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Construction and measuring combination of KDR-targeted ultrasound contrast agent in vitro for evaluating endometrial receptivity

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Summary

Objective: To investigate the preparation of a new kind of targeted lipid ultrasound contrast agent with anti-KDR antibody based on biotin-avidin bridge (MB-BAB-KDR) which could combine specifically with KDR that increases during the time of embryo implantation. Then its binding capability in vitro was evaluated. *Materials and Methods:* The agitation of high-speed method was employed to prepare biotin-microbubbles (MB-B), and biotin–avidin mediated technique was used to produce MB-BAB-KDR. MB-BAB-KDR, MB-B, and biotin-microbubbles-KDR (MB-B-KDR) were incubated with fluorescein-conjugated affiniPure goat anti-rat IgG (H+L) to assess the linked condition. Second, MB-BAB-KDR and control groups (MB-B and MB-B-KDR) were incubated with human umbilical vein endothelial cell (HUVEC). Rosette formation rate was observed and calculated. Then, the parallel plate flow chamber technology was used to access attachment efficiency to KDR Fc. *Results:* The surface of bubbles could carry KDR antibody through "biotin-avidin" bridge. After incubated with second antibody, bright green fluorescence (II grade) could be observed in MB-BAB-KDR group, as compared with weak fluorescence in control groups of MB-B (0 grade) and MB-B-KDR (I grade). The surrounding rosette formation rate on HUVEC was 89.86% in MB-BAB-KDR group and that of control groups were 7.13% (MB-B-KDR) and 3.02% (MB-B) ($p < 0.05$). The number of MB-BAB-KDR bound to KDR Fc increased as the KDR Fc density increased ($p < 0.05$). Under the same concentration, the MB-BAB-KDR bound to KDR Fc increased as time extended. *Conclusion:* The successful preparation of MB-BAB-KDR with anti-KDR antibody which shows specially targeting binding capability with HUVEC and stability in shear stress may be served as a noninvasive detection of endometrial vascular KDR expression and provide an experimental foundation for evaluating endometrial receptivity in vivo.

Key words: Targeted contrast; KDR; HUVEC; Parallel plate flow chamber; Endometrial receptivity.

Introduction

The human endometrium undergoes a complex series of proliferative and secretory changes in each menstrual cycle and displays only a short period of receptivity, known as the "window of implantation", necessary for the implantation of the blastocyst in uterus [1]. About 60% of failures in the process of in vitro fertilization-embryo transfer (IVF-ET) are due to defected endometrial receptivity [2]. Recent evidences show that the increasing vascular endothelial growth factor (VEGF) and its receptor-KDR, which can improve the vascular angiogenesis, remodeling, dilatation and permeability, as well as conduct the signal indication, are necessary in the successful embryo implantation [3-5]. However, detecting the expression level of VEGF and KDR to assess the endometrial receptivity, nowadays usually depends on the endometrial biopsy. Because of its invasiveness, it is suboptimal in clinical practice. In recent years, targeted contrast-enhanced ultrasound imaging, also named molecular ultrasound imaging has emerged as a promising new non-invasive imaging strategy for imaging biological processes at molecular level [6]. KDR is an ideal target for molecular imaging because of its overexpression and upregulation at the stage of the "window of implantation". In this study, the authors prepared the perfluorocarbon-filled lipid target microbubbles-biotin-avidin-biotin-KDR (MB-BAB-KDR), based on the biotin-avindin bridge, which could generate multilevel biological amplification to increase the KDR consistency around bubbles. Then WE used human umbilical vein endothelial cell (HUVEC) and the parallel plate flow chamber system [7] to characterize microbubbles special attachment efficiency which would be helpful in the prediction of in vivo adhesion behavior of targeted MB-BAB-KDR as well as its clinic applications in future.

Materials and Methods

Ordinary microbubbles-biotin (MB-B) preparation

DSPE-PEG2000-Biotin, DPPC, F68 and so on were mixed into distilled water and dissolved fully in the constant temperature (75°C) water bath and were shaken well with a determinate ratio [8]. The agitation of high-speed method was employed to prepare

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the perfluorocarbon-filled lipid microbubbles with biotin (MB-B) with piping into the whole fluorine propane (C3F8).

