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Preneoplastic Lesions of the Human Mammary Gland Transplanted into the Nude Athymic Mouse[1]

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Summary

Morphologically normal lobules and atypical lobules postulated precancerous to ductal carcinoma were transplanted to test their biological behavior. Supravital staining disclosed the 1 to 4-mm microorgans. "Cleared" mammary fat pads of nude mice were optimal transplantation sites. Of the total of 217 transplants from 19 cancer-associated and 13 non-cancer-associated breasts, 151 survived after 2 to 27 weeks. Of 61 surviving normal-appearing lobules from cancer-associated breasts transplanted without prior *in vitro* maintenance, 20 (30%) dedifferentiated, and of 48 surviving lobules from noncancerous breasts, 11 (20%) dedifferentiated. Fifteen of 28 histologically normal-appearing lobules (60%) obtained from cancer-associated breasts after age 50 dedifferentiated. Thirty of 36 atypical lobules isolated from the breast tissue were obtained from cancer-associated breasts and the 5 of those that dedifferentiated came from cancer-associated breasts. Twenty of 22 (90%) dedifferentiating transplants from cancer-associated breasts showed a vascularization response, whereas 3 of 7 (43%) from non-cancer-associated breasts did so. If dedifferentiation in this experimental setting is indicative of a precancerous potential, the data on normal-appearing lobules obtained from cancer-associated breasts from women over age 50 suggest that these lobules carry the greatest precancerous potential. Such lobules probably belong to a type persisting after menopause and they may be hormonally autonomous. Such lobules might undergo further atypia *in vivo* and, eventually, cancerous transformation.

Introduction

This transplantation study was undertaken to examine the behavior of lobules of the human breast that showed atypical morphology. Earlier studies placed the site of origin of most ductal carcinomas in the lobule and its draining duct. Support for this hypothesis was based on the finding of minute foci of ductal carcinoma *in situ*, located in the terminal ductal-lobular unit of the human breast (16). A morphological link between normal lobules and ductal carcinoma *in situ* has been described (17), and the epithelial proliferations within such atypical lobules are believed to be potentially preneoplastic. Previous studies suggested similar lesions to be precancerous (see Ref. 17 for review). Quantitative studies on whole human breasts indicated that ALA[3] often persisted after monopause and they were more numerous and had a higher degree of atypia in high-cancer-risk breasts (17). Mammary glands containing ALA belonged to a population group different from that without ALA (7). Lesions similar to ALA were described in the mouse mammary gland several years ago. They were designated HAN and were found more frequently in mice with a high incidence of mammary carcinomas. When transplanted into "cleared" mammary fat pads of isologous mice, HAN grow to fill the pad but display a type of outgrowth different from that of normal mammary parenchyma (2). A similar experimental approach was followed to examine possible biological differences between ALA and normal-appearing lobules of the human mammary gland. Cleared fat pads of athymic nude mice were used as transplantation sites. The congenital absence of the thymus (11) endows the nude mice with a degree of immunoincompetence (12, 15, 18) sufficient to permit growth of human heterotransplants (4-6, 10, 13-15).

This paper presents observations indicating that, upon transplantation, morphologically normal lobules derived from cancerous breasts or breasts contralateral to cancerous breasts have an elevated degree of dedifferentiation that might be related to a precancerous state not otherwise apparent.

Materials and Methods

The nude athymic mice receiving the transplants were of Swiss strain, bred and reared in a pathogen-free environment and handled in a laminar flow hood following a protocol described previously (3). The colony was started with breeders donated by Dr. B. Giovanella. Mammary tissue was obtained sterilely from 29 women, ages 22 to 78. The specimens for transplantation were derived from 17 mastectomies and 18 biopsies. Tissue slices, about 5 mm thick, were placed in culture media at 4° (Medium L-15 + penicillin, 400 μg/ml, + streptomycin, 400 μg/ml, + insulin, 10 μg/ml, + estrogen, 0.01 μg/ml). Lobules were removed using a dissecting microscope (× 2 to × 4). For facilitating identification of ALA, a supravital stain was used to color the tissue slices (methylene blue chloride chroma 11045, 1 mg/100 ml in Medium L-15 for 45 min at 20° (Chromagesellschaft, Stuttgart-Unterpurkheim, West Germany).

