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Persistence of Stem-like Cells in Glandular Structures in Mammary Cell Grafts

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Abstract

The mammary gland contains a subpopulation of epithelial cells with large proliferative potentials which are the likely targets for carcinogens. These clonogenic cells can proliferate and differentiate into functional glandular structures. Multicellular secretory alveolar units (AU) develop from these clonogens in grafts of monodispersed rat mammary epithelial cells (RMEC) in gland-free mammary fat pads in intact recipient F344 rats co-grafted with mammotropic hormone-secreting pituitary tumors (MtT F4). Multicellular nonsecretory ductal units (DU) develop in grafts of monodispersed RMEC in gland-free fat pads in adrenalectomized recipient WF rats co-grafted with MtT W10. However, this effect were reversed by hydrocortisone replacement therapy. RMEC were isolated from appropriate donor rats as monodispersed mixed cells or, alternatively, PNA+ cells were sorted by flow cytometry of mixed RMEC stained with FITC-PNA and PE-anti-Thy-1.1 monoclonal antibody. We grafted mixed or sorted PNA+ cells in gland-free mammary fat pads in recipient rats that were endocrinologically manipulated to induce AU or DU. Cells were also isolated from these AU or DU as mixed or sorted PNA+ cells and sub-transplanted in recipient rats treated appropriately to induce AU or DU, respectively. Cells obtained from AU in grafts gave rise to clonal AU and from DU in grafts to DU on sub-transplantation in appropriate recipients. When adrenalectomized recipient WF rats co-grafted with MtT W10 received daily subcutaneous injections of hydrocortisone for periods of 21 days following the PNA+ cell transplantation, AU, instead of DU, were developed. The histologies of these secondary AU and DU were not different from those of the primary AU and DU. Casein and laminin proteins were demonstrated by immunocytochemical staining of primary and secondary AU. Electron micrographs also demonstrated that AU were composed of secretory cells with milk protein in the cytoplasm. DU were composed of little or non-secretory ductal epithelial cells. These AU and DU also secreted large amounts of lipids. Clonogenic cells were more common in DU than in AU. Thus, AU and DU contain persistent subpopulations of clonogenic stem-like cells.

Key words – RMEC(rat mammary epithelial cells, stem, stem-like, clonogen

Introduction

Mammary clonogen transplantation assays have been developed to quantitate the biological effects of hormonal manipulation on donor cells and/or on the host animals, to identify the host mechanisms regulating the proliferation and function of the tissues of origin, to investigate the carcinogenic process, and to determine the roles of the products of these cells in the host [1-3]. The assays

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are based on the fact that when a mammary clonogen is transplanted into a subcutaneous fat pad of a syngeneic recipient and is hormonally stimulated, it will proliferate and differentiate to form a clonal glandular structure. In recipient rats with elevated prolactin (Prl+) and normal or elevated glucocorticoids (Glc-n or Glc+), the glandular structure formed is an alveolar unit (AU). AU are histologically and ultrastructurally similar to alveolar structures found in intact glands; they are lined with secretory epithelium surrounded by myoepithelial cells. Moreover, three weeks after mammary cell transplantation, most AU are filled with secretion [4].

Mammotropic pituitary tumors MtT F4 and MtT W10 were originally induced by chronic exposure to diethylstilbestrol in F344 and WF rat strains, respectively [6,7]. These pituitary tumors are derived from the prolactin-secreting cells of the anterior pituitary [8] and initially secrete the primary pituitary mammotropic hormone, prolactin [9] with lesser amounts of growth hormone [6]. On serial transplantation, MtT F4 acquired the ability to secrete adrenocorticotropin as well [10,11]. MtT F4 produces predominantly Prl (serum levels of 1-3 (g/ml), adrenocorticotropin, and growth hormone. MtT W10 also secretes Prl and growth hormone but had little or no adrenocorticotropic effect.

We have concentrated rat mammary clonogens using AU and DU formation in grafts as the index of clonogenic fraction sizes. AU and DU are very similar to the mammary glands in secretory and developing states, respectively. However, the cells composing these clonal structures have not been tested for the persistence of clonogens within them. If these AU and DU are truly clonal structures, they would be expected to contain a persistent subpopulation of clonogenic or stem-like cells.

Primary mammary epithelial cells were cultured in serum medium with several lactogenic hormones [22]. Four cell subpopulations were then sorted by flow cytometry from suspensions of the cultured rat mammary cells after staining with fluorescein isothiocyanate (FITC)-PNA and phycoerythrin (PE)-anti-Thy-1.1 monoclonal antibody. These subpopulations included PNA positive cells (PNA+), Thy-1.1 positive cells (Thy-1.1+), cells positive to both labels (both positive, B+), and cells negative to both reagents (B-). The flow cytometry sorted PNA+ cell populations contained the highest clonogenic fraction and the Thy-1.1+ cells the lowest. The B+ and B- cells contained slightly lower clonogenic fractions than the PNA+ cells [22,23,24]. Electron micrographs showed that PNA+ cells contained secretory vacuoles; Thy-1.1+ cells appeared to be myoepithelial with prominent myofibril bundles in the cytoplasm; B- cells had invaginations of the nuclear membrane. Moreover, when we grafted each of 4 different RMEC subpopulations after fluorescence activated cell sorting in hyperprolactinemic recipient rats, the PNA+ cell subpopulation included most of the clonogenic cells. Thy-1.1+ cells appeared to be terminally differentiated and they are likely to be myoepithelial cells. B+ cells may be a transitional from one type of cell to another. Moreover, when mammary organoids, monodispersed epithelial cells, flow cytometry-sorted PNA+, Thy-1.1+, B+, and B- cells from immature virgin rats were grown in Engelbreth-Holm-Swarm (EHS) reconstituted basement membrane, ductal, lobulo-ductal, stellate, webbed, and squamous morphogenesis occurred [23,24].

In the present studies, we have investigated the hormonal control of formation and differentiation of AU and DU from transplanted mammary clonogens, the concentration of clonogenic cells in AU and DU that developed in mammary cell grafts and the persistence of clonogenic cells in glandular structures. Therefore, mixed cells or PNA+ cells from clonal structures were subtransplanted to determine their capacity to form AU and/or DU in hormonally manipulated syngeneic recipient rats.

