Differential Control of Alveolar and Ductal Development in Grafts of Monodispersed Rat Mammary Epithelium (43190)

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Abstract. **Multicellular secretory alveolar units (AU) develop in grafts of monodispersed mammary cells in intact recipient rats co-grafted with mammotropic hormone-secreting pituitary tumor (MtT). The cumulative evidence is consistent with a postulated clonal origin of these structures. Small numbers of multicellular structures of a second type, mammary ductal units (DU), were found in mammary cell grafts in intact Wistar/Furth recipients co-grafted with MtT W10 but not in intact F344 recipients co-grafted with MtT F4. Studies in (Wistar/Furth × F344) F_1 hybrid recipients grafted with mammary cells from either parent strain demonstrated that this difference in DU formation is dependent on the strain of grafted MtT, and is not a genetic characteristic of the rat strain. DU formation is stimulated and AU formation is inhibited by elevation of mammotropic hormones from MtT coupled with glucocorticoid deficiency induced by adrenalectomy. Cortisol treatment reverses this effect. Finally, in mammary glands *in situ* in intact rats, the total numbers of AU-forming clonogens decrease during 6 weeks after MtT transplantation. In contrast, during the same period, elevated mammotropins from grafted MtT coupled with glucocorticoid deficiency from adrenalectomy cause an increase in the total number of cells that are capable of AU formation when transplanted to intact recipients co-grafted with MtT. Thus, the same hormonal combination that stimulates DU formation in mammary cell grafts and has previously been shown to promote cancer in mammary glands *in situ* also stimulates an increase in the glandular content of assayable AU-forming cells *in situ*.** [P.S.E.B.M. 1991, Vol 196]

In a program designed to investigate the origins of radiogenic mammary cancer at the cellular level *in vivo*, we have found that a subpopulation of monodispersed mammary epithelial cells from normal young adult female rats gives rise to multicellular glandular structures when grafted in gland-free fat pads in hormonally modified recipients. When such grafts in intact females are stimulated by hormones from co-grafted mammotropic pituitary tumor strain (MtT) F4, secretory spherical or ovoid alveolus-like mammary structures are formed in the graft sites. More recently, nonsecretory mammary ductal structures as well as alveolar structures have been observed in similar grafts in Wistar-Furth (W/Fu) female rats co-grafted with MtT W10. The current experiments were undertaken to clarify the physiologic and/or genetic control of the formation of these two types of mammary structures with the ultimate aim of gaining information on their relationship to the genesis of mammary cancer.

Autoradiographic and light scanning and electron microscopic studies (1) have shown that by 3 to 4 weeks after grafting, the ~0.3- to 0.7-mm alveolar structures contain a central lumen filled with secretion that contains lipid droplets and electron-dense particles. When such a structure is pricked with a dissecting needle, white fluid is released. The single layer of epithelial cells lining the lumen contain secretory vacuoles with lipid or the electron-dense material at the lumenal pole. The apical cell membranes bulge into the lumen and form folds and microvilli. Myoepithelial cells with myofibrils surround the secretory epithelium and are in turn surrounded by connective tissue (1). The similarity of these

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structures to the swollen alveoli of intensely secretory normal mammary glands *in situ* led to their designation as alveolar units (AU).

The cumulative evidence is most consistent with the postulate that these AU arise from single cells, i.e., are clonal in origin (2). First, during the first few days after transplantation and hormonal stimulation of monodispersed mammary cells, light microscopy reveals scattered single epithelial appearing and inflammatory cells at the graft site (1). Epithelial cell clusters comprised of two to a few cells are present by 5 days after grafting when inflammation is subsiding. During the first 4 days, about 98% of the radioactivity in grafted cells that had been prelabeled with tritiated thymidine in the mammary donor animal before transplantation disappears from the graft site, indicating considerable cell loss (1). The cell clusters increase in size by proliferation during succeeding days and develop a central lumen.

Second, quantitative transplantation assays have shown that the grafted cell dose-AU formation response relationship is that which would be expected if an AU arises from a single cell (1, 2); it is inconsistent with AU origin from two or more cells. Third, quantitative transplantation assays of cells prepared from x-irradiated mammary glands in which the formation of AU is used as the end point have shown that the capacity of such cells to form AU decreases with the x-ray dose according to the relationship to be expected if AU are derived from single cells (3, 4). Furthermore, the recovery of AU-forming capacity after radiation exposure *in vivo* follows the kinetic pattern typical of repair of potentially lethal intracellular damage in irradiated mammalian cells in culture as measured by clonal colony-forming capacity (5). Fourth, as reaffirmed herein, the number of grafted cells required to produce at least one AU in 50% of the graft sites, the alveolar dose-50% (AD_{50}) is remarkably constant in cells prepared from the glands of normal young adult female rats of both Fischer (F344) and W/Fu strains.