A total of 159 lobules was transplanted within 2 to 72 hr of

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[3] The abbreviations used are: ALA, atypical lobules, type A; HAN, hyperplastic alveolar nodules.

the surgical removal of the gland. A group of 58 lobular explants was held at 37° in Medium L-15 + penicillin, 100 μg/ml + streptomycin, 100 μg/ml, + insulin, 10 μg/ml, + estrogen, 0.01 μg/ml for a period of 12 hr to 5 days prior to transplantation to test the role of preculture on lobular survival and morphology. All lobules were cut in half before transplantation, and 1 part was maintained as controls of the morphological architecture of the transplant. Of the transplants, 75% were placed in female mice and 25% in male mice. The transplantation sites were the inguinal fat pad cleared of the female mouse mammary gland (2), the inguinal s.c. tissue, and the peritoneal cavity of both females and males.

One to 27 weeks after transplantation, the lobules were removed, and their size and vascularization were recorded. They were fixed in 10% buffer formaldehyde, dehydrated, embedded in methacrylate or paraffin, serially sectioned, and stained with aldehyde fuchsin or hematoxylin and eosin for light microscopy. The transplants were analyzed for structural integrity, morphological characteristics of the cell population, and behavior of the stroma.

Transplants not observed grossly or microscopically at transplantation sites were classified as "not found" (NF). Transplants classified as not surviving contained no epithelium and were called "found stroma" (FS). Transplants classified "found epithelium unchanged" (FEU) displayed well preserved epithelium of similar architecture as the controls. Dedifferentiation was defined as hyperplasia of epithelium showing variable degrees of disorganization and immaturity within enlarged ductular units. Transplants showing such changes were called "found epithelium dedifferentiated" (FED). Thus transplants found consisted of FS, FEU, and FED. Surviving transplants were those designated FEU and FED.

Of the host animals 5 to 10% developed runt disease common among nude mice (12). Most lived beyond age 7 months, and few experiments were terminated due to debilitation of the host. The animals displayed vigor as did those from which they descended (3). At autopsy, when experiments were terminated, no unusual findings were noted.

Results

None of the transplanted lobules grew to fill the cleared fat pad as HAN does in the isologous mouse. However, in those transplants defined as dedifferentiating, multiplication of immature-appearing cell populations had occurred within existing ductular units. Transplants appeared as rounded gray structures with a glistening surface. Of the 159 transplants without prior maintenance *in vitro*, 27 were not found, 21 did not survive, 82 did not show epithelial change, and 29 dedifferentiated. Of the 58 transplants previously maintained *in vitro*, 15 were not found, 3 did not survive, 33 did not show epithelial change, and 7 dedifferentiated.

The data concerning the fate of transplants as related to *(a)* transplantation site and sex of the host animals; *(b)* size of the explant; *(c)* total hours held at 4° before transplantation; *(d)* effect of supravital staining; and *(e)* variations in transplantation period are reported in Tables 1 to 5. The optimal site of transplantation was the cleared fat pad, at which site more transplants dedifferentiated. The poorest site was the peritoneal cavity, where transplants remained floating and avascular. The sex of the host played little role in transplant survival (Table 1). The size of the explant did not affect either survival or the angiogenic response of the host. There appeared to be no loss of angiogenic response after *in vitro* maintenance (Table 2). Tissue slabs held up to 48 hr in tissue culture medium at 4° survived as well as those transplanted as soon as possible after removal of the gland

Table 1
Fate of transplants in relation to transplantation site and sex of host

	i.p. male	i.p. female	s.c. male	s.c. female	CFP[a] female	Total
Transplants without prior in vitro maintenance						
Tr-FEU	2	4	24	26	26	82
Tr-FED	0	0	2	2	25	29
Tr-FS	0	4	4	7	6	21
Tr-NF	3	4	7	6	7	27
Total	5	12	37	41	64	159
Transplants with prior in vitro maintenance						
Tr-FEU	0	1	7	16	9	33
Tr-FED	0	0	4	1	2	7
Tr-FS	0	0	2	0	1	3
Tr-NF	0	0	3	11	1	15
Total	0	1	16	28	13	58

[a] CFP, cleared fat pad; Tr, transplanted and atypical lobules; FEU, found epithelium unchanged; FED, found epithelium dedifferentiated; FS, found stroma; NF, not found.

