CHAPTER III

Anti-angiogenic effect of retinoid is partially mediated by TGF-β

III-1. Introduction

Angiogenesis is a fundamental process by which new blood vessels are formed. It is essential in development, reproduction, and wound healing (82, 83). The process of angiogenesis is completed through the following sequence of events (Fig. III-1): (1) local degradation of the basement membrane of the vessel by proteases produced from the endothelial cells; (2) migration and invasion of endothelial cells underlying this regional disruption; (3) proliferation of the endothelial cells trailing behind the advancing front; (4) formation of capillary tubes and reconstitution of the basement membrane. It is possible that retinoid enhances the angiogenesis via stimulating the production of endothelial proteases including PA and thereby plasmin. On the other hand, it is also possible that retinoid inhibits the angiogenesis via stimulating the production of TGF-β and its receptors in the endothelia cells. In fact, controversial results have been reported as to the effect of retinoid depending upon what model of angiogenesis was used for appreciating it. Lansink et al. (19) reported that RA stimulates *in vitro* angiogenesis developed on the fibrin gels associating with its ability to enhance PA production. On the other hand, several reports described the anti-angiogenic property of retinoids *in vivo* (84, 85), although underlying mechanism has been unclear. Especially, Oikawa et al. (85) described that retinoid is one of the most potent natural low molecular weight substance with anti-angiogenic activity in the chorioallantoic membranes (CAMs) of growing chick embryos, the most popular methods for *in vivo* angiogenic assay.

In the present study, I examined a potential involvement of TGF-β in the anti-angiogenic activity of retinoid using *in vivo* CAM assay system.

