RESEARCH COMMUNICATION

The Antitumor Activity of Exogenous and Endogenous Canstatin on Colorectal Cancer Cells

Ya-Nan Xing[1], Hong-Wei Liang[1], Li Zhao[2], Hui-Mian Xu[1]*

Abstract

Colorectal cancer is the third most common malignancy and the third-leading cause of cancer-related deaths worldwide. In the last several years, recombinant DNA technology has made cancer gene therapy feasible in the clinic. In our studies, we used both exogenous and endogenous canstatin, a type IV collagen genetically distinct product. We detected the effects of canstatin on colorectal cancer cells HCT-15 and HCT-116. DAPI staining, FCM and migration analyse were used to detect the apoptotic cells, cell cycle and mobility. As shown in the results, the apoptotic cell numbers ($p<0.05$) and G1 arrest cell numbers ($p<0.05$) were higher than in the non-treatment case. The mobility of the cells was also decreased obviously ($p<0.05$). Simultaneously, combination effects of exogenous and endogenous canstatin were identified.

Keywords: Colorectal cancer - canstatin - apoptosis - cell cycle - mobility

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Introduction

Tumor angiogenesis is a significant process for tumor development and aggravation (Kerbel, 1997). The balance between endogenous angiogenic stimulators and inhibitors play an important role in tumor angiogenesis (Hanahan et al., 1996; Malin et al., 2005). In the previous studies, tumstatin and canstatin have been identified as two endogenous inhibitors of angiogenesis in vivo (Kamphaus et al., 2000; Maeshima et al., 2000). The type IV collagen is one of the components that form the basement membrane consists of six genetically distinct products: α1-6 (Prockop, 1995; Timpl, 1996). α2 (IV) has been named canstatin which has a 24 kDa peptide. Canstain has antitumor activity in the prostate and renal cell carcinoma xenograft models (Kamphaus et al., 2000). In addition, Panka et al. (2003) found that canstatin can inhibit Akt activation and induce Fas-dependent apoptosis in endothelial cells. Magnon et al. (2005) identified that canstatin suppress endothelial and tumor cells though mitochondria-dependent pathways.

Colorectal cancer is the third common malignancy and the third leading cause of cancer-related deaths worldwide (Weitz et al., 2005). WHO estimates that more than 945,000 people develop colorectal cancer, accounting for 8.5% of all new cases, and around 492,000 patients die (Potter et al., 1999; Weitz et al., 2005). The formation of CRC is a slow process, with accumulating genetic alterations resulting in the so-called adenoma-carcinoma sequence (Vogelstein et al., 1993). Conventional therapy such as surgery, radiation, and chemotherapy have been widely used, but have not improved survival rates. If the cancer is diagnosed at a later stage, the five-year relative survival rate is 6% (O'Connell et al., 2004). In the last several years, recombinant DNA technology has made cancer gene therapy feasible in the clinical treatment. p53 (Miyake et al., 2000), p73 (Tuve et al., 2006) and Bax (Okumura et al., 2007) have been used to treat cancer directly or enhance the effects of anticancer drugs.

To our knowledge, we first detected the antitumor activity of canstatin in colorectal cancer cells. In our studies, we prepared recombinant canstatin using the lipofectamine method (Lee et al., 2007). Then we add the recombinant canstatin to the culture of HCT-15 (ATRA sensitive) and HCT-116 (ATRA resistant). Simultaneously we tansfected the plasmid pMT-canstatin to HCT-15 and HCT-116. We observed the antitumor activity of exogenous and endogenous canstatin on colorectal cancer cells. In our studies, apoptosis rates, cell cycle and mobility of cells was analyzed by DAPI staining, PI staining and transwell analysis.

The results showed that both exogenous canstatin and endogenous canstatin play a significant role in suppressing proliferation and inducing apoptosis of cancer cells.
We also found that combining these two therapies had greater antitumor activity than either treatment alone in the colorectal cancer cells. Canstatin may be a novel drug to treat colorectal cancer.

Materials and Methods

Cell lines and culture

Human colorectal cancer cells HCT-15 and HCT-116 were all obtained from the American Type Culture

*[1]Department of Surgical Oncology, [2]Department of Radiology, First Affiliated Hospital of China Medical University, Shenyang, China *For correspondence: cmu_directorHM@163.com*

Collection (Bethesda, MD, USA). The cell lines were grown in RPMI-1640 medium (Hyclone, USA) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 ug/ml streptomycin) and maintained in a humidified cell incubator with 5% CO2 at 37 ºC.