Materials and Methods

Animals and treatment

Fifty- to 55-day-old virgin female F344 and WF (Wistar-

Furth) rats were used throughout. The rats were fed commercial laboratory chow and were housed in a temperature (23 ¡ ¾2℃) and humidity (50%) controlled room with a 12 h light-12 h dark cycle. When required, adrenalectomies (Adx) were performed under ether anesthesia 15 days prior to the mammary cell transplantation. The adrenalectomized animals were given weekly subcutaneous injections of 2.5 mg deoxycorticosterone acetate (DOCA, Sigma, St. Louis, MO) suspended in steroid menstruum after surgery, and all were given their choice of acidified tap water or physiologic saline ad libitum. Designated recipients received daily subcutaneous injections of hydrocortisone for periods of 21 days following the mammary cell transplantation. One thousand mg of crystalline hydrocortisone (Sigma) was partially dissolved and suspended in ~20 ml of absolute ethanol and the suspension was brought to a final volume of 100 ml with peanut oil. Aliquots of 0.1 ml of the suspension containing 1.0 mg of hydrocortisone were injected per day.

MtT tumor preparation for transplantation

For transplantation, MtT F4 or MtT W10 tumors were removed from donor animals, minced in serum free medium (SFM: Dulbecco's Modified Eagle Medium with 50 (μg/ml gentamicin sulfate and 0.33 mg/ml glutamine; without phenol red, Gibco, Grand Island, NY), and passed through cytosieves. Two weeks before mammary cell transplantation, five week-old, syngeneic recipient rats were each grafted intramuscularly in a hind leg with 0.1 ml of a 50% cytosieved suspension of MtT as indwelling sources of mammotropin [4].

Mammary cell preparation for in vivo transplantation

To induce primary AU and DU, virgin female F344 or WF rats, 50-55 days old were killed and their inguinal mammary fat pads were removed, and digested with collagenase solution (Type III, 2 mg/ml, Worthington Biochemical, Freehold, NJ) in SFM. After digestion, the suspension was washed in serum medium (SM: SFM with 10% fetal bovine serum, FBS, Hyclone, Logan, UT), centrifuged, and the pellet which contained cells, cell clumps, and mammary organoids was collected. The pellet was washed, resuspended, and passed onto a 40 (m pore nylon mesh filter (Tetko, Briarcliff Manor, NY) which allowed the dispersed cells and small cell clumps to pass. The organoids trapped on the filter surface were collected by backwashing the filter with SM and were again washed and recentrifuged twice at ~20 g for 2 min. The pelleted organoids were then washed once with SFM, resuspended in 0.05% trypsin-EDTA, and incubated at 37℃ for 9 min with shaking. The resultant, dispersed cells were washed and resuspended in SFM. Three ml 0.05% DNase (Worthington Biochemical, Freehold, NJ) was added per 10 ml suspension, and the mixture was triturated and filtered in sequence through 40, 20 and 10 (m pore size nytex filters.

The concentration of morphologically intact cells was determined by mixing 1 vol of cell suspension with 1 vol of 0.5% trypan blue in 0.85% saline and counting by phase microscopy in a hemacytometer. The prepared cells were then used for *in vivo* transplantation or were further subdivided. When only PNA+ cells were desired for transplantation, the cell suspension was immunostained and sorted as described below.

Immunostaining of monodispersed cells

The details of the immunostaining have been described previously [22,23]. Briefly, the concentration of monodispersed cells in suspension was adjusted in PBS with 1.0% bovine serum albumin (BSA, Sigma) to 2×10^7 cells/ml. Fifty μℓ aliquots of the cell suspension (1×10^6 cells) were stained with 50 μℓ of FITC (fluorescein isothiocyanate)-peanut lectin (PNA-FITC, 1.25 μg/ml, Vector Laboratories, Burlingame, CA) and/or 8 μℓ of Phycoery-

thrin-conjugated anti-Thy-1.1 (Thy-1.1-PE) monoclonal antibody (Bioproducts For Science, Indianapolis, IN) at 4℃ for 30 min. Some cell samples were single-labeled with either PNA-FITC or Thy-1.1-PE and others with both. A negative control for the lectin staining was prepared by pre-incubation with 0.2 M galactose (Sigma) for PNA-FITC before staining the cells as above. A negative control for anti-Thy-1.1-PE antibody was an aliquot of cell suspension incubated with PE-conjugated IgG_1 isotype (Beckton Dickinson, Mountain View, CA). The stained cells were then washed and the final concentrations were adjusted to 1×10^6 cells/ml in PBS with 1% BSA.

Flow cytometric analyses and sorting

The technique of flow cytometric analysis and sorting has been described previously [22,23]. Briefly, immunostained cells were excited at 488 nm and analyzed and/or sorted with FACScan or FACStar^{PLUS} fluorometers (Beckton Dickinson). Green FITC fluorescence was measured with a 530/30 band pass filter, and orange PE fluorescence with either a 585/42 or 575/26 band pass filter. Forward light scatter, side scatter, and fluorescence signals were collected in list mode files. For the analysis, dead cells were excluded on the basis of propidium iodide (PI) uptake (1.0 μg/ml, Sigma). Data were analyzed with Lysis II version 1.0 software (Beckton Dickinson). For cell sorting, dead cells and debris were excluded by forward and side scatter, and sort windows were set on the appropriate fluorescence signals. Cells were suspended in PBS with 1% BSA, and sorted fractions were collected into the same solution. Sorted cells were recovered, resuspended in SFM, counted in a hemacytometer, diluted appropriately, and transplanted.