Table 2
Fate of transplants in relation to size of explant

	1 mm	2 mm	3–4 mm	5–6 mm	7–10 mm	Total
Transplants without in vitro maintenance						
Tr-FEU	2	18	47	12	3	82
Tr-FED	1	9	16	1	2	29
Tr-FS	2	7	8	3	1	21
Tr-NF	0	10	15	2	0	27
Total	5	44	86	18	6	159
No. of Tr-F vascularized	3	19	29	4	3	
Transplants with prior in vitro maintenance						
Tr-FEU	1	7	13	10	2	33
Tr-FED	0	2	5	0	0	7
Tr-FS	0	0	3	0	0	3
Tr-NF	3	0	8	3	1	15
Total	4	9	29	13	3	58
No. of Tr-F vascularized	1	1	9	5	0	

[a] Tr, transplanted and atypical lobules; FEU, found epithelium unchanged; FED, found epithelium dedifferentiated; FS, found stroma; NF, not found; Tr-F, Tr-found.

(Table 3). Supravital staining was very useful for identification of lobules and did not affect survival (Table 4). The length of the transplantation period necessary for dedifferentiation to occur appeared variable, but dedifferentiation was not observed after 26 weeks (Table 5).

The light microscopic characteristics of lobules removed at various intervals after transplantation were evaluated. Mitoses and regeneration of the epithelium were already evident during the 1st week (Figs. 1 and 2). In 1 to 2 weeks vascularization had taken place (Figs. 3 and 4). About 50% of the transplants contained fewer ductules than did their controls and most displayed increased stromal sclerosis (Figs. 5 and 6). In explants where ALA had undergone dedifferentiation, the cytological atypia was most severe (Fig. 7).

Most lobules from non-cancer-associated breasts were derived from women below age 50. The number of lobules obtained from cancer-associated breasts was reflecting the known age distribution of breast cancer (Table 6). Of lobular transplants without prior *in vitro* maintenance from cancer-associated breasts surviving, 20 of 61 dedifferentiated compared with 11 of 48 from the non-cancer-associated breasts. However, of 28 lobules from cancer-associated breasts past age 50, 15 dedifferentiated. Only 36 atypical lobules were isolated in all breast tissue studied, and the data obtained from transplantation were too scanty for analysis (Table 6). Dedifferentiating transplants from cancer-associated breasts showed stromal vascularization in 20 of 22 and stromal vascularization occurred in 3 of 7 of those from non-cancer-associated breasts (Table 7).

Table 3

Fate of transplants without in vitro maintenance in relation to hr held at 4° prior to transplantation

	1–12 hr	13–24 hr	25–48 hr	49–72 hr	Total
Tr-FEU"	18	51	6	7	82
Tr-FED	13	9	7	0	29
Tr-FS	6	14	1	0	21
Tr-NF	8	12	6	1	27
Total	45	86	20	8	159

" Tr, transplanted and atypical lobules; FEU, found epithelium unchanged; FED, found epithelium dedifferentiated; FS, found stroma; NF, not found.

Discussion

Nude mice born and maintained in a germ-free environment live longer than other nude mice (9). The mice in our colony raised in a pathogen-free environment lived long enough for our needs, since dedifferentiation was found to occur within 25 weeks.

In spite of the immunological incompetence of the host animal, normal lobules and ALA failed to grow into the

Table 4

Fate of transplants in relation to supravital staining

	Stained	Unstained	Total
Transplants without prior in vitro maintenance			
Tr-FEU"	71	11	82
Tr-FED	23	6	29
Tr-FS	19	2	21
Tr-NF	25	2	27
Total	138	21	159
Transplants with prior in-vitro maintenance			
Tr-FEU	10	23	33
Tr-FED	2	5	7
Tr-FS	0	3	3
Tr-NF	4	11	15
Total	16	42	58

" Tr, transplanted and atypical lobules; FEU, found epithelium unchanged; FED, found epithelium dedifferentiated; FS, found stroma; NF, not found.

Table 5

Fate of transplants in relation to transplantation period in weeks

	0–3 wk	4–6 wk	7–10 wk	11–14 wk	15–18 wk	19–21 wk	22–25 wk	26–29 wk	Total
Transplants without prior in vitro maintenance									
Tr-FEU"	3	17	17	16	4	10	9	6	82
Tr-FED	2	1	8	3	5	5	5	0	29
Tr-FS	4	3	8	4	2	0	0	0	21
Tr-NF	3	1	2	6	8	4	2	1	27
Total	12	22	35	29	19	19	16	7	159
Transplants with prior in vitro maintenance									
Tr-FEU	5	4	9	1	5	2	5	2	33
Tr-FED	1	1	0	0	3	2	0	0	7
Tr-FS	0	0	2	0	1	0	0	0	3
Tr-NF	0	3	0	2	5	3	2	0	15
Total	6	8	11	3	14	7	7	2	58

" Tr, transplanted and atypical lobules; FEU, found epithelium unchanged; FED, found epithelium dedifferentiated; FS, found stroma; NF, not found.