Construction of prokaryotic pET-3a-canstatin and eukaryotic pMT-canstatin expression plasmids

The canstatin cDNA was amplified using the reverse transcription polymerase chain reaction (RT-PCR) from the total RNA of HCT-15 and HCT-116 respectively. The sense primer was 5'-GTCAGCATCGGCTACCTCCTGGTGAA-3' and the antisense primer was 5'-CAGGTTCTTCATGCACACCTGGCAG-3'(the primers were designed by software primer premier 5.0). The cDNA was digested both with Nde I and BamH I and ligated into predigested pET-3a (Novagen), then transformed into Escherichia coli BL21 (DE3) for the expression of canstatin (Figure 1). pMT/BiP/Can-V5-His was constructed by inserting the cDNA fragment digested by Bgl II and Xhol (Figure 2).

Expression and purification of recombinant canstatin

As the method of Lee et al (2007) and He et al (2003), the expression protein was isolated predominantly from inclusion bodies. The soluble protein was purified by Sephadex G-100 gel filtration. Then we used western-blot to identified the recombinant canstaitin. Anti-human canstatin primary antibody was purchased from Novus Biologicals. The method was not described in details.

Stable cell lines

Cells were transfected using Lipofectamine™ 2000 (Invitrogen, American) according to the manufacturer's instructions. Canstatin expression cells were obtained by transfecting with pMT-canstatin. Then the cells were selected and cultured in normal condition for further analyze.

Cell growth assay

Cell growth was determined by MTT assay. The cells (1500 cells/well) including HCT-15, HCT-116, HCT-15 and HCT-116 treated with recombinant canstatin(100 ng / ul), HCT-15 and HCT-116 transfected with pMT-canstatin were plated in 96-well plates and incubated with the normal conditions. Then the cells were treated with 10 µl of MTT solution (5 mg/ml, Sigma). Finally, absorbance were measured at 550-560 nm.

4'-6-Diamidino-2-phenylindole staining assay

4'-6-Diamidino-2-phenylindole (DAPI) staining was applied for determining the apoptotic cells. Cells at a density of 1×105 cells/well were maintained on six-well plates and then were treated under the normal condition. Cells in each well were individually fixed in 4% (v/v) paraformaldehyde (Sigma) for 15min and then stained using DAPI (Invitrogen) for apoptotic cells. Cells were then examined and photographed using a fluorescence microscope(OLYMPUS CX71, Japan).

Cell cycle studies

In vitro, the cells described above were harvested. Cells were suspended in PBS and then fixed with 70% ethanol at 4°C. After centrifugation, the supernatants were discarded, and cellular DNA was stained in a solution containing 10 µmol/l of propidium iodide (Keygene, China). Then the mixture was analyzed by a FACSCalibur machine(Model FACSC420, American). Data were analyzed using Cell Quest software WinMDI Version 2.9.

Migration assay

The transwell cell culture chambers (8 µM pore size polycarbonate membrane) were purchased from Costar. After trypsinizing, washing, and diluting, the cells were resuspended in 1640 with no FBS to a concentration of 3 × 105 cells/ml. The lower chamber was loaded with 600 µl of 1640 with 10% FBS and 100 µl of cell suspension were seeded on the upper chamber wells. The chamber was incubated at 37°C with 5% CO2 and 95% humidity for 24 h. After discarding the non-migrated cells and washing the upper wells with PBS, the filters were scraped with a plastic blade, fixed in 4% paraformaldehyde in PBS, stained with crystal violet (CV), and placed on a glass slide. Ten microscopic fields (400×) were randomly selected to count cells. Each assay was done in triplicate.

Statistical analysis

All numerical data were expressed as means ± SD. Differences among the mean values were evaluated using Student's t-test. All statistical analyses were conducted by

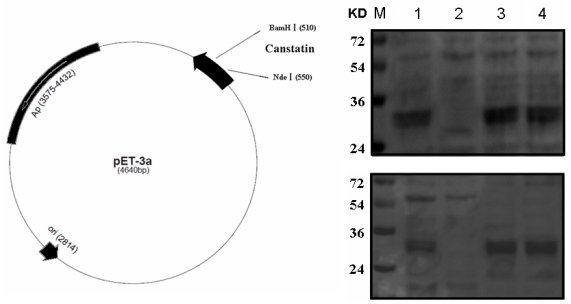

Figure 1. Production and Immunodetection of Recombinant Canstatin. a) Ideograph of pET-3a-canstatin. b) SDS–PAGE Coomassie blue staining. c) Western-blotting of canstatin with anti-human canstatin antibody. Lane 1, induced BL21; Lane 2, uninduced BL21; Lane 3, isolated inclusive bodies of canstatin; Lane 4, purified canstatin protein