In vivo cell transplantation for AD50 or DD50 assays and for AU or DU development

The assay methodology and statistical evaluation procedures have been described [1,22]. For the transplantation assay of primary "whole" mammary cell suspensions or of sorted PNA+ cells, appropriate serial dilutions were prepared in SFM and mixed with an equal volume of 50% rat brain homogenate [1,22]. Addition of brain homogenate significantly increases retention of inoculated cells at the graft sites [4]. Aliquots of 0.06 ml of these cell suspensions were inoculated at each of three sites in the interscapular (IS) white fat pads and of two sites in each of the latero-dorsal inguinal fat pads of MtT F4-grafted recipient F344 rats or adrenalectomized recipient WF rats co-grafted with MtT W10. Generally, each cell concentration was inoculated into a total of 20 graft sites. Thus, each assay of AU- or DU-forming ability of cells from grafts in intact F344 rats co-grafted with MtT F4 or in adrenalectomized WF rats co-grafted with MtT W10, respectively, involved transplantation of 5 cell concentrations and scoring a total of ~100 graft sites from ~20 recipients of each. All recipients were killed and autopsied 3 weeks after cell transplantation, and the fat pads were removed, fixed, stained, and examined under a dissecting microscope for the presence of AU and/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.* [25]. AD50 and DD50 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 or DU in 50% of the sites [1,22]. In MtT grafted intact or adrenalectomized recipient rats, AD50 or DD50 values are inversely proportional to the clonogenic fractions, respectively. Tissues for cross-sectional histology were fixed in 10% neutral-buffered formalin, dehydrated, and embedded in paraffin. Six micrometer sections were cut and stained with hematoxylin and eosin.

When primarily AU or DU were desired in the graft sites for sub-transplantation, a known number of mixed or PNA+ monodispersed mammary cells ($1\text{-}2 \times 10^5$) prepared in SFM and mixed an equal volume of 50% rat

brain homogenate were inoculated only at each of three sites in the IS white fat pads of MtT F4-grafted F344 recipients or adrenalectomized MtT W10-grafted WF recipients. For this, a total of ~120 graft sites from 40 recipient rats of each strain were used.

Cell preparation from AU or DU for sub-transplantation

All recipients were killed 3 weeks after primary cell transplantation, and the IS fat pads were removed, scissors-minced in chilled serum free medium. The minces were then incubated in collagenase solution (Type III, 2 mg/ml) with gentle shaking at 37℃ for overnight. Monodispersed cells from the digested organoids and cells were prepared as described above. To isolate the PNA+ cells from the cell suspension, the monodispersed cells were immunostained and sorted as described above. To obtain information about the fractions of each subpopulation from the AU or DU, immunostained cells were analyzed with the Lysis II version 1.0 software. Some of the IS fat pads were removed, fixed, and stained as whole mounts or prepared for light and electron microphotographs for morphological examination of clonal structures from sub-transplantation.

In vivo sub-transplantation assay

The sub-transplantation procedures are summarized in Fig. 1. To determine the concentration of clonogens which persisted in AU or in DU that arose in primary grafts of mixed or sorted PNA+ cells, cells were prepared from AU or DU as described above. Appropriate serial cell dilutions were prepared in SFM and routine AD50 or DD50 assays were performed as described above. Briefly, cells were inoculated at each of three sites in the interscapular white fat pad and two sites in each of the latero-dorsal inguinal fat pads of each recipient rat. All recipients were killed and autopsied 3 weeks after cell transplantation, and the fat pads were removed, fixed, stained, examined, and scored under a dissecting microscope for the presence of AU and/or DU as described above. Tissues for cross-sectional histology were removed, fixed, and prepared for light and/or electron microphotographs.

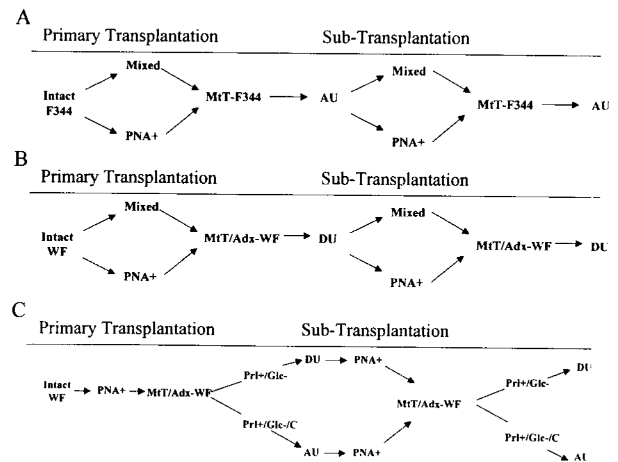

Fig. 1. Sub-transplantation procedures for preparation, separation, and transplantation of know number of monodispersed, sorted rat mammary cells for survival assay. Mixed, mixed cell population; PNA+, PNA+ cell subpopulation; AU, alveolar unit; MtT, mammotropic pituitary tumor; Adx, adrenalectomized; Prl+, prolactin sufficient; Glc-, glucocorticoid deficient; C, hydrocortisone replacement therapy.

As shown in Fig. 1, we designed three different experiments. A) We isolated RMEC from intact F344 rats as mixed or PNA+ cells and injected them on MtT-grafted F344 rats (Fig. 1A). Mammary IS fat pads of recipients which contained AU were removed and digested as described above to isolate RMEC as mixed or sorted PNA+ cells. The isolated cells were then injected on MtT-grafted F344 rats as sub-transplantation to give rise AU. B) We used WF rats as cell donors and MtT W10-grafted and adrenalectomized WF rats as recipients to induce DU (Fig. 1B). C) We isolated RMEC from intact WF rats as PNA+ cells and injected them on MtT W10 grafted-Adx WF recipient rats (Fig. 1C). One group of recipients were untreated (Prl+/Glc-) and the other groups were treated with hydrocortisone replacement therapy (Prl+/Glc-/C). From untreated and hydrocortisone-treated

recipients, DU and AU were isolated, respectively. PNA+ cells from DU or AU were then inoculated on MtT W10 grafted-Adx WF recipients. The recipients which were inoculated with PNA+ cells from AU were then untreated (Prl+/Glc-) further to give rise DU. However, The recipients which were inoculated with PNA+ cells from DU were then treated with hydrocortisone replacement therapy (Prl+/Glc-/C) to give rise AU as described above.

