RESEARCH ARTICLE

Role of TGF-β1 in Human Colorectal Cancer and Effects after Cantharidinate Intervention

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Abstract

　　Effects of transforming growth factor-beta (TGF-β) were investigated in human colorectal cancer, and the influence of cantharidinate in inhibiting TGF-β1 expression was explored. Relationships among TGF-β1 and sex, age, tumor size, tumor location, tumor stage were also analyzed. H&E and immunohistochemistry staining were employed to assess colorectal cancer and TGF-β1 expression, respectively. Then, HCT-116 CRC cells were randomly divided into four groups, controls, no serum-treated, chemotherapy and cantharidinate-treated. Immunohistochemistry and real-time PCR were employed to assess the expression of TGF-β1 in CRC cells. Our data showed that the expression of TGF-β1 might be associated with tumor size and tumor location ($P<0.05$). The expression of TGF-β1 in CRC groups was higher than in adjacent groups (P<0.05). In addition, the expression of TGF-β1 in cantharidinate-treated group was much lower than in CRC group (P<0.05). Taken together, these results suggest that TGF-β1 plays an important role in CRC development. Cantharidinate might inhibit the expression of TGF-β1 and control the development of colorectal cancer.

Keywords: Colorectal cancer - TGF-β1 - cantharidinate

Introduction

　　Colorectal cancer (CRC) is a kind of frequently-occurring cancer that starts in either the colon or the rectum. CRC and rectal cancer have many features in common (Jemal et al., 2011). At present, the etiology of CRC is poorly understood. Recent studies have found that modifiable lifestyle factors, such as smoking, alcohol drinking, physical activity, obesity, and diet, are associated with CRC risk, but each seems to explain only a small proportion of the total CRC incidence (Huxley et al., 2009). Untill now, the exact pathogeny and cure of CRC are still unclear. Transforming growth factor beta 1 (TGF-β1) is a polypeptide member of the transforming growth factor beta superfamily of cytokines (Mimeault et al., 2010). It is a secreted protein that performs many cellular functions, including the control of cell growth, cell proliferation, cell differentiation and apoptosis (Aihara et al., 2010). In addition, TGF-β1 plays an important role in controlling the immune system, and shows different activities on different types of cell, or cells at different developmental stages. In the past, fluorouracil is one of the standard chemotherapeutic drugs in the treatment of cancer (van Hazel et al., 2009). Proper use of these therapies can have a major impact on patients' prognoses (Dehmer et al., 2010). Recent studies have shown that emerging data in treat cancer on cantharidinate has influenced some

cancer setting with regard to the identification of specific molecular markers (Liu et al., 2007). However, whether cantharidinate can be used to inhibit the expression of TGF-β1 in CRC? In the present study, we investigated the expression of TGF-β1 in CRC, and investigate the effect of cantharidinate in inhibiting TGF-β1 expression.

Materials and Methods

Tissues and cells

　　This study was approved by the Jilin University. Adjacent normal (N) and tumor (T) tissues were collected from patients with cancer. Tissues were quick frozen in liquid nitrogen till further use. The histology of the tissues was confirmed by the pathologist. Thirty sets of tumor (T) tissue, adjacent normal (N) tissue when available were collected for the analysis in this study. HCT-116 cells were obtained from Jilin University.

H&E staining

　　CRC tissues were harvested and fixed in neutral buffered 10% paraformaldehyde. After paraffin embedding, colon cancer tissue cross sections were prepared (1.5 μm) followed by Hematoxylin-Eosin (H&E) staining for morphological observation of the colon cancer layers. The HCT-116 cells with the application of cantharidinate were assessed by light microscopy with H&E staining.