In view of these findings, the progenitor cells of mammary AU are referred to as *clonogens* in this report, and the data are analyzed accordingly. It is of interest to note that functional structures develop in similar grafts of monodispersed rat thyroid cells (2) and hepatocytes (6) with appropriate physiologic stimulation. The data from experiments with these latter systems are consistent with the conclusion that these structures are also clonal in origin.

The transplantation and hormonal manipulation procedures and results from the mammary cell transplantation and irradiation studies noted above have been applied in estimation of the frequency of radiogenic malignant initiation in studies of carcinoma incidence per irradiated grafted mammary cell (7). The methodology developed for such experiments offered an opportunity for quantitative study of the control of mammary growth and differentiation at the cellular level *in vivo*.

The ductal mammary structures described herein were first observed in grafts of large numbers of mammary cells in intact W/Fu females co-grafted with MtT W10. These mammary ductal units (DU) are comprised of branching ducts and are not distended with secretion. DU had not been noted in similar grafts in intact F344 females co-grafted with MtT strain F4. MtT F4 and MtT W10 were both originally induced by chronic exposure to diethylstilbestrol (8, 9). Such estrogen-induced pituitary tumors are derived from the prolactin-secreting cells of the anterior pituitary (10) and initially secrete the primary pituitary mammotropic hormone, prolactin (11) with lesser amounts of growth hormone (12). On serial transplantation, MtT F4 acquired the ability to secrete adrenocorticotropin as well (12, 13). In this report, the mammotropic MtT secretions will be referred to as *mammotropin*.

The current studies were undertaken in part to determine (i) whether the development of DU is a genetically controlled physiologic or cellular characteristic of W/Fu and not of F344 rats, or (ii) alternatively, whether the difference in DU development is dependent on differences in the secretory products of the two MtT lines used in the two rat strains as the source of mammotropin; and (iii) what other endocrine factors are important in DU formation.

A combination of chronically elevated mammotropin combined with glucocorticoid deficiency is the most effective hormonal condition of which we are aware for the promotion of radiogenic rat mammary cancer (14–16). Endocrine promotion of neoplasia is perforce dependent on the effects of the hormones on cell population size, differentiation, and proliferation. Hence, experiments were also performed (iv) to determine how elevated mammotropin plus glucocorticoid deficiency affects the number of AU-forming clonogenic cells in the rat mammary gland *in situ*. In sum, we wished to gain further insight into the relationship between formation of AU and DU and the control of the number and nature of their cell(s) of origin. Our ultimate goal is to relate the findings to the cellular origins of radiogenic mammary cancer and its hormonal promotion.

Materials and Methods

Animals and Treatment. Fifty- to 60-day-old virgin female F344 and W/Fu rats (Harlan Sprague-Dawley, Madison, WI) and their F_1 hybrids (W/Fu × F344) bred in our facilities were used. They were housed in temperature (23 ± 2°C)- and humidity (50%)-controlled quarters with a 12-hr light/12-hr dark cycle and were fed commercial rodent chow *ad libitum*. When required, adrenalectomies were performed under ether anesthesia. When used, crystalline cortisol (Sigma

Chemical, St. Louis, MO) was dissolved in peanut oil with a small volume of ethanol and was administered subcutaneously at a dose of 1 mg/day, or in a preliminary experiment, 0.5 mg/day. Adrenalectomized rats were given their choice of acidified tap water or physiologic saline; rats with intact adrenals were given only acidified tap water. For transplantation, MtT were removed from donor animals, minced in Eagle's minimal α-medium (α-MEM; Flow Laboratories, McLean, VA), and passed through cytosieves. The slurries were transplanted by intramuscular injection in a hind leg (1).

Preparation of Monodispersed Mammary Cell Suspensions for Transplantation. Two different methods were employed for the preparation of monodispersed mammary cell suspensions in these experiments. For maximum recovery of mammary cells, the original collagenase-Pronase method (1, 2) was used in the studies of the effects of glucocorticoid deficiency on the number of clonogenic cells in the mammary gland *in situ* in MtT-grafted rats. In the series of experiments on the control of AU and DU formation in grafts of mammary cells, a method modified from the original procedure by the inclusion of epithelial purification steps was employed.

