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— SHORT COMMUNICATION —

Chick yolk sac membrane assay: a novel angiogenesis model

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Many angiogenesis models have been developed so far, however most of them have a number of limitations such as requiring skilled operation and quite long testing times. Based on the conventional chick chorioallantoic membrane (CAM) angiogenesis assay, we developed a chick yolk sac membrane (YSM) model, which provides a simple and easy method *in vivo* for screening of angiogenesis-affecting drugs. Fertilized chick eggs (60 hours of incubation) were opened, and their contents were poured into beakers for 12 hour-incubation and then put the test solution into silicon rings laid on YSM for another 12 hours of incubation. The influences of the tested drugs on YSM angiogenesis were observed dynamically. The results showed that the YSM vascular growth was obviously inhibited by an angiogenesis inhibitor, the recombinant human angiostatin (rh-AS). Specially designed software makes YSM assay simple, timesaving and accurate.

Key words: yolk sac membrane (YSM), chorioallantoic membrane (CAM), angiogenesis model, silicon ring.

INTRODUCTION

Since Folkman (1971) proposed the anti-angiogenesis theory, a considerable progress has been made on the mechanism of tumor blood formation, endogenous angiogenic stimulators and inhibitors and the anti-angiogenic drug screening, most of which is relied on the methods for the proliferation of endothelial cells and angiogenesis *in vivo* and *in vitro* (Taraboletti & Giavazzi, 2004; Arsenou *et al.*, 2005). In quantitative and qualitative studies on angiogenesis *in vivo*, many test models have been adopted (Gimbroney, 1974; Gabison *et al.*, 2004; Tufan & Satiroglu-Tufan, 2005), among which the chick embryo chorioallantoic membrane (CAM) model is the most convenient and practical method widely used (Ribatti *et al.*, 2000, 2001). However, the CAM model has considerable limitations, such as i) designating and observing only a local vascular area, ii) difficulties in keeping the uniformity of the test site, iii) using of a drug adsorbing carrier which is apt to adhere and irritate the CAM, iv) hardly keeping the test area level and calm, and v) low contrast of CAM against its blood vessel to make direct observation difficult. Based on the chick CAM assay, we developed the chick embryo YSM angiogenesis model. In this study, rh-AS was used as an antiangiogenic drug to affect YSM angiogenesis, and glycerin was used as a penetrant to facilitate the absorption of the test drugs.

MATERIALS AND METHODS

Recombinant human angiostatin (rh-AS) was expressed and purified in our laboratory. All other chemicals used were commercial of analytical grade.

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Incubation of eggs

Fertilized eggs were purchased from a local hatchery. The eggs were incubated with blunt side up in a humidified 37.8 °C incubator for 60 hours. Under sterile conditions, the eggs were cleaned with 75% alcohol, carefully cracked, and their contents were poured into sterile glass beakers, which were caped with sterile Petri dishes and further incubated for 12 hours. Before cracking the egg contents into the beakers, small amount of Ringer solution is placed in the beakers and a spoon can be used to help the embryo disks turn upside, if necessary.

Selection of chick embryos

The incubated embryos in the beakers have intact YSM, the embryonic disks are towards upside and in the center of the yolk level, the embryos developed well with heart beat strong, and the YSM vascular net circle nearly round were chosen for YSM assay.

Anti-angiogenesis assay

A sterile silicon ring (OD 26 mm, ID 22 mm) was placed on the center of each YSM to make an isolated circle. A circular depression under the ring was formed due to the weight of the ring in which the test solution could be placed (Fig. 1). Embryos were randomly divided into groups, at least 10 embryos per group. 200 μl of the control solution (8% glycerin in 20 mM PBS, pH = 7.2) or the test solution (10, 100 and 1000 μg ml^{-1} rh-AS in the control solution) were slowly applied into each silicon ring on the YSM, incubated at the above conditions except for removing the humidity pan for 12 hours, and then carefully removing the silicon rings.

