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EXPRESSION OF VEGF RECEPTOR KDR IN DIFFERENT ORIGINATED CARCINOMAS*

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Objective: To detect the expression of KDR in different originated carcinomas and to explore its expressed ways and the relationship with tumor progression. Methods: KDR cDNA (V-VII domains) fragment was cloned from human umbilical vein with RT-PCR and was expressed in Ecoli.Jm109. The fusion protein of GST-KDR was used for immunizing Balb/c mice to prepare monoclonal antibodies against KDR. The different tumor tissues and related normal tissues were examined with KDR McAb by S-P immuno-histochemistry. Results: the rate and intensification of KDR expression among different originated cancers are very different, bladder cancers from transmigrated epidermis are 100% positive and highest intensification. The expression of KDR in breast cancer and intestinal cancer lie in the second-rate, the weakest expression of KDR is in lung squamous carcinoma. Moreover, expression of KDR in tumor tissues lie both in endothelial cells (EC) of tumor blood vessels and tumor cells. Conclusion: VEGF may be not only the para-secretory factor making EC proliferation but also auto-secretory factor stimulating the proliferation of tumor cells to benefit the growth and metastasis of malignant tumors. The different expression of KDR in different originated carcinomas may relate with malignant degree of tumor.

Key words: VEGF receptor II (KDR), Expression, Tumor.

Angiogenesis is the prerequisite of solid tumor growth.[1] During the process of tumor progression, tumor cells can secrete various angiogenic factors such as bFGF, PDGF, VEGF and TGF-β etc., which stimulate the formation of tumor blood vessels. Among these angiogenic factors, VEGF, which plays an important role in the tumor angiogenesis, is the direct and pivotal factor. Studies show that VEGF is expressed in various tumor tissues and tumor cell lines.[2,3]

KDR is one of the main receptors of VEGF, which medicate the biological responses of VEGF. Generally thinking, KDR and flt-1 are selectively expressed on endothelium.[4] However, it's reported recently that KDR is also expressed on macrophages and some hemocytoblast.[5] Boocock et al.,[6] demonstrated, by RT-PCR, the expression of KDR and flt-1 in ovarian carcinoma cell lines. They also found that VEGF receptor expression not only in the vascular endothelial cells but also in the tumor cells in ovarian carcinoma tissues.[6] The results raise the possibility that VEGF may act not only as an angiogenic factor but also by autocrine or paracrine mechanisms on receptor-expressing tumor cell to stimulate the growth of tumor cells. To detect the expression of KDR in different originated tumors, we cloned the extra-cellular V-VII domains of KDR from umbilical vein endothelial cell and prepare its monoclonal antibodies, which were used to investigate the KDR expression in different originated tumor tissues.

MATERIALS AND METHODS

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Isolation of Human Umbilical Vein Endothelial Cells (HUVEC) and RT-PCR

HUVEC was isolated according to the described method.[7] Total RNA was extracted from cultured EC cells by RNA extraction Kit (GIOTRX Co. USA). According to KDR DNA sequence, 5'- primer: 5'-TAAGGATCCCACTCAAACGCTGAC-3' and 3'-primers: 3'-1: 5'GGAGAATTCTCAACTGC ATG-CCTGGCAG-3' and 3'-2: 5'-TCCTGGGCACCTT-CTA-3' were designed and synthesized. M-ALV reverse transcriptase (GIBIO) was used to synthesize complementary DNA (cDNA) from total RNA primed with 3'-2 prime. KDR (V-VII) DNA fragment was amplified with 2.5 μl cDNA as template and 5'-1 and 3'-1 as specific primes. The condition of amplification as follows: 94°C, 45 seconds, 48°C 1 minute, 72°C 1.5 minute. After two cycles, the annealing temperature was raised to 60°C from 48°C and carried out 30cycles. The product of PCR was analyzed with endorestric-nuclase mapping (*Bam*HI/*Eco*RI) and sequencing.