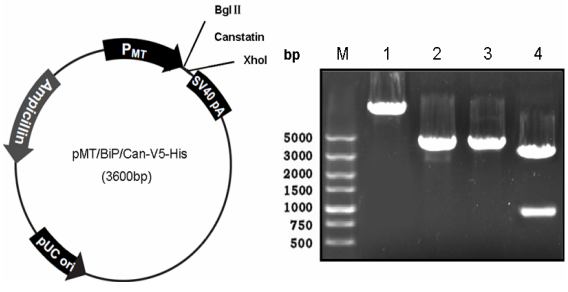

Figure 2. Construction and Identification of pMT-canstatin. a) Ideograph of pMT-canstatin. b) Lane1, integrated pMT-canstatin; Lane2, pMT-canstatin digested by Bgl II; Lane3, pMT-canstatin digested by Xhol; Lane4, pMT-canstatin digested by Bgl II and Xhol

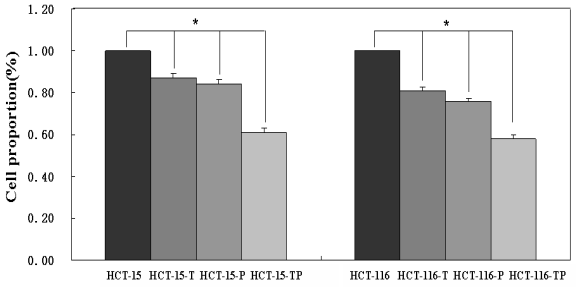

Figure 3. Inhibitory Effects of Canstatin on Colorectal Cells. The percentages of growth inhibition of canstatin on HCT-15 and HCT-116 were determined by MTT. Canstatin showed an inhibitory effects on cell growth ($p<0.05$). T: the cells tansfected with pMT-canstatin. P: the cells treated with recombinant canstatin. TP: the cells treated with recombinant canstatin and tansfected with pMT- canstatin simultaneously

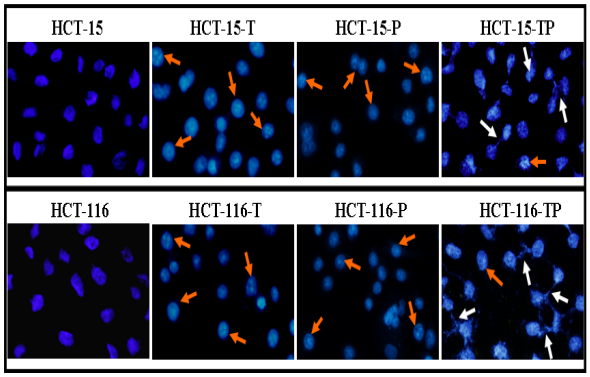

Figure 4. Apoptosis was Induced by Canstatin. Apoptotic body formation was observed under a fluorescence microscope after DAPI staining. Yellow arrows indicate apoptotic bodies. White arrows indicate nuclear fragmentation

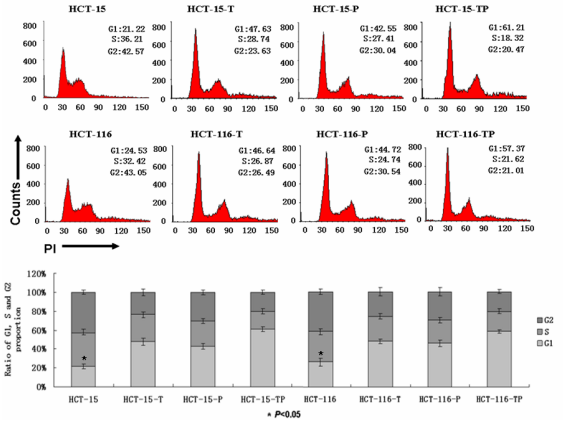

Figure 5. Cell Cycle Analysis by FCM. a) Propidium iodide staining showed changes in the cell cycle. b) The histogram shows the results has statistical significant ($p<0.05$)

SPSS 11.0 (SPSS Inc., American). P-values <0.05 were considered to statistically significant.

Results

Expression and verification of recombinant canstatin

We isolated recombinant canstatin from DL21 carrying pET-3a-canstatin. Gene expression at 72 h after induction using 1 mM CuSO4 was analyzed by SDS-PAGE analysis and Western-blotting analysis. As shown in Figure 1, recombinant canstatin protein a molecular weight of 29 kDa was detected in DL21 isolated mixture. The soluble protein was used for the following experiments.

