Angiogenic Responses Elicited From Chorioallantoic Membrane Vessels by Neoplastic, Preneoplastic, and Normal Mammary Tissues From GR Mice

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Neoplastic tumors are able to elicit the ingrowth of new capillaries, a process known as angiogenesis. The chorioallantoic membrane (CAM) of chicken embryos was used in an assay for this response, and normal mammary glands and various mammary growths from GR mice, including plaques, hyperplasic alveolar nodules, and hormone-dependent and hormone-independent tumors were tested. Fifteen percent of the male mammary glands tested were positive, as were 28% of the resting female mammary glands. Fifty percent of the plaques and 63% of the hyperplastic alveolar nodules tested induced neovascularization. Eighty percent of the hormone-dependent tumors and 97% of the hormone-independent tumors tested elicited angiogenesis. A fine-structural study revealed that capillaries invaded to within less than 0.5 μ of the tumor cells, but no penetration of tumor cells through the basal lamina was observed. Positive responses were directly correlated with the neoplastic potential of the tissues tested, indicating that angiogenesis can predict mammary gland growths most likely to become malignant. (Am J Pathol 1983, 111:282–287)

IN 1945 Algire and Chalkley[1] observed that actively growing tumors continuously elicited the growth of new capillaries from the host, a process called tumor angiogenesis. It is this event that often initiates the rapid (exponential) growth of the tumor,[2] and malignant progression (characterized by invasiveness and metastases) frequently follows.[3] The first experimental evidence indicating that a diffusible factor was involved in tumor angiogenesis came from a study by Greenblatt and Shubik,[4] who demonstrated that a tumor on one side of a Millipore filter could induce new vessels to grow toward it from connective tissue stroma on the opposite side of the filter. Other studies have shown that tumors can stimulate capillary proliferation from distances up to 5 mm.[5] In 1971 Folkman et al.[6] isolated a diffusible factor from tumor cells which is mitogenic to vascular endothelial cells and stimulates capillary growth, but attempts to purify this factor have thus far been unsuccessful.

Angiogenesis is a property of mammary carcinomas and approximately 30% of mammary hyperplasias from mice[7] and human breasts.[8] It is also a marker for neoplastic transformation of mouse mammary papillary hyperplasias and appears *before* any morphologic or clinical evidence of malignant transformation can be detected.[9] In contrast, it is rare for resting mammary glands to elicit neovascular responses.[7]

We undertook the present study to establish the neoplastic potential of various mammary gland lesions in GR mice, using angiogenesis as an assay technique. This mouse strain[10] provides an excellent model because of the various types of mammary tumors it develops. Hormone-dependent mammary tumors arise during pregnancy from disk-shaped lesions called plaques.[11,12] They reach a maximum size shortly before parturition and then regress.[11,12] If repeated matings take place, these tumors reappear in the same location and eventually progress to become hormonally independent (malignant).[13,14] We wished to test plaques and hormone-dependent GR

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mammary tumors to determine whether or not they produced an angiogenesis factor. Spontaneous hormone-independent mammary tumors also arise in GR mice from lesions called hyperplastic alveolar nodules. In the present study we have tested these various mammary gland growths, as well as resting mammary glands from GR mice, for their ability to elicit angiogenesis from the chorioallantoic membrane of chicken embryos.

Materials and Methods

The chorioallantoic membranes (CAMs) of 8-day-old white leghorn chicken embryos were used as recipients of tumor grafts. A total of 85 GRS/AJs mice (GR mice) were used in this study. Ninety-nine separate mammary tissues were examined, including 13 plaques, 20 hyperplastic alveolar nodules, 19 hormone-independent mammary tumors, and 14 hormone-dependent mammary tumors. Tumors were identified as being hormone-dependent by the following procedure. Minced pieces of tumor were injected subcutaneously into several castrated male GR mice. Some of the mice were injected with estrogen and progesterone, and others were not. Tumors that grew *only* in castrated mice given injections of hormones were designated hormone-dependent. In addition, 19 normal mammary glands and 6 livers from nonpregnant female mice, and 8 mammary glands from male GR mice were also tested for their ability to elicit angiogenesis.

