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Enhanced *in Vitro* Proliferation and *in Vivo* Tumorigenic Potential of Mammary Epithelium from BALB/c Mice Exposed *in Vivo* to γ-Radiation and/or 7,12-Dimethylbenz[*a*]anthracene[1]

Laurel M. Adams,[2] Stephen P. Ethier,[3] and Robert L. Ullrich

Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831

ABSTRACT

Virgin female BALB/c mice were exposed *in vivo* to whole body γ-radiation and/or to 7,12-dimethylbenz[*a*]anthracene (DMBA) p.o. Mammary epithelial cells were isolated and assayed for carcinogen altered cell populations both *in vitro* by an epithelial focus assay and *in vivo* by injection into cleared fat pads of syngeneic host mice. Five groups of mice were exposed as follows: (*a*) sham controls; (*b*) 50-rad γ-radiation; (*c*) 100-rad γ-radiation; (*d*) 75 μg DMBA; or (*e*) 50-rad γ-radiation followed in 1 week by 75 μg DMBA. Mammary epithelial cells were isolated and assayed at 24 h and at 1, 4, 16, and 52 weeks after *in vivo* exposure. Four to 12 mice per treatment per time point were individually assayed. Altered *in vitro* growth potential was characterized by the proliferation of carcinogen exposed (but not control) cells as epithelial foci which persisted at least 12 weeks in primary culture. Epithelial foci which could then be subcultured at least four times were termed subculturable epithelial foci. Altered *in vivo* morphogenic potential was characterized by dysplastic or neoplastic growth in host fat pads. With increased time *in situ* between exposure and assay, cell populations emerged which exhibited both increased *in vitro* subculturability and enhanced tumorigenic potential including a host response upon injection *in vivo*. Further, combined radiation and DMBA resulted in higher frequencies of subculturable epithelial foci than either treatment alone. The relevance of these progressive cellular changes to the process of mammary tumor development is discussed.

INTRODUCTION

The multistage nature of the carcinogenic process in mammary tissue has been suggested by the results of experiments in both mouse mammary gland (1–3) and rat mammary gland (4, 5) model systems. Evidence for a similar process in humans involving the development of preneoplastic lesions and their subsequent progression has been presented by several investigators (6–9). A study of the dynamics of this process and of the relative contribution of the carcinogenic insult to initiating events in target mammary cells and to effects involved in progression of these cells toward the neoplastic phenotype is difficult in the intact rodent and virtually impossible in humans. Accordingly, several investigators have utilized the transplantation of mammary tissue or dissociated mammary cells into syngeneic hosts as a model for dissecting the many factors involved in mammary carcinogenesis. Medina (10) and DeOme *et al.* (1) have shown that normal mammary cells have a controlling influence on the expression of hyperplastic alveolar nodules from murine mammary tumor virus positive infected mice *in vivo*. Guzman *et al.* (11) and Medina *et al.* (12) have also demonstrated that transplanting preneoplastic dissociated cells rather than intact mammary fragments results in enhanced lesion frequency in transplants from mice exposed to DMBA at high doses. Gould and Clifton (13) have reported the influence of hormones on the survival and growth of irradiated mammary cells transplanted in Fisher rats, and Faulkin *et al.* (14) have shown a differential effect of radiation on the growth of transplanted normal *versus* hyperplastic mammary tissue.

Using the cell dissociation technique originally developed by DeOme *et al.* (1), we have recently described some of the early events in mammary tumorigenesis following exposure of mice to low doses of γ-radiation or DMBA[4] (15–19). These studies have shown that carcinogen altered cell populations can be detected by their expression as ductal dysplasias within outgrowths derived from mammary epithelial cells from carcinogen treated mice. Further, such altered cells were found to be present in mammary tissue dissociated within 24 h after carcinogen treatment. Although this early alteration was shown to be a stable characteristic of these cells, the expression of this altered phenotype was found to be dependent upon the mammary outgrowth being in a state of active growth. When the gland was not actively growing the dysplasias regressed but could be detected upon retransplantation. If cells remained *in situ* for longer times following carcinogen exposure prior to cell dissociation (1–16 weeks), cell populations emerged which gave rise to ductal dysplasias which persisted. These results suggested that altered growth potential is acquired early after carcinogen treatment, while the acquisition of the ability to autonomously maintain the dysplastic phenotype is a separate, second event in the process of progression in mammary tumor development.