Table 6

Fate of transplants placed s.c.or in cleared fat pads, without prior in vitro maintenance in relation to patient age

	20–29	30–39	40–49	50–59	60–69	70–79	Total
			Cancer-associated breasts				
L-FEU[a]	0	0	28	10	1	2	41
L-FED	1	0	4	10	1	4	20
L-FS	0	3	5	1	2	0	11
L-NF	0	0	3	3	0	0	6
Total	1	3	40	24	4	6	78
			Non-cancer-associated breasts				
L-FEU	29	9	9	1	0	0	48
L-FED	7	2	0	2	0	0	11
L-FS	1	1	2	0	0	0	4
L-NF	13	5	5	0	0	0	23
Total	50	17	16	3	0	0	86
			Cancer-associated breasts				
ALA-FEU	0	0	7	6	2	1	16
ALA-FED	0	0	1	2	0	2	5
ALA-FS	0	0	3	0	0	2	5
ALA-NF	0	0	2	0	0	2	4
Total	0	0	13	8	2	7	30
			Non-cancer-associated breasts				
ALA-FEU	1	0	2	1	0	0	4
ALA-FED	0	0	0	0	0	0	0
ALA-FS	0	0	0	0	0	0	0
ALA-NF	0	1	1	0	0	0	2
Total	1	1	3	1	0	0	6

[a] L, transplanted lobules; FEU, found epithelium unchanged; FED, found epithelium dedifferentiated; FS, found stroma; NF, not found.

Table 7

Fate of transplants placed s.c. or in cleared fat pads without prior in vitro maintenance in relation to presence or absence of stromal vascularization

	Cancer-associated breasts		Non-cancer-associated breasts		
	Vascularized	Nonvascularized	Vascularized	Nonvascularized	Total
Tr-FEU[a]	19	29	9	19	76
Tr-FED	20	2	3	4	29
Tr-FS	5	8	1	3	17
Total	44	39	13	26	122

[a] Tr, transplanted and atypical lobules; FEU, found epithelium unchanged; FED, found epithelium dedifferentiated; FS, found stroma; NF, not found.

cleared fat pad as do normal parenchyma and HAN from mice (2). However, when normal lobules become atypical and evolve into ductual carcinoma *in situ in vivo*, all cell proliferations do occur within existing ductular units (17), and this pattern might well persist in the transplantation setting until the lobules have progressed into frank carcinoma, a condition not yet achieved using the described protocol. Possibly, the denser stroma of human lobules, especially dense in ALA (17), would mechanically hinder such outgrowth. It is possible that the presence of immunoglobulin M (1, 8) or other residual immunological competence of the β-lymphocyte system as suggested by others (15) was responsible for this lack of carcinomatous transformation and of outgrowth into the cleared fat pad.

Insufficient numbers of ALA for data analysis were obtained. Our previous study showed that there is only an average of 30 ALA within a whole breast from high-cancer-risk patients (17). So these lesions will take a long time to obtain in greater numbers.

The high rate of dedifferentiation in lobules obtained from high-cancer risk patients after age 50 suggests that it is the lobules persisting after menopause that are potentially precancerous in the human breast. Such histologically normal-appearing lobules consistently evoked a vascularization response from the host that may be another indication of their precancerous potential.

References

1. Bloemmen, J., and Eyssen, H. Immunoglobulin Levels of Sera of Genetically Thymusless (Nude) Mice. European J. Immunol., *3:* 117–118, 1972.

