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Multiple drug resistance phenotype of human endothelial cells induced by vascular endothelial growth factor 165[1]

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KEY WORDS endothelial growth factors; endothelium; multiple drug resistance; apoptosis; cytotoxicity; drug therapy; Western blotting; polymerase chain reaction; electrophoresis

ABSTRACT

AIM: To investigate the effect of vascular endothelial growth factor 165 ($VEGF_{165}$) on sensitivity of endothelial cells to anticancer drugs. **METHODS**: Human dermal microvessel endothelial cells (HDMEC) were incubated with anticancer drugs in the presence of $VEGF_{165}$. Survival of endothelial cells was assayed by MTT method. DNA fragments of apoptosis were detected by agarose electrophoresis. Potential mechanisms underlying the effect of $VEGF_{165}$ on endothelial cells were investigated with RT-PCR and Western blot analysis. **RESULTS**: $VEGF_{165}$ induced the multidrug resistance phenotype of HDMEC to a wide variety of anticancer drugs such as epirubicin, cisplatin, etoposide, mytomycin C, vincristine, CPT-11, and taxol *in vitro*. This protective effect was partly due to the up-regulation of lung drug resistance protein (LRP) and multidrug resistance-associated protein (MRP), as well as the down-regulation of Bax protein induced by $VEGF_{165}$. **CONCLUSION**: $VEGF_{165}$ induced multidrug resistance phenotype of endothelial cells, which implicated the anti-angiogenic effect of anticancer drugs might depend on microenvironment of tumors *in vivo*.

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INTRODUCTION

Growth and metastasis of solid tumors depend on angiogenesis, the process leading to the formation of new blood vessels. Suppression of angiogenesis induces apoptosis of tumor cells *in vivo*. The endothelial cells lining in tumor vessels are normal cells, revealing stable genotype and being exposed to drugs directly. Therefore, targeting endothelial cells is superior to targeting tumor cells theoretically. This concept promoted the research on anti-angiogenic agents such as TNP-470, angiostatin, and endostatin[1-4]. Up to now, since inhibitors of angiogenesis are not commercially available for clinical usage, attention has been focused on the anti-angiogenic potential of conventional anticancer drugs[5]. Anticancer drugs such as taxol, vincristine, vinblastine, 2-methoxyestradial, bleomycin, epirubicin, doxorubicin, bripirimine, and linomide have the potential to induce apoptosis of endothelial cells *in vitro* and inhibite angiogenesis *in vivo*[6-8]. However, no evidence showed that the anti-angiogenic effect of epirubincin and doxorubicin is involved in the response of chemotherapy in breast cancers[9]. More evidence was needed to support the development of cytotoxic agents as angiogenesis inhibitors.

Tumor angiogenesis is under the control of angiogenic growth factors and anti-angiogenic growth factors secreted by tumor cells and stroma cells. Vascular endothelial growth factor (VEGF) plays an important role in this process. VEGF is a poly-functional protein containing at least four isoforms, eg, $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$, which stimulate the mitogenesis and motion of endothelial cells (ECs), increase the permeability of microvessels and protect endothelial cells from apoptosis induced by TNF-α[10]. In addition, VEGF receptors, flt-1 and KDR, are also expressed in hematopoietic stem cells. $VEGF_{165}$ can protect hematopoietic stem cells from apoptosis induced by etopo-

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side, doxorubicin, and γ-ray radiation[11,12]. Enlightened by the protective effect of $VEGF_{165}$ on EC and hematopoietic stem cells, this study was designed to testify whether $VEGF_{165}$ modify the sensitivity of EC to anticancer drugs.

MATERIALS AND METHODS

Drugs and cell line Commercially available anticancer drugs including epirubicin (Farmitalia Carlo Erba, Milan, Italy), vincristine (Eli Lilly, Indianopolis, USA), mitomycin C (Kyowa Hakko Kogyo, Tokyo, Japan), cisplatin (David Bull Laboratories, Vaudruil, Canada), 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxy camptothecin (CPT-11) (Rhne-Poulenc Rorer, Antony, France), taxol (Bristol-Myers Squibb, New York, USA), and etoposide (Clontech, Palo Alto, USA) were dissolved in tissue culture medium before use. Recombinant human $VEGF_{165}$ was obtained from Pepro Tech EC Ltd, London, England. Human dermal microvessel endothelial cells (HDMEC) was prepared as previous description[13]. Cells were maintained in MCDB131 (Sigma, St Louis, USA) supplemented with 10 % fetal bovine serum (Gibco BRL, Rockville, USA), $VEGF_{165}$ 5 μg/L, and antibiotics in a humidified atmosphere containing 5 % carbon dioxide at 37 ℃.