Eggs containing embryos were candled to identify large branched vessels in the CAM, and the position of these vessels was marked on the shells. The air sac at the large end of the egg was punctured with a needle to allow the CAM to drop away from the shell. A window was then made over the marked area, and a 1-sq cm piece of shell was carefully removed to expose the underlying CAM. Tumors, mammary gland lesions, and normal mammary glands from donor mice were cut into small pieces (approximately 1 cu mm), and one piece was placed on the CAM near the Y-branch of a large blood vessel. The window in the shell was then sealed shut with a piece of Scotch tape. The eggs bearing grafts were incubated at 38 C in an incubator having an atmosphere of 50% humidity. After a few days we examined the grafts under a dissecting microscope to establish whether or not angiogenesis had occurred. On the seventh day, the responses were recorded, and some preparations were photographed. A number of grafts eliciting positive angiogenesis were fixed at room temperature by the addition of 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3[15] to the CAM. After 2 hours the grafts were carefully excised and placed into a vial of the same fixative overnight. The following morning the tissues were cut into small pieces and transferred to cold 0.2 M sodium cacodylate buffer, pH 7.3. The samples were postfixed at 4 C for 1 hour in 1% OsO_4 buffered by 0.1 M sodium cacodylate, stained *en bloc* with 2% uranyl acetate in 50% ethanol, dehydrated, and embedded in Epon. Semithin sections were cut and evaluated by light microscopy. Thin sections were then cut from selected areas and stained with uranyl acetate[16] and lead citrate[17] for examination in a Philips EM 201 electron microscope.

Results

The chorioallantoic membranes bearing grafts were examined and evaluated under a dissecting microscope for evidence of angiogenesis. By 3 days, convincing evidence of angiogenesis was apparent; but the grafts were allowed to grow for 7 days, at which time the responses were recorded.

The angiogenic responses of various tissues are indicated in Table 1. Ninety-seven percent of the hormone-independent mammary tumors arising spontaneously in older GR mice displayed clear evidence of angiogenesis (Figure 1). Moreover, 80% of the hormone-dependent mammary tumors tested gave positive evidence of neovascularization. Sixty-three percent of the hyperplastic alveolar nodules (Figure 2) and 50% of the plaques tested were also positive. Liver tissue from GR mice did not stimulate angiogenesis, and neither did most mammary glands from male mice (Figure 3), but 15% of the mammary glands from male GR mice were positive, indicating the background level of this assay (for

Table 1—Angiogenic Response From CAM Vessels Elicited by Implants of Various GR Mouse Mammary Tissues

Type of tissue	Number of donor tissues	Number of positive responses/ number of CAMs tested	% Positive grafts
Normal mammary gland:			
Male mice	8	2/13	15.4
Resting female mice	19	7/25	28.0
Liver	6	0/6	0
Plaques	13	11/22	50.0
Preneoplastic tissues			
Hyperplastic alveolar nodules	20	19/30	63.3
Hormone-dependent tumors	14	35/44	79.5
Neoplastic tissue			
Hormone-independent tumors	19	34/35	97.1

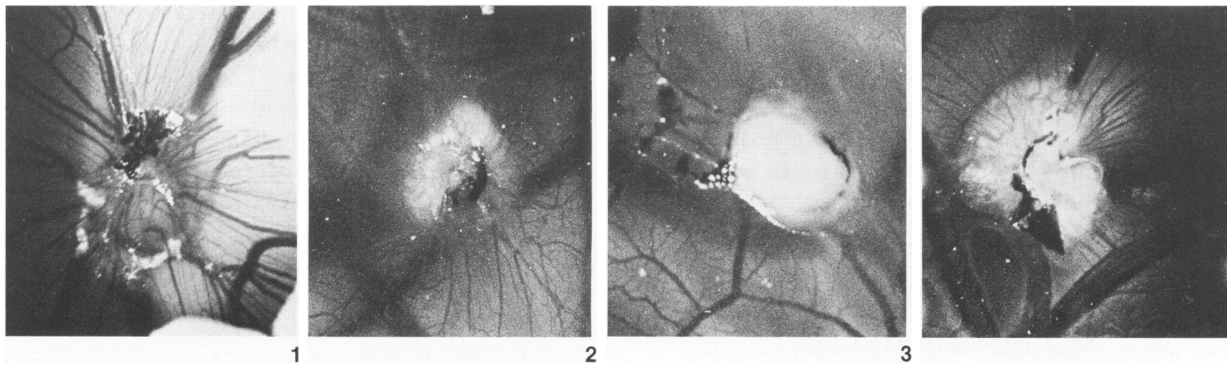


Figure 1 — Positive angiogenic response elicited by a hormone-independent mammary tumor from a GR mouse. (×6) **Figure 2** — This hyperplastic alveolar nodule induced angiogenesis from vessels in the CAM. (×8) **Figure 3** — Negative angiogenic response by mammary gland from a male GR mouse. (×8) **Figure 4** — Twenty-eight percent of resting mammary gland from female GR mice elicited angiogenesis, as shown here, (×8)

