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• GASTRIC CANCER •

The role of KDR in the interactions between human gastric carcinoma cell and vascular endothelial cell

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Abstract

AIM:**To study the interactions between human gastric carcinoma cell (HGCC) and human vascular endothelial cell (HVEC), and the role of KDR in these interactions.**

METHODS:**Antisense oligodexynucleotide(ASODN) specific to KDR gene was devised and added to the culture medium of HGCC and HVEC. After the action of ASODN, the proliferation of two cells was measured by MTT method. The role of KDR in regulating the proliferation of two kinds of cells was known through observing the effect of ASODN on them. The conditioned mediums (CMs) of HGCC and HVEC were prepared. The CM of one kind of cell was added acting on the other kind of cell, then the cell proliferation was measured by MTT. After the action of ASODN or CM, the cellular expression of KDR gene was detected with *in situ* hybridization(*ISH*) for mRNA level and with immunohistochemical staining for protein level. *ABC*-ELISA was used to detect *h*VEGF in the CMs of two cells.**

RESULTS: **KDR ASODN could specifically inhibit the proliferation of HGCC and HVEC significantly. The growth inhibitory rate amounted to 55.35 % and 54.83 %, respectively ($P < 0.01$). HGCC and HVEC could secret a certain level of hVEGF(92.06±1.69 ng/L, 77.70±8.04 ng/L). The CM of HGCC could significantly stimulate the growth(2.70±0.01 times) and KDR gene expression of HVEC($P < 0.01$) while the CM of HVEC could significantly inhibit the growth(52.97±0.01 %) and KDR gene expression of HGCC ($P < 0.01$).**

CONCLUSION: **KDR plays a key role in regulating the proliferation of HGCC and HVEC. There exist complicated interactions between HGCC and HVEC. HGCC can significantly stimulate the growth of HVEC while HVEC can significantly inhibit the growth of HGCC. KDR is involved in the interactions between them.**

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INTRODUCTION

There exist many kinds of cells besides tumor cell in the solid neoplasm. The relations among all kinds of cells are very complicated. These cells depend on each other and contribute together to the genesis, development, invasion and metastasis of tumor. During the tumor angiogenesis and hematogenous metastasis, there exist complicated interactions between tumor cell (TC) and vascular endothelial cell(VEC). In the pre-angiogenesis, how does TC induce VEC to establish the tumor vascular system? How does TC influence the proliferation, degeneration, morphogenesis and functions of its neighbouring VEC? On the other hand, the interactions between the two cells play a role in the tumor hematogenous metastasis. There exist some complicated mechanisms in these processes. The study of tumor angiogenesis mainly focuses on the interactions among the vascular component cells while the study of tumor metastasis mainly focuses on the interactions between TC and its surrounding stroma. Seldom does anyone notice the interactions between TC and VEC. To better understand some mechanisms in gastric carcinoma angiogenesis and hematogenous metastases, we select human gastric carcinoma cell (HGCC) and human vascular endothelial cell(HVEC) to study their interrelations and some factors in these interactions[1]. Conditioned mediums (CMs) of HGCC and HVEC were prepared. The CM of one kind of cell was added to the other kind of cell, then the cell proliferation was measured by MTT. Many studies have found that KDR, VEGF receptor 2, played an important role in regulating the biological functions of TC and VEC. In order to make clear the role of KDR in regulating the growth of HGCC and HVEC, antisense oligodexynucleotide(ASODN) specific to KDR mRNA was devised and was added acting on the two cells[2,3]. There has been no one to devise KDR ASODN up to now. After the action of CM, the expression of KDR gene was detected. The purpose is to probe into the interactions between HGCC and HVEC and if KDR is involved in the interactions.

MATERIALS AND METHODS

Materials

Cell line HGCC line SGC7901 and HVEC line Eahy926 were employed. The expression of KDR on two cells was (++).

KDR antisense oligodexynucleotide(ASODN) and the action of KDR ASODN ASODN, sense oligodexynucleotide (SODN) and mismatch oligodexynucleotide(MODN) specific to KDR mRNA were designed by the software "Primer 3". The sequence of ASODN was: 5' CAC CTT GCT CTG CAT CCT G 3' , The sequence of SODN was 5' CAG GAT GCA GAG CAA GGT G 3' , The sequence of MODN was 5' CAC TTT GAT CTA CAC CCT G 3' ; The way of KDR ASODN action: cells were placed in serum free medium for growth arrestting. At different periods, ASODN in different doses and culture medium with serum were added at the same time. After different periods:the proliferation of cell was measured by MTT. The cell without the action of ODN was taken as control.

