** The protein expression of Bax. Compared with the control group, CD40L gene expression can promote the expression of Bax and inhibit the expression of Bcl-2.**p<0.01.

Discussion

In the specific immune response system of human body, CD40 and its ligand, CD40L, are members of the TNF receptor and TNF superfamily, respectively. They are a pair of co-stimulatory molecules that play an important role in the specific *in vivo* immune response system. The binding of CD40 to CD40L can activate T and B cells, which can participate in cellular immune and humoral immune processes [11,12]. A previous study has shown that CD40 and CD40L play important roles in various diseases such as autoimmune diseases and inflammatory responses [13]. In recent years, increasing attention has been paid to the function of CD40/CD40L in the occurrence, progression and treatment of tumors [14].

The expression of CD40 can be detected in the different developmental stages of B lymphocytes, T lymphocytes, and mononuclear macrophages. CD40 not only plays important roles in B cell antibody secretion, proliferation, and differentiation, but also participates in changes of endothelial cell adhesion molecules and the process of inflammatory factor release [15]. CD40 was found to be highly expressed in tumor cells in acute and

chronic lymphocytic leukemia, lung cancer, and liver cancer, and its expression plays a key role in the occurrence, development and metastasis of tumors [16]. The high expression of CD40 in tumor cells can activate the CD40 signaling pathway, leading to apoptosis of tumor cells [17,18]. The Bcl-2 gene plays an important role in the mechanism of apoptosis. Bcl-2 inhibits apoptosis [19], and can protect cells from various forms of death, and improve cell survival, which in turn increases cell number. The increased expression of the Bcl-2 gene in some tumor cells can protect tumor cells from death, or extend the life of cells [20], indicating that the Bcl-2 gene is closely related to tumors. Bax can promote apoptosis [21]. Bax and Bcl-2 belong to the same gene family, but the function of Bax is opposite to that of Bcl-2. Bax can inhibit apoptosis induced by Bcl-2, and has direct effects on promoting apoptosis.

In this study, we investigated the effect of CD40/CD40L on the proliferation and apoptosis of SW48 cells by gene transfection. Immunohistochemical analysis showed that the positive staining of CD40 was mainly localized in the cell membrane and scarcely in the cytoplasm. The positive staining of CD40 was almost absent in the adjacent normal tissue, while there was high expression of CD40 in colon cancer tissue. RT-PCR analysis showed that the CD40 gene was highly expressed in SW48 cells. The expression of CD40L mRNA was significantly increased after CD40L gene transfection. The results of MTT assay showed that CD40L gene transfection significantly inhibited the proliferation rate of CD40L cells compared with the control and empty plasmid groups, indicating that CD40L gene transfection activated the CD40/CD40L signaling pathway, which in turn inhibited cell proliferation. The inhibitory effects of the activated CD40/CD40L signaling pathway have been supported by previous studies. A previous study reported that CD40 activation in epithelial tumor cells can significantly inhibit cell pro-

liferation, and transfection of the CD40L gene in HeLa cervical cancer cells can significantly inhibit cell growth [22]. CD40L showed no significant inhibitory effect on CD40-negative breast cancer [22] cells, indicating that this effect of CD40L is dependent on CD40 [23]. Western blot analysis showed that Bcl-2 expression was downregulated, and Bax expression was upregulated after CD40L gene transfection. A previous study showed that reduced expression of Bcl-2 can induce tumor cell apoptosis [23,24]. Another study [25] showed that increased Bax protein expression can lead to decreased Bcl-2 expression in gastrointestinal epithelial tumor cells, and the changes may be closely related to the formation of digestive tract tumors. Consistent with previous studies, our results showed that activation of the CD40/CD40L signaling pathway can induce apoptosis by upregulating the expression of Bax protein, and downregulating the expression of Bcl-2 protein. Similar findings from both previous studies and our study provide a basis for our future studies.

In conclusion, the present study showed that CD40 was mainly expressed in the cell membrane and scarcely in the cytoplasm. CD40 was also found to be highly expressed in colon cancer tissue, but was not expressed in normal adjacent tissue. In addition, CD40/CD40L signaling can inhibit the proliferation of SW48 cells and induce SW48 cell apoptosis possibly by upregulating the expression of Bax protein and downregulating the expression of Bcl-2 protein.

Acknowledgments

Conflict of interests

The authors declare no conflict of interests.

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