mammary glands from mice containing mammary tumor virus). Although 28% of the glands tested from female GR mice elicited a response (Figure 4), this percentage is not statistically significant when compared with positive responses by male glands.

Tumors examined by light microscopy revealed capillaries penetrating among the islands of tumor cells (Figure 5). The elliptical shape of the erythrocytes within these capillaries suggested these cells were of host origin. This was confirmed in electron micrographs, where nucleated chicken erythrocytes were clearly seen (Figure 6). In areas where capillaries were invading, or had just invaded, the clusters of tumor cells, many leukocytes were observed. The most common cell in these regions was a polymorphonuclear granulocyte (Figure 7). Its cytoplasm contained large dense granules, similar in appearance to those observed in basophils. Elliptical granules were also present in these cells. Macrophages were also abundant in these regions (Figure 8) and were characterized by many lysosomes and prominent phagocytic vacuoles of various shapes and sizes.

Hormone-independent tumors that elicited marked angiogenic responses were examined by electron microscopy for evidence of the penetration of tumor cells through the basal lamina. However, after 7 days

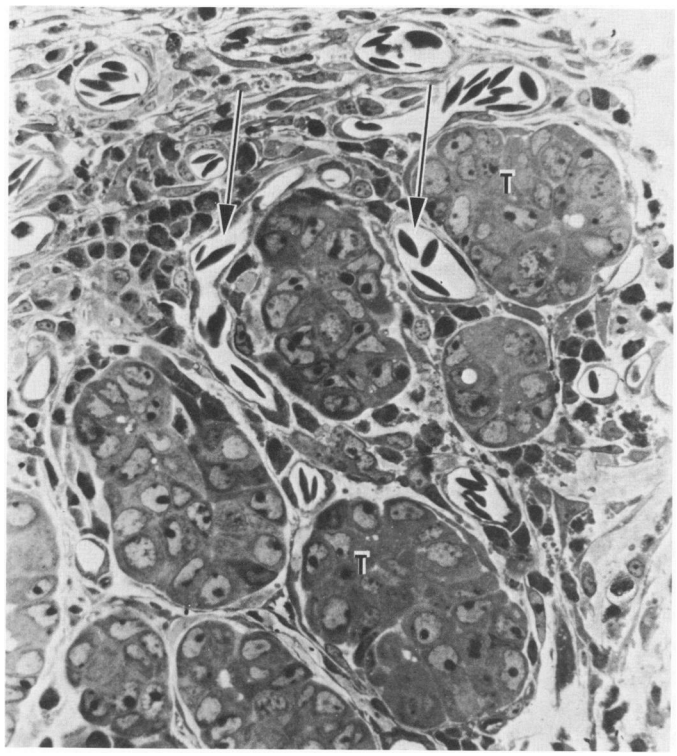


Figure 5 — Light micrograph showing a positive angiogenic response 7 days after grafting of the tumor to the CAM. Capillaries (*arrows*) are seen penetrating among the clusters of tumor cells (*T*). (×130)