Immunohistochemical staining of AU, DU, and autologous lactating mammary gland with anti-casein, laminin, or collagen IV antibodies

Lactating mammary glands, AU, and DU were fixed in methanol/chloroform/acetic acid (6 : 3 : 1) at 4℃ overnight, routinely processed, and embedded in paraffin. Sections of 4 ㎛ were cut and mounted on poly L lysine-coated slides. The avidin-biotin-peroxidase complex (ABC) immunoperoxidase procedure [22] was followed by using a StreptABComplex kit (Dako, Carpinteria, CA). The production of casein, laminin, or collagen IV was assessed using antiserum or affinity-purified antibodies. Rabbit anti-rat casein antiserum was the kind gift of Dr. Margot M. Ip (Roswell Park Cancer Institute, Buffalo, NY) and used at a dilution of 1 : 1000. Rabbit anti-laminin (Sigma) and goat anti-collagen IV antibodies (Southern Biotechnology Asso., Birmingham, AL) were purchased and used at dilutions of 1: 100.

All sections were deparaffinized and treated with 3% H_2O_2 in 100% methanol for 5 min to inhibit endogenous peroxidase. For protease treatment to detect laminin, sections were incubated with Tris buffer (TBS, 0.05 M, pH 7.4) at 37℃ for 20 min followed by a further 20 min incubation at 37℃ with protease type XXIV (Sigma). After washing with TBS, the sections for casein and laminin staining were incubated with 10% normal swine serum and the sections for collagen IV staining were incubated with 10% normal rabbit serum. The sections were then incubated with primary antibodies at 4℃ overnight. After incubation, the sections were washed with TBS, incubated with biotinylated swine anti-rabbit for casein and laminin, or with biotinylated rabbit anti-goat for collagen IV for 30 min. After rinsing, the sections were incubated with strept-avidin peroxidase complex for 30 min. After washing in TBS, the sections were treated with a solution of 0.025% diamino-benzidine, 0.05 % H_2O_2 in TBS. Sections were rinsed, stained with hematoxylin and mounted with Permount.

Electron microscopy of AU and DU

The tissue fragments which contained AU or DU from primary grafts and from sub-transplantations were prepared for electron microscopy as described previously [22]. Briefly, the tissues were fixed in chilled 2.5% glutaraldehyde in Sorensen's phosphate buffer (0.1 M, pH 7.4) at 4℃ for 1 hr. After rinsing, the tissues were post-fixed in Caulfield's 2% osmium tetroxide for 30 min. The samples were then rinsed, dehydrated, and embedded in Epon 812. Thin sections were cut with an LKB ultra-microtome. Copper grids were stained with lead citrate and uranyl acetate and observed in a Hitachi H-300 electron microscope.

Statistics

Statistical significance was determined using Student's *t* test. $P<0.05$ was judged to be statistically significant.

Results

Flow Cytometric Analysis and Sorting of Immunostained Cells from Intact Rats

Multiparameter flow cytometric analysis of cells from F344 or WF rats stained with both PNA-FITC and Thy-1.1-PE showed four subpopulations of cells: cells negative to both reagents (B-), PNA+ cells, Thy-1.1+ cells, and cells positive to both reagents (B+). PNA+ cells from F344 or WF rats were sorted by fluorescence-activated techniques with the FACStar^{PLUS} using the sort windows. The sorted cells were counted and diluted for transplantation assay *in vivo*.

AD50 Values in MtT F4-Grafted F344 Females

AD50 values of monodispersed F344 epithelial cells were determined in intact young adult female F344 rats co-grafted with MtT F4. The AD50 value of unsorted mixed cells from intact F344 rats was ~484 cells (Table 1). The AD50 value of sorted PNA+ cells was slightly reduced to ~367 cells. The very high AD50 value (~20,632 cells) of unsorted mixed cells prepared from AU structures that had developed in MtT F4-bearing rats grafted with mixed cells reflected about a forty five-fold lesser clonogen concentration than in mixed cells or PNA+ cells from intact glands. When AU structures arose in grafts of PNA+ cells which were sub-transplanted from AU that arose in grafts of sorted, PNA+ cells, the AD50 values were dramatically decreased. The AD50 value of mixed unsorted cells from AU structures which had developed from grafts of PNA+ cells was ~7,400 cells which reflects a two- to three-fold greater clonogen concentration; the AD50 value of PNA+ cells from AU structures which developed from PNA+ cells was ~2,060 cells, reflecting about a ten-fold greater clonogen concentration. However, the lowest AD50 value (~2,060) of PNA+ cells from AU structures is still about five-fold greater than the AD50 of PNA+ cells from mammary glands of intact untreated rats (~367).

DD50 Values in MtT W10-Adx WF Females

The efficiency of DU formation in grafts of known numbers of monodispersed mammary epithelial cells was determined in adrenalectomized young adult female WF rats co-grafted with MtT W10. The DD50 value of unsorted mixed cells from intact WF rats was ~793 cells (Table 2). The DD50 value of sorted PNA+ cells was ~332 reflecting about a 2.4-fold increase in clonogen concentration. The DD50 value of unsorted mixed cells from DU structures that had developed in Adx-MtT W10-bearing rats grafted with mixed cells was ~4,065 cells. The DD50 value of mixed unsorted cells from DU structure which had developed from grafts of PNA+ cells was ~7,688 cells. Although this number was slightly increased, there was no statistical difference between two groups. However, the DD50 value of PNA+ cells from DU structures which developed from PNA+ cells was ~1195 cells, reflecting about four to seven-fold greater clonogen concentration than later two groups. However, this value (~1195) of PNA+ cells is still higher than the AD50 of PNA+ cells from mammary glands of intact untreated rats (~332).

Table 1. AD50 assay of F344 mammary cells prepared by the direct method and grafted in F 344 recipients co-grafted with MtT F4. Monodispersed cells were injected as unsorted mixed cells or sorted PNA+ cells

Cell Inocula for Primary AU[a]	Donor Mammary Structure	Cell Inocula for AD50 Assay[b]	AD50	95% Confidence Limits
--	Intact gland	Mixed	484 ± 42[c]	ND[d]
--	Intact gland	PNA+	367 ± 6c	ND
Mixed	AU in graft	Mixed	20632e	14594; 27864
PNA+	AU in graft	Mixed	7400e	4577; 11387
PNA+	AU in graft	PNA+	2060e	1386; 2864

[a]$1\text{-}2 \times 10^5$ monodispersed mixed or sorted PNA+ cells grafted per site. There were three graft sites in the IS fat pad per rat in a total of 40 recipient rats.
[b]Each AD50 assay involved transplantation of 5 cell concentrations and scoring a total of ~100 graft sites from ~20 recipient rats.
[c]Three separate runs (mean ¡ ¾ SEM).
[d]Not determined.
[e]Single run for each experiment.