Recombinant and Expression of KDR Extra-cellular V-VII Domains

After being digested with *Bam*HI/*Eco*RI, KDR DNA fragment was extracted with DNA purified kit (QIAGEN) and cloned in the GST express vector pGEX2T (*Bam*HI/*Eco*RI). Ecoli.Jm109 Bacteria was used for transformation. Positive clones were selected by protein expression and the analysis of digested plasmid DNA .The desired clone was cultured in LB (Amp^+) 37°C overnight and then were induced by IPTG (0.5 mM). Foreign expressed fusion protein GST-KDR was extracted from induced bacteria by basic denature method and was purified by SDS-PAGE gel and elect-elution method.

Preparation of KDR (V-VII) Monoclonal Antibodies

Balb/c mice were immunized subcutaneously with the purified fusion protein, GST-KDR, (30 μg/ injection) in complete Freund's adjuvant. Three weeks later, the mice were boosted 3 times at intervals with the same amount of respective antigen in incomplete adjuvant. Three days after the final injection in mice tail vein, mice were sacrificed and spleen cells were fused with mouse sp 2/0 myeloma cells at a ratio of 10:1 as described previously.

Positive hybridoma cell clones were identified by rapid ELISA two screening, with respectively recombinant fusion protein GST-KDR and GST-P21 as antigen. Immunohistochemistry was used to select the positive clones.

Immunoblot for Identification of KDR McAb

Western blot analysis was performed according to established methods. 5ug GST-KDR protein were analyzed under SDS-PAGE and transferred to nitro-cellulose membrane, blocked with PBS containing 1% BSA, and incubated with KDR McAb SSW2 (1 μg/ml in PBS). Immunoreactive bands were visualized by labeled secondary antibodies and OPD as the chromogenic substrate.

Expression of KDR in Different Originated Tumors

One hundred and fifteen cases paraffin-embedded tumor specimens were collected from Department of Pathology at the Beijing Institute for Cancer Research (25 cases lung carcinomas, 30 cases breast cancers and 25 cases intestinal carcinomas) and from Institute of Urinology at Beijing Medical University (35 cases bladder carcinomas). All specimens were cut into two sections at consecutive 4-um section. One section was stained with hematoxytin and erosin, and additional sections were immunostained for KDR with KDR McAb SSW2.

Immounohistochemical staining was performed by Biotin-Streptavidin immounoperoxidase technique following blocking native oxidase with 0.3% H_2O_2 (methanol dilution) and handling with microwave oven in 0.01M Sodium Citrate buffer (PH6.0) for 5-10 min then respectively add KDR McAb, Biotin-conjugated second antibody and Streptavidin-peroxidase were added respectively, and the sections were colored with DAB (0.5 mg/ml PBS plus 1 μl 30% H_2O_2/1 ml DAB).

RESULTS

Amplification and Identification of KDR (V-VII) cDNA Fragment

KDR (V-VII) cDNA was amplificated under 3.75 mM concentration of Mg^{++}, after 30 circles PCR. Following 1% agarose gel analysis, there was a clear band about 900 bp, which tally with the ideal data

(Figure 1).

The vector pGEX2T -KDR constructed with specific KDR V-VII cDNA fragment can release about 900bp band after *Bam*HI/*Eco*R I digestion (Figure 1).

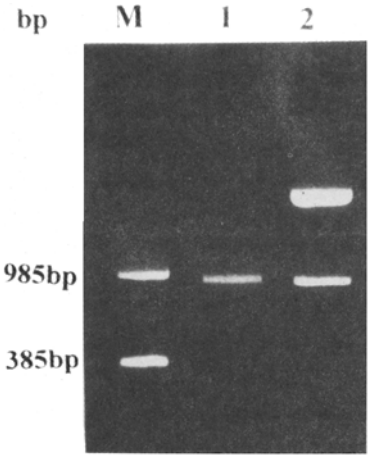

Fig. 1. RT-PCR of KDR gene and its identification of cloning in plasmid pGEX2T. M. DNA marker. 1. RT-PCR product of KDR V-VII. 2. pGEX2T-KDR digested with *Bam*HI/*Eco*RI.