Briefly, the modified method was as follows: After removal of the inguinal mammary fat pads, mincing, dispersion in collagenase and DNase, and washing, the digested suspension contained cells, cell clumps, and mammary organoids (ductal fragments and end buds) (2, 17). The suspension was washed with α-MEM with 10% fetal bovine serum (FBS; Sterile Systems, Logan, UT) followed by two washes in α-MEM with centrifugation at ~350*g* for 6 min. The pellet was resuspended in a 1:1 mixture of α-MEM:Ham's F-12 (Gibco, Grand Island, NY) with 10% FBS, 0.5 mg of insulin/ml (Collaborative Research, Waltham, MA), 0.005 μg of estradiol/ml, and 0.5 μg of cortisol/ml (Sigma), 5 μg of bovine prolactin/ml (Hormone Distribution Office, National Institute of Arthritis, Digestive Disorders and Kidney Diseases), and antibiotics (100 IU of penicillin/ml, 100 μg of streptomycin/ml, 50 μg of gentamicin sulfate/ml). The suspension was transferred to petri dishes and incubated at 37°C for 120 min. The cells that adhered to the plastic surfaces during this time were predominantly fibroblasts and other nonepithelia (17). The supernatant, which contained the cells and organoids that did not adhere, was removed from the dishes and centrifuged at ~20*g* for 2 min. The pellet that contained predominantly organoids was washed and recentrifuged twice in α-MEM-10% FBS, resuspended, and passed onto a 40-μm pore nylon mesh filter, which allowed the free cells and clumps to pass but retained the organoids on the filter surface. The trapped organoids were collected by backwashing the filter with α-MEM-FBS and again washed and recentrifuged twice.

The pelleted organoids were resuspended in 0.05% trypsin (Gibco):0.02% EDTA (Sigma) in α-MEM and incubated at 37°C for 10 min with shaking. The resultant monodispersed cells were centrifuged at ~350*g* and washed twice and resuspended in α-MEM. Three milliliters of 0.05% DNase (Cooper Biomedical, Freehold, NJ) were added per each 17-ml suspension, and the mixture was incubated for 10 min at room temperature and filtered twice through a 40-μm pore filter.

The concentration of cells in the suspensions from the collagenase-Pronase and the modified methods were determined by phase microscopy in a hemocytometer, and appropriate dilutions were prepared. Before transplantation, an equal volume of 50% brain suspension was added to each suspension (1, 2). As noted below, the two methods of preparation of monodispersed mammary cells result in different final concentrations of clonogens as measured by AU-forming ability.

Transplantation Assay of Clonogen Concentrations. The assay methodology and statistical evaluation procedures have been described (2). Aliquots of 0.06 ml of the serially diluted mammary cell suspensions were inoculated at each of three sites in the interscapular white fat pads of recipient rats. Generally, each cell concentration was inoculated into a total of 15 graft sites. Thus, the assay of AU- and DU-forming ability of cells from normal glands grafted in intact MtT F4-grafted F344 rats (Fig. 2) involved transplantation of 13 cell concentrations and scoring a total of ~195 graft sites from ~65 recipient rats.

In the assays used for comparisons of the effects of elevated mammotropin with elevated glucocorticoid versus elevated mammotropin with glucocorticoid deficiency on total AU-forming clonogens *in situ* (Figs. 4 and 5), 90–150 graft sites from 30 to 50 recipients were scored. In all such assays, 2 weeks before grafting of mammary cells, F344 recipient rats were grafted with MtT F4, or W/Fu recipients with MtT W10, as indwelling sources of mammotropin. Some groups of recipients were also adrenalectomized within 24 hr of the MtT grafting. Within a few weeks of grafting of either MtT F4 or MtT W10, radioimmunoassays of serum prolactin show titers in excess of peak normal levels (data not shown). The growth rates of the two MtT strains are comparable. All recipients were killed by ether overdose and autopsied 3 weeks after mammary cell transplantation. The fat pads were removed, fixed, stained, and examined with a dissecting microscope for the presence or absence of AU or DU. The fractions of transplant sites in each dilution group, which contained at least one AU or one DU, and the mean numbers of morphologically intact cells grafted per site in each group were then computer fit to the transplantation model of Porter *et al.* (18). AD_{50} values were calculated from the data; these values are the numbers of morphologically intact cells per graft site

required to produce at least one AU in 50% of the sites (2). In MtT grafted intact recipient rats, AD_{50} values are inversely proportional to the clonogenic fractions. As the relationship between the fractions of graft sites with DU and the numbers of grafted cells followed the same pattern, comparable ductal dose-50% (DD_{50}) values were also calculated.

The total numbers of AU-forming clonogens per inguinal mammary fat pad *in situ* were estimated from the clonogenic fractions and the total mammary cells recovered by the collagenase-Pronase method (1, 2). As noted below, the AD_{50} values of suspensions prepared by this original method are greater than those obtained by insertion of the clonogen purification steps in the modified cell suspension procedure.

Results

AU and DU Morphology. The light and electron microscopic morphology of AU have been described (1). AU are spherical structures distended with secretion and lined with a single layer of secretory epithelial cells associated with myoepithelia (Fig. 1, A and C). The secretory cells are rich in lipids and vacuoles. In contrast, DU resemble developing mammary glands comprised of ducts with walls two to four cell layers deep with multilayered end buds (Fig. 1, A, B, and D). Mitoses are common in the epithelia in the end buds, and are also found in the duct walls and in the stroma surrounding both end buds and ducts.