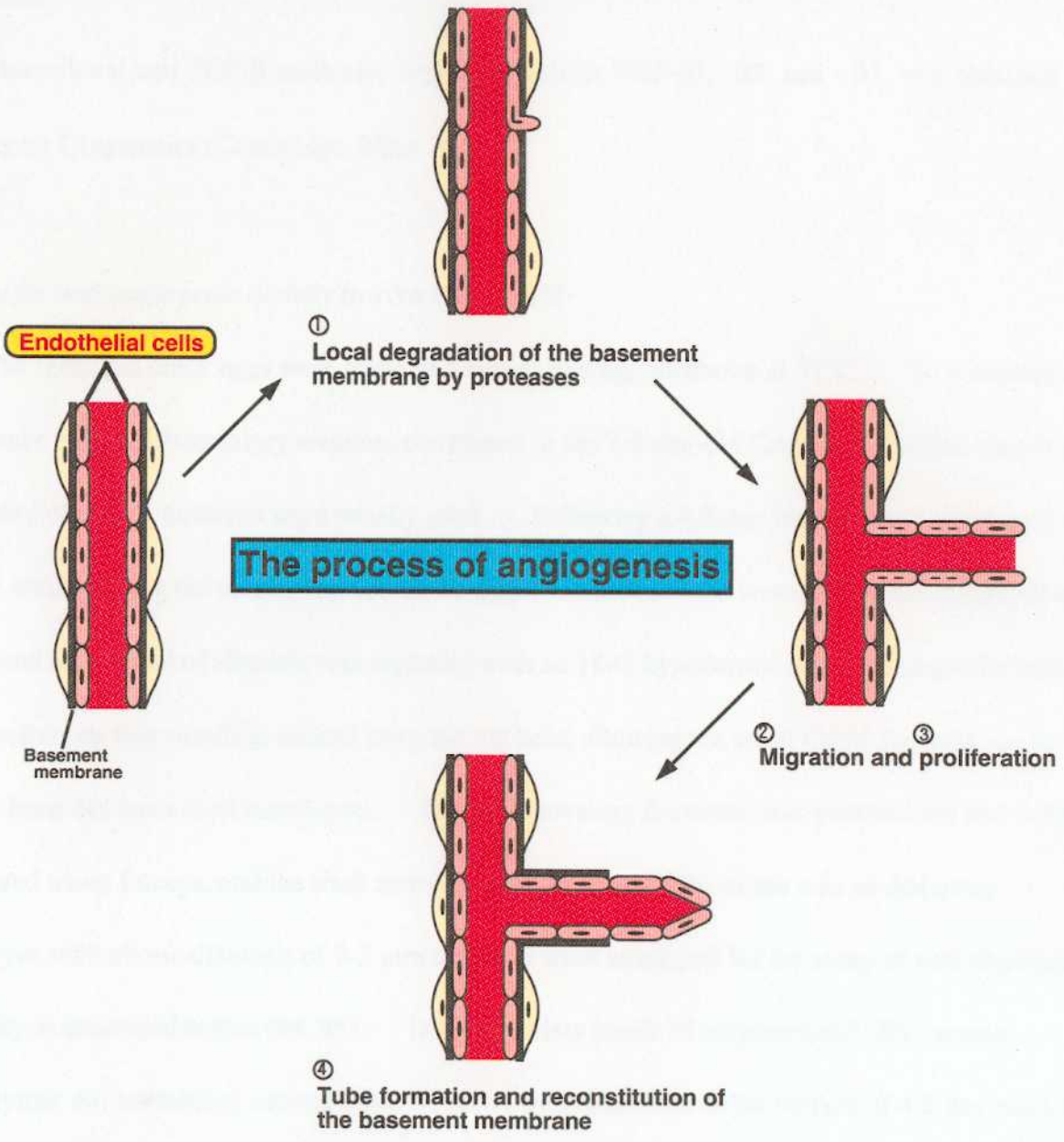


Fig. III-1. Sequential events required for angiogenesis. Vascular endothelial cells play an important role in angiogenesis. There are four steps to complete a new vessel formation as explained in the text.

III-2. Materials and Methods

Material

Monoclonal anti-TGF-β antibody, which neutralizes TGF-β1, -β2, and - β3, was obtained from Genzyme Diagnostics (Cambridge, MA).

Assay for anti-angiogenic activity in vivo *using CAM*

The fertilized chick eggs were stood in a humidified egg incubator at 37°C. To eliminate an influence of the inflammatory response developed in the 7-8-day-old CAM, the vitelline membrane of 4-day-old chick embryos were usually used. Following a 4.5-day incubation, a small hole was made with a drill at the narrow top end of the eggs. Another hole was made at the middle of the eggs and about 2 ml of albumin was aspirated with an 18-G hypodermic needle through the hole. Thereafter, air was carefully sucked from the top hole, allowing the small CAM and yolk sac to drop away from the inner shell membrane. The shell covering the airsac was punched out and carefully removed using forceps, and the shell membrane on the floor of the airsac was peeled away. Embryos with chorioallantosis of 2-3 mm diameter were employed for the assay of anti-angiogenic activity as described before (84, 85). Briefly, pellets (made of ethylenevinyl (EV) acetate copolymer 40) containing various doses of 9cRA were placed on to the surface of 4.5-day-old CAMs. The eggs were covered with teflon-coated metal cups and incubated in a humidified egg incubator at 37°C. Two days later, approximately 0.5 ml of a 10% fat emulsion was injected into the chorioallantois, so that the vascular networks formed on the CAM stood out against the white background of the lipid. Inhibition of angiogenesis was assessed by measuring the area of avascular zones within the CAM. The anti-angiogenic response was scored when the avascular

zone exceeded 4 mm in a diameter. Frequency of avascular zone was calculated as a percentage of the numbers of eggs scored as anti-angiogenic response against total numbers of eggs examined.