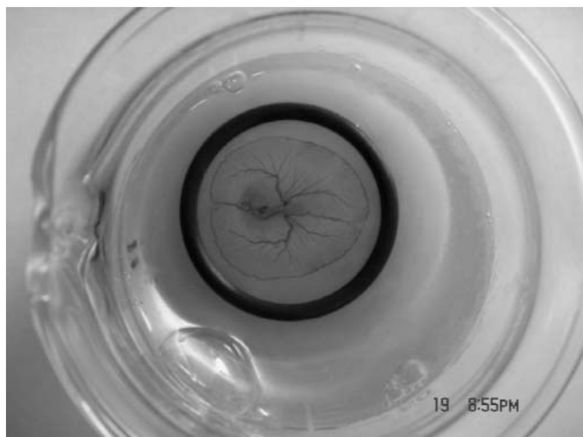

FIG. 1. A sterile silicon ring (OD 26 mm, ID 22 mm) placed on the center of the YSM to make an isolated circle. The test solution was applied into the silicon ring.

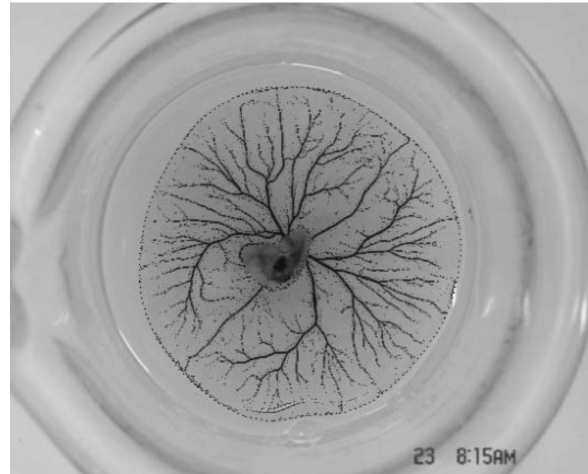

FIG. 2. The operation interface of the image analysis software. The measured data from this figure are given below. Embryo: perimeter 10.81 mm, area 6.90 mm^2, shape coefficient 1.16. Net CAM: perimeter 54.90 mm, area 236.01 mm^2, shape coefficient 1.01. Blood vessel area: 28.36 mm^2. Blood vessel density: 12.38%.

Morphological observation

Incubated embryos were dynamically observed for the development of a YSM vascular net every 4 hours, photographs were taken with a digital camera (5 Mpixel, SONY).

Statistical analysis

A self-designed image analysis software was used to analyze the changes of the YSM area, the total YSM vascular area and the density. The embryo-occupied area on the center of the YSM was deleted from the interface of the image before the analysis (Fig. 2). Experimental results were obtained from the equation bellow:

$$I = (A - B / B) 100\%$$

where, I is the increase rate of the YSM vascular area or the YSM area or the YSM vascular density, A is the total vascular area or the YSM area or the YSM vascular density after administration, B is the same as A but before administration. The data were evaluated against control using the t-test.

RESULTS AND DISCUSSION

Morphological observations

In this assay, rh-AS was chosen to act on the growing blood vessel of the chick embryo YSM. After application (12-16 hours), there were remarkable differences between the control and the test groups in the

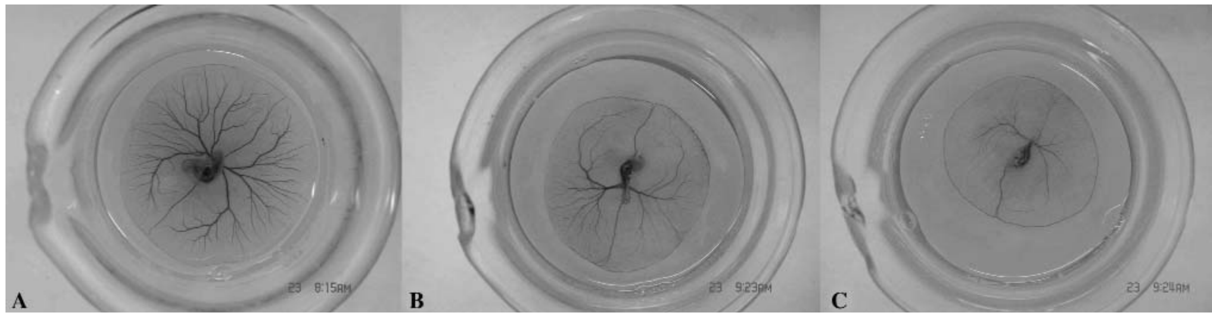

FIG. 3. The effects of rh-AS on angiostasis of chicken embryo YSM since administration (12-16 hours). A. Control, B. rh-AS 20 μg/egg, C. rh-AS 200 μg/egg.