Table 2. DD50 assay of WF cells prepared by the direct method and grafted in adrenalectomized WF recipients co-grafted with MtT W10. Monodispersed cells were injected as unsorted mixed cells or sorted PNA+ cells

Cell Inocula Primary DU[a]	Donor Mammary Structure	Cell Inocula for DD50 Assay[b]	DD50[c]	95% Confidence Limits
--	Intact gland	Mixed	793	517; 1082
--	Intact gland	PNA+	332	179; 470
Mixed	DU in graft	Mixed	4065	1261; 6035
PNA+	DU in graft	Mixed	7688	5036; 12931
PNA+	DU in graft	PNA+	1195	727; 1744

[a]1-2 $\times 10^5$ monodispersed mixed or sorted PNA+ cells grafted per site. There were three graft sites in the IS fat pad per rat in a total of 40 recipient rats.
[b]Each DD50 assay involved transplantation of 5 cell concentrations and scoring a total of ~100 graft sites from ~20 recipient rats.
[c]Single run for each experiment.

Effect of Hydrocortisone Replacement Therapy on AU and DU Development in Sub-Transplantation

The effect of hydrocortisone replacement therapy on the efficiency of AU or DU formation was tested in two experiments with adrenalectomized WF rats co-grafted with MtT W10, which does not secrete ACTH. At first, PNA+ cells were sorted from intact WF rats and transplanted on adrenalectomized WF rats co-grafted with MtT W10. The recipients were then divided into two groups: the first group was untreated and therefore to develop into DU. The second recipient group was treated with hydrocortisone. The hydrocortisone dose was chosen tomaximize the glucocorticoid effect. At the first experiment, all of the grafted sites of hydrocortisone-treated group were AU, instead of DU.

In the second, AU or DU structures from the primary grafted sites were taken to separate PNA+ cells with flow cytometry. The isolated PNA+ cells from each groups were also sub-transplanted on the IS fat pad of adrenalectomized WF rats co-grafted with MtT W10. The recipients grafted with PNA+ cells from DU were then treated with hydrocortisone as the first experiment to run AD50 assay. However, the recipients grafted with PNA+ cells from AU were untreated to run DD50 assay. In the second experiment of DD50 assay, DD50 value of PNA+ cells from AU in grafts was ~3,259 cells (Table 3).

Table 3. Effect of hydrocortisone on AU and DU development in sub-transplantation of cells in adrenalectomized WF recipients co-grafted with MtT W10. Monodispersed cells were injected as sorted PNA+ cells

Cell Inocula for Primary AU[a] or DU[a]	Donor Mammary Structure	Cell Inocula for AD50[b] or DD50 Assay[c]	AD50/DD50[d]	95% Confidence Limits
PNA+	AU in graft	PNA+	3,259 (DD50)	2129; 4613
PNA+	DU in graft	PNA+	1,717 (AD50)	949; 2794

[a]1-2 $\times 10^5$ monodispersed sorted PNA+ cells grafted per site in MtT/Adx-WF recipient rats. There were three graft sites in the IS fat pad per rat in a total of 40 recipient rats per group. For AU development, 1.0 mg of hydrocortisone was injected subcutaneously per day.
[b]Each AD50 assay involved transplantation of 5 cell concentrations and scoring a total of ~60 graft sites from ~20 recipient rats. For AU development, 1.0 mg of hydrocortisone was injected subcutaneously per day.
[c]Each DD50 assay involved transplantation of 5 cell concentrations and scoring a total of ~100 graft sites from ~20 recipient rats.
[d]Single run for each experiment.

AD50 value of PNA+ cells from DU in grafts was ~1,717 cells. Although these two values were not significantly different from each other, PNA+ cells from DU contained slightly greater clonogen concentration.

Immunocytochemical Staining of AU, DU, and Autologous Lactating Mammary Gland with Anti-Casein, Laminin, and Collagen IV Antibodies

We stained for the presence of casein proteins in the lumina and in the cytoplasm of secretory cells lining AU, DU, and in lactating mammary gland (Fig. 2). The secretory cells in AU (Fig. 2B) and in lactating mammary gland (Fig. 2D) stained strongly for with anti-casein antiserum. The secretion in the lumen of AU also stained positively (Fig. 2B). Ductal structures of DU showed lower activity of milk secretion with narrowed lumina (Fig. 2F). The cells lining the lumina or apical surfaces of these cells were only positive to anti-casein antiserum. Controls exposed to non-immune rabbit serums failed to show any specific staining (Fig. 2A, 2C, 2E).

Laminin was specifically located in the basal lamina surrounding the AU (Fig. 3A), the mammary gland alveoli (Fig. 3B), and the DU (Fig. 3D). However, immunostaining of AU with the anti-collagen IV antibody was very weak and focal (not shown). Controls exposed to non-immune rabbit or goat serums failed to show any specific staining (Fig. 3C). The basal lamina, as visualized by these two markers, appeared as a thin band adjacent to the outer surface of lining epithelial cells.