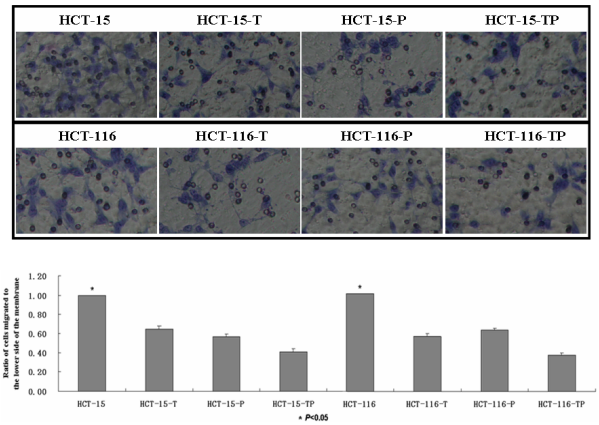

Figure 6. Mobility of Colorectal Cancer Cells After Treated with Canstatin. a) Migrated cells found on the bottom side of the membrane were stained. b) Numbers of migrated cells on the membrane bottom were counted. The histogram shows the results has statistical significant ($p<0.05$)

The antitumor functions of exogenous and endogenous canstatin on colorectal cancer cells

To further investigate the functions of canstatin, we constructed pMT- canstatin. Full-length canstatin was exogenously expressed in colorectal cell lines. The data were shown in Figure 2. Then we detected the antitumor activity of exogenous and endogenous canstatin. MTT assay showed that the proliferation rates of HCT-15 and HCT-116 treated with exogenous canstatin or transfected with pMT- canstatin were lower than untreated ones ($p<0.05$, Figure 3). The results of DAPI staining indicated that the number of apoptotic cells was higher than untreated ones (Figure 4). We can see apoptotic bodies in the cells only treated with exogenous canstatin or endogenous canstatin. Apoptotic bodies and nuclear fragmentation were observed in the cells treated with both methods. As shown in FCM, both the exogenous canstatin and endogenous canstatin can lead HCT-15 and HCT-116 to G1 phase arrest ($p<0.05$, Figure 5). In order to have a better understanding of the effects of canstatin on cell phenotype, we used migration assay to examine the mobility of HCT-15 and HCT-116. It showed that fewer cells treated with the exogenous canstatin and endogenous canstatin migrated to the lower side of the membrane than untreated ones ($p<0.05$, Fig. 6). In all our results, the combining the exogenous canstatin and endogenous canstatin had greater antitumor activity than either treatment alone in the colorectal cancer cells.

Discussion

In this study, we demonstrate that canstatin is crucial for development and cell cycle progression of colorectal cancer cells. We utilized recombinant canstatin and pMT-canstatin transfection to treat HCT-15 and HCT-116.

Earlier observations showed that α1 and α2 type IV collagen chains may be inhibitory to capillary endothelial cell proliferation in the Engelbreth-Holm-Swarm sarcoma tumor (Madri et al., 1997). Similarly with our results, both the exogenous canstatin and endogenous

canstatin can inhibit proliferation of HCT-15 and HCT-116. Canstatin has a great potential for inhibiting tumor growth in experimental models of prostate cancer (Kamphaus et al., 2000), pancreatic cancer (He et al., 2003), breast cancer (Wang et al., 2008), and lung caner (Li et al., 2006). However, Kamphaus et al (2000) also found that 40 μg/ml canstatin has no significant on the proliferation of renal carcinoma cells, prostate cancer cells and human embryonic kidney cells. Dosage of canstatin and tissue specific may cause this difference. In previous studies, canstatin has been demonstrated that can induce endothelial cells to apoptosis, but no evidence showed in non-endothelial cells (Panka et al., 2003). But very interesting, in our studies, we found not only exogenous canstatin but also endogenous canstatin can cause apoptosis to colorectal cancer cells. In endothelial cells, Panka et al (2003) found canstatin induces apoptosis through a Fas-dependent signal pathway. The mechanism of canstatin induces apoptosis in colorectal cancer cells remains unclear. In our further studies, we will detect the related signal pathway. To our knowledge, we first analyzed the effects of canstatin on the cell cycle. As shown in the results, the colorectal cancer cells showed G1 arrest. The evidence indicated that canstatin has an inhibitory activity on colorectal cancer. Since metastasis depends on mobility of cancer cells, we detected the effects of canstatin on the colorectal cancer cells using transwell migration assay. We found that canstatin can inhibit mobility of the cells. Narazaki et al (2006) also found the mobility of endothelial cells was inhibited by canstatin. The results were coincident with each other. In all our studies, we detected the combining affection of exogenous canstatin and endogenous canstatin. The apoptotic cell number and G1 arrest cell number were higher than the cell number that treated with one of them. The mobility of the cells was also decreased obviously.

In summary, our studies showed the effects of canstatin on colorectal cancer cells first time. In our studies, we utilized the exogenous canstatin and endogenous canstatin treated colorectal cancer cells simultaneously. We found the antitumor activity of canstain. No evidence showed the difference between exogenous canstatin and endogenous canstatin. However, the combining affection of exogenous canstatin and endogenous canstatin was identified. The results may provide a novel method to clinical treatment.

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