AD_{50} and DD_{50} Values in Intact MtT-Grafted Females. The efficiency of AU and DU formation in grafts of known numbers of monodispersed mammary epithelial cells was determined in intact young adult female F344 and W/Fu rats co-grafted with MtT F4 and MtT W10, respectively. The cell dose-AU formation response curves for the two rat strains are virtually superimposable (Fig. 2). AU formation efficiencies are expressed in terms of AD_{50} values, i.e., the average numbers of inoculated cells per graft site required to yield at least one AU in 50% of the sites. The AD_{50} in W/Fu rats was 704 mammary cells and in F344 rats, 786 cells (Fig. 2).

In these MtT-grafted intact recipients, the formation of DU was much less efficient. In W/Fu rats, the cell dose-DU response data followed the same relationship as AU formation, but the curve was far to the right (Fig. 2). The DD_{50} calculated in the same manner as the AD_{50} values was 13,012 cells, more than 18-fold the AD_{50}. In this experiment, no DU were seen in F344 rats at doses up to 2×10^5 mammary cells/graft site.

AU and DU Formation in (W/Fu × F344) F_1 Hybrid Rats. Experiments were designed to determine whether the differences in formation of DU are characteristics of the grafted mammary cells or of the recipient rat strains, and if the latter, whether such differences are dependent on differences in the spectra of secretions of the different MtT lines. F_1 hybrid rats were thus grafted with 1.2×10^5 mammary cells from either F344 or W/Fu rats per graft site. The F_1 recipients had been grafted 2 weeks previously with MtT W10, MtT F4, or with MtT of both strains.

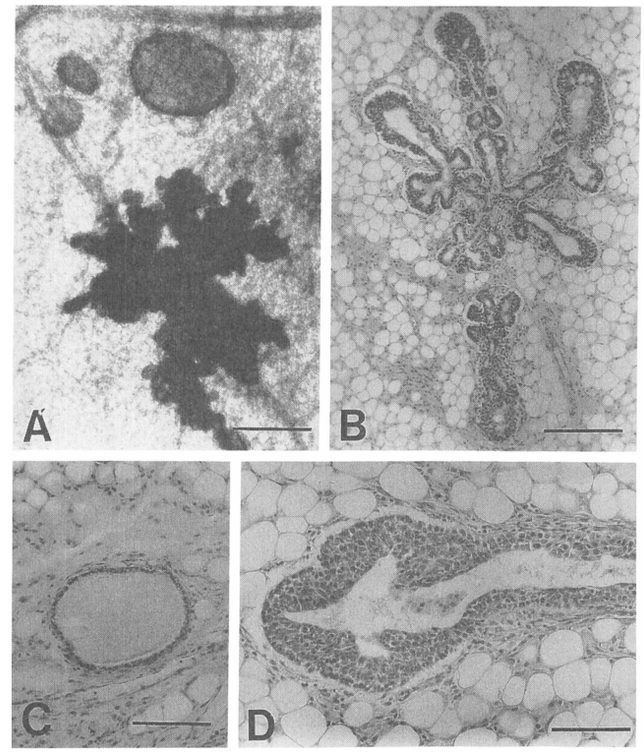

Figure 1. Morphology of AU and DU 3 weeks after grafting of monodispersed mammary cells in fat pad sites of recipient rats that had been grafted with MtT 2 weeks before mammary cell transplantation. Whole mounts were fixed in 70% ethanol, cleared in xylene, and passed into mineral oil. Illustrated whole mount was stained with hematoxylin; 5-μm paraffin sections of whole mounts were stained with hematoxylin and eosin. (A) Photomicrograph of three AU (upper) and one DU (lower) in a whole mount of a fat pad grafted with ~1800 mammary cells in an intact F344 rat. (original magnification 40×; bar = 500 μm). (B) Section through a DU after grafting of ~1250 mammary cells in an adrenalectomized W/Fu recipient treated with 0.5 mg of cortisol/day (original magnification ×330; bar = 60 μm). (C) Section through an AU in a graft site in a recipient treated as in B (original magnification ×660; bar = 30 μm). (D) Section through a mammary duct and end bud in a graft site in a recipient treated as in B (original magnification ×660; bar = 30 μm).