Methods

Preparing CM, measuring activity of conditioned medium (CM) and measuring level of hVEGF Cells in different confluent states were washed twice with PBS, then 3 mL culture medium was added to the cells. The medium what was taken as CM was collected after different periods. After the growth of HGCC and HVEC was arrested for 24 h and 6 h respectively, CM was added. The proliferation of cell was measured by MTT after different culturing periods. The cell without the action of CM was taken as control. The level of *h*VEGF in CMs of two cells was measured by ABC-ELISA kit(*Jingmei, Beijing*).

MTT (methyl tetrazolium colorimetry) 20 μl MTT solution (5 g/L) was added to 200 μl medium in each well of 96 well plate. 4 h later, the supernant was discarded, 150 μl DMSO (Dimethylsulphoxide)was added. After the crystal was dissolved completely, absorption spectrum was measured at 490 nm in the enzyme linked immunosorbent assay meter. The inhibitory rate of cell proliferation=[1-(the mean *A* of experimental group /The mean *A* of control group)] ×100 %

Detecting the expression of KDR gene After the action of KDR ASODN or the action of CM of one kind of cell on the other kind of cell, the expression of KDR gene was measured by *in situ* hybridization for KDR mRNA level and immunohistochemical staining for KDR protein level. Probe for KDR mRNA was designed by the software "Primer 3". The sequence is 5' GGT AGG AGA GGA TAT CCA GCC TG 3' ; Probe labeling and *in situ* hybridization(*ISH*) were carried on according to the manuals of the Dig Oligodexynucleotide Tailing Kit and Dig Detection Kit (*Boehringer Mannheim, Germany*) respectively. PBS was substituted for anti-Dig-Ap as negative control. Immunohistochemical staining for KDR protein on the cells was carried on according to the manual of the S*ABC* Kit (*Huamei, Henang*). KDR polyclonal antibody(*Santa cruz, USA*) was diluted 1:100. Secondary mouse-anti-rat antibody (*Huamei, Henang*) was diluted 1:25. PBS was substituted for primary antibody as negative control. The sections were analyzed for *A* value in the image analysis apparatus.

Statistical analysis

t test was used to compare the means.

RESULTS

Effects of KDR ASODN on the proliferation of HGCC and HVEC

KDR ASODN inhibited the proliferation of HGCC and HVEC significantly. It produced effects in 0.5-1 μmol/ L and 3-6 h later. The cell proliferation inhibitory rate could amount to more than 50 %. The inhibitory rate was related to the dose and action periods(Table 1, 2).

Difference between the effects of KDR ASODN and SODN, MODN on HGCC, HVEC

There existed significant difference between the effects of KDR ASODN and SODN, MODN on the proliferation of HGCC and HVEC (Table 3).

Effects of KDR ASODN on the expression of KDR gene in HGCC and HVEC

Through *ISH* for detecting KDR mRNA level and immunohistochemical staining for KDR protein level, it was found that KDR ASODN inhibited the expression of KDR gene in HGCC and HVEC significantly(Table 4) (Figure 1).

Table 1 Dose-effect of KDR ASODN on HGCC and HVEC for 48 h (n=8, $\bar{x}\pm s$)

	Inhibitory rate of cell proliferation(%)	
KDR ASODN dose / (μmol/L)	HGCC	HVEC
0(control)	0	0
0.5	21.32[b]	0
1	28.31[b]	15.33[b]
5	34.56[b]	30.53[b]
10	45.59[b]	39.67[b]
15	55.35[b]	54.83[b]
20	50.74[b]	48.79[b]

[b]P<0.01 *vs* control.

Table 2 Time-effect of 15 μmol/L KDR ASODN on HGCC and HVEC(n=8, $\bar{x}\pm s$)

	Inhibitory rate of cell proliferation(%)	
KDR ASODN action time/h	HGCC	HVEC
0(control)	0	0
3	16.41[b]	0
6	18.99[b]	6.67[b]
12	28.96[b]	10.36[b]
24	38.90[b]	37.52[b]
48	55.35[b]	54.83[b]
72	50.45[b]	46.18[b]

[b] P <0.01 *vs* control.