Construction of microbubbles carrying KDR

After being washed to remove excess free unincorporated lipid, streptavidin in a determinating ratio were added to MB-B. Then they were washed to remove excess free unincorporated streptavidin and the biotin conjugated rat anti-mouse-KDR monoclonal antibodies were added to prepare MB-BAB-KDR. At last, the MB-BAB-KDR was washed to remove excess free unincorporated antibodies. Biotin-microbubbles-KDR (MB-B-KDR) was made only by adding KDR directly into MB-B without streptavidin. Prepared MB-B, MB-B-KDR and MB-BAB-KDR, they were stored in refrigerator at 4°C.

Detection of property of microbubbles

The three types of microbubbles were observed under light microscope. The mean diameter and density in all MB-B, MB-B-KDR, and MB-BAB-KDR were measured by Coulter particle counting instrument.

Determination of biological activity of microbubbles

Fluorescein-conjugated affiniPure goat anti-rat IgG (H+L) was considered as the second antibody. MB-BAB-KDR was incubated with the second antibody to assess linked condition. With the MB-B and MB-B-KDR as control groups, all the three microbubbles were observed under fluorescence microscope after washing excess free second antibody. Fluorescence degree was observed under a fluorescence microscope. A fluorescent-based classification method was made on fluorescence degree. The fluorescence intensity of very bright, little bright, and not bright were defined as II, I, and 0 grade, respectively.

Detection of KDR expression of HUVEC

HUVEC in logarithmic growth phase were incubated with 200 µl biotin-KDR monoclonal antibody for 30 minutes at 4°C. Then they were washed three times and incubated with fluorescein-conjugated affiniPure goat anti-rat IgG (H+L) for 30 minutes. After being washed three times and added with 500 µl distilled water, they were analyzed with a flow cytometer, with the same amount of suspended HUVEC as in the control group called NC group.

Evaluation of specific binding capability of microbubbles

MB-BAB-KDR, MB-B-KDR, and MB-B labeled with FITC were incubated with HUVEC which were labeled by DiI for ten minutes. The excess free unincorporated microbubbles were washed away and rosette formation rate was observed and calculated under a light microscope and fluorescence microscope with 20 power field.

Flow-chamber adhesion

Thirty-five-mm Petri dishes were air-dried after methanol rinsing and were incubated with 200 µl KDR Fc with different concentrations (10 ng/ml, 100 ng/ml, 200 ng/ml, 600 ng/ml, 1000 ng/ml) in PBS all night under the condition of 4°C. The blocked group and the 0 ng/ml KDR Fc group were control groups. After being washed six times with 0.05% twain 20, they were closed by 3% of TBS-calf serum albumin liquid. Petri dishes were mounted on a parallel plate flow chamber with controlled gasket thickness and channel width. The flow chamber was placed in an inverted position on a microscope with a high-resolution charge-coupled device camera for video recording. A suspension of MB-BAB-KDR (3.0×10^6/ml) was drawn through the flow chamber with an adjustable withdrawal pump. The number of microbubbles attached to KDR FC was determined after six minutes of continuous flow at rates of 1.2 dyn/cm^2. Each flow chamber was imaged with a microscope. Sequences of images were sampled to collect the binding condition.