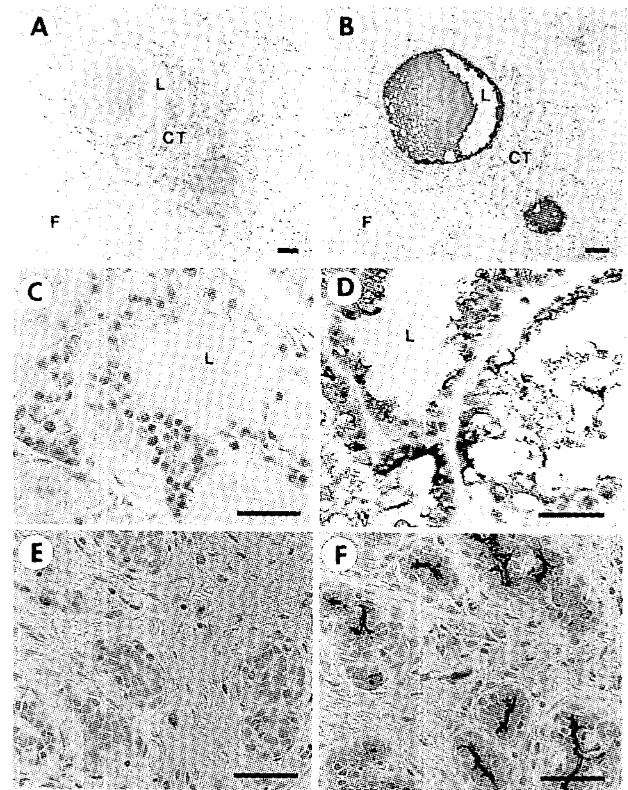

Fig. 2. Immunocytochemical staining of AU, DU, and lactating autologous mammary gland, and DU with anti-casein antiserum. Sections of AU [Fig. 2B], DU lactating mammary glands [Fig. 2B], and DU [Fig. 2E] were stained with antiserum. Large AU developed in grafts in the interscapular white fat pad were surrounded by connective tissue (CT) and adipose tissue (F). Secretion in the lumina (L) of AU and in the cytoplasm of secretory cells were clearly positive. The control sections of AU [Fig. 2A], mammary gland [Fig. 2C], and DU [Fig. 2E] were negative to the antiserum. Bars: 50 ㎛.

Histology of AU and DU in Grafts from Primary and from Sub-Transplantation

The light and electron microscopic morphology of AU and DU derived from the unsorted mixed mammary cells have been described [4,5,22]. The microscopic morphologies of AU and DU derived from the sub-transplantation of mixed or PNA+ RMEC subpopulations were not detectably different from the cross-sections of AU or DU from primary transplants of mixed cells. AU from the sub-transplantation of PNA+ cells were also spherical structures (Fig. 4A) distended with secretion and lined with a single layer of secretory epithelial cells surrounded by myoepithelia (Fig. 4B). The secretory cells contained prominent lipid vacuoles and secretory droplets in their cytoplasm and had microvilli on their apical surfaces (Fig. 4B). The lumina contained granular material which is presumed to be casein. The cells surrounding the lumina were simple cuboidal epithelium and were con-

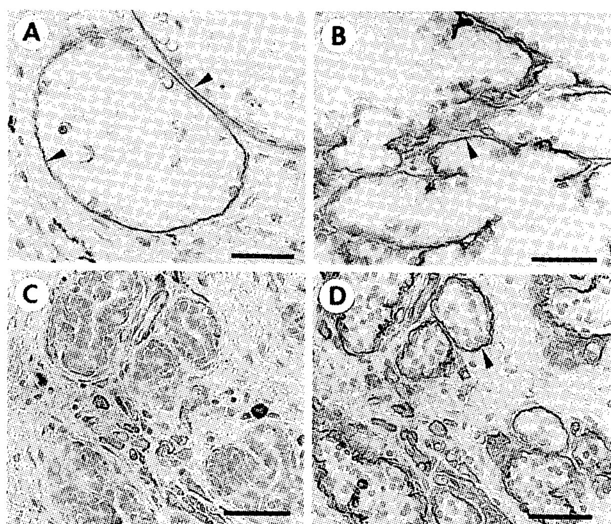

Fig. 3. Immunocytochemical staining of AU, lactating gland, and DU with anti-laminin antibody. Sections of AU [Fig. 3A], lactating mammary gland [Fig. 3B], DU [Fig. 3D] were stained with the antibody. Note the basal lamina stained with anti-laminin antibody surrounding the alveolar units [Fig. 3A], the mammary alveoli [Fig. 3B], and the ductal units [Fig. 3D] (arrow head). The control section of DU [Fig. 3C] was negative to the antiserum. Bars: 50 μm.

nected by tight junctions. AU were surrounded by connective tissue, i.e., collagen fibers and fat tissue. DU from the primary and sub-transplantations of mixed or PNA+ cells were indistinguishable from each other. DU resemble developing mammary glands comprised of ducts with walls two to four cell layers deep with multilayered end buds (Fig. 4C). Electron micrographs of DU showed a lumen lined with simple cuboidal epithelium surrounded by myoepithelia (Fig. 4D). The ductal cells contained abundant lipid and almost no secretory granules in their cytoplasm, had microvilli on their apical surfaces and were connected by tight junctions. DU were also surrounded by connective tissues.