Three weeks after mammary cell transplantation, the graft sites were examined for the presence or absence of mammary structures. One-hundred percent of the graft sites in all groups contained AU (Table I). DU were present in 90% of graft sites with W/Fu mammary cells in hybrid recipients co-grafted with MtT W10, which does not secrete ACTH; in contrast, there were no DU in W/Fu AU cell grafts in recipients co-grafted with MtT F4, which secretes ACTH (Table I). Similarly, 81% of the F344 mammary cell grafts in hybrid rats co-grafted with MtT W10 contained DU, but no DU were found in the F344 cell grafts in rats co-grafted with MtT F4. In rats co-grafted with both MtT strains,

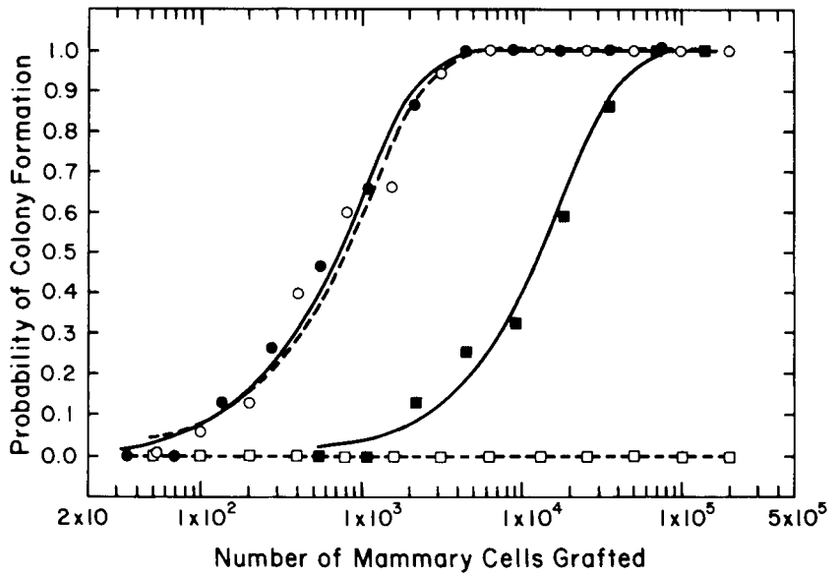

Figure 2. AU and DU formation in mammary cell grafts in MtT co-grafted W/Fu and F344 recipients. Fractions of graft sites with AU: ●——●, W/Fu rats, AD_{50} = 704 grafted cells/site (95% confidence limits, 488–964); ○– – –○, F344 rats, AD_{50} = 786 cells (95% confidence limits, 529–1091). Fractions of graft sites with DU: ■——■, W/Fu rats, DD_{50} = 13,012 cells/site (95% confidence limits, 9,097–17,745); □– – –□, F344 rats, $DD_{50} > 2 \times 10^5$ cells. Symbols are observed data points; lines are best fit of Porter *et al.* (18) model (see Materials and Methods).

Table I. Dependence of DU Formation in Grafts in F_1 Recipients on Co-Grafted MtT Strain

Strain of mammary cell donors	Strain of recipient rats	Strain of MtT	Graft recipient weights ± SD			% Graft sites with[a]	
			Body (g)	Adrenal gland (mg)	Uterus (mg)	AU	DU
W/Fu	F_1[b]	MtT-W10	313 ± 19	63 ± 12	289 ± 58	100 (21)[c]	90 (21)
W/Fu	F_1	MtT-F4	250 ± 33[d]	228 ± 32[e]	358 ± 193	100 (21)	0 (21)[f]
W/Fu	F_1	MtT-W10 + MtT-F4	253 ± 31[d]	233 ± 43[e]	348 ± 160	100 (21)	10 (21)[f]
F344	F_1	MtT-F4	—	—	—	100 (18)	0 (18)[g]
F344	F_1	MtT-W10	—	—	—	100 (21)	81 (21)
F344	F_1	MtT-F4 + MtT-W10	—	—	—	100 (15)	0 (15)[g]

[a] All graft sites received 1.2×10^5 monodispersed mammary cells. There were three graft sites per recipient rat for a total of five to seven recipient rats per group.
[b] F_1 = (W/Fu × F344).
[c] Number of transplanted sites in parentheses.
[d] Versus MtT W10, $P < 0.01$.
[e] Versus MtT W10, $P < 0.001$.
[f] Versus W/Fu mammary cells and MtT W10, $P < 0.001$.
[g] Versus F344 mammary cells and MtT W10, $P < 0.001$.

DU were found in only 10% of the W/Fu mammary cell graft sites and in none of the F344 mammary cell graft sites.

The body weights of recipient F_1 rats grafted with W/Fu mammary cells and co-grafted with MtT W10 alone were significantly greater, and the adrenal weights were highly significantly less than those co-grafted with MtT F4 or with both MtT strains (Table I). Body weights and adrenal weights of rats grafted with MtT F4 and those grafted with both MtT strains were insignificantly different from each other. Thus, grafts of MtT F4, which secretes ACTH, affected both body weight and adrenal weight whether or not the rats were co-grafted with MtT W10. Differences in uterine weights among groups were not significant.