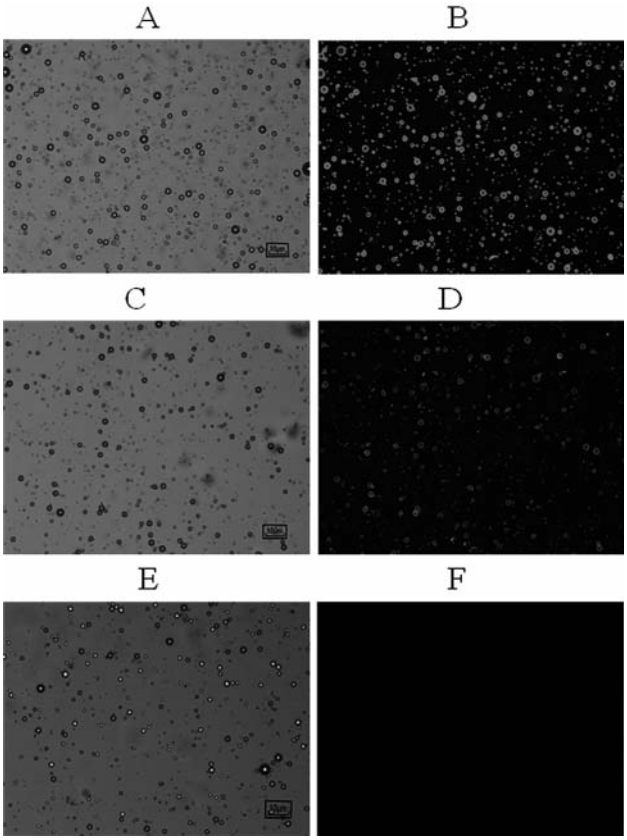

Figure 1. — Micro-bubbles incubated with fluorescein-conjugated AffiniPure Goat Anti-Rat IgG (H+L) under light microscope and fluorescence microscope. (×200). A - B: MB-BAB-KDR group; C - D: MB-B-KDR; E- F: MB-B group.

Statistical analysis

All the data were expressed as mean ± SD. K independent samples test was applied to compare the rosette formation rate among groups, ANOVA for repeated measures was applied to compare adhesion situation among groups, which were performed by the software SPSS 13.0. All $p < 0.05$ were considered as statistically significant.

Results

Properties of microbubbles

Microbubbles were observed under the microscope. Coulter particle counting instrument showed that the size of MB-B, MB-B-KDR, and MB-BAB-KDR, mainly 2.19±1.35 µm, 2.38±1.60 µm, and 2.42±1.71 µm. The density was about 9.8×10^8/ml, 9.6×10^8/ml, and 9.6×10^8/ml. There was no difference after the authors added biotin-avidin bridge and biotin-KDR antibody. All of three types of microbubbles were spherical and well-distributed.

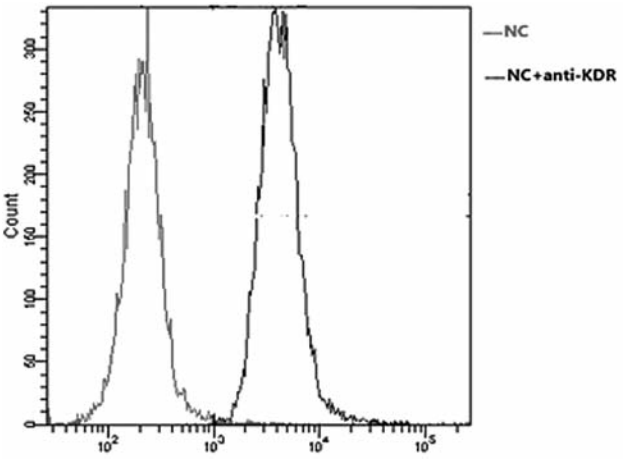

Figure 2. — The result of KDR expression on the HUVEC by flow cytometry (NC is the control group of cell background fluorescence, NC+anti-KDR is the experimental group).

Measurement of the biological combination

Bright green fluorescence (II grade) could be observed in MB-BAB-KDR incubated with second antibody, as compared with weak fluorescence in control groups (MB-B: 0 grade, MB-B-KDR: I grade) (Figure 1).

KDR expression

Control cell background fluorescence was NC group, the experimental group was HUVEC immune staining for anti - KDR, FITC HUVEC carrying rate was 99%, which expressed exoressin of KDR in HUVEC in the high status (Figure 2).

Specific binding capability

MB-BAB-KDR combined around or on the HUVEC and formed the wreath sample structure. The surrounding rosette formation rate on vascular endothelial cells was 89.86% (Figure 3), which were higher than the control groups: 7.13% (MB-B-KDR) and 3.02% (MB-B).There was a significant difference between MB - BAB - KDR group and the control groups, $p < 0.05$.

Adhesion studies

Shown in the flow shear stress of 1.2 dyn/cm2, the MB - BAB - KDR could combine to KDR Fc with a concentration of 1000 ng/ml, 600 ng/ml, 200 ng/ml, 100 ng/ml, and ten ng/ml groups. The recorded video was disposed with image pro-plus (IPP) software (Figure 4). Black hollows were stabilized adhesion MB - BAB - KDR and the trajectory of rolling were the mobile bubbles. MB - BAB - KDR combined with different concentration KDR Fc package and showed that adherence of MB-BAB-KDR increased with increasing KDR Fc density ($F = 571.926$, $p < 0.01$). Observed within six minutes the number of MB-BAB-KDR bound to KDR Fc increased as the time going. MB - BAB - KDR hardly combined with the blocked control microbubble group and the 0 ng/ml group. Throughout the six-minute period, there were only three to five totally combined on the flow chamber Petri dish and in different places and different time points, namely the combination of random and could not resist the shear force (Figure 5).