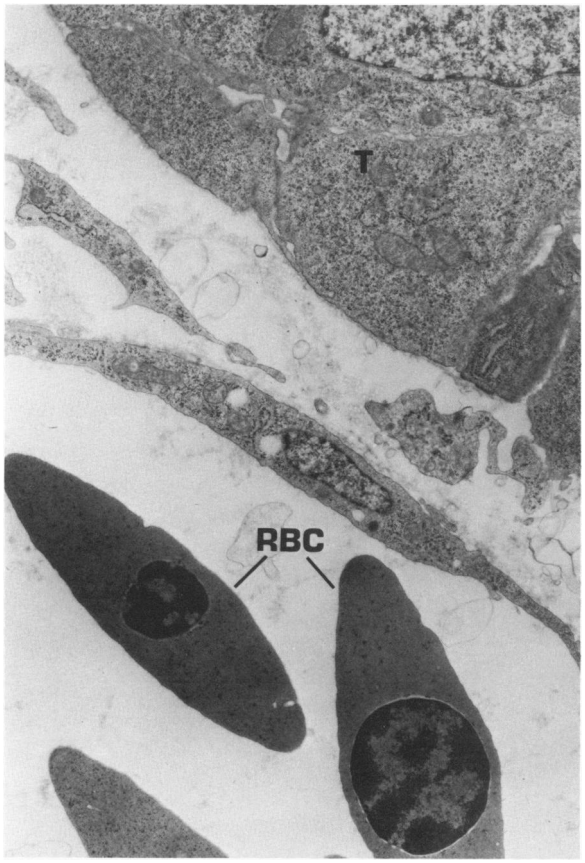
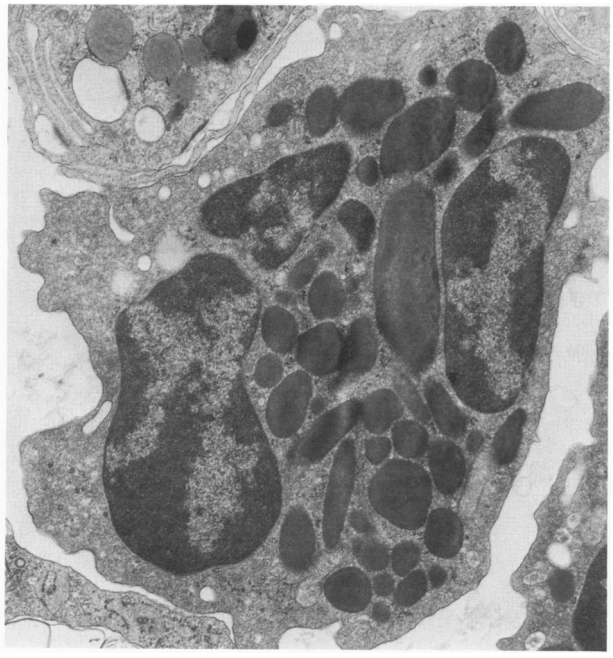


Figure 6—Electron micrograph of nucleated chicken erythrocytes (*RBC*) in capillary near tumor cells (*T*) grafted onto CAM. (×8300)

in contact with the CAM, no such penetration was observed. This might have occurred elsewhere in the graft, or could conceivably take place at later time periods. It is noteworthy that some capillaries had invaded to within less than 0.5 μ of the plasma membrane of tumor cells (Figure 9), suggesting the importance of these vessels in providing nutrients for tumor cell growth and maintenance. Hormone-dependent GR mammary tumors and hyperplastic alveolar nodules displaying positive angiogenic responses showed a similar relationship of capillaries invading the area close to the epithelial cells. The tumor cells grew well on the CAM, and mitotic figures were often observed. Mammary tumor virus was also synthesized and released by the cells into small lumens and/or vacuoles present within the clusters of tumor cells.

Discussion

This study has focused on the ability of various mammary gland tissues from GR mice to induce angiogenesis. Normal mammary glands from male mice were usually negative, as was liver tissue taken from this strain. However, 28% of the mammary glands from resting female GR mice were positive. This was an unexpected finding and could indicate that the capacity to induce angiogenesis is already present in these tissues, even though no morphologic changes are yet detectable. However, since the back-



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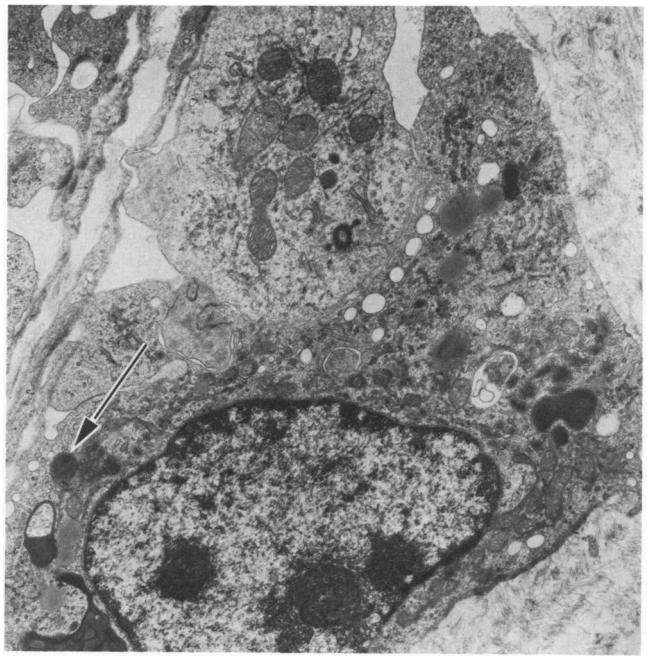