Table3 Difference between the effects of KDR ASODN and SODN,MODN(n=8, $\bar{x}\pm s$)

	Inhibitory rate of cell proliferation(%)	
Types of KDR ODN	HGCC	HVEC
No ODN(control)	0	0
ASODN	45.07[b]	31.18[b]
SODN	3.15[d]	2.61[d]
MODN	2.88[d]	2.02[d]

[b]P<0.01, *vs* control. [d] P<0.01, *vs* ASODN group.

Table4 Expression of KDR mRNA and protein in HGCC and HVEC after the action of KDR ASODN (n=8, $\bar{x}\pm s$)

	A of HGCC		*A* of HVEC	
action of KDR ASODN	mRNA	protein	mRNA	protein
Before(control)	0.35±0.03	0.33±0.02	0.37±0.03	0.34±0.03
After	0.16±0.02[b]	0.15±0.02[b]	0.16±0.02[b]	0.15±0.02[b]

[b]P<0.01, *vs* control.

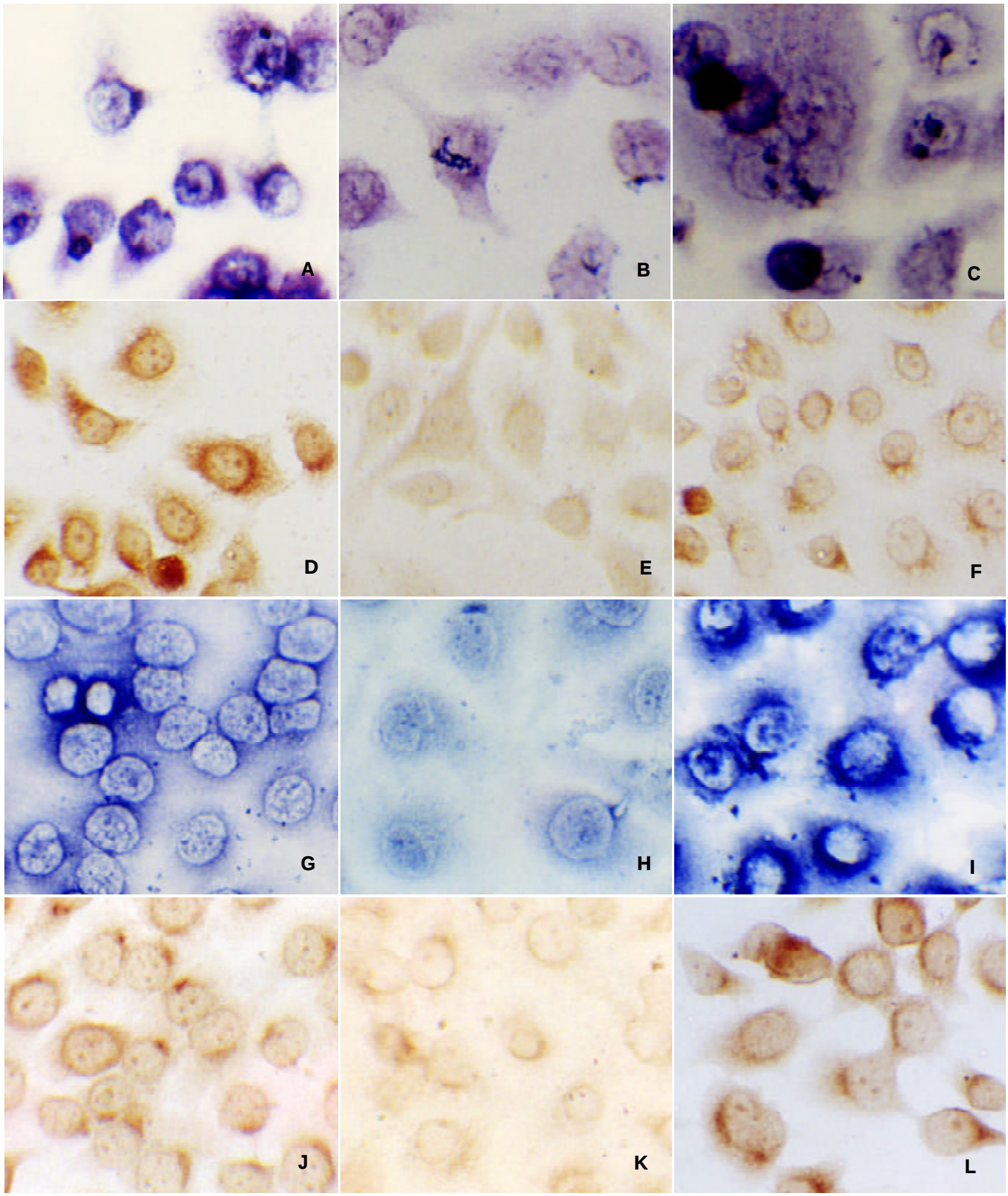

Figure 1 Expression of KDR gene in HGCC and HVEC before and after the action of KDR ASODN and CM
A.KDR mRNA in HGCC(Control) (*in situ* hybridization); B. KDR mRNA in HGCC after the action of KDR ASODN (*in situ*hybridization); C. KDR mRNA in HGCC after the action of CM of HVEC(*in situ* hybridization); D. KDR protein in HGCC (Control) (immunohistochemical staining); E. KDR protein in HGCC after the action of KDR ASODN (immunohistochemical staining) ; F. KDR protein in HGCC after the action of CM of HVEC(immunohistochemical staining); G. KDR mRNA in HVEC (Control) (*in situ* hybridization); H. KDR mRNA in HVEC after the action of KDR ASODN (*in situ* hybridization); I. KDR mRNA in HVEC after the action of CM of HGCC(*in situ* hybridization); J: KDR protein in HVEC (Control) (immunohistochemical staining); K. KDR protein in HVEC after the action of KDR ASODN (immunohistochemical staining); L. KDR protein in HVEC after the action of CM of HGCC(immunohistochemical staining)