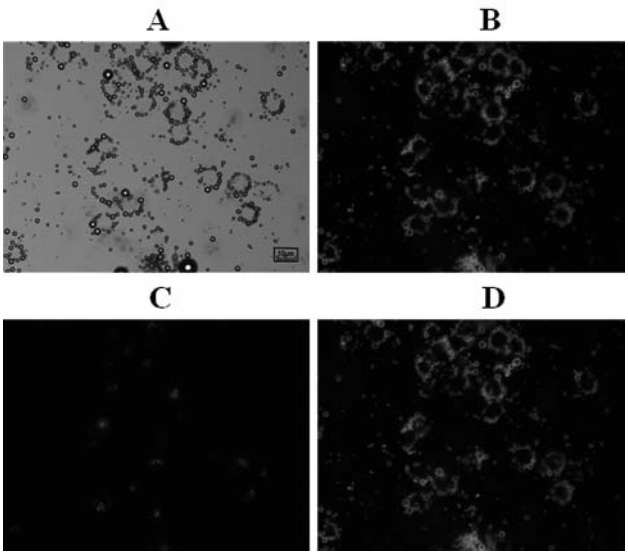

Figure 3 — MB-BAB-KDR combined around or on formed the HUVEC (×200). A: the wreath sample structure under light microscope; B: green fluorescence emitted by FITC-labeled MB-BAB-KDR under fluorescence microscope; C: red fluorescence emitted by DiI-labeled HUVEC; D: the wreath sample structure merged by C and D.

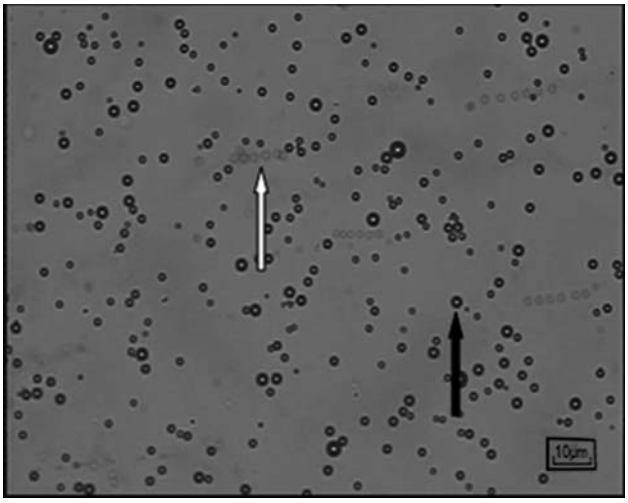

Figure 4. — MB-BAB-KDR could combine on KDR Fc package (Black hollow are stabilized adhesion MB-BAB-KDR and the trajectory of rolling tracks trailing are the mobile bubbles).

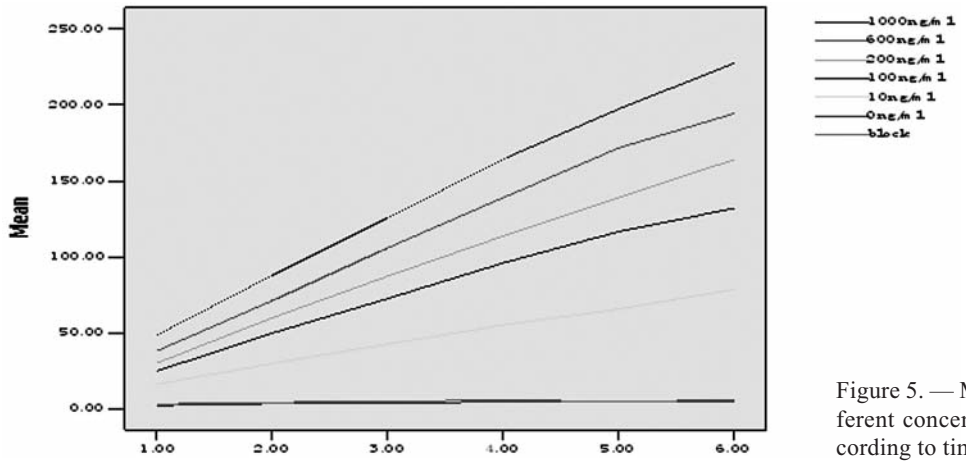

Figure 5. — MB-BAB-KDR combined on different concentration of KDR Fc package according to time.