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Figure 7—Electron micrograph of most common cell type seen near capillaries invading among clusters of tumor cells. This chicken polymorphonuclear leukocyte most closely resembles the mammalian basophil, except for its content of elongated granules. (×13,800)
Figure 8—Macrophages displaying many phagocytic vacuoles (*arrow*) and lysosomes were common near areas of neovascularization. (×9200)

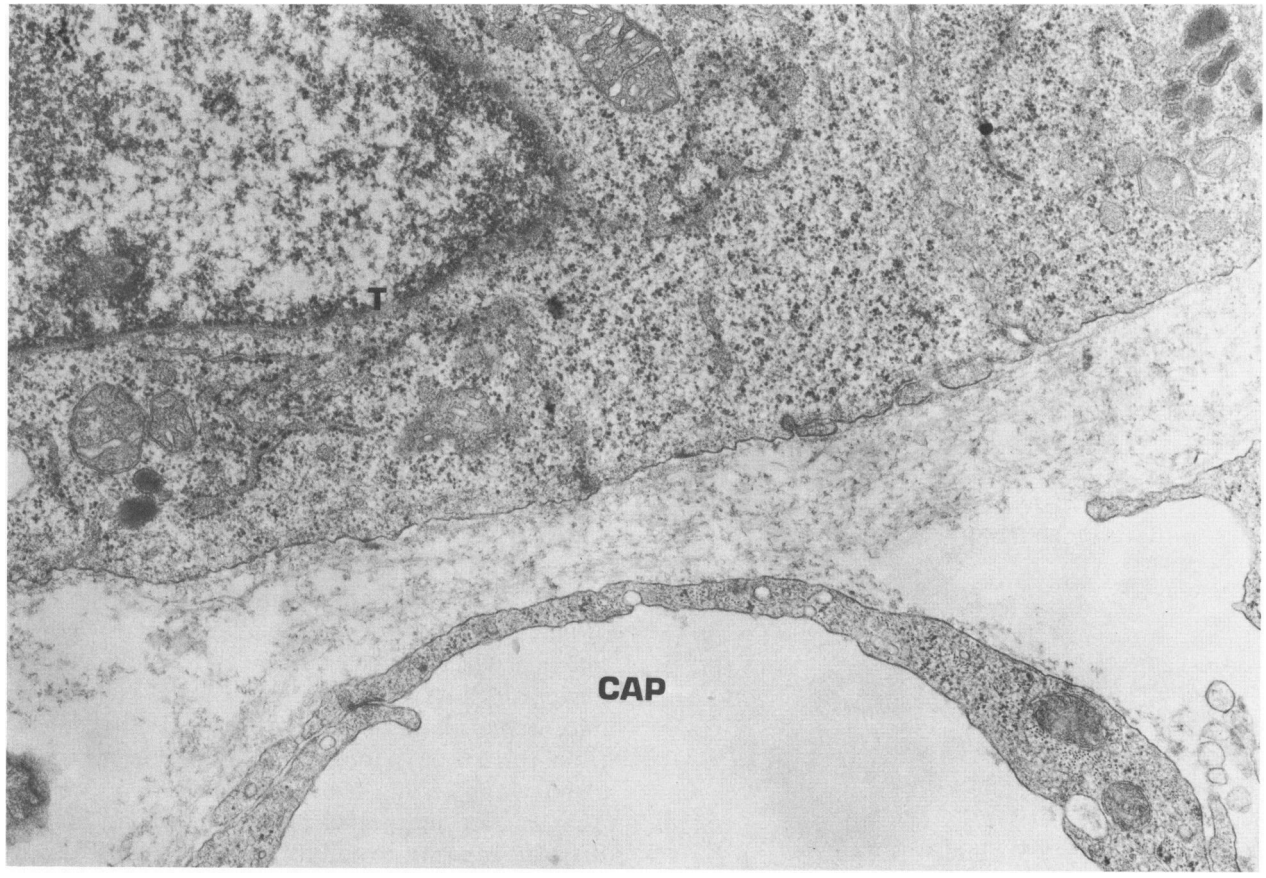

Figure 9—Electron micrograph illustrating a CAM capillary (*CAP*) at a distance of 0.5 μ from a grafted tumor cell (*T*). (×25,400)

