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• *GASTRIC CANCER* •

Effect of Weikangning on gastric cancer cell growth and expression of vascular endothelial growth factor and its receptors KDR and Flt-1

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Abstract

AIM: To observe the effect of Chinese traditional herbal decoction Weikang-ning (WKN) on cell growth and expression of VEGF and its receptors KDR and Flt-1 in gastric cancer cell line MGC-803.

METHODS: A total of 120 male Wistar rats were divided into control group, high dose, medium dose and low dose groups fed with natural saline, 20, 10, and 5 g/kg of WKN, respectively. The experimental animals were finally killed for the preparation of drug-containing serum. The gastric cancer cell MGC-803 was cultured with the drug-containing serum drawn from the rats in different groups. We observed the growth condition of the cancer cells with light microscope and flow cytometer. The expression of mRNA of VEGF and its receptors KDR and Flt-1 was detected with RT-PCR.

RESULTS: The proportion of cells in G_0-G_1 phase was (65.40±0.41)%, (56.92±0.62)%, (55.89±0.69)% in high, medium and low dose groups respectively *vs* (41.35±0.55)% in control group (P<0.01), while the cells in G_2-S and S phases were (11.62±0.62)% and (22.99±0.69)%, (17.08±0.80)% and (26.00±0.71)%, (19.37±0.57)% and (24.74±0.64)% in high, medium and low dose groups, respectively, *vs* (23.65±0.56)% and (35.00±0.60)% in control group (P<0.01). The expression of mRNA of VEGF and its receptors was significantly decreased, the area of electrophoresis bands (AREA), the absorptivity of mean optical density (A) and the product of AREA and A were significantly lower in WKN-administered groups than that in control group (P<0.01).

CONCLUSION: The decoction of WKN suppresses the growth of gastric cancer cell MGC-803 and decreases the expression of mRNA of both VEGF and its receptors KDR and Flt-1.

Key words: Weikang-ning; Gastric cancer; VEGF; KDR; Flt-1; MGC-803

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INTRODUCTION

Gastric cancer continues to be one of the most common malignancies in humans worldwide[1-4], severely influencing the people's health. In China, its mortality accounts for the leading cause of death among malignant tumors[5]. The occurrence and development of gastric cancer is a complicated process involving multi-factors and multi-genes[6]. During this course, the malfunction of proliferation of cells resulting from the inactivation, mutation or irregulation of pro-oncogenes, suppressive oncogenes and apoptosis-related genes leads to malignant conversion[7,8]. Our study investigated the expression of mRNA of both VEGF and its receptors in gastric cancer cell line MGC-803, and the effect of traditional herbal decoction Weikang-ning (WKN) on it.

MATERIALS AND METHODS

Preparation of WKN decoction

WKN was made up of *Codonopsis Pilosula*, *herba scutellariae barbatae*, *etc.* (the powder preparation was supplied by Jiangyin Pharmaceutical Factory, Jiangsu Province, China). Each milliliter of the preparation of WKN decoction solution contains 1 g crude drug equivalently. The preparation added with benzyl acid (0.1 g/100 mL) was stored at 4 ℃ for animal feeding.

Preparation of drug-containing serum

A total number of 120 Wistar rats (clean rank, supplied by Sun Yat-Sen University Experimental Animal Center,

Table 1 Primers for PCR

	Positive	Negative
VEGF	5′AATGCTTTCTCCGCTCTG3′	5′TTGCTGCTCTACCTCCAC3′
Flt-1	5′CAAGTGGCCAGAGGCATGGAGTT3′	5′GATGTAGTCTTTACCATCCTGTTG3′
KDR	5′GAGGGCCTCTCATGGTGATTGT3′	5′TGCCAGCAGTCCAGCATGGTCTG3′
β-actin	5′CTAYYGGCAACGAGCGGTTC3′	5′CTTAGGAGTGGGGGTGGCTT3′

Guangzhou, China) weighing 120-150 g were randomly divided into four groups. The high dose, medium dose and low dose groups were fed with the decoction of WKN containing equivalently 20, 10, 5 g/kg crude drugs, respectively, while the control group was fed with saline of the same volume. All groups were fed once a day for 1 wk. During the first hour after the last feeding, blood was aseptically drawn from the abdominal aortas of the animals in different groups and immediately centrifuged at 1 700 r/min to harvest the serum. The preparation of drug-containing serum was blended according to different groups and stored at -70 ℃, inactivated at 56 ℃ before use.