2. DeOme, K. B., Faulkin, L. J., Jr., Bern, H. A., and Blair, P. B. Development of Mammary Tumors from Hyperplastic Alveolar Nodules Transplanted into Gland-free Mammary Fat Pads of Female C3H Mice. Cancer Res., *19:* 515–520, 1959.
3. Giovanella, B. C. and Stehlin, J. S. Heterotransplantation of Human Malignant Tumors in "Nude" Thymusless Mice. I. Breeding and Maintenance of Nude Mice. J. Natl. Cancer Inst., *51:* 615–619, 1973.
4. Giovanella, B. C., Stehlin, J. S., and Williams, L. J. Heterotransplantation of Human Malignant Tumors in "Nude" Thymusless Mice. II. Malignant Tumors Induced by Injection of Cell Cultures Derived from Human Solid Tumors. J. Natl. Cancer Inst., *52:* 921–930, 1974.
5. Giovanella, B. C., Yim, S. O., Morgan, A. C., Stehlin, J. S., and Williams, L. J. Metastases of Human Melanomas Transplanted in "Nude" Mice. J. Natl. Cancer Inst., *50:* 1051–1053, 1973.
6. Giovanella, B. C., Yim, S. O., Stehlin, J. S., and Williams, L. J. Development of Invasive Tumors in the "Nude" Mouse after Injection of Cultured Human Melanoma Cells. J. Natl. Cancer Inst., *48:* 1531–1533, 1972.
7. Jensen, H. M., Rice, J. D., and Wellings, S. R. Preneoplastic Lesions in the Human Breast. Science, *191:* 295–297, 1976.
8. Luzzati, A. L., and Jacobson, E. B. Serum Immunoglobulin levels in Nude Mice. European J. Immunol., *2:* 473–474, 1972.
9. Outzen, H. C., Custer, R. P., Eaton, G. J., and Prehn, R. T. Spontaneous and Induced Tumor Incidence in Germ Free "Nude" Mice. RES J. Reticuloendothelial Soc., *17:* 1–9, 1975.
10. Ozzello, L., Sordat, B., Merenda, C., Carrel, S., Hurlimann, J., and Mach, J. P. Transplantation of a Mammary Carcinoma Cell Line (BT 20) into Nude Mice. J. Natl. Cancer Inst., *52:* 1669–1672, 1974.
11. Pantelouris, E. M. Absence of Thymus in a Mouse Mutant. Nature, *217:* 370–371, 1968.
12. Pantelouris, E. M. Observations on the Immunobiology of Nude Mice. Immunology, *20:* 247–252, 1971.
13. Povlsen, C. O., and Rygaard, J. Heterotransplantation of Human Adenocarcinomas of the Colon and Rectum to the Mouse Mutant Nude. A Study of Nine Consecutive Cases. Acta Pathol. Microbiol. Scand. A, *79:* 159–169, 1971.
14. Rygaard, J., and Povlsen, C. O. Heterotransplantation of a Human Malignant Tumour to "Nude" Mice. Acta Pathol. Microbiol. Scand., *77:* 758–760, 1969.
15. Schmidt, M., and Good, R. A. Transplantation of Human Cancers to Nude Mice and Effects of Thymus Grafts. J. Natl. Cancer Inst., *55:* 81–87, 1975.
16. Wellings, S. R., and Jensen, H. M. On the Origin and Progression of Ductal Carcinoma in the Human Breast. J. Natl. Cancer Inst., *50:* 1111–1118, 1973.
17. Wellings, S. R., Jensen, H. M., and Marcum, R. G. An Atlas of Subgross Pathology of the Human Breast with Special Reference to Possible Precancerous Lesions. J. Natl. Cancer Inst., *55:* 231–273, 1975.
18. Wortis, H. H. Immunological Responses of "Nude" Mice. Clin. Exptl. Immunol., *8:* 305–317, 1971.