Cytotoxicity of anticancer drugs to HDMEC Drug sensitivity of HDMEC to anticancer drugs was measured by MTT assay. The percentage of inhibition rate was defined as: [1-(absorption of the treated well/absorption of the control well)] × 100 %. The 50 % inhibitory concentration (IC_{50}) was calculated by Bliss software. Cell apoptosis was detected by DNA ladder electrophoresis. All drugs were tested in triplicate wells and the experiments were repeated at least four times.

Expression of drug resistance-associated genes The isolation of total RNA (Gibco BRL, Rockville, USA) and RT-PCR analysis followed the standard method. Primers used in this study included 5′-CCCATCATTGCAATAGCAGG-3′ and 5′-GTTCAAACTTCTGCTCCTGA-3′ for multidrug resistance gene 1 (MDR-1, 157-bp fragment), 5′-CAGGCCATCCCTCTAGACGAG-3′ and 5′-GGTAGCTGACCACACGGGTCT-3′ for lung drug resistance protein (LRP, 250-bp fragment), 5′-TGAAGGACTTCGTGTCAGCC-3′ and 5′-GTCCATGATGGTGTTGAGCC-3′ for multidrug resistance-associated protein (MRP, 257-bp fragment), 5′-ACAGGTGGAGGCAAATCTTCG-3′ and 5′-GAATGACCCTGTTAATCCGTTCG-3′ for breast cancer resistance protein (BCRP, 253-bp fragment), 5′-ATCCCATCACCATCTTCCA-3′ and 5′-CCTGCTTCACCACTTTCTTG-3′ for internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 580-bp fragment). RT-PCR productions were subjected to electrophoresis through 2 % agarose.

Expression of Bax and Bcl-2 proteins Western blot analysis was used to examine the expression of Bax and Bcl-2 protein. Nitrocellulose membrane was incubated with polyclonal rabbit antibody against human Bax (1∶300) and mouse antibody against human Bcl-2 (1∶50) goat antibody (Santa Cruz, USA) respectively. After incubation with horseradish peroxidase-conjuncted secondary antibodies, protein bands were visioned with enhanced chemoluminescence kit (Amersham, Piscataway, USA).

RESULTS

$VEGF_{165}$ increased survival of HDMEC treated with anticancer drugs In the presence of either 5 or 25 μg/L of $VEGF_{165}$, HDMEC was treated with anticancer drugs including epirubicin, cisplatin, etoposide, CPT-11, taxol, vincristine, and mytomycin C for 72 h, respectively. The results revealed that $VEGF_{165}$ protected HDMEC from cytotoxicity of these drugs. The protective effect of $VEGF_{165}$ varied among them. $VEGF_{165}$ showed potent protection of HDMEC from epirubicin, intermediate protection from cisplatin, CPT-11, taxol, and mytomycin C, weak protection from etoposide and vincristine (Tab 1).

Tab 1. Protective effect of $VEGF_{165}$ on HDMEC from anticancer drugs. $n = 4$ independent experiments. $\bar{x} \pm s$.

Drug	IC_{50}/mg·L^{-1}		RR[1)]
	$VEGF_{165}$ 5 μg·L^{-1}	$VEGF_{165}$ 25 μg·L^{-1}	
Epirubicin	0.5±0.4	9.5±2.1	19.1
Taxol	1.6±0.3	7.6±1.2	4.9
Cisplatin	2.8±0.7	9.4±0.6	3.4
CPT-11	6.9±1.4	21.0±2.3	3.1
Mytomycin C	2.6±0.5	6.1±1.2	3.0
Etoposide	3.4±0.5	7.7±1.9	2.2
Vincristine	3.5±1.5	6.2±1.4	1.8

1): Relative resistance, the ratio of IC_{50} for HDMEC in the presence of $VEGF_{165}$ 25 μg·L^{-1} to IC_{50} in the presence of $VEGF_{165}$ 5 μg·L^{-1}.