morphology of the YSM blood vessels. The whole vascular net was growing normally in the control groups with the blood vessels filled well, and the vascular branches were dense and symmetrically distributed. In the rh-AS 20 μg/egg groups, the YSM vascular net showed a developmental retardation, blood vessel branches were much less than those of the control. In the rh-AS 200 μg/egg groups, the YSM blood vessels grew slowly and atrophied with the vascular branches sparse and disorderly, especially in the peripheral vascular area (Fig. 3).

Image and statistical analyses

The quantitative analysis of the effect of the rh-AS on chick embryo angiogenesis was performed with special image analysis software (Fig. 2). The increasing rate of the YSM blood vessel area, the blood vessel density and the YSM area acted as quantitative criteria for the angiogenesis. The increasing rate of the YSM blood vessel density in the rh-AS groups was remarkably lower than that of the control ($p < 0.05$). The increasing rate of the YSM area and the YSM blood vessel area were much lower than those of the control ($p < 0.01$). The results showed that the angiogenesis of the chick embryo YSM might be remarkably inhibited by rh-AS (Fig. 4).

In a conventional chicken embryo CAM angiogenesis assay, a window is opened on the egg shell. The test drug is planted on CAM through an adsorbing carrier such as filter paper or gel sponge, most of which is apt to adhere or irritate to CAM. The whole CAM test takes 11 to 15 days (Auerbach *et al*., 2000). Chicken embryos can frequently move after six days

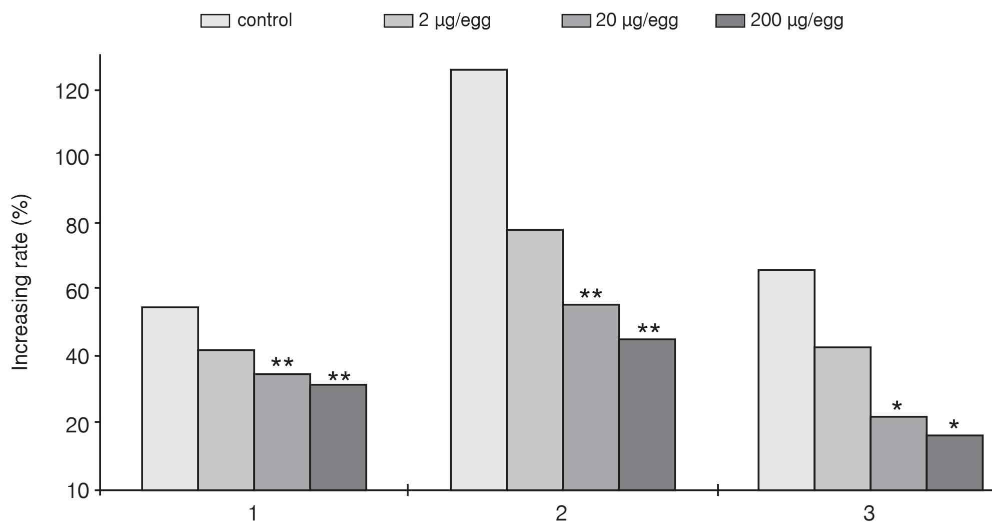

FIG. 4. The comparison of the control and the rh-AS groups on the inhibition of YSM angiogenesis and YSM development. 1. Increasing rate of the YSM area, 2. Increasing rate of the YSM blood vessel area, 3. Increasing rate of the YSM blood vessel density. ** $p < 0.01$, * $p < 0.05$, n = 10.