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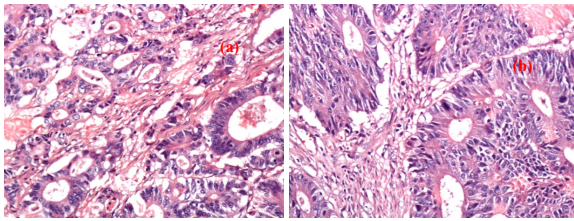
Figure 1. The Pathological Examination of the Primary Human CRC from a 60-years. a, b. atypic glands arranged in dense and cancer cell with large and deeply stained nuclei arranged in disorder. HE staining ×400

Table 1. TGF-β1 Expression and Clinicopathological Characteristics of Colon Cancer Correlation Analysis

Clinical and pathological features		cases	TGF-β1 expression		X^2	*P* value
			Masculine 21 Negative 9			
Sex	male	16	11	5	1.067	>0.05
	woman	14	10	4		
Age (years)	>60	18	12	6	0.267	>0.05
	<60	12	9	3		
Tumor size (cm)	>5	12	10	2	4.923	<0.05
	<5	18	11	7		
Tumor location	left	13	12	1	4.9	<0.05
	right	17	9	8		
Duke's stage	A+B	17	12	5	0.643	>0.05
	C+D	13	9	4		

Immunohistochemical staining

Tissues from paraneoplastic and tumor area were fixed in buffered formalin overnight. Fixative was removed by keeping tissue in tap water followed by dehydration of tissues in 70% ethanol, 80% ethanol and 90% ethanol (5 min each). Tissues were kept in xylene for 20 min twice and then in mixture of xylene and paraffin (1:1) for 1 h. Tissues were paraffinized in fresh paraffin for 2 h twice and then embedded in paraffin blocks. Sections from these blocks were examined by the pathologist and areas identified for generating a tissue microarray. Tissues were incubated with a 1:200 dilution of primary antibodies polyclonal anti-human TGF-β1 antibodies (Sigma), followed by incubation with a universal secondary antibody. A positive reaction was visualized using chromagen 3, 3'-diaminobenzidine (DAB substrate kit). Sections were counterstained with hematoxylin and mounted with glass coverslips.

Cell culture

The HCT-116 cells were cultured in cantharidinate. Human CRC cells were stored at 4°C, while the cell cultures were incubated at 5% CO_2 and 37°C. The cells were grown as monolayers, containing 10% heat-inactivated fetal calf serum, 50 U/ml penicillin and 50 U/ml streptomycin.

Quantitative PCR analysis

The total RNA (1ug) was reverse transcribed to cDNA using AMV reverse and the stem-loop RT primer. Real-time PCR was performed using TaqMan miRNA probes (Applied Biosystems). The sense primer was 5'-CATGGAGACCACCTGGGAC-3'and the antisense primer was 5'-CATTGCCGTAACCCTGGTA-3' (84 bp). The housekeeping gene GAPDH was used as an internal control. The sense primer was 5'-CAGAACATCATCCCAGCGT-3' and the antisense primer was 5'-GCAGGTCAGGTCAACAACAG-3' (132 bp).

Statistical analysis

Results are expressed as mean ± SEM deviation. Statistical comparison was made with Student s *P*-Values of less than 0.05 were considered to be of statistical significance. Statistical analysis of the data was performed via SPSS for Windows Version 11 (SPSS, Chicago, IL, USA).

Results

There were no significant differences between adjacent tissues and cancer tissues with respect to age, gender, nodal involvement or pathological stage; however, tumor size was greater in CRC (Table 1). Overall, TGF-β1 mutations were observed in 21 of 87 cases. TGF-β1 expression were closely related with tumor size and Tumor location (X^2=4.923, 4.900. *P*<0.05). However, there was no statistical significance for tumor grade, which may be due to the number of cases. These results showed that TGF-β1 might play an important role in CRC (Table.1).

H&E staining

To keep the CRC tissue specimens histologically representative and to avoid areas of necrosis, aliquots of CRC tissues from 30 CRC patients (patient consent) in the human biobank of the third Hospital of Jilin University were sectioned and stained with H&E. After staining the samples with conventional H&E, the CRC tissues were observed to have representative histological structures under a microscope. We randomly selected 21 CRC tissues and matched adjacent tissues for RNA quality evaluation (Figure 1).

The expression of TGF-β1

The expression of TGF-β1 was detected by method of immunohistochemical staining. The pattern of TGF-β1 protein expression was mixed nuclear/cytoplasmic staining. Experimental results showed that TGF-β1 was expressed in their membrane and cytoplasm (Figure 2 a). The expression of TGF-β1 increased obviously in CRC tissues. It is expressed mainly in tumor region and tumor interstitial regions (Figure.2b). There was significant difference between CRC groups and adjacent groups (*P*<0.01). The expression of TGF-β1 on color is brownish yellow.