Effect of Adrenalectomy on AU and DU Formation. The effect of adrenalectomy on the efficiency of AU and DU formation was tested in two experiments with W/Fu rats co-grafted with MtT W10, which does not secrete ACTH. In the first, aliquots of serially diluted monodispersed mammary cells were grafted in groups of intact or adrenalectomized recipients with MtT W10 co-grafts as in standard AD_{50} assays. In the second, 2×10^5 mammary cells were grafted in intact or adrenalectomized rats co-grafted with MtT W10; half of the adrenalectomized recipients were treated

with cortisol. The cortisol dose was chosen to maximize the glucocorticoid effect.

In the first experiment, adrenalectomy increased the AD_{50} more than 100-fold from 703 cells in intact rats to $>10^5$ cells/graft site; indeed, AU were only sporadically seen in adrenalectomized recipients (Fig. 3). In contrast, the DD_{50} was reduced about 10-fold from 11,377 cells/graft site in intact rats to 1,244 cells/site in adrenalectomized recipients. The DD_{50} in these adrenalectomized rats was insignificantly different from the AD_{50} in intact animals. The AD_{50} and DD_{50} values in the intact rats in this experiment (Fig. 3) were insignificantly different from those in intact W/Fu rats in the strain comparison study above (Fig. 2).

In a preliminary experiment with adrenalectomized, MtT-grafted F344 recipients treated with 0.5 mg of cortisol/day, both AU and DU were found in the graft sites (Fig. 1). In the second experiment of this series, all mammary cell grafts in intact W/Fu recipient rats co-grafted with MtT W10 contained both AU and DU (Table II). Only DU were found in all mammary cell graft sites in adrenalectomized MtT-grafted recipients that received no cortisol. In contrast, only AU were found in such adrenalectomized rats, which were treated with 1 mg of cortisol/day (Table II).

Effect of Adrenalectomy on Total Clonogens in Mammary Glands *in Situ* in MtT-Grafted Rats. It was of interest to determine how adrenalectomy might affect the total number of clonogenic cells in the mammary glands of MtT-grafted rats *in situ*. In a preliminary study, AD_{50} values of cells from the glands of adrenal-

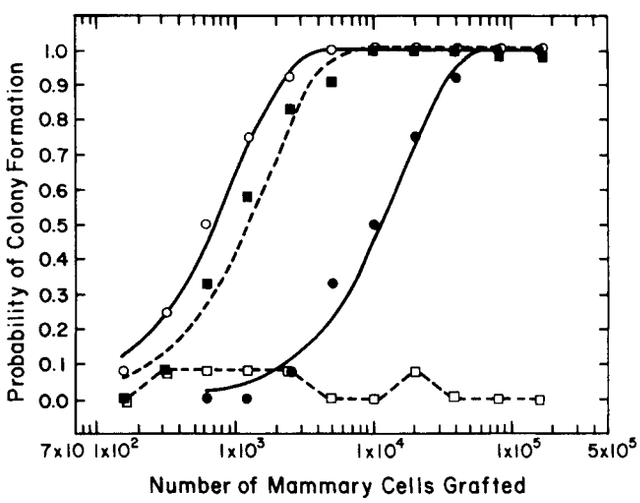

Figure 3. Effect of adrenalectomy on AU and DU formation in mammary cell grafts in W/Fu recipients co-grafted with MtT W10. Fractions of graft sites with AU: ○——○, intact recipients, AD_{50} = 703 cells/graft site (95% confidence limits, 423–1,004 cells); □- - -□, adrenalectomized recipients, $AD_{50} > 1 \times 10^5$ cells/site. Fractions of graft sites with DU: ●——●, intact recipients, DD_{50} = 11,377 cells/site (95% confidence limits, 7,547–15,932); ■- - -■, adrenalectomized recipients, DD_{50} = 1244 cells/site (95% confidence limits, 792–1755). Symbols are observed data points; lines are best fit of Porter *et al.* (18) model.

Table II. Effect of Cortisol on AU and DU Development in W/Fu Recipients Co-Grafted with MtT W10[a]

Treatment of recipients		% Graft sites with[b]	
Surgery	Hormone	AU	DU
None	None	100 (21)	100 (21)
ADX[c]	None	0 (15)[d]	100 (15)
ADX	Cortisol[e]	100 (15)	0 (15)[f]

[a] 2.0×10^5 monodispersed mammary cells grafted per site. There were three graft sites per rat for a total of five or seven recipient rats per group.
[b] % with number of graft sites in parentheses.
[c] ADX, adrenalectomized.
[d] Versus intact or ADX-cortisol groups, $P < 0.001$.
[e] 1.0 mg of cortisol injected subcutaneously per day.
[f] Versus intact or ADX alone groups, $P < 0.001$.