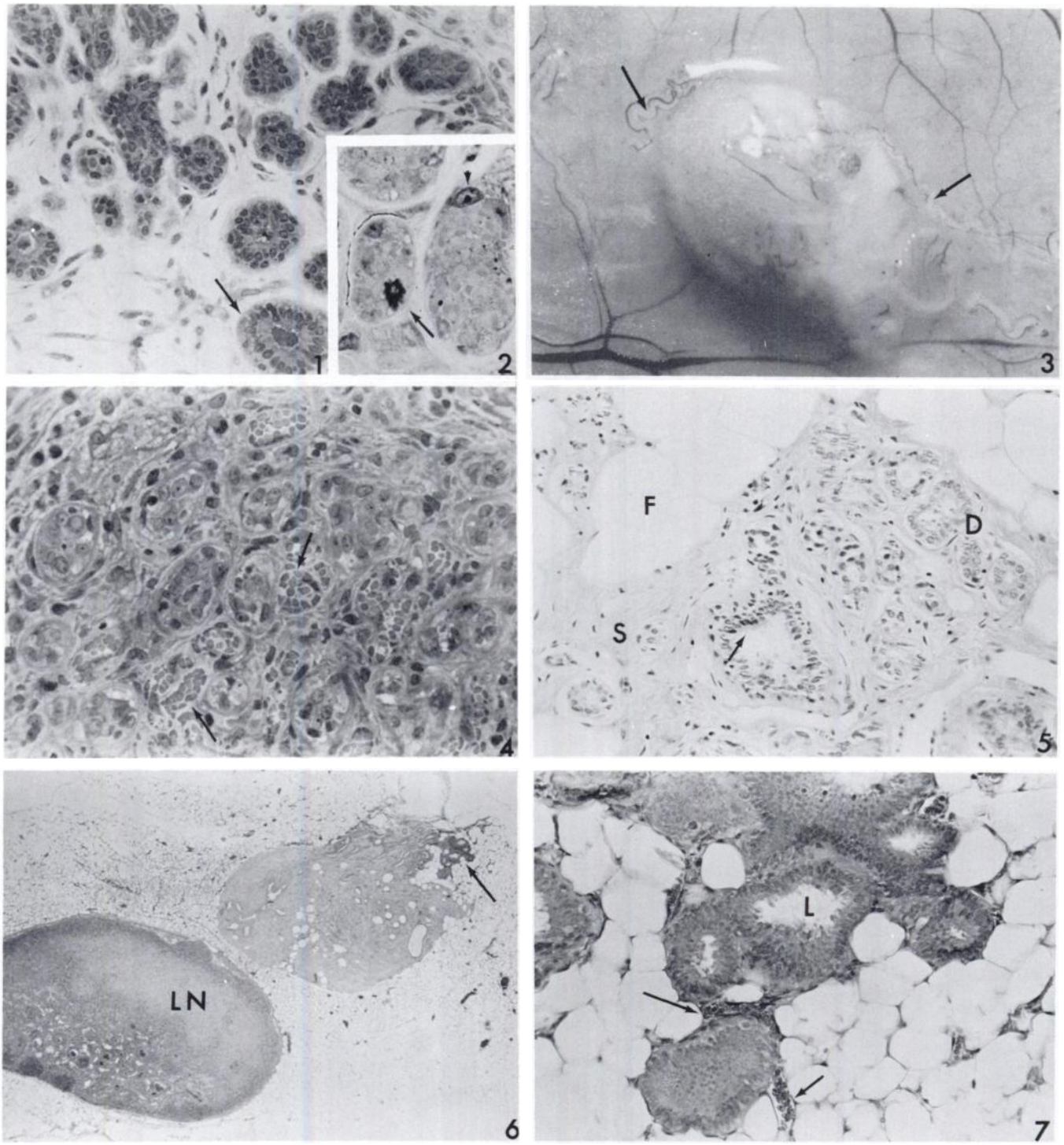

Fig. 1. Transplant, s.c., for 8 days of lobule from cancerous breast displays well-preserved ductules in loose stroma peripherally. *Arrow*, ductule with luminal secretion. Plastic-embedded, 2-μm section. Aldehyde fuchsin, × 160.

Fig. 2. The centrally located ductules of lobule seen in Fig. 1 show extensive necrosis. *Arrow*, mitotic cell; *arrowhead*, well-preserved cell. × 250.

Fig. 3. Transplant, s.c., for 16 days of lobular tissue from noncancerous breast *in vivo*. *Arrows*, thick-walled arterioles coursing to, over, and into the 5-mm transplant. Thin-walled venules appear dark. × 8.

Fig. 4. Transplant, s.c., for 16 days of lobule from breast depicted in Figs. 1 and 2. *Arrows*, thin-walled vessels filled with RBC of probable murine type. Well-preserved ductules show distortion but no hyperplasia. Plastic-embedded, 2-μm section. Aldehyde fuchsin, × 160.

Fig. 5. Part of a lobule from a cancerous breast, not transplanted, displays fatty *(F)* and sclerotic *(S)* stroma. Most ductules *(D)* are normal. One ductule is enlarged and slightly hyperplastic. *Arrow*, cytoplasmic blebs protruding into lumen. A Grade I atypical lobule. Paraffin-embedded, 7-μm section. H & E, × 160.

Fig. 6. Same lobule as in Fig. 5 but after 13 weeks in a cleared fat pad. The fat pad displays congested vessels and its large lymph node *(LN)*. Transplant shows more sclerosis than control. *Arrow*, part of the hyperplastic area of transplant located in fat pad. Paraffin-embedded, 7-μm section. H & E, × 16.

Fig. 7. Part of transplant at *arrow* in Fig. 6 shows hypercellularity of enlarged ductules, but lumina *(L)* are still present in some. *Arrows*, capillaries "hugging" the most cellular ductule. Grade II atypical lobule. × 160.