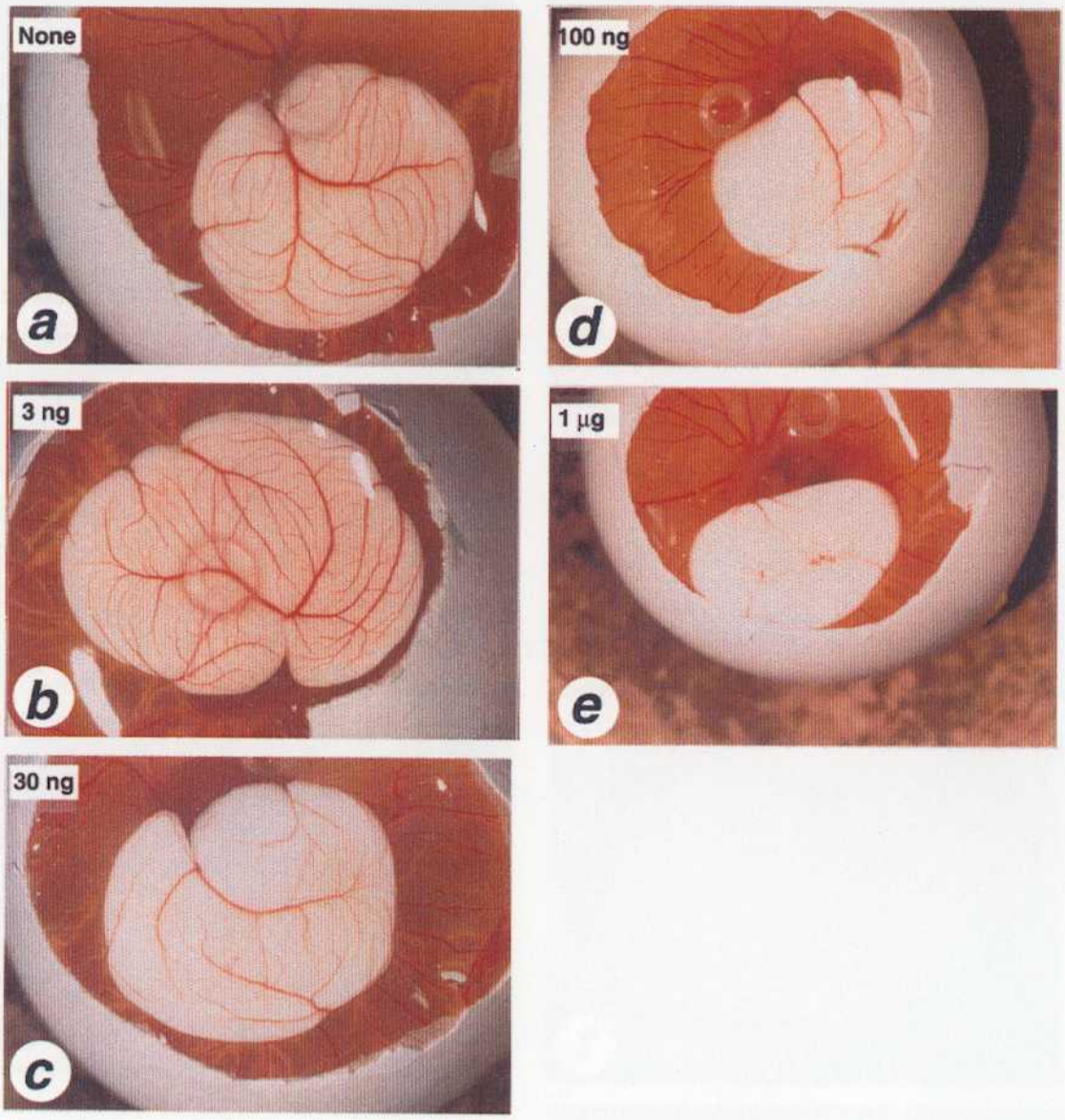
III-3. Results

Neutralization of anti-angiogenic activity of retinoid with anti-TGF-β antibody

The effect of 9cRA on embryonic angiogenesis was examined by implanting an EV pellet containing various doses of 9cRA on the 4.5-day CAM. Figure III-2A shows the results on day 2 after initiating the treatment. In the control samples containing empty EV pellet (*panel a*), the intricate vascular networks were developed on the CAM. 9cRA inhibited this angiogenesis in a dose-dependent manner (*panels b-e*).; it suppressed the angiogenesis in 6 eggs out of total 10 eggs examined at 30 ng (*panel c*), 8 eggs out of 10 eggs examined at 100 ng (*panel d*), and in all none eggs examined at 1 μg of 9cRA (*panel e*). Figure III-2B is the result of plotting the incidence of eggs in which anti-angiogenic activity of 9cRA was observed against the dosages of the reagent in EV pellet. The effect of 9cRA exhibited the sigmoid curve and the ID_{50} values determined from this curve was 23 ng/CAM, which was comparable with the ID_{50} values of other retinoids as reported before (84, 85).

I next tested whether TGF-β is involved in this anti-angiogenic effect by including the neutralizing anti-TGF-β antibody with the retinoid. Eggs were incubated for 2 days with EV pellets containing 100 ng of 9cRA plus/minus 5 μg or 10 μg of anti-TGF-β antibody or non-immune antibody, and blood vessel formation on CAM was observed (Fig. III-3). Although addition of anti-TGF-β antibody alone to the control eggs had no obvious stimulating effect (*panels b* and *c*), simultaneous addition of the antibody with 9cRA reverted anti-angiogenic effect of 9cRA (*panels e* and *f*). This neutralizing effect was not observed when the same amount of non-immune antibody was included together with 9cRA (*panel g*). These data suggest that the anti-angiogenic effect of 9cRA on CAM may be partially mediated by TGF-β.