Real-time PCR analysis

Real-time PCR detection of the expressions of TGF-β1 genes. On adjacent and CRC tissues, the total RNA was extracted. The real-time RT-PCR analysis was performed. Experimental results showed that the expression of TGF-β1 mRNA in CRC groups was higher than in adjacent groups (*P*<0.01). These results further confirmed that TGF-β1 plays an important role in CRC.
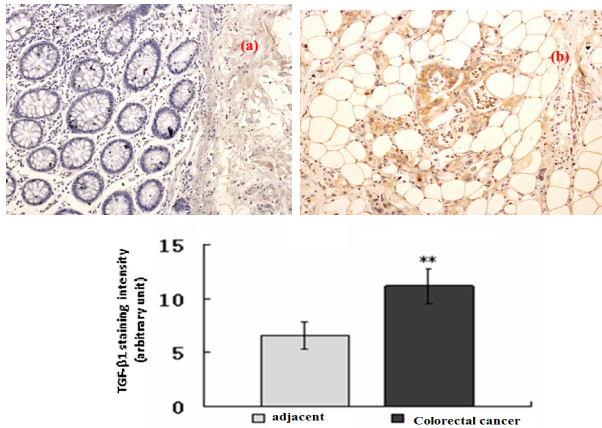
Figure 2. TGF-β1 Staining in Adjacent and CRC Groups. a. Immunohistochemical staining of TGF-β1 in adjacent groups. b. Immunohistochemical staining of TGF-β1 in CRC groups. Values are expressed as means±SEM, Immunohistochemical staining ×200, **P<0.01

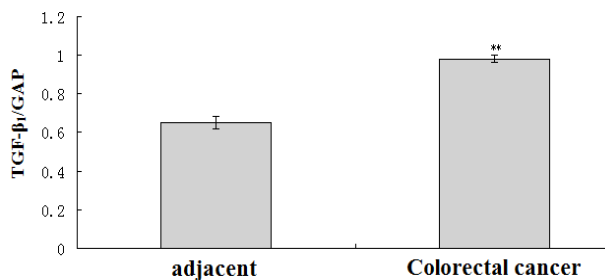**Figure 3. TGF-β1 Real-time PCR in Adjacent and CRC Groups.** Values are expressed as means±SEM, **P<0.01
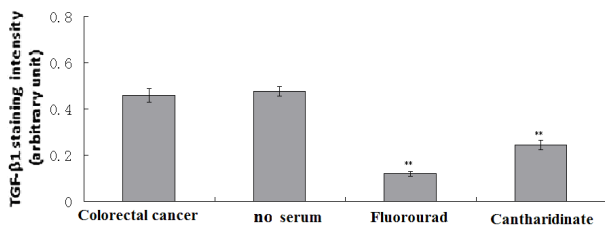

Figure 4. TGF-β1 Staining in CRC Cells. a. CRC cells. b. CRC cells treated with no serum-free medium. c and d. CRC cells treated with fluorouracil and Cantharidinate respectively. Values are expressed as mean ±SEM, immunohistochemical staining ×200, **P<0.01

Expression of TGF-β1 on human colon cancer cells

Immunohistochemical staining is widely used in the diagnosis of abnormal cells such as those found in cancerous tumors. In this experiment, Figure.4 illustrates representative examples of human CRC cells immunostaining of TGF-β1. In order to determine whether cantharidinate can reduce the expression of TGF-β1 in CRC cells. The results of immunohistochemical staining showed that TGF-β1 had strong expression in CRC cells and CRC cells of no containing serum, $P<0.05$ (Figure 4 ab). These results showed cantharidinate might inhibit the expression of TGF-β1 and block tumor deterioration. Immunohistochemical pictures and the results of the statistical analysis presented in Figure 4.