Expression and Purification of KDR V-VII cDNA Fragment

After inducing by IPTG, the positive transformed Ecoli. Bacteria Jm 109 can stably express fusion protein GST-KDR. The proportion of expressed KDR in total bacteria protein is about 10%, molecular weight of KDR fusion protein is about 60 KD which presented as include body in bacteria. It was prepared and purified with basic denature protocol and SDS-PAGE gel (Figure 2).

Preparation and Identification fo KDR Monoclonal Antibodies

After fourth subcloning, twelve clones secreting KDR antibodies were obtained in which five clones were selected for preparing ascite. The KDR antibodies were purified through protein A affinity chromatography. Effect values of the ascites reach 1×10^{-6}. ELISA shown that the purified antibodies have strong reaction with KDR but not with GST.

Immunoreactivity of KDR McAb on vascular endothelial cells is also well. Western blot results suggested that purified KDR McAb SSW2 specifically reacted with KDR antigen (Figure 3).

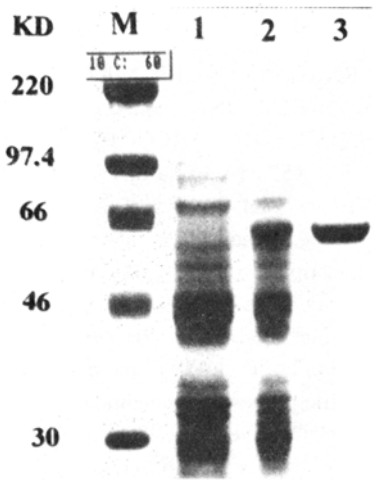

Fig. 2. The expression of GST-KDR in Ecolo. Jm 109 and purified GST-KDR fusion protein. M. Protein standards. 1. Bacterial proteins from bacteria transformed with pGEX2T-KDR uninduced with IPTG. 2. Bacterial protein from bacteria transformed with pGEX2T-KDR induced with IPTG. 3. Purified GST-KDR protein with basic denature and SDS-PAGE gel.

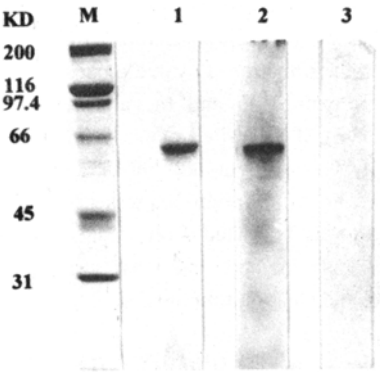

Fig. 3. Western blot results of KDR McAb with KDR protein. M. Standard molecular weight protein. 1. Positive reaction of SSW2 supernate with KDR. 2. Specific reaction of purified KDR McAb with KDR. 3. Negative control. Reaction of normal mouse IgG with KDR.

Expression of KDR in Different Tumor Tissues

One hundred and fifteen tumor specimens were

studied by immunohistochemistry for expression of VEGF receptor KDR. The results show that immunoreactivity of KDR was almost restricted to tumor cells and vascular endothelial cells (EC) in most tumor tissues, although rare lymphocytes and vascular smooth muscles within tumor stroma appeared to be positive as well. Among different originated tumors, 35 bladder tumor cells from transmigrant epithelium show 100% positive and the degree of KDR immunostaining is from middle to high. While intensity of KDR in EC of the tumor tissues appears low to middle reactivity, rare with high reactivity. Immunoreactivity of breast cancer cells and intestinal tumor cells from adenoepithelium appear little inferior to the bladder tumor cells, positive rates of which respectively 92% and 90%. While most EC in tumor stroma appear low reactivity. Some show middle reactivity. The immunoreactivity of lung cancer from squamous epithelium is much weaker than that from adenoepithelium and transmigrant-epithelium whose expression rate is about 77%.