of incubation, especially when the eggs are disturbed, so the stability of the test area on the CAM is difficult to keep. After nine days of incubation, CAM adheres closely to the shell membrane due to the yolk swelling pressure (Jiang, 1983), and so it is apt to rupture and bleed while unveiling the shell membrane. Moreover, CAM has less contrast against its blood vessel and it looks gray and opaque due to the egg content underneath, which makes a direct observation difficult. Thus, a contrast-increasing reagent should be injected under CAM. In a strict CAM assay, tested CAM has to be fixed in situ and taken out to stain before analysis (Papadimitriou *et al*., 2001; Hatziapostolou *et al*., 2003). The YSM model developed in this paper has many advantages: a) since the chick embryo is removed from the shell into the beaker, the embryonic disc automatically turns up in the center of the yolk as a result of the opposite side of the yolk gravity, b) the embryonic disc grows in a level due to the liquid tension in the beaker, which is convenient to operation and observation, c) it is an advantage for locating the test area and observing the function of the test drug for the whole blood vessel net exposed thoroughly, and d) the whole YSM blood vessel net contrasts well against the yellow yolk underneath, which makes observation and photographing easy.

After the chick embryo is moved into the beaker, its growth and survival are affected to some degree by the mechanical irritation and the environmental changes. Under normal incubation, the CAM gradually replaces the respiration of the YSM blood vessel net after five days of incubation. After five days of incubation in the beaker, the CAM was only about 3 mm of diameter with no obvious respiration function. On the contrary, YSM angiogenesis in the container was affected much less by environment changes, and the YSM blood vessel increased at a high speed. On the forth day of incubation in the beaker, YSM blood vessel covered two thirds of the yolk and at the sixth day it nearly covered the whole yolk plane. The individual differences of the CAM blood vessels are obvious and the local administration on CAM is difficult to keep the uniformity of the administration site. Thus, we tried to use an YSM angiogenesis model to take place of the CAM model. A nontoxic silicon ring was used as a drug carrier, which can fix the test area and bear the drug solution. A round cavity under the ring brought the ring in close contact with the YSM, and so the solution in the ring leaked out less and was absorbed well. Administration in the silicon ring resulted in avoiding of the carrier displacement and adhesion to the membrane. There are amniotic and serous membranes covered on the YSM blood vessels, which can affect the penetration of the test drugs. So, we chose glycerin as a penetrant after comparing different penetrants (data not shown). Glycerin at a concentration of 8% has less toxicity to chick embryo and can facilitate the absorption of the test solution by the blood vessels. Glycerin is also a good wetting agent to keep the test drug at a constant concentration. Removing of the humidity pan from the incubator can also keep constant drug concentration. These measures make YSM angiogenesis assay more sensitive.

In the present study, we used a chick YSM model to examine the anti-angiogenesis function by recombinant angiostatin. The results showed that in a short time, YSM vascular development was remarkably inhibited (vascular branches sparse and thin are obvious), especially in the peripheral vascular area, because angiostatin could inhibit the growth of the precursor vascular endothelial cells (Ito *et al*., 1999) and much more new-born blood vessels were distributed to the periphery of the YSM. We also observed that the development of the whole YSM blood vessel net exhibited considerable individual differences, especially in the test groups. Some embryos in the vascular net grew negatively and thus the test sensitivity was influenced to a certain extent.

In a conventional angiogenesis assay, the angiogenic response was measured macroscopically or with computer-assisted counting of the local new-born vessel, or with histological analysis of the blood vessel distribution (Nikiforidis *et al*., 1999; Youn *et al*., 2006). This takes more time and labor and it is difficult to compare data with other laboratories. In this assay, the self-designed image analysis software was used to analyze the vascular area on the chick embryo YSM, in a fast, objective and quantitative manner. The increase rate of the YSM blood vessel area and the density can truly reflect the angiogenesis variations and to a certain degree, also reflect the changes in blood vessel diameter and number and even the whole YSM area. In this assay, angiogenesis aimed at the whole YSM blood vessel network instead of the local area as in the other angiogenesis assay, which remarkably diminishes the errors as a result of the designation of the test area. For the observation of the whole YSM vascular network, the images analyzed in this study were aimed at larger blood vessels instead of capillary ones. Though angiogenesis-affecting reagents mainly affect the precursors of the vas-

cular endothelial cells or the new-born capillary, larger blood vessels originate from new-born vessels, so the variations of larger blood vessels can also reflect the growth state of the new-born capillary, especially for the rapid growing YSM blood vessels.

In conclusion, the YSM model is timesaving, more objective and an accurate angiogenesis observation method *in vivo*. The YSM model will provide a novel method to promote the testing and screening efficiency of the angiogenesis-affecting drugs.

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