A)



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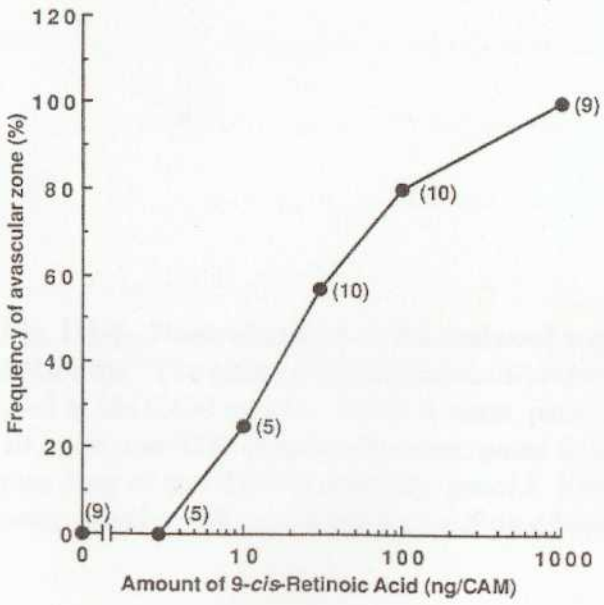

Fig. III-2. Dose-dependent suppression of angiogenesis developed on CAM by 9cRA. A) The effect of 9cRA on embryogenic angiogenesis was examined by implanting EV pellets containing the indicated amounts of 9cRA on the 4.5-days-old CAM. Panel a, none; panel b, 3 ng of 9cRA; panel c, 30 ng of 9cRA; panel d, 100 ng of 9cRA; panel e, 1 μg of 9cRA. B) The anti-angiogenic activity of 9cRA was evaluated by measuring the avascular zones. The values in parentheses express the number of CAMs examined for each point.