Effect of conditioned medium of gastric carcinoma cell on vascular endothelial cell The conditioned medium of HGCC could stimulate the proliferation of HVEC(^aP <0.05 *vs* no-CM action group) significantly. The stimulation effect was related to the CMs of different cell confluent state, different preparing periods, different volume fraction and different action periods (Table 5, 6).

Table 5 Different dose-time-effects of CMs of subconfluent and confluent HGCC on HVEC (n=8, $\bar{x}\pm s$)

	A of CM group / *A* of control group									
CM volume fraction	100%CM		80%CM		50%CM		30%CM		10%CM	
t / h cell confluent state	subconfluent	confluent	subconfluent	confluent	subconfluent	confluent	subconfluent	confluent	subconfluent	confluent
24	2.38±0.01^a	1.29±0.00^{ab}	2.70±0.01^a	1.55±0.02^{ab}	2.21±0.01^a	1.49±0.02^{ab}	2.10±0.03^a	1.44±0.01^{ab}	1.92±0.02^a	1.11±0.01^{ab}
48	1.60±0.01^a	1.22±0.01^{ab}	1.54±0.01^a	1.33±0.02^{ab}	1.38±0.02^a	1.23±0.02^{ab}	1.37±0.02^a	1.21±0.01^{ab}	1.31±0.01^a	1.15±0.02^{ab}
72	0.89±0.00	0.85±0.00	0.99±0.01	1.03±0.01	1.07±0.01^a	1.11±0.01^a	1.27±0.01^a	1.01±0.00^b	1.01±0.00	1.15±0.01^a

^aP <0.05 *vs* No-CM action group, ^{b} P<0.05 *vs* subconfluent group.

Table 6 Different dose-time-effect of CMs of different preparing periods of confluent HGCC on HVEC (n=8, $\bar{x}\pm s$)

	A of CM group / *A* of control group									
CM volume fraction	100%CM		80%CM		50%CM		30%CM		10%CM	
t / h Preparing periods	24hCM	48hCM	24hCM	48hCM	24hCM	48hCM	24hCM	48hCM	24hCM	48hCM
24	1.29±0.01^a	1.38±0.01^{ab}	1.55±0.02^a	1.39±0.01^{ab}	1.49±0.01^a	1.38±0.01^{ab}	1.44±0.02^a	1.30±0.01^{ab}	1.11±0.01^a	1.00±0.01^b
48	1.22±0.01^a	1.11±0.02^{ab}	1.33±0.01^a	1.14±0.02^{ab}	1.23 ±0.01^a	1.03±0.01^b	1.21±0.01^a	1.03±0.01^b	1.15±0.01^a	1.02±0.01^b
72	0.85±0.01	0.78±0.00	0.99±0.01	0.92±0.00	1.07 ±0.02^a	1.14 ±0.01^{ab}	1.27±0.01^a	1.20±0.01^{ab}	1.15±0.01^a	1.16±0.01^a

^aP <0.05 *vs* No-CM action group, ^{b} P<0.05 *vs* preparing for 24 hCM group.