To further characterize the cellular changes occurring during the carcinogenic process in the mammary gland, we have developed an EF assay in which mammary epithelial cells exposed *in vivo* to carcinogens are assessed *in vitro* for altered growth potential as a function of time following exposure. This *in vitro* assay was adapted from one first described by Terzaghi and Nettesheim (20) for examining the dynamics of neoplastic development in rat tracheal epithelium. In this paper we describe the results of studies using this EF assay to examine the progressive cellular changes involved in the process of mammary tumor development following DMBA and/or radiation exposure. In addition, the results of these *in vitro* studies are compared with results obtained using the cell dissociation technique.

MATERIALS AND METHODS

Animals. The animals used in these experiments were specific pathogen-free virgin female BALB/cAnNBd mice bred and maintained in the Biology Division barrier facility. The animals were housed eight/cage and fed Purina Laboratory chow and water *ad libitum*.

Carcinogen Exposure. Mice were exposed to γ-radiation, to DMBA, or to both γ-radiation and DMBA at 12 weeks of age. Whole body γ-

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[2] To whom requests for reprints should be addressed, at Ayerst Laboratories Research, Inc., CN 8000, Princeton, NJ 08543-9990.

[3] Present address: Michigan Cancer Foundation, 110 East Warren Avenue, Detroit, MI 48201.

[4] The abbreviations used are: DMBA, 7,12-dimethylbenz[*a*]-anthracene; EF, epithelial focus (foci); EF_s, subculturable epithelial focus (foci); CD, cell dissociation (assay); FCS, fetal calf serum.

radiation was carried out with a 2000-Ci ^{137}Cs source at a dose rate of 31.7 rads/min. During exposure the unanesthetized animals were kept in individual plastic tubes and rotated in the beam. Details of the irradiation procedure have been published previously (21). DMBA (Sigma Chemical Co., St. Louis, MO) was dissolved in stripped corn oil (Eastman Kodak Co., Rochester, NY) and administered to unanesthetized mice by gastric intubation via a 20-gauge gavage needle as previously described. Control mice were sham irradiated and/or received stripped corn oil alone.

Experimental Protocol. These studies were designed to examine the influence of DMBA and/or γ-radiation and the influence of time that cells remain *in situ* following these treatments on the acquisition of increased *in vitro* growth potential. In these studies mice were exposed to one of the following treatments: (*a*) controls; (*b*) 50-rad γ-radiation; (*c*) 100-rad γ-radiation; (*d*) 75 μg DMBA; or (*e*) 50-rad γ-radiation followed in 1 week by 75 μg DMBA. Studies that led to selection of these particular doses have been published previously (15–17). Mice from each of these treatment groups were killed and their mammary tissue was analyzed by the epithelial focus assay (described below) at 24 h, 1 week, 4 weeks, 16 weeks, and 52 weeks following treatment. Altered growth potential *in vitro* was categorized as follows: (*a*) epithelial foci that persisted and grew 12 weeks in primary culture without undergoing senescence (*i.e.*, demonstrated an increased *in vitro* growth capacity) were termed EF; and (*b*) EF that could be subcultured at least four times were termed EF_s. To further characterize these EF_s they were injected into cleared mammary fat pads and 10 weeks later the subsequent outgrowth was classified as either ductal, dysplastic, or neoplastic. For comparison of the *in vitro* results with the results obtained by using the cell dissociation technique, cells from the same preparations used for the EF assay in the 100-rad, DMBA, and control groups were also injected into gland-free fat pads.

Preparation of Mammary Tissue. All 10 mammary glands were removed from a single mouse anesthetized with 0.05 mg/kg Diabutal (Diamond Laboratories, Inc., Des Moines, IA). The mammary tissues were weighed, minced, and enzymatically dissociated as previously described (16). The resultant cell suspension was filtered through 20-μm Nytex nylon bolting cloth and viability was determined by trypan blue dye exclusion. For the EF studies, 5×10^4 cells/60-mm dish (~$2 \times 10^3/cm^2$) were plated in Ham's F-12 medium supplemented with 10% FCS, insulin (10 μg/ml), penicillin (100 IU/ml), and streptomycin (100 μg/ml). Ten dishes/mouse were plated and maintained at 37°C in 5% CO_2. For the CD studies, 10^4 cells from the same cell preparations were suspended in Ham's F-12 medium (no FCS) and injected with no intervening *in vitro* culture bilaterally into the gland-free inguinal fat pads of 3-week-old recipients.