$VEGF_{165}$ protected HDMEC from apoptosis induced by epirubicin Since $VEGF_{165}$ showed the

most potent protection of HDMEC from cytotoxicity of epirubicin, epirubucin was used to damage HDMEC, to investigate mechanism of the protective effect of $VEGF_{165}$. Results showed that $VEGF_{165}$ 25 μg/L prevented HDMEC from apoptosis induced by epirubicin 1 mg/L after 24 h of incubation (Fig 1).

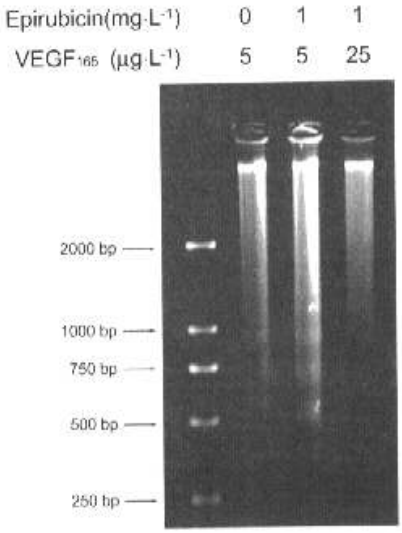

Fig 1. Protective effect of $VEGF_{165}$ on HDMEC. HDMEC was incubated with epirubicin and $VEGF_{165}$ for 24 h. $VEGF_{165}$ 25 μg/L protected HDMEC from apoptosis induced by epirubicin 1 mg/L.

$VEGF_{165}$ induced expression of drug resistance genes In the presence of epirubicin 1 mg/L, HDMEC was incubated with either $VEGF_{165}$ 5 or 25 μg/L for 24 h. RT-PCR analysis revealed that $VEGF_{165}$ induced expression of LRP and MRP, except for BCRP (Fig 2). In addition, HDMEC constitutively expressed MDR-1 gene, $VEGF_{165}$ could not modulate the expression of MDR-1.

$VEGF_{165}$ reduced expression of Bax In the presence of epirubicin 1 mg/L HDMEC was incubated with either $VEGF_{165}$ 5 or 25 μg/L for 24 h. Western blot analysis revealed that expression of Bax protein progressively decreased during the 24-h incubation, although the alteration of Bcl-2 protein was not observed (Fig 3).

DISCUSSION

This study showed that $VEGF_{165}$ induced resistance of EC to diverse anticancer drugs including epirubicin, CPT-11, taxol, vincristine, cisplatin, etoposide, and

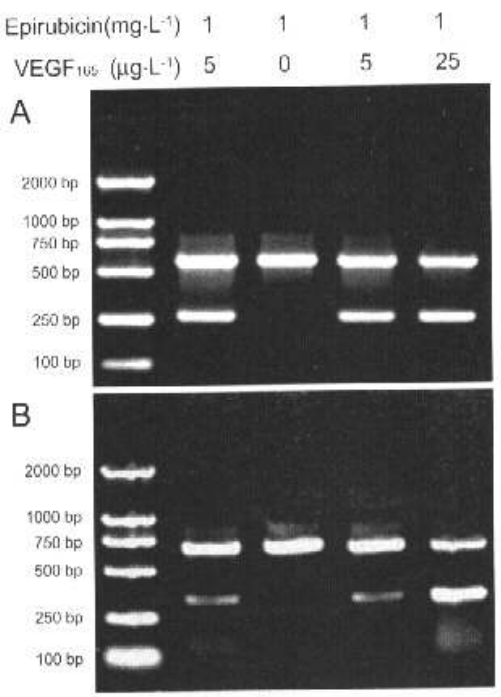

Fig 2. Expression of LRP and MRP induced by $VEGF_{165}$. HDMEC was incubated with epirubicin and $VEGF_{165}$ for 24 h. $VEGF_{165}$ induced the expression of LRP (A) and MRP (B) regardless of the presence of epirubicin.