Culture of gastric cancer cells

Gastric cancer cell line MGC-803 (supplied by Experimental Animal Center of Sun Yat-Sen University, Guangzhou, China) was cultured in RPMI-1640 medium (purchased from NBS, USA) containing 100 g/L fetal bovine serum (FBS, purchased from Invitrogen Biotech, Shanghai, China) in an incubator (purchased from NAPCO, USA) containing 50 mL/L CO_2 at 37 ℃ for 48-72 h after resuscitated. The medium was changed every 24 h and discarded after the bottom of the flasks was covered with cells. Culture medium RPMI-1640 supplemented with 100 g/L FBS was used to adjust the cell density to 2×10^6/mL after the cells were trypsinized with 2.5 g/L trypsin, and then the cells were inoculated into serum.

The cells in control group were grown in routine culture medium. The cells in serum group were grown in the serum from normal rats. The cells in high, medium and low dose groups were grown in the sera from high, medium and low dose group animals, respectively. After the cells were grown for two generations *in vitro*, we observed the morphological changes of the cells and the index of doubling proliferation with reverse microscope and detected the cell cycle change with flow cytometer (purchased from Becton-Dickinson, France).

Evaluation of cell cycle

After the cells (1×10^6/mL) were evenly inoculated into the culture medium containing 10% drug-containing serum and grown for two generations, we collected the suspension of cells after being trypsinized with 2.5 g/L trypsin. Then the cells were washed with PBS, embedded in alcohol at -20 ℃, and dyed by PI, and the change of cell cycle was observed with flow cytometry.

Effect of WKN on the expression of VEGF and its receptors KDR and Flt-1

Guanidinium isothiocyanate-phenol single-step method was applied to extract the total RNA of cells using TRIzol (purchased from GIBCO BRL, Gaithersburg, MD, USA) following the instruction of the kit. The integrity, purity and concentration of the mRNA extracted were detected with ultraviolet spectral photometer and agarose gel electrophoresis. The RNA extract dissolved in DEPC was stored at -70 ℃.

Design and synthesis of the primers

According to the sequences of human mRNA of VEGF, KDR and Flt-1, the primers were designed (Table 1), synthesized and supplied by Boya Biotech, Shanghai, China.

RT-PCR

First-strand cDNA was synthesized from 2 μg RNA dissolved in water administered with DEPC, in which 0.2 μL oligo dT was added at 70 ℃ for 5 min, and placed on ice for 1 min, then 1 μL MMLV reverse transcriptase (200 IU/μL), 0.5 μL RNasin (40 IU/μL), 5 μL 5×RT buffer, and 1.5 μL dNTPs (10 mmol/L) were added at 42 ℃ for 60 min. The reverse transcriptase was inactivated at 65 ℃ for 5 min. Five microliters of cDNA, 12.5 mL 10× buffer, 3 μL 2 mmol/L dNTPs, 0.5 μL 5 IU/L LA Taq enzyme and water were added to a volume of 25 μL. The final concentrations of positive and negative chain primers were both 0.8 μm/L. VEGF was amplified for 30 cycles at 94 ℃ for 1 min, at 55 ℃ for 1 min, at 72 ℃ for 2 min, and extended at 72 ℃ for 7 min. KDR and Flt-1 were both amplified for 30 cycles at 95 ℃ for 2 min, at 94 ℃ for 1 min, at 60 ℃ for 1 min, at 72 ℃ for 2 min, and extended at 72 ℃ for 7 min. The area of electrophoresis bands (AREA), the absorptivity of mean optical density (*A*) and the product of AREA and *A* were quantitatively analyzed after the product of PCR was detected with auto image manipulating system following agarose gel (2%) cataphoresis and dyed with EB. The samples were controlled with blank and β-actin.

Statistical analysis

Software SPSS was applied for the statistical analysis of the data from the experiments.

RESULTS

Effect of WKN on cell cycle

Our results showed that there was significant difference in the proportion of cells in different phases between drug-administered groups and control group in a dose-dependent manner (Table 2, Figure 1).

Effect of WKN on the expression of mRNA of VEGF and its receptors KDR and Flt-1

The mRNA expression of VEGF and its receptors KDR and Flt-1 mRNA was seen in all groups; however, compared to the control group, the expression was decreased in drug-administered groups in a dose-related manner (Table 3, Figure 2).

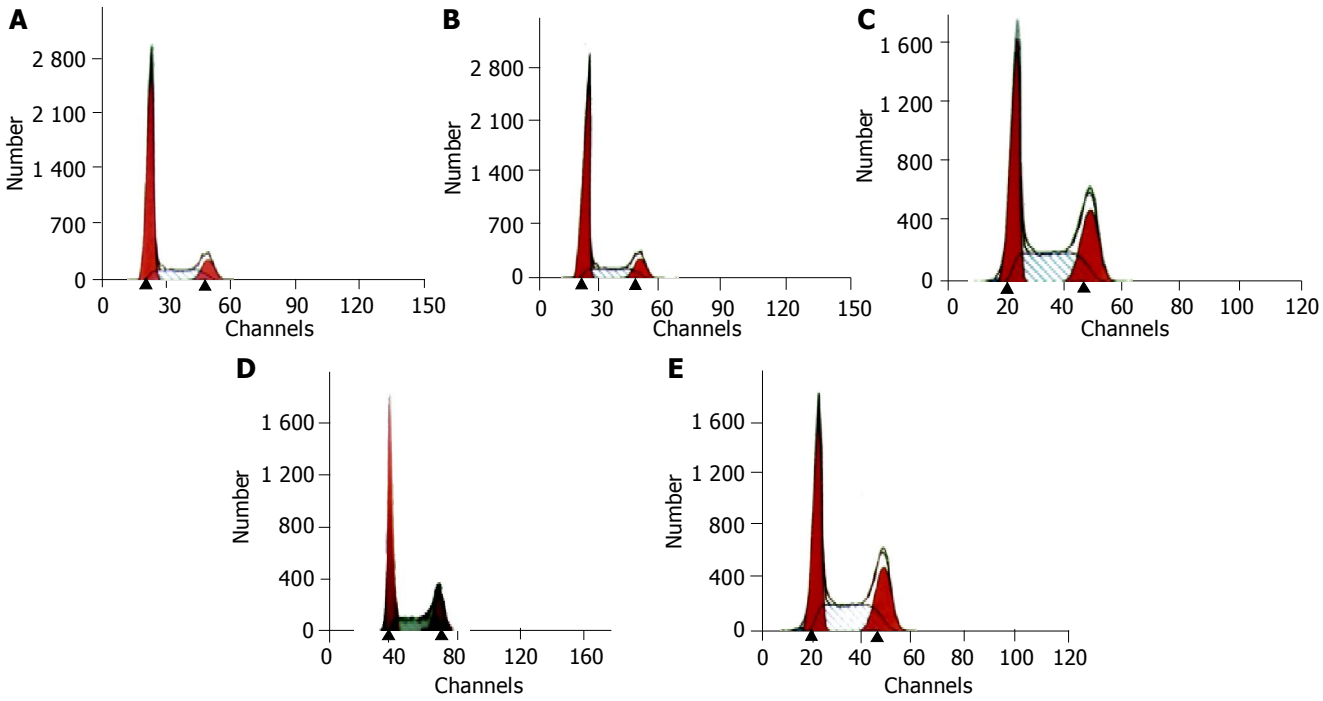

Figure 1 Effect of WKN on growth of gastric cancer cell MGC-803.

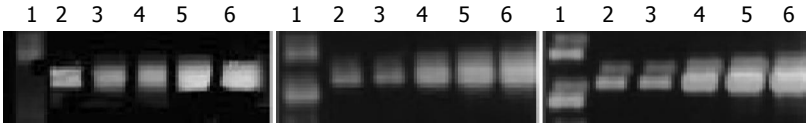

Figure 2 Expression of VEGF, KDR and Flt-1 mRNAs. Lanes 1 to 6 were loaded with the marker, high dose, medium dose, low dose, serum groups and control group.

Table 2 Proportion of cells in different phases of cell cycle of different groups

Groups	Proportion of cells (%)		
	G_0-G_1 phase	G_2-S phase	S phase
High dose	65.40±0.41[a,b]	11.62±0.62[a,b]	22.99±0.69[a,b]
Medium dose	56.92±0.62[a,b]	17.08±0.80[a,b]	26.00±0.71[a,b]
Low dose	55.89±0.69[a,b]	19.37±0.57[a,b]	24.74±0.64[a,b]
Serum	42.25±0.25	22.51±0.58	35.34±0.56
Control	41.35±0.55	23.65±0.56	35.00±0.60

[a]P<0.05 between high, medium, and low dose groups, [b]P<0.01 *vs* control group.

Table 3 Effect of WKN on the mRNAs of VEGF and its receptors KDR and Flt-1

Groups		High dose	Medium dose	Low dose	Serum	Control
VEGF	AREA	120[b]	134[a,b]	192[a,b]	261	297
	OPTDM	0.072[b]	0.107[a,b]	0.140[a,b]	0.152	0.174
	APTDI	8.588[b]	14.373[a,b]	25.856[a,b]	39.555	50.850
KDR	AREA	79[b]	178[a,b]	239[a,b]	272	408
	OPTDM	0.237[b]	0.112[a,b]	0.150[a,b]	0.162	0.282
	APTDI	18.752[b]	19.883[a,b]	35.764	44.183	115.242
Flt-1	AREA	111[b]	197[a,b]	247[a,b]	271	306
	OPTDM	0.061[b]	0.275[a,b]	0.340[a,b]	0.368	0.407
	APTDI	6.805[b]	54.256[a,b]	84.040[a,b]	99.600	124.531

[a]P<0.05 *vs* high dose group, [b]P<0.01 *vs* control group.

DISCUSSION

The proliferation of tumor cells as normal cells goes through the cell cycle, which is known to consist of four phases: G_1, G_2, M and S. Drugs with the effect of tumor suppression by blocking any one or more of the four phases can effectively inhibit the growth of tumors[9]. Our results showed that WKN-containing serum arrested the gastric cancer cell MGC-803 in G_0-G_1 phase, blocked or delayed the entry into S phase, disturbed the synthesis of DNA. The growth and proliferation of cancer cells were inhibited in a dose-dependent manner.

VEGF is a new type growth factor in tumor cells, which specifically acts on endothelial cells, and prompts the proliferation of endothelial cells, playing an important role in the formation and growth and metastasis of tumors. In the course of occurrence and development of gastric cancer, angiogenesis plays a crucial role in which VEGF is believed to be the most important factor in neovascularization[10]. The expression of VEGF is commonly shown in gastric cancer cells in a number of studies. One mechanism of invasion and metastasis of gastric cancer is related to the pro-angiogenesis action performed by VEGF, and the extent of its expression is closely related to the occurrence, development and prognosis of gastric cancer[11-14] especially the expression of VEGF-C and VEGF-D is the important

marker of lymph node metastasis of the cancer[15,16]. Some studies in rats showed that the level of VEGF in serum was not only related with the prognosis of gastric cancer but also with the efficacy of therapy[17]. Both the kinds of specific receptors of VEGF, KDR and Flt-1 are commonly distributed in vascular endothelial cells. VEGF's combination with any one or both of its receptors activated the intrinsic tyrosinase. By tyrosinase signal transduction pathway, the tyrosine remnant in the cytoplasm was activated and then vascular-derived factors were secreted to function[18]. Our study confirmed that KDR and Flt-1 also exist in the surface of cancer cells, consistent with other recent studies[19]. Most investigators believe that VEGF secretes in an autocrine way[20,21].

Some scholars have proposed anti-cancer therapies targeting at VEGF and its receptors. Studies have shown that monoclonal anti-VEGF antibodies can inhibit the progression and metastasis of cancers[22]. The anti-sense mRNA of VEGF inhibits the expression and secretion of VEGF by complimentarily combining with the bases of VEGF mRNA, inhibiting the transcription and increasing the degradation of VEGF mRNA[22]. Soluble receptors or antibodies combining with the excessive VEGF in serum are also effective. Soluble VEGF receptor sFlt-1 combines with VEGF with a high affinity through the formation of isogenical homodimer or combines with integral Flt-1 or integral KDR to form heterogenical alloploid, competitively inhibiting the combination of VEGF with integral Flt-1 or the autopolyploid of KDR, resulting in the blockage of stimulation VEGF on endothelial cell proliferation, and finally suppression of angiogenesis[23]. Flt-1 coupled with small peptides can combine with human umbilical vein blood cells and cancer cells to inhibit the angiogenesis, prompt the necrosis of tumor tissues and significantly inhibit the growth of cancer cells[24].

Studies on Chinese traditional medicine recently show that herbs belonging to spleen boosting and Qi tonification as well as their compounds not only improve the immune function and quality of life of patients with gastric cancer but also increase the curative effect of anticancer drugs, and decrease the toxic side effects[25]. Herbs with spleen boosting, Qi tonifying, blood circulation stimulating and thrombosis dissolving functions as well as their compounds could induce or prompt the differentiation and maturation of cells with intestinal metaplasia and heterogeneous-type proliferation[26], inhibit and remedy the abnormal propagation and protect the gastric mucosa[27,28]. Our previous studies had shown that WKN could inhibit not only the growth of gastric cancer but also the expression of p53 and VEGF in gastric cancer tissues[29]. The current study shows that the expression of VEGF and KDR, Flt-1 decreased in drug administered groups, indicating that WKN could inhibit the expression of receptors in a dose-dependent manner. It also shows that the therapeutic action of herbs on gastric cancer involves multiple factors and at multiple levels. The herbs act not only on related genes but also on relevant proteins or receptors. The expression of VEGF and its receptors is regulated by a variety of cytokines and tumorigenic genes[30]. Further studies are required to determine the exact molecular mechanism of WKN's effect on VEGF and its receptors KDR and Flt-1.

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