Epithelial Focus Assay. Culture medium was changed and each dish was examined weekly for the presence of epithelial foci. Any colony of epithelial cells still proliferating at 12 weeks *in vitro* was considered to be an EF. Such colonies were easily distinguished by their size and cell number (see "Results") from the occasional large, spread, senescing cells which sometimes remained on the dish at 12 weeks. Fibroblasts did not proliferate under these conditions. The percentage of EF was determined by dividing the number of dishes with an EF at 12 weeks by the total number of dishes plated.

Cell populations from individual EF were derived as follows. When each focus reached a diameter of 1.5–2 cm, any other cells remaining on the plate were scraped with a sterile rubber policeman and discarded. Care was taken in addition to remove ~1 mm of cells at the periphery of each focus so as to preclude any cells not associated with the epithelial focus. The plates were rinsed 3 times in Dulbecco's phosphate buffered saline and the EF was then removed from the dish as follows. Two ml Corry's EDTA were added to the dish and incubated at 37°C for 15–30 min until about 80% of the cells appeared rounded up. The cells were removed from the plate and dissociated by repeatedly dispersing them via a 5-ml syringe fitted with a 20-gauge needle. The suspension was washed once in 10 volumes of complete medium and all the cells from a single focus were repeated in a single 60-mm dish. This was designated *in vitro* passage 1. Subsequent passages were similarly performed when dishes were about 90% confluent (2-4 weeks) at a split ratio of 1:3. Foci that could be subcultured at least four times were designated EF_s. The percentage of EF_s was determined by dividing the number of subculturable foci by the total of foci proliferating at 12 weeks *in vitro*.

To further characterize the EF_s obtained, at the fourth passage these cells were suspended in Ham's F-12 medium (no FCS) and 2×10^5 cells were injected into cleared fat pads of syngeneic mice. Subsequent outgrowths were examined and classified at 10 weeks after injection as in the cell dissociation technique.

Cell Dissociation Assay. The procedure for enzymatic dissociation of the mammary tissues and transplantation of the cells into gland-free fat pads of host mice has been described previously (18). At either 10 or 16 weeks after the injection of the cells, the host animals were killed and the outgrowths were removed, fixed, and stained. The stained mammary outgrowths were then examined at the whole mount level and classified as being normal or as having ductal or lobular dysplasia. All dysplasias observed in this study were ductal in nature. The dysplasias were characterized by epithelial hyperplasia within mammary ducts. The hyperplasias ranged from mild, in which only a few extra layers of epithelium were present, to severe, in which the ducts and end buds were occluded and distended with epithelial cells. A complete description of the morphological and biological characteristics of the dysplasias observed in mammary outgrowths has been published (19). By examining outgrowths at 10 and 16 weeks after injection of cells we could evaluate the expression of dysplasias in actively growing mammary glands (10 weeks) or their ability to persist in full mammary glands (16 weeks) which were not actively growing.

RESULTS

Morphology of EF and EF_s *in Vitro*. Cell populations designated EF were epithelial in nature. These cells grew in a characteristic cobblestone monolayer on plastic, and often formed domes spontaneously in primary culture and at high density even after five *in vitro* passages. In addition, some EF_s exhibited intermediate junctions typical of mammary epithelium in culture even after five subcultures, as shown in the electron micrograph in Fig. 1. With passage, however, most EF_s exhibited fewer cell/cell contacts and less frequent dome formation, especially when plated at low cell density.

Frequency of EF. To determine the effect of carcinogen exposure and the effect of time *in situ* after exposure on the *in vivo* growth capacity of mammary epithelial cells, the frequency of EF was determined following exposure to DMBA and/or radiation over the time period of 24 h through 52 weeks after treatment. The frequency of proliferating epithelial foci at 12 weeks after *in vitro* plating (EF) for the four treatment groups as a function of time *in situ* following treatment is shown in