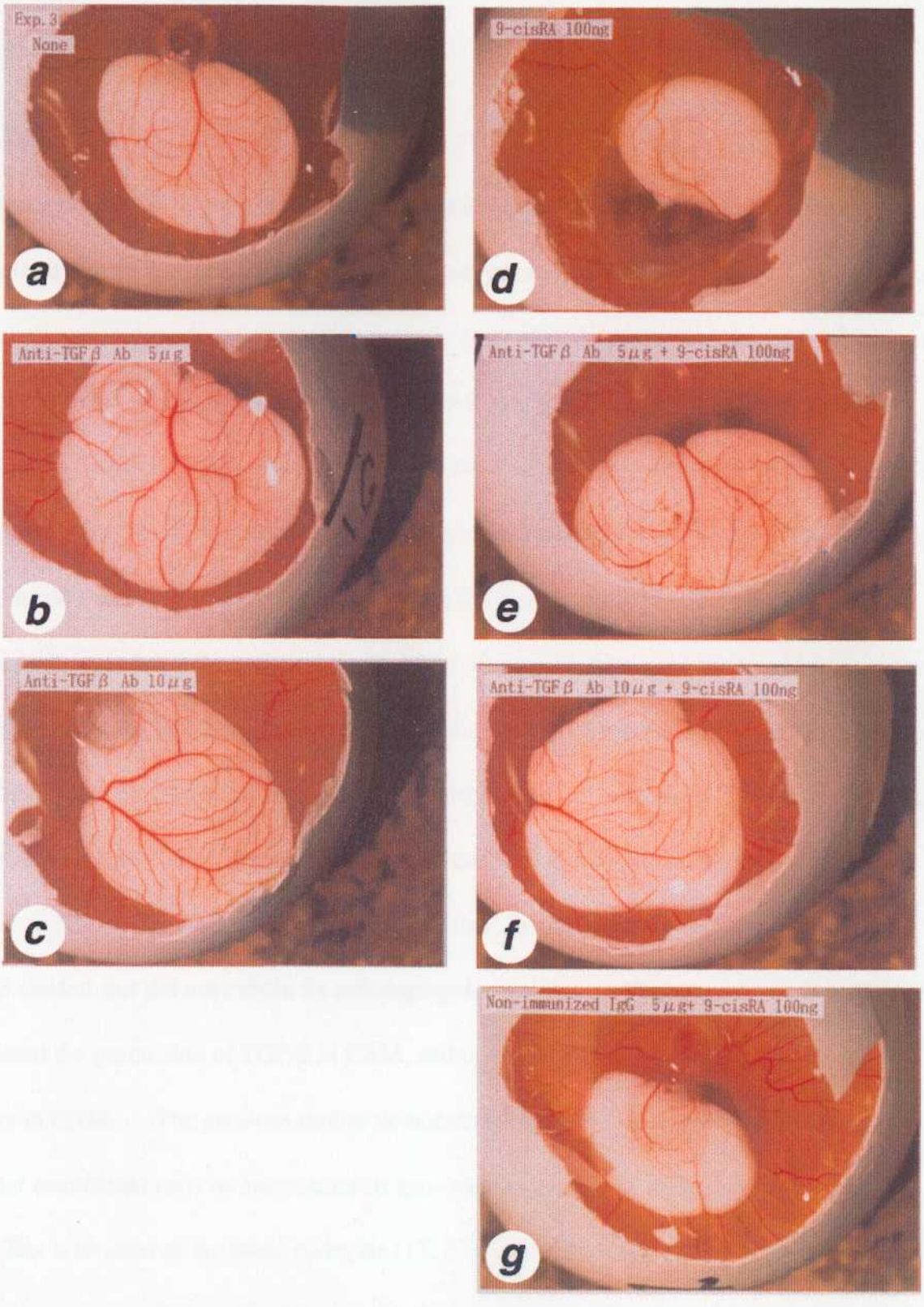

Fig. III-3. Neutralization of RA-induced suppression of angiogenesis by anti-TGF-β antibody. The effect of simultaneous addition of anti-TGF-β antibody and 9cRA was examined in the CAM assays. Panel a, none; panel b, 5 μg of anti-TGF-β antibody alone; panel c, 10 μg of anti-TGF-β antibody alone; panel d, 100 ng of 9cRA alone; panel e, 100 ng of 9cRA plus 5 μg of anti-TGF-β antibody; panel f, 100 ng of 9cRA plus 10 μg of anti-TGF-β antibody; panel g, 100 ng of 9cRA plus 5 μg of non-immune IgG.

III-4. Discussion

A potential link between retinoids and angiogenesis has been documented in several reports. Egg yolk of vertebrates stores a large amount of retinal, and eggs from a vitamin A-deficient hen fail to hatch due to abnormal angiogenesis at an early stage of embryogenesis (4). Mendelsohn et al. (86) reported that compound null mutations of RAR genes lead to numerous developmental abnormalities, especially abnormalities in the cardiovasucular development. Furthermore, Oikawa et al. reported that several synthetic retinoids exhibit anti-angiogenic activity in CAM assay (84). I reproduced a similar anti-angiogenic activity of 9cRA (Fig. III-2). However, molecular mechanisms underlying the anti-angiogenic effect of retinoids have not been elucidated. The present data suggest that at least the anti-angiogenic activity of 9cRA may be partially mediated by the TGF-β. It is notable that anti-TGF-β antibody did not affect so much the basal angiogenesis in the CAM (Fig. III-3, *panels b* and *c*), suggesting that TGF-β may be not involved in this basal process. There are two possibilities to explain this; 1) TGF-β did not exist in the CAM, or 2) TGF-β existed, but did not exhibit its anti-angiogenic activity. Namely, it is predicted that 9cRA stimulated the production of TGF-β in CAM, and/or enabled TGF-β to exhibit its anti-angiogenic activity in CAM. The previous studies demonstrated that retinoid produces active TGF-β in vascular endothelial cells by stimulating its gene expression as well as inducing PA/plasmin-dependent activation of the latent molecule (15, 23). Furthermore, retinoids up-regulates TGF-β receptor expression (24). Combining with the current data, it is suggested that 9cRA may inhibit the angiogenesis in CAM by stimulating the TGF-β production as well as its receptor expression probably through RAR-Sp1 interaction. In order to verify more directly the biological consequences of the transcriptional regulation via RAR/RXR-Sp1 interaction in CAM, I am planing

to construct dominant-negative mutants, which specifically inhibits the RAR/RXR-Sp1 interaction, and see the changes upon introducing it into CAM.

III-5. Summary

The effect of 9cRA on embryonic angiogenesis was assessed using 4.5-day CAMs of growing chick embryos. 9cRA inhibited the angiogenesis in a dose-dependent manner. This anti-angiogenic activity was neutralized by simultaneous addition of anti-TGF-β antibody, suggesting that the anti-angiogenic activity of 9cRA may be at least in part mediated by TGF-β.