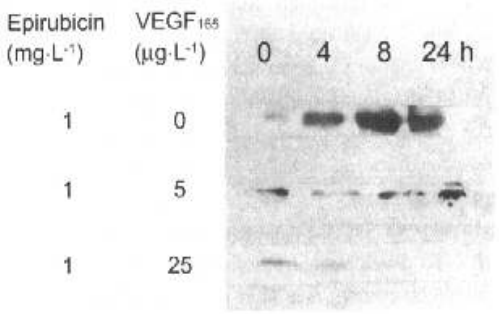

Fig 3. Effect of $VEGF_{165}$ on the expression of Bax protein. HDMEC was incubated with epirubicin and $VEGF_{165}$ for 24 h. $VEGF_{165}$ progressively inhibited the expression of Bax protein induced by epirubicin.

mytomycin C. Among them $VEGF_{165}$ revealed potent protection of HDMEC from epirubicin, intermediate protection from cisplatin, CPT-11, taxol, and mytomycin C, weak protection from etoposide and vincristine.

The mechanism underlying multidrug resistance phenotype of EC induced by $VEGF_{165}$ is unknown. One of

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the possible mechanism is the expression of drug resistance genes. LRP, MRP, and P-glycoprotein are energy-dependent efflux pumps, their substrates include the anthracyclines, the vica alkaloids, the epipodphyllotoxin, taxols, *etc*. P-Glycoprotein and MRP are membrane-bound proteins extruding substrates from the inside of cell while LRP plays a role in nucleo-cytoplasmic transport[14-17]. HDMEC constitutively expressed MDR-1 gene and $VEGF_{165}$ could not modulate its expression, suggesting that the expression of MDR-1 gene was not related to the protective effect of $VEGF_{165}$. However, $VEGF_{165}$ induced the expression of MRP and LRP, paralleling to the protective effect of $VEGF_{165}$, which implicated that MRP and LRP were involved in this drug resistance phenotype. It was intriguing that $VEGF_{165}$ also protected HDMEC from the cytotoxicity of cisplatin, mytomycin C, and CPT-11, in which MRP and LRP did not relate to their drug resistance phenotype[18,19]. So alternative mechanism of drug resistance may exist.

Anticancer agents can induce apoptosis and the ratio of Bax to Bcl-2 is the check point upstream of caspases cascade[20,21]. In this study, $VEGF_{165}$ decreased the expression of Bax induced by epirubicin, it was conceivable that $VEGF_{165}$ modified the ratio of Bax to Bcl-2, diminished the death signal, and then suppressed the toxicity of chemotherapy on HDMEC.

Tumor cells are subjected to chronic and acute hypoxia[22]. Chemotherapy would enhance hypoxia of tumor cells[23] and then hypoxia hasten expression of VEGF[24], so residual tumor cells up-regulate expression of VEGF during chemotherapy. This study showed evidence that $VEGF_{165}$ protected ECs from a wide variety of anticancer drugs *in vitro*, it was reasonable to infer that the anti-angiogenic effect of conventional anticancer drugs might depend on the microenvironment of tumors *in vivo*. To develop angiogenesis inhibitors, it was worthy of researching alternative kind of agents which could circumvent the protective effect of VEGF on endothelium.

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血管内皮生长因子 165 诱导人血管内皮细胞多药耐药表型[1]

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关键词 内皮生长因子；内皮；多种抗药性；细胞凋亡；细胞毒性；药物疗法；蛋白质印迹；聚合酶链反应；电泳

目的：研究血管内皮生长因子 165 ($VEGF_{165}$)对血管内皮细胞药物敏感性的影响. **方法**：人真皮微血管内皮细胞(HDMEC)与抗癌药物和 $VEGF_{165}$混合培养，MTT 法分析药敏变化，琼脂糖电泳检测细胞凋亡，RT-PCR、免疫印迹法分析 HDMEC 产生多药耐药表型的机制. **结果**：$VEGF_{165}$体外诱导 HDMEC 对多种抗癌药物耐药，如表柔比星、顺铂、足叶乙甙、丝裂霉素 C、长春新碱、CPT-11、泰素等，其机制与 $VEGF_{165}$诱导 HDMEC 上调表达多药耐药相关蛋白和肺耐药相关蛋以及下调表达 Bax 蛋白有关. **结论**：$VEGF_{165}$诱导血管内皮细胞的多药耐药表型，提示化疗时抗癌药物的抗血管生成活性可能取决于肿瘤微环境.

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