ground level of this assay is 15%, as revealed by the positive responses from male glands, only 13% of the female glands should be considered as having elicited the ingrowth of capillaries. Whether or not these positive responses are early indications of glands at risk for developing neoplastic mammary tumors remains to be determined.

There were many leukocytes in the areas where blood capillaries had recently invaded islands of tumor cells. The most common cell-type was a polymorphonuclear cell containing large granules, similar in appearance to those found in basophils. Since basophils release histamine, a substance associated with vascular leakage, these cells might be involved in blood vessel changes associated with angiogenesis. However, a biochemical characterization of the contents of these granules would be necessary to establish whether or not this is true. In the cornea, it has been reported that blood vessels will invade only if the tissue has first been infiltrated by leukocytes of the polymorphonuclear series,[18,19] but the specific cell types involved were not identified. In the present study macrophages were also associated with the ingrowth of capillaries from the CAM. Although these cells phagocytized debris in the area, they also might have been associated with the actual angiogenic response, since activated macrophages have been shown to induce the proliferation of blood vessels.[20]

Fifty percent of the plaques tested caused an ingrowth of capillaries, suggesting that they contained cells which produced tumor angiogenesis factor. The wide variation in the neovascular responses of plaques might be related to how far advanced the lesion was in giving rise to a hormone-dependent tumor. Although the size of the plaque was not directly correlated with a positive response, larger plaques elicited a marked degree of neovascularization.

Sixty-three percent of the hyperplastic alveolar nodules transplanted to the CAM elicited an angiogenic response. This is in agreement with the study of Gimbrone and Gullino, where 30% of the hyperplastic alveolar nodules from other mouse strains showed positive angiogenic responses when tested with the use of the rabbit iris as an assay.[7] The preneoplastic nature of hyperplastic alveolar nodules was established many years ago by the studies of DeOme et al,[21] and our positive angiogenic results confirm the preneoplastic character of these lesions.

Eighty percent of the hormone-dependent GR mammary tumors tested induced neovascularization.

This was somewhat surprising, because these tumors rapidly regress after parturition and are highly sensitive to hormones. We were not able to establish the number of times each hormone-dependent tumor had appeared and regressed, but while these tumors were still subject to hormonal control, they were definitely producing tumor angiogenesis factor. It has been reported that after about six matings these tumors usually become unresponsive to hormones.[11,12] This progression to independence was also demonstrated in a series of transplant studies in GR mice by Aidells and Daniel.[22] Thus, angiogenesis appears to signal the ultimate destiny of these hormone-dependent tumors.

It was not surprising that 97% of the hormone-independent (malignant) mammary tumors in GR mice stimulated neovascularization. These tumors are autonomous and grow rapidly in the absence of any hormonal stimulation, eventually killing the host. They often grow large (30 mm or more in diameter) and occasionally metastasize to the lung.[23] In this study we saw no evidence of tumor cells penetrating the basal lamina, but our grafts had been in contact with the CAM for only 7 days.

Our findings indicate that the ability to elicit angiogenesis is directly correlated with the known neoplastic potential of the GR mammary tissues tested: as the risk increases, the angiogenic response also increases. The progression of growth in mammary tumor development from preneoplasia to neoplasia is thought to be a multistep process. Hyperplastic alveolar nodules are well recognized as being one morphologic step in this progression. However, we believe that the ability to induce angiogenesis is another step in the process of malignant transformation, as others have also suggested.[7,9] The present study indicates that some plaques are preneoplastic, and so are most hormone-dependent mammary tumors in GR mice. Based upon these results, the CAM assay for angiogenesis has proved to be a simple and reliable tool for identifying mammary gland growths at highest risk of becoming malignant.

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