Effect of conditioned medium of vascular endothelial cell on gastric carcinoma cell

The conditioned medium of HVEC could inhibit the proliferation of HGCC(^aP<0.05 *vs* no-CM action group) significantly. The inhibitory effect was related to the different cell confluent states and different volume fractions (Table 7).

Table7 Effects of CMs of subconflunt and confluent HVEC on HGCC for 48 h (n=8, $\bar{x}\pm s$)

	Inhibitory rate of cell proliferation(%)	
Volume fraction	CM of subconfluent HVEC	CM of confluent HVEC
100%CM	52.97±0.01^a	31.62±0.02^{ab}
80%CM	54.26±0.01^a	30.46±0.01^{ab}
50%CM	23.46±0.01^a	19.00±0.01^{ab}
30%CM	21.70±0.00^a	2.13±0.01^b
10%CM	14.36±0.00^a	1.61±0.00^b

^aP<0.05 *vs* No-CM action group;^bP<0.05 *vs* subconfluent group

Expression of KDR gene in HVEC before and after the action of HGCC CM

The mRNA level(*A* value of *in situ* hybridization) before and after the action of HGCC CM was (0.37±0.03), (0.48±0.01^b) respectively (^bP<0.01 *vs* before the action). The protein level (*A* value of immunohistochemical staining) before and after the action of HGCC CM was (0.34±0.03), (0.48±0.02^b) respectively,(^{b}P<0.01 *vs* before the action).So, after the CM of HGCC acted on HVEC, the expression level of KDR gene in HVEC was increased significantly (Figure 1).

Expression of KDR gene in HGCC before and after the action of HVEC CM

The mRNA level(*A* value of *in situ* hybridization) before and after the action of HVEC CM was (0.35±0.03), (0.22±0.02^b) respectively.The protein level(*A* value of immunohistochemical staining) before and after the action of HVEC CM was(0.33± 0.03), (0.23±0.02^b) respectively,(^bP<0.01 *vs* before the action). So, after the CM of HVEC acted on HGCC, the expression level of KDR gene in HGCC was inhibited significantly (Figure 1).

The hVEGF levels in the CMs of HGCC and HVEC

The *h*VEGF level in the CMs of HGCC and HVEC was (92.06 ±1.69 ng/L), (77.70±8.04 ng/L) respectively.

DISCUSSION

An important advance in oncology is that the importance of the tumor angiogenesis in the tumor genesis, growth and metastases, and the importance of the vascular targeting therapy in the tumor treatment have been proved. One of the most important factors in the tumor angiogenesis is vascular endothelium growth factor(VEGF). VEGF can promote and maintain the establishment of tumor vascular system. And it can promote the tumor growth directly. VEGF can induce the mitogenesis and chemotaxis of vascular endothelial cell(VEC)

and tumor cell(TC) intensely. Almost all types of TC and tumor VEC can secret VEGF. But the expression of VEGF in the normal tissue is very low. In the four VEGF receptors, R_2: KDR is the main receptor which gives play to VEGF functions, while other receptors play little role in cell growth. KDR is highly expressed on the TC and tumor VEC while lowly expressed on the normal tissues. In order to make clear the role of KDR in regulating the growth of HGCC and HVEC, We devised the antisense oligodexynucleotide(ASODN) specific to KDR mRNA. There has been no one to devise KDR ASODN up to now. The results showed that KDR ASODN could inhibit the growth and expression of KDR gene of HGCC and HVEC significantly. There was a great difference between the effects of ASODN and SODN, MODN. The CMs of two cells had a certain level of *h*VEGF. This illustrated that KDR ASODN could really enter into the cells and specifically block the expression of KDR gene to interrupt the selfscrine and parascrine growth-stimulation pathway of VEGF. The results showed that VEGF and its receptor KDR played a key role in regulating the proliferation of HGCC and HVEC.