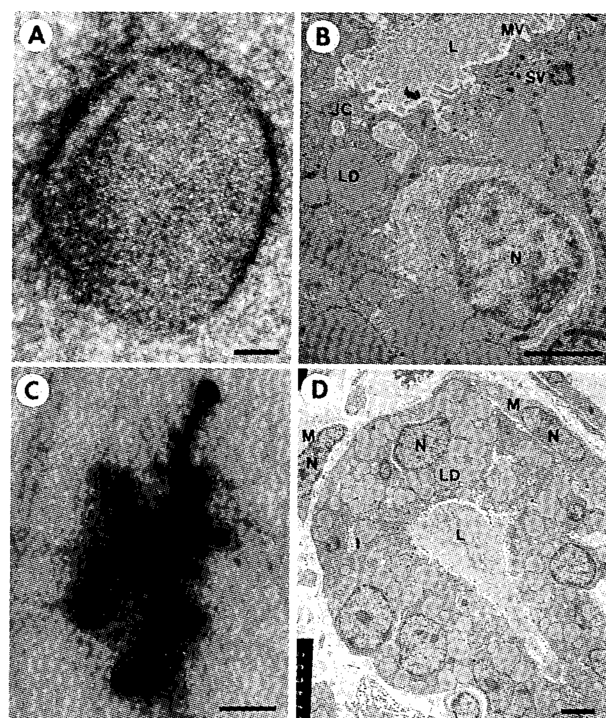

Fig. 4. Light and electron micrographs of AU in an interscapular white fat pad 3 weeks after injection of PNA+ cells in an intact F344 rat co-grafted with MtT F4 [Fig. 4A, 4B] and DU in the interscapular white fat pad 3 weeks after injection of PNA+ cells in adrenalectomized WF rat co-grafted with MtT W10 [Fig. 4C, 4D]. Electron micrographs of AU [Fig. 4B] showed well polarized simple cuboidal secretory epithelial cells interconnected by junctional complexes (JC). The secretory cells were rich in lipids droplets (LD) and secretory vesicles (SV) and had microvilli (MV) on their apical surfaces. Cross section of a DU was well illustrated by lumen (L) formed by mammary epithelial cells associated with a myoepithelial cell (M) [Fig. 4D]. N, nucleus; LD, lipid droplet; JC, junctional complex; SV, secretory vesicle, MV, microvilli; M, myoepithelial cell, L, lumen. Bar in Figs. 4A and 4C: 200 (m; Bars in Figs. 4B and 4D: 2 μm.

Discussion

A large body of data from these laboratories is consistent with the conclusion that the multicellular glandular structures that develop from grafts of monodispersed mammary cells are clonal in origin, i.e. can develop by proliferation and differentiation from single stem-like cells we have termed *clonogens* [2]. The current studies show that clonogens persist within the AU and DU that

develop in grafts of mammary cells, and that the clonogens are contained within the subpopulation of cells that are PNA+, i.e. are stainable with fluorescein-labeled peanut agglutinin. When cell suspensions prepared from AU and from DU are transplanted to appropriate recipients, they give rise to new AU or DU. The latter structures have been shown by fluorescence-activated analyses to contain the same cell types as are found in the ducts, endbuds or alveoli of the normal mammary gland. Furthermore, they are light and electron microscopically indistinguishable from AU or DU that arise in grafts of mammary cells prepared directly from normal mammary tissue.

The development of two types of clonal structures induced in grafts of mixed monodispersed mammary epithelial cells in hormonally manipulated host rats have already been demonstrated in our laboratory [5]. It has been suggested that these two types of mammary structure are very likely clonal in origin, and perhaps are derived from the same cell subpopulation. *In vivo*, 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. These results of the experiments reflect the proliferative and differentiative potentials of individual clonogens under different hormonal conditions in graft recipients. Morphologically, AU strongly resemble secretory alveoli; DU are composed of nonsecretory ducts and cellular end buds that strongly resemble developing mammary glands. If the AU and DU that develop in primary grafts are both clonal in origin, one would expect that subpopulations of clonogens would persist within them which could give rise to further AU or DU on sub-transplantation to appropriate recipients.

AD50 values of cells from AU that developed in grafts of mixed cells were significantly higher than those of cells from intact mammary glands (AD50 values: ~20,632 cells vs. ~484 cells). AU structures strongly resemble secretory alveoli; most cells composing AU are well differentiated. The AD50 value of cells from F344 rats in which lactation had been induced by grafts of mammotropic pituitary tumors (MtT F4) grafts were markedly increased, i.e., prolactin-induced proliferation and secretory differentiation markedly reduced the concentration of clonogens (AD50 value: 15,720 cells; 95% conf. lim., 11,136-21,264 cells) [26]. Sub-transplantation of the mixed unsorted cell population from AU that had developed in primary grafts of mixed unsorted mammary cells showed a marked decrease in clongenicity (AD50 of primary cells, ~484 cells; AD50 of sub-transplantation cells from AU, ~20,632 cells). Concentration and transplantation of the PNA+ primary cells reduced the AD50 of the sub-transplantation of mixed cells from AU to 7,400 cells. When only PNA+ cells were used for both primary grafts and for sub-transplantation from AU, the AD50 was further reduced to 2,060 cells. Thus, transplantation of higher concentrations of clonogens leads to maintenance of higher clonogenic fractions in the resulting glandular structures.

Flow cytometric analysis of cells from AU that developed from PNA+ cells showed that there was almost the same pattern of cell subpopulations as in the whole mixed population, i.e., B-, PNA+, Thy-1.1+, and B+ cells. Morphological study showed that myoepithelial cells were present surrounding the single layer of secretory epithelial cells in AU (Fig. 4B). Furthermore, AU in grafts produced casein in their cytoplasm and secreted into the lumina. Typically, the presence of casein in the cytoplasm and lumina demonstrated that these structures are fully functional as in mammary glands. PNA+ cells thus contain clonogens with a broad differentiative potential.

In vivo mammary ductal and alveolar structures are composed of well-polarized epithelial cells which develop in association with a visible basal lamina composed of collagen IV, laminin, fibronectin, entactin, and heparan sulfate proteoglycans [27-29]. Deposition of a basal lamina

containing these proteins beneath epithelial cells may contribute to the maintenance of normal cell morphology. The presence of a basal lamina with laminin and collagen IV proteins beneath the basal plasmalemma of the epithelial cells in AU further demonstrated that AU are morphologically and functionally similar to if not indistinguishable from the mammary gland *in vivo.*

The DD50 value of mixed and PNA+ cells from DU structures which developed from unsorted mixed cells was ~4,065 and ~7,688 cells, respectively. These values were also significantly higher than those of cells from intact mammary glands (AD50 values of mixed and PNA+ cells: ~793 and ~332 cells, respectively). However, DD50 values of the PNA+ cells from DU structures which developed from PNA+ cells were ~1,195 cells. Although these values are still about one to three times higher than the cells from intact glands, DU from subtransplantation contained more clonogenic fraction. The comparison of these values to the AD50 values of PNA+ cells from AU (AD50 values: ~2,060 cells) also suggested that DU possibly contained higher fractions of clonogenic cells than that of AU. Moreover, from the hydrocortisone replacement experiment, PNA+ cells from DU contained higher fraction of clonogenic cells (AD50 values: ~1,717 cells) than PNA+ cells from AU (DD50 values: ~3,259 cells). These results also suggested that AU which are well- or terminally differentiated structures contained less fraction of clonogenic cells than undifferentiated DU.