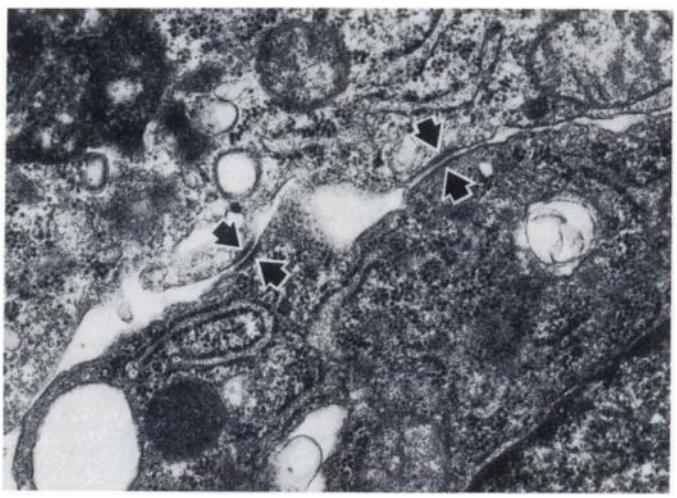

Fig. 1. Electron micrograph of EF_s at *in vitro* passage 5. Hemidesmosomes (*arrows*) characteristic of epithelial cells *in vitro* are illustrated. × 20,000.

Table 1 *Effect of treatment and expression time in situ on the frequency and subculturability of epithelial foci*

Mice were exposed to DMBA and/or radiation *in vivo*. Mammary epithelial cells were isolated for *in vitro* EF assay at 24 h and at 1, 4, 16, and 52 wk after exposure. Percentage of EF and percentage of EF_s were determined as described in "Materials and Methods."

Time *in situ* prior to assay of treatment groups	% of treated mice yielding EF	% of EF[a,b]	% of EF_s[b]
24 h			
All groups	0	0	0
1 wk			
Control	0 (0/3)	0 (0/30)	[c]
DMBA, 75 μg	80 (4/5)	15.5 ± 5.4 (7/45)	42.8 ± 18.7 (3/7)
100-rad γ-radiation	0 (0/4)	0 (0/40)[d]	[c]
50-rad γ-radiation	0 (0/4)	0 (0/40)[c]	[c]
50-rad γ-radiation, and 75 μg DMBA after 1 wk	100 (4/4)	12.5 ± 5.2 (5/40)	60.0 ± 22 (3/5)
4 wk			
Control	0 (0/3)	0 (0/30)	[c]
DMBA, 75 μg	100 (4/4)	35.5 ± 7.6 (15/40)	60.0 ± 12.6 (9/15)
100-rad γ-radiation	100 (4/4)	65.0 ± 7.5 (26/40)[e]	30.8 ± 9.1 (8/26)[e]
50-rad γ-radiation	0 (0/4)	0 (0/40)	[c]
50-rad γ-radiation, and 75 μg DMBA after 1 wk	100 (4/4)	30.0 ± 7.2 (12/40)	75.0 ± 12.5 (9/12)
16 wk			
Control	0 (0/3)	0 (0/30)	[c]
DMBA, 75 μg	75 (9/12)	27.4 ± 4.1 (32/117)	75.0 ± 7.7 (24/32)
100-rad γ-radiation	41.7 (5/12)	12.7 ± 3.2 (14/110)[d]	42.9 ± 13.2 (6/14)[e]
50-rad γ-radiation	0 (0/8)	0 (0/80)	[c]
50-rad γ-radiation, and 75 μg DMBA after 1 wk	50 (4/8)	13.9 ± 3.9 (11/79)	100.0 (11/11) [95% CI[f] (75,100)]
52 wk			
Control	25 (1/4)	2.5 ± 2.5 (1/40)	0 (0/1)
DMBA, 75 μg	100 (4/4)	28.2 ± 7.2 (11/39)	81.8 ± 12.9 (9/11)
100-rad γ-radiation	75 (3/4)	15.6 ± 6.4 (5/32)	80.0 ± 17.9 (4/5)

[a] Number of dishes with EF at 12 wk/number of dishes plated at 5×10^4/60-mm dish, excluding dishes lost to contamination.
[b] Mean ± SE calculated assuming a binomial distribution.
[c] No EF obtained.
[d] Significantly different from DMBA treatment, $P < 0.01$.
[e] Significantly different from DMBA treatment, $P < 0.05$.
[f] CI, confidence interval.