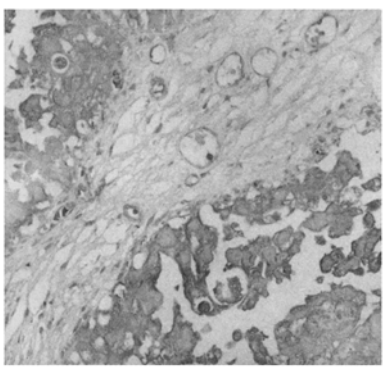

Fig. 4. Bladder cancer (20×10). Positive reactivity of KDR McAb with tumor cell and small vascular EC. Bladder tumor cell show high immunoreactivity, EC show weak reactivity.

DISCUSSION

Vascular endothelial cell growth factor (VEGF) is a secret sugar protein which not only stimulates endothelial cell growth and angiogenesis, but as a potent inducer of microvascular hyperpermeability as well. VEGF is synthesized and secreted by a variety of cultured tumor cell lines and expressed in most tumor tissues. In general knowledge, two known receptors of VEGF, KDR and flt-1 are located in the vascular endothelial cells (EC).[8] However, nonendothelial expression of VEGF receptors has been reported recently for invasive cell types including placental trophoblasts, macrophage and malignant melanoma cell lines.[5] The studies of Boocock et al.[6] indicated the presence of flt-1 and KDR mRNA in ovarian carcinoma cell lines and in tumor cells at primary ovarian carcinoma. All of this unexpected results raised the possibility that VEGF may act not only as an angiogenic factor but also more fundamentally as an autocrine or paracrine growth regulator, acting via receptors expressed by tumor cells.

Our results suggested no matter origination of the tumors, expression of KDR is not only restricted to EC in tumor tissue, but also in the tumor cells. Moreover, the intensity of KDR expression on tumor cells is almost higher than that in EC. Our studies demonstrated KDR was also expressed in vascular smooth muscle cells, some tissue cells such as macrophage in lung tumors, parts of lymphocytes in intestinal and bladder cancer besides in EC and tumor cells. These results indicated that VEGF is not only an angiogenic factor, its function is much more complicated than general believing. It may act via KDR expressed in tumor cells to stimulate the proliferation or motility of tumor cells and also improve the formation of tumor stromal matrix and local immune reaction via KDR expressed in macrophage, tissue cells and lymphocytes. Recently we also detected both KDR and VEGF expression by RT-PCR in Gastric adenocarcinoma cell line MGC803 which responded to recombinant VEGF165 (2 ng/ml) (data not shown). All our results are similar to that of Boocock which further suggest VEGF act as growth factor to affect the growth of tumor cell and formation of tumor stromal matrix via autocrine and paracrine passways, which also indicate the relationship between the proliferation of tumor cells and tumor stromal matrix.

Analysis of KDR expression on different tumors shows that immunoreactivities of KDR on different originated tumors are variable obviously. The bladder tumor cells from transmigrant epithelium express KDR exclusively and the degree of KDR immu-staining on bladder tumor cells is much higher than that of lung tumor. While breast cancer and intestinal cancer from adenoepithelium also have strong positive staining in tumor cells. The significance of different KDR expression on different originated tumor tissues is not very clear which need intensively studied.

To our knowledge, it is the first reported that KDR

expression is different on different originated tumors. Simultaneous coexpression of KDR on EC and tumor cells raises the possibility that VEGF secreted by tumor cells could not only facilitate their growth indirectly, via its effects on endothelial cells, but also directly via tumor cell receptor KDR. Therefore VEGF and its receptor KDR are potentially important targets for therapeutic strategies against tumor especially for bladder cancer.

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