ectomized, otherwise untreated rats were ~50% of those from intact rats 2 weeks after surgery (19), suggesting about a 2-fold increase in clonogen concentrations in such animals; total clonogens per gland were not determined, however. In the current experiments, AD_{50} assays and estimates of the total assayable clonogens were made with cells prepared from the mammary glands of untreated F344 rats (Day 0), and with cells from glands of intact or adrenalectomized F344 rats 3, 5, or 6 weeks following surgery and/or grafting of MtT F4. To ensure recovery of as many clonogenic cells as possible for estimation of the total number of clonogens per mammary chain, epithelial cell purification steps were not used during preparation of the cell suspensions for assay. Rather, the original collagenase-Pronase cell dispersion method (1, 2) was employed. With this dispersion method, the AD_{50} value of cells prepared from untreated glands of young adult virgin females is routinely ~2000 cells/graft site (Fig. 4).

AD_{50} values of cell suspensions prepared from the mammary glands of intact rats progressively increased with time to 10-fold the Day 0 value by 6 weeks after grafting of MtT (Fig. 4). This increase in AD_{50} reflects a 10-fold decrease in the concentration of clonogens. The estimated total clonogen number per inguinal mammary chain was similar to the Day 0 control number 3 weeks after grafting; total clonogens then decreased 5-fold by the sixth week (Fig. 5).

The AD_{50} values of mammary cells from adrenalectomized MtT-grafted rats remained near the Day 0 control value for 5 weeks after surgery, and then increased 2- to 3-fold by 6 weeks (Fig. 4). More importantly, the total clonogen numbers in the inguinal glands of the adrenalectomized rats were 4-fold those of intact animals 3 weeks after MtT grafting, 15-fold greater at 5 weeks and 8-fold greater after 6 weeks of exposure to elevated mammotropin (Fig. 5). The decrease in the concentration of clonogens at 6 weeks

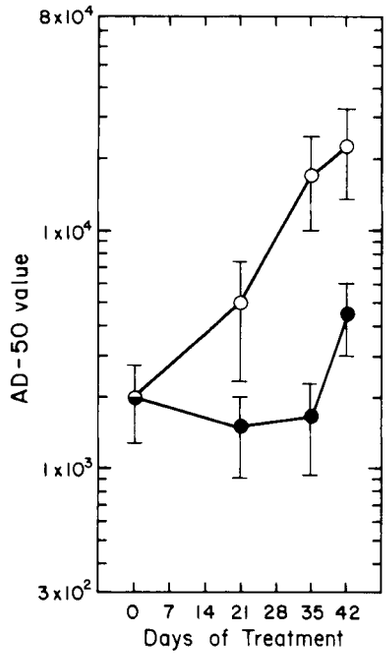

Figure 4. AD_{50} values of mammary cells from untreated F344 female rats (Day 0, ◒) and from rats at intervals after grafting of MtT F4 (○) or grafting of MtT F4 and adrenalectomy (●). Mammary glands were removed from the treated rats on the indicated days, monodispersed mammary cell suspensions were prepared, and the concentrations of clonogenic cells were estimated by AD_{50} transplantation assay. Vertical bars are 95% confidence limits.

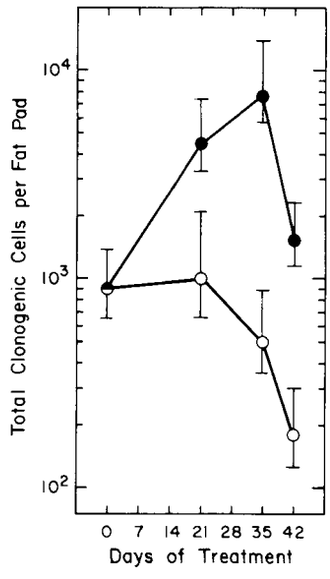

Figure 5. Effect of adrenalectomy on the total recoverable mammary clonogens in the three inguinal mammary glands of F344 females at intervals after surgery and grafting of MtT F4. MtT grafts and adrenalectomies on Day 0. Intact females with no MtT (◓), intact with MtT (○), and adrenalectomized with MtT (●). Total mammary clonogens = total cells recovered after dispersion × clonogenic fraction from AD_{50} transplantation assay (see Fig. 4). Vertical bars are 95% confidence limits.

may be related to the large MtT tumor burden during the last week of the experiment.

Discussion

The results of the first series of these experiments show that development of two types of mammary structures can be induced in grafts of monodispersed mammary epithelial cells by different hormonal combinations. We conclude that both types of mammary structure are likely clonal in origin, although not necessarily from the same cell subpopulation. AU formation occurs in grafts in intact recipients in response to elevated mammotropins; few or no ductal structures occur in such grafts. In glucocorticoid-deficient graft recipients with elevated mammotropin, DU formation is favored; few or no alveolar structures develop.