Table 1. Foci were evenly distributed among the mice within a given treatment group with an average of 1 to 4 foci/mouse. Since cells from untreated mice undergo four to five cell doublings (*i.e.*, a "focus" of 16–32 cells) before senescing, only those foci with clearly altered proliferative potential were scored as true EF. For such EF the average colony diameter was 2 cm and the average number of cells was 10,000–20,000/colony. In every case but one, there was only a single focus in each dish. The exception was a second focus in a single dish. This focus was comprised of fibroblastic looking cells (the only such focus seen) which could not be subcultured. For all groups giving rise to EF, the frequency peaked at 4 weeks and declined thereafter until reaching a steady rate between 16 and 52 weeks. This rise and decline was most marked following γ-irradiation. At 4 weeks the frequency of EF from irradiated animals was significantly higher than from those treated with DMBA, while at 16 weeks the EF frequency from irradiated animals was significantly lower than from DMBA treated animals. Since 50-rad γ-radiation alone did not result in any detectable EF during the first 16 weeks, this treatment was not carried to 52 weeks. Interestingly, although 50 rads of γ-radiation alone resulted in no EF, in combination with DMBA it actually slightly reduced the yield of EF elicited by DMBA alone, although the difference was not statistically significant. Cells from untreated, age-matched virgin mice produced only 1 EF in 124 dishes (1 of 13 mice), and this occurred at the 52-week time point.

The percentage of mice which gave rise to at least one EF as a function of time *in situ* following treatment is shown in Table 1. As was the case for the frequency of EF, the percentage of mice yielding EF peaked at 4 weeks following carcinogen exposure. The frequency remained relatively high for animals exposed to DMBA but was more variable following radiation or DMBA plus radiation treatments. Only a single untreated control mouse, one of four at that time point, gave one focus at 52 weeks (Table 1). Cultures established from the combined DMBA plus radiation treated mice at the 52-week time point were unfortunately contaminated during a move to a new laboratory, and the data points are not included in this report.

Subculture of EF_s. To further characterize the effects of carcinogen and of time following carcinogen exposure on the *in vitro* growth capacity of mammary cells, we examined the subculturability of all the EF obtained. As with EF, the EF_s were evenly distributed among the mice, and at no time point were EF_s derived from only a single donor mouse. In contrast to the frequency of EF and the percentage of mice yielding EF, both of which peaked soon after carcinogen treatment, the percentage of epithelial foci which could be subcultured (EF_s) as a function of time *in situ* following treatment increased continuously for all treatment groups assayed through 52 weeks (Table 1). Although the percentage of subculturable foci was higher for DMBA alone than for 100 rads alone at 1, 4, and 16 weeks, the two groups were approximately equal by 52 weeks. In contrast to the EF data, the percentage of EF_s derived from the combined 50-rad plus DMBA treatment exceeded that of DMBA alone at all time points, although not significantly. No data were collected beyond 52 weeks. No untreated control mice gave rise to EF_s.

***In Vivo* Growth of EF_s.** To further characterize these EF_s, at the fourth *in vitro* passage cells were injected into cleared inguinal fat pads of syngeneic virgin mice. Ten weeks later the cell injected fat pads were removed, stained, and examined for growth. Any outgrowths obtained were classified as normal ductal, dysplastic, or tumorous. The results are shown in Table 2. Of 31 EF_s injected, 16 gave rise to growth in the mammary

Table 2 *In vivo growth of EF_s at in vitro passage 4 following injection into cleared fat pads*

At the fourth *in vitro* passage, EF_s cells from each treatment group were suspended in Ham's F-12 medium (no FCS) and 2×10^5 cells/fat pad were injected into cleared fat pads of syngeneic mice. Outgrowths were classified after a 10-wk growth period *in vivo*.

Time between treatment and dissociation (wk)	Positive growth *in vivo*[a]	Type of outgrowth		
		Normal ductal	Dysplastic	Tumorous
1–16[b]	12/23	12/12	[c]	[c]
52	4/8	[c]	3/4	1/4

[a] Number of EF_s cell lines growing in fat pads/number of EF_s lines injected. Positive growth was defined as at least one normal, dysplastic, or tumorous ductal structure in the fat pad.
[b] No significant difference among 1- to 16-wk groups.
[c] No growth of this type seen.

Table 3 *Tumorigenicity of EF_s*

EF_s cell lines were injected into cleared fat pads of syngeneic mice at increasing *in vitro* passage levels (every fourth passage). Outgrowths were classified after a 10-wk growth period *in vivo*.

Time between treatment and dissociation (wk)	No. of EF_s producing tumors/ no. of EF_s lines injected	*In vitro* passage when tumorigenic (range)
1–16[a]	4/12[b]	20–40
52	4/4[c]	4–12

[a] No significant difference among 1- to 16-wk groups.
[b] These twelve cell lines originally gave rise to normal ductal outgrowths at *in vitro* passage 4.
[c] Three of these four cell lines originally gave rise to ductal dysplasias at *in vitro* passage 4.