Histological examination of these structures showed that DU were composed of nonsecretory ducts and cellular end buds that strongly resemble developing mammary glands. These ducts and cellular end bud-like structures might contain a higher concentration of clonogenic stem-like cells than AU which are secreting milk. The distal tips of terminal end buds consist of three to six layers of cells, including loosely packed, undifferentiated cap cells overlying more closely packed masses of epithelial cells morphologically similar to those lining the ducts. These cells located at the tips of the endbuds have been considered as putative stem cells [30-33]. Utilizing a pulse dose of 3H-thymidine on the rat mammary gland, these authors described the presence of mitogenic cells in the end buds and the differentiation of these cells along the duct. From these results, they suggested that the cells in the end bud generated the lineage of luminal cells.

The comparisons of subpopulation fractions of AU or DU which developed from mixed or PNA+ cells to subpopulation fractions of intact or hormonally manipulated mammary glands suggest that the patterns of subpopulation distributions of AU and DU were very similar to the developmental stages of intact or hormonally manipulated mammary glands. Roughly, the subpopulation fractions of AU and DU developed three weeks after cell grafts were similar to the fractions of cells from mammary glands three or four weeks after hormonal stimulation.

Investigators in several laboratories have attempted to identify mammary stem cells with animal and culture models. Stem cells can be defined as those cells which have the capacity to repopulate the mammary fat pads with parenchyma that undergoes the entire range of morphological and differentiative changes exhibited in the normal developmental cycle of the mammary gland. Some of the progeny produce milk proteins, carbohydrates, and lipids, undergo involution and start the complete cycle again upon the renewal stimulus of pregnancy and lactation [34].

Stem-like mammary cells which have been described include the RAMA 25 cell line [35], cap cells [30], basal cells [36], and pale-staining cells [37]. The RAMA 25 cell line is an undifferentiated cell from a rat mammary tumor induced by DMBA. Cap cells were proposed as the putative stem cells of mouse mammary epithelium. They are located at the tips of the endbuds, just beneath the basal lamina, and are cuboidal in shape, becoming flattened toward the neck of the endbud and then becoming indistinguishable from myoepithelial cells. Sonnenberg *et*

al. [36] proposed that basal cells were probably stem cells. They divided the epithelium into three types, i.e., basal, myoepithelial, and epithelial cells. The epithelial cells were further subdivided into three types, luminal type I, luminal type II, and alveolar cells. These five cell types were identified by immunohistochemistry using various monoclonal antibodies. The basal cells possess unique antigens and synthesize laminin, which is deposited at the cell base replacing the basement membrane after the growth of the ductal endbud. Pale-staining cells have been identified cytologically in mouse mammary glands, and are distributed sporadically among the mammary epithelial cells at all stages of development: fetal, virgin, pregnant, lactating, and involuting.

The detection and characterization of mammary gland stem cells, if present, is of more than academic interest. Because the properties of stem cells are relevant to the behavior of mammary cancers, elucidation of the nature of stem cells has implications regarding the origins and behavior of preneoplasms and neoplasms of the mammary gland. Although the number of stem cells in the various differentiative stages of the mammary gland have been difficult to quantify, there is little doubt that such cells exist. Phenotypic markers specific for stem cells would be valuable in following their fate and their cellular progeny. The current results demonstrate that cells from AU or DU which developed from mixed or PNA+ cells in primary grafts or on sub-transplantation contained four different RMEC subpopulations like those in mammary glands in situ. The mixed unsorted cells from AU and DU and PNA+ cells sorted from them also contained the clonogenic potential to develop AU or DU on sub-transplantation in appropriate recipient rats. Cells from DU have relatively more clonogenic potential than cells from AU.

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초록 : 유선상피세포 이식편으로부터 생성된 유선구조물 내의 상피간세포 지속성 연구

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흰쥐의 유선조직에는 증식과 분화능력을 가지는 일련의 세포군, 즉 clonogen이라 불리는 간세포군들이 존재하는 것으로 알려져 있으며 이들은 발암물질의 표적이 되기도 한다. 이들 간세포는 기능을 가진 다양한 세포로 분화할 수 있다. 단일 세포로 분리된 유선 간세포를 유선자극호르몬을 분비하는 MtT F4 종양 이식 F344 흰쥐(생체 내 높은 코르티손 농도 유지)에 이식할 경우 포상조직(AU)를 생성한다. 그러나 단일 세포로 분리된 유선 간세포를 다른 종류의 유선자극호르몬 분비 MtT W10 종양 이식 WF 흰쥐(생체 내 낮은 코르티손 농도 유지)에 이식할 경우 관상조직(DU)를 생성한다. 그러나 MtT W10 종양 이식 WF 흰쥐에 하이드로코르디손을 주사하고 간세포를 이식하면 DU가 생성되지 않고 AU가 생성된다. 유선 간세포를 순수 분리하기 위해 유세포분석기로 PNA 양성 세포(PNA+)를 분리한 뒤 내분비 환경을 변화시킨 흰쥐에 이식하면서 어떤 구조물이 생성되는가를 확인한 결과 AU 조직으로부터 분리한 PNA+ 세포는 AU를, DU에서 분리한 세포는 DU 조직을 재이식한 조직에서 만들었다. 그리고 생체 내 낮은 코르티손 농도 유지 WF 흰쥐에 코르티손을 주사하면서 PNA+ 세포를 이식하면 AU 대신 DU 조직이 생성되었다. 간세포 재이식 후 AU 조직에서 얻은 PNA+ 세포를 생체 내 낮은 코르티손 유지 흰쥐에 이식하면 AU 대신 DU, 이와 반대로 간세포 재이식 후 DU 조직에서 얻은 PNA+ 세포를 생체 내 높은 코르티손 유지 흰쥐에 이식하면 DU 대신 AU를 생성하였다. 그리고 재이식 후 얻은 AU 혹은 DU 조직은 처음 이식 후 얻은 AU 혹은 DU 조직과 일치하였으며 재이식 후 얻은 조직에서도 유선 상피세포의 특징인 유단백, 높은 지질분비물 등을 함유하고 있었다. 이러한 실험의 결과는 유선 조직에 존재하는 간세포가 이식, 재이식 과정을 거치면서도 지속적으로 존재하면서 조직뿐 아니라 장기의 증식과 분화에 관여함을 알 수 있었다.