Morphologically, AU strongly resemble secretory alveoli; DU are composed of nonsecretory ducts and cellular end buds that strongly resemble developing mammary glands. The cumulative evidence from previous experiments is most consistent with the postulate that AU arise by proliferation and differentiation from single mammary epithelial clonogens (1, 2). The current results suggest that DU are also clonogenic in origin. The grafted cell dose:DU formation response curve follows the expected relationship for a single-cell origin of DU as it does for AU formation. Furthermore, when hormonal conditions are optimal for AU formation, few DU are formed. When hormonal conditions are optimal for DU formation, AU formation is markedly suppressed. Under the latter conditions, DU are formed with almost the same efficiency as AU are under conditions optimal for AU formation.

In accord with the above, DU formation is not genetically limited to the W/Fu rat strain in which they were first observed; they can be induced in F344 rats or in F_1 hybrids. The lack of development of DU in intact MtT F4-grafted F344 rats is attributable to ACTH secretion by MtT F4 (12, 13); this in turn brings about an elevation in glucocorticoid levels that favors development of AU. Adrenalectomy prevents the elevation of glucocorticoids and greatly enhances the development of DU; the latter effect is reversed by high level cortisol treatment.

Assuming that AU and DU are both clonal in origin, the results of the experiments discussed above reflect the proliferative and differentiative potentials of individual clonogens under different hormonal conditions in graft recipients. In contrast, the second series of experiments discussed below was devoted to measurements of hormonal effects on the total number of AU-forming clonogens in mammary glands *in situ*. AU formation in graft recipients was used as the end point in measurements of the sizes of the subpopulations of clonogens in cell suspensions prepared from the glands of hormonally manipulated donors.

In adrenalectomized F344 rats cografted with MtT F4, the combination of elevated pituitary mammotropin with glucocorticoid deficiency for 3 to 6 weeks significantly increased the total number of assayable AU-forming clonogens *in situ* per mammary chain, whereas MtT secretions in intact rats caused a decrease in total clonogens. A significant increase in total clonogens per mammary chain as measured by AU formation has also been described in adrenalectomized F344 rats 48 days after intrasplenic implantation of a pituitary gland and estrone capsule as a source of mammotropin (20). We interpret these data to indicate that in the presence of glucocorticoids, mammotropin-stimulated AU-forming clonogens *in situ* give rise to nonclonogenic differentiated cells, and hence the total AU-forming clonogen population decreases. When secretory differentiation is blocked by glucocorticoid deficiency, mammotropin-stimulated proliferation of AU-forming clonogens results in more daughter cells, which retain clonogenic capacity.

These findings are consistent with previous studies in which extensive mammary development was observed *in situ* in adrenogonadectomized male F344 rats, which were grafted with MtT and given no glucocorticoid replacement therapy or gonadal steroids (21). Milk secretion occurred only when such animals were treated with cortisol. A glucocorticoid requirement for secretory differentiation in mouse mammary glands was reported earlier by Nandi (22). In mice, however, gonadal hormones were required in addition to pituitary hormones for mammary gland development (22).

The combination of elevated mammotropins and glucocorticoid deficiency has been shown to promote cancer in irradiated rat mammary glands *in situ* more effectively than elevated mammotropins alone, and given time, to cause an increase in mammary tumors without other treatment (7, 14–16, 20). The effect of adrenalectomy was reversed by glucocorticoid treatment (15). This finding was interpreted to indicate that in irradiated rats with elevated mammotropins and adequate glucocorticoid levels, terminal secretory differentiation removed some potential cancer-forming cells from the irradiated cell population.

In our early carcinogenesis studies, MtT were used as the source of chronically elevated mammotropins (14, 15). More recently, intrasplenic implantation of a single pituitary gland adjacent to a silicone capsule containing estrin was found to produce sustained elevation of prolactin without producing peripheral hyperestrinism (16); this procedure avoids the large MtT tumor burden and the necessity for resection and regrafting of MtT in experiments of long duration. In adrenalectomized animals with such implants, with time, prolactin titers reach 10^1–10^3 times normal and appear to exceed the maximum cancer-promoting level (7, 16); e.g., although this hormonal combination promoted cancer in grafts of preirradiated monodispersed mammary cells, there was no correlation between cancer incidence and final prolactin levels in these rats (7).

Thus, the efficient hormonal promoting combination for mammary cancer in the rat, elevated pituitary mammotropins plus glucocorticoid deficiency, also induces ductal differentiation from grafted mammary cells as well as a marked increase in the number of assayable AU-forming cells in mammary glands *in situ*. The nature of the ductal differentiative process and its relationship to the control of alveolus-forming capacity and to tumor promotion are under continuing investigation.

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