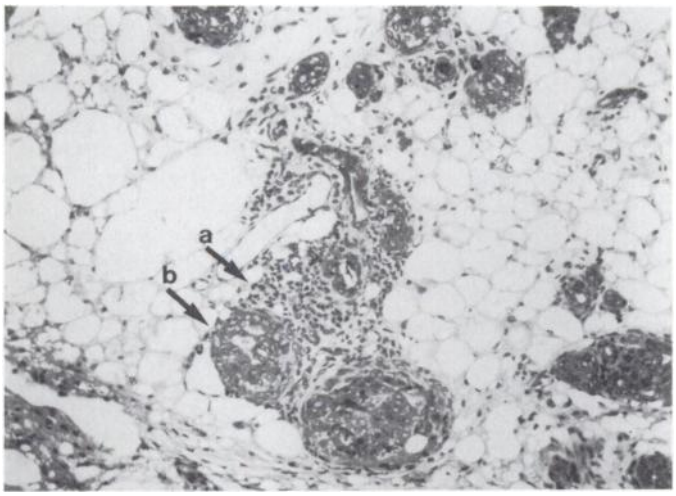

Fig. 2. Histological section of EF_s injected into gland-free fat pad. Cells derived from mice 52 weeks following treatment elicit a host response (*a*) in the vicinity of dysplastic ducts (*b*). × 100.

gland. At least two EF_s from each treatment at each time point were injected, but it did not appear that the type of carcinogen influenced whether or not the cells were able to grow *in vivo*. Injection of those EF_s derived from mammary tissue treated 1–16 weeks prior to dissociation resulted in normal ductal outgrowths in every case in which growth was observed. Upon further *in vitro* passage of these EF_s, 8 of the 12 which had originally produced normal ductal outgrowths no longer produced any growth *in vivo*, while 4 of the 12 continued to produce ductal outgrowths for many passages. With continued *in vitro* passage, these 4 EF_s eventually became tumorigenic. The range of passage levels at which these EF_s became tumorigenic is shown in Table 3. Injection of those EF_s derived from mammary glands 52 weeks after carcinogen treatment resulted in dysplastic outgrowth (3 of 4) or tumor formation (1 of 4) upon initial injection at the fourth *in vitro* passage (see Table 2). The dysplastic outgrowths were characterized by ductal dysplasias accompanied by leukocytic infiltration and angiogenesis in the vicinity of the dysplastic regions (Fig. 2). The EF_s which initially produced dysplastic outgrowth became tumorigenic quite rapidly compared with those EF_s derived 1–16 weeks after treatment which had resulted initially in normal ductal outgrowths (see Table 3). All tumors were either adenocarcinomas or carcinomas, except one which had characteristics of both adenocarcinoma and adenoacanthoma.

***In Vivo* Growth of Dissociated Cells.** To correlate the results of the *in vitro* studies with those obtained by using the cell dissociation assay, cells from animals treated with radiation or DMBA from the same preparations used for the EF assay were injected into cleared inguinal fat pads of 3-week-old virgin female mice. The resultant outgrowths were examined as previously described. The frequency of ductal dysplasia and the ability of these dysplasias to persist as a function of time after carcinogen exposure are shown in Table 4. These results confirm those from our previous study which showed that the potential for the expression of ductal dysplasia is acquired soon (within 24 h) after carcinogen exposure, and that more autonomous cell populations with the ability to persist as dysplastic outgrowth in a full mammary outgrowth emerge with time. The morphological and histological appearance of the dysplasias which resulted from cells disassociated 1–16 weeks following carcinogen exposure were similar to those from our previous studies. A portion of the outgrowths which were derived from cells disassociated 52 weeks after carcinogen treatment differed. Histological examination of these dysplastic outgrowths revealed that like dysplastic outgrowths from 52-week EF_s, these dysplasias were surrounded by leukocytic infiltration. Those infiltrations usually consisted of both lymphocytes and polymorphonuclear leukocytes. Increased number of mast cells and apparent angiogenesis in the area around those dysplasias were also observed. Not all dysplastic ducts within an outgrowth were involved. The frequency of outgrowths which contained dysplasias provoking this host response is shown in Table 5.