Discussion

Targeted ultrasound imaging is an emerging research field of the burgeoning in recent years, and gradually becomes a hot topic in the world. Targeted contrast agent of microbubble carries the specific antibody or ligand which can combine with antigen or receptor on cell surface specially [9]. Ultrasonic testing targeted contrast agent, which distributes in tissues and organs, can obtain the molecular characteristics, so it is referred to as "ultrasonic molecular imaging" [10]. Along with the in-depth study of vascular endothelial cell function, recent studies have shown that ultrasonic molecular imaging research mainly focuses on the specific molecule expressed in the endothelial cells. Local echo is strengthened consecutively and succeeds in targeting imaging through the combination or aggregation of the ligand-carrying contrast agents with the specific molecular targets on the surface of endothelium. It shows great prospects in the research fields of cancer, atherosclerosis, thrombus, inflammation, and so on [11-14]. Some researchers have achieved good ultrasonic imaging on the new-born vascular region of tumors on mice by targeting KDR, the receptor of VEGF [11, 12].

Studies have found that the combination of VEGF and KDR exerts many biological functions on embryo implantation, such as vascular endothelial cells proliferation, angiogenesis, vascular permeability, signal transduction, changes of gene expression in endothelial cells, regulation of endometrial development, and so on [15-19], especially allocated in epithelial cell, stroma cell, myometrium, and vascular endothelial cells [20, 21]. Experiments showed that there was a significantly decrease in the pregnancy rate of the Rhesus monkey after administered VEGF antagonists five to ten days after ovulation [22]. At present, detecting the VEGF and KDR level in the endometrium depends on biopsy, which is not practical in the IVF-ET cycle because of is invasiveness.

It will be an simple and non-invasive way to access the endometrial receptivity relative factors-VEGF and KDR by using the targeted ultrasonic imaging technology, which takes the KDR of the endometrial vascular epithelial cell as targets. If quantitive analysis of the expression of KDR in the endometrium during the IVF-ET cycle is successful, it will avoid blind embryo transfers and increase the successful implantation rate. Meanwhile, it will reduce the economic and spiritual pressure of the patients.

In this study, biotin–avidin-mediated technique was used for the attachment of anti-mouse KDR monoclonal antibody to produce MB-BAB-KDR successfully. There was little effect on the consistency of microbubbles after the authors added biotin-avidin bridge and biotin-KDR antibody. Coulter Particle Counting instrument showed that the size and the density of MB-BAB-KDR were well-distributed; about 94.1% were smaller than five μm in diameter. After incubation with second antibody, the fluorescence intensity of MB-BAB-KDR group was brighter than MB-B-KDR and MB-B groups, which signified that biotin-avidin bridge mode not only could build a targeted microbubble successfully, but also could maintain the higher biological activity. In the in vitro experiment which tested the ability of targeting combination, MB-BAB-KDR could specifically combine with the HUVEC which with positive KDR expression and form the typical abundant garlands samples. That indicated again that MB-BAB-KDR can remain the activity and has the character of specific combination. Parallel Plate flow chamber is one of the important techniques to study the cellular mechanical behavior (especially the cell adhesion) in vitro, and its use of mimicking the blood flow situation can almost takes the place of in vivo experiments. This study applied the fluid mechanics calculation method to test the stability of the cell adhesion, under the condition of same velocity, which means in the same shear stress in Parallel Plate flow chamber. The human body microvessels and capillary blood flow shear stress is very small, generally less than 1.0 dyn/cm^2. The present experiment adopted the shear stress as 1.2 dyn/cm^2 and MB-BAB-

KDR stable adhesion was observed on the KDR Fc. With the increase of the concentration of antigen, targeted adsorption showed increasing trend, which makes it possible for microcirculation targeted molecular imaging of endometrium.

The present study constructed the targeted liposome microbubble MB-BAB-KDR successfully, tested the basic physical properties, proved its biological activity, targeting specific binding ability, and stability of adhesion in vitro. The study laid a foundation for a noninvasive and half-quantitative detection of KDR of endometrial endothelial cells and made an effect on promoting the targeted molecular imaging of other endometrial receptivity relating cytokines. All these achievements provided a technical support to further evaluate the targeted therapy and its effect, based on ultrasound mediating drug or gene which would improve the endometrial receptivity.

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