The result of Real-time PCR analysis

To determine whether TGF-β1 mRNA in CRC cells was changed after injection with cantharidinate, the real-time RT-PCR analysis was performed. As shown
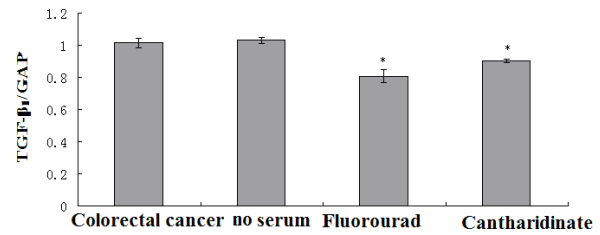

Figure 5. TGF-β1 Real-time PCR in CRC Cells. a. CRC cells. b. CRC cells treated with serum-free medium. c and d. CRC cells treated with fluorouracil and cantharidinate respectively. Values are expressed as means ±SEM, *P<0.05

in Figure 5a, the expression of TGF-β1 mRNA in CRC cells and CRC cells of no containing serum was higher than in treatment with 2.5 μg/ml of cantharidinate for 48 hours ($P<0.05$). These results showed the expressional result of TGF-β1 in cantharidinate groups was similar to fluorouracil groups. The observation suggested that cantharidinate might inhibit TGF-β1 mRNA expression via killing the tumor cells (Figure 4).

Discussion

Cancer can be disclosed in a number of ways, including the presence of certain signs and symptoms, screening tests. Once a possible cancer is investigated it is diagnosed by microscopic examination of a tissue sample. The causes of cancer are multiplex, complex, and only partially understood. At present, many things are known to increase the risk of cancer, including dietary factors, certain infections, other factors, genetic factors, exposure to radiation, lack of physical activity, and environmental pollutants (Su et al., 2013). However, CRC is cancer of the large intestine, the lower part of one's digestive system (Haggar et al., 2009). Rectal cancer is cancer of the last several inches of the colon. Together, they're often referred to as CRC. Most CRC occurs due to lifestyle and increasing age with only a minority of cases associated with underlying genetic disorders (Fearon., 2011). But the role of these mutations in the pathogenesis of CRC remains unclear. Therefore, the prevention and treatment of mutant gene has an important significance. Recent studies showed, TGF-β could increase the invasiveness of cancer cells by increasing their proteolytic activity and promoting their binding to cell-adhesion molecules (Imai et al., 2013). In an earlier study, tumor-derived TGF-β1 induced dendritic cell apoptosis within lymph nodes in non-small cell lung cancer (Imai et al., 2013). Consistent with those findings, the effects of TGF-β1 on angiogenesis, stroma formation and immune function appear to further support tumor progression and invasion (Balasubramanian et al., 2002; Botaitis et al., 2013). The present study demonstrated that TGF-β1 increased in the process of tumor occurrence in a positive regulatory manner. In this experiment, whether TGF-β1 plays an important role in CRC? The results showed that TGF-β1 expression was closely related with tumor size and tumor location. In addition, expression of TGF-β1 in CRC groups was higher than in adjacent groups.

Cancer is usually treated with chemotherapy, radiation therapy and surgery. The chances of surviving vary greatly

by the type, location of the cancer and the extent of disease at the start of treatment (Jemal et al., 2011). At present, more and more studies indicate that cantharidinate was a reasonable and effective treatment for liver cancer. However, whether it can be used for the inhibition of other cancers? For years, cantharidinate entered gradually our field of vision and become a research hot spot. Our data demonstrated that cantharidinate could initiate the inhibitory process of TGF-β1 involved in CRC. It seems that the effects of cantharidinate are similar to fluorouracil. In addition, we demonstrated that cantharidinate reduced the expressions of mRNA and protein levels of TGF-β1 in CRC cells and the effects of cantharidinate are similar to fluorouracil.

In conclusion, the increased frequency of TGF-β1 expression in carcinomas and in CRC compared to adjacent tissues indicated that increasement of TGF-β1 expression might be an early event in CRC carcinogenesis. TGF-β1 plays an important role in CRC and TGF-β1 expression might be a complementary mechanism in the onset of colorectal cancer. In addition, cantharidinate could induce the CRC apoptosis through reducing TGF-β1 expression. But the exact machenisms that cantharidinate inhibits the expression of TGF-β1 still need futher studies.

Acknowledgements

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