DISCUSSION

The results of the present studies both confirm and extend our previous findings with the *in vivo* CD assay. Moreover, several interesting parallels can be drawn among the alterations in growth potential detected in the *in vitro* EF assay, the expression of altered morphogenic potentials in the *in vivo* CD assay, and the development of mammary carcinogenesis in intact carcinogen exposed mice.

First, altered cell populations with the ability to form ductal dysplasias were detected by the CD assay early after carcinogen treatment. With increased time *in situ* between carcinogen exposure and assay, cell populations could be detected which had acquired the ability to maintain this dysplastic phenotype in the form of lesions which persisted in the absence of a growth stimulus, *i.e.*, in a full, resting mammary gland. Likewise, cells with altered *in vitro* proliferative potential were detected by the EF assay early after *in vivo* carcinogen exposure. With increased time *in situ* between carcinogen exposure and assay, an increasing proportion of the epithelial foci isolated *in vitro* acquired the ability to maintain this phenotype via their subculturability, *i.e.*, EF_s. These data suggest that altered morphogenic potential is acquired early, whereas escape from normal growth controlling factors *in vivo* is a second, separate event in neoplastic progression. Likewise, *in vitro* epithelial focus forming ability

Table 4 *Effect of treatment and expression time in situ on the frequency and persistence of ductal dysplasia in the cell dissociation assay*

Mice were exposed to DMBA or radiation *in vivo*. Mammary epithelial cells were isolated at 24 h and at 1, 4, 16, and 52 wk after exposure, and 10^4 cells/fat pad were injected into cleared fat pads of syngeneic mice with no intervening *in vitro* culture period. Outgrowths were classified after a 10-wk or 16-wk growth period *in vivo* (one-half of the treated mice at each time point in each group).

Time between treatment and dissociation	Control	Radiation, 100-rad γ-radiation	DMBA, 75 μg
Dysplasias in 10-wk outgrowths			
24 h	6.5 ± 2.8 (5/77)[a]	11.7 ± 4.2 (7/60)	11.7 ± 4.2 (7/60)
1 wk	ND[b]	13.6 ± 4.5 (8/59)	20.0 ± 5.2 (12/60)
4 wk	ND	27.6 ± 5.9 (16/58)	29.8 ± 6.1 (17/57)
16 wk	12.9 ± 4.3 (8/62)	41.7 ± 6.4 (25/60)[c]	42.2 ± 6.2 (27/64)[c]
52 wk	17.7 ± 3.4 (22/124)	48.1 ± 4.3 (63/135)[c]	53.3 ± 4.5 (65/122)[c]
Dysplasias in 16-wk outgrowths			
24 h	0 (0/80)[a]	0 (0/63)	1.7 ± 1.7 (1/58)
1 wk	ND	3.3 ± 2.3 (2/60)	12.1 ± 4.3 (7/58)
4 wk	ND	6.6 ± 3.2 (4/61)	16.1 ± 4.7 (10/62)
16 wk	5.0 ± 2.8 (3/60)	9.5 ± 3.7 (6/63)	20.3 ± 5.2 (12/59)[c]
52 wk	5.5 ± 2.0 (7/128)	29.6 ± 3.9 (40/135)[c]	33.1 ± 4.1 (42/127)[c]

[a] Mean ± SE of number of dysplasias in one-half of the outgrowths from 128–272 fat pads given injections (4–12 donor mice at each time point). Calculation assumed a binomial distribution.

[b] ND, not done.

[c] Significantly different from controls, $P < 0.05$, by χ^2 analysis.

Table 5 *Persistent dysplasias at 52 wk which provoked a host response*

Outgrowths in which dysplasias persisted in full and resting outgrowths 16 wk after transplant in the CD assay (see Table 4) were examined for evidence of a host response as shown in Fig. 2.

	Overall frequency	Positive donors/total no. of donors	Frequency in outgrowths from positive donors
Radiation	3.7	1/5	15.6
DMBA	8.7	2/4	17.5
Control	0	0	0

and subculturability are separate but sequential events at the level of the individual mammary cell. Two differences were apparent, however, between the EF and CD data. (*a*) No EF were obtained from cells isolated 24 h after *in vivo* carcinogen exposure, whereas a small percentage (11.7%) of recipient fat pads exhibited ductal dysplasia in the CD assay at this early time point. One explanation could be that initiated mammary cells require interaction with mesenchymal cells *in vivo* during a critical early period in order to "fix" the initiating event. Cells isolated *in vitro* soon after exposure are