In the human solid neoplasm, there exist many kinds of cells besides the tumor cell, such as: interstitial cell,immunocyte and vascular cell. They depend on each other to contribute to the tumor genesis, development, invasion and metastasis. The interactions among them are complicated. During the tumor angiogenesis and tumor hematogenous metastasis, both VEC and TC contribute to finishing the pathology processes. The interactions between them act in close coordination in establishing tumor vascular system and finishing hematogenous metastasis. There exist very complicated interrelations between TC and VEC. In the pre-angiogenesis, how does TC induce VEC to establish the tumor vascular system? That is, how does TC influence the proliferation, degeneration, morphogenesis and functions of its neighboring VEC? Or, how does VEC influence these characters of its neighboring TC? These are all unclear. On the other hand, the interactions between two cells are involved in the tumor infiltration and hematogenous metastasis. Tumor vascular provides the passage for tumor infiltration and metastasis. The integrated vascular endothelium is the barrier to the tumor infiltration and metastasis. How do these two kinds of cells interact reciprocally to render the tumor cell to adhere to and destroy the vascular endothelium to enter vascular lumen and damage it again to enter stroma? There exist some complicated mechanisms in this process. The study of tumor angiogenesis mainly focuses on the interactions among the vascular component cells while the study of tumor invasion and metastasis mainly focuses on the interactions between TC and surrounding stroma. Seldom has anyone noticed the interactions between TC and VEC. The relations between TC and VEC are not clear. Studies on this aspect are very few. Studies abroad always choose melanoma, glioblastoma, cephalo-cervical squamous carcinoma and hepatocellular carcinoma [4-19], but not gastric carcinoma as the target research yet. And the results are also controversial. We have not found anyone who studies gastric carcinoma yet. There are lots of studies on gastric carcinoma [20-45], but seldom in this aspect. To better understand some mechanisms in gastric carcinoma angiogenesis and hematogenous metastases, we select HGCC and HVEC to study their interrelations and the mechanisms. Considering the results showed that KDR was an important regulator to the growth of HGCC and HVEC, we study the role of KDR in the interactions between the two cells.

Results showed that CMs of HGCC with different confluent state, different preparing periods, different volume fraction and different action periods stimulated the growth of HVEC significantly. The activity of CM of subconfluent cell was stronger than that of CM of confluent cell. The activity of CM preparing for 24 h was stronger than that of CM preparing for 48 h. After the nutrition exhaustion was replenished, the more of the volume fraction, the stronger of the activity. This is consistent with the results of some studies. Someone found CM of bladder carcinoma stimulated the growth of HVEC. Others found that cephalo-cervical squamous carcinoma cell stimulated the growth of HVEC through secreting FGF and VEGF. But there were other contrary viewpoints. Zhao found bladder carcinoma cell inhibited the growth of HVEC through a 10-16bp tRNA fragment. Albini found some kinds of tumor cells inhibited HVEC to form vascular through secreting IFN-γ. There was also a neutral objection: TC has little effect on the proliferation of HVEC. Some researchers found that although TC had no effect on the growth of HVEC, TC could change morphology of HVEC or its sensitivity to TNF-α. we think these different results are due to different cell types. Results showed that HGCC could secret a certain level of *h*VEGF. CM of HGCC could up-regulate the expression of KDR gene in HVEC. It illustrated that KDR played an important role in the growth-stimulation of HGCC to HVEC. It needs further study to show if there exist other factors and mechanisms involved in this effect of HGCC on HVEC.

Our results also showed that CM of HVEC could significantly inhibit the growth of HGCC. The activity of CM of subconfluent cell was stronger than that of CM of confluent cell. After the nutrition exhaustion was replenished, the more of the volume fraction, the stronger of the activity. We do not know what this inhibition means exactly. Maybe in the tumor angiogenesis, the inhibition could prevent HGCC to occupy the place of vascular or participate into the vascularition. Then the stimulation of vascular system to growth of gastric carcinoma is not through the direct interaction between HGCC and HVEC, but the establishment of the vascular system passes nutrition to HGCC and excretes its metabolism waste. HVEC also secreted a certain level of *h*VEGF and VEGF could stimulate the proliferation of HGCC. But CM of HVEC inhibited the growth of HGCC and the KDR gene expression in HGCC. Although HVEC produced growth-stimulator, VEGF, HVEC could interrupt the role of VEGF on HGCC through reducing the level of its main functional receptor,KDR. It illustrated that KDR play an important role in the growth-inhibition of HVEC to HGCC. Whether HVEC secretes some other growth-inhibiting factors to inhibit the proliferation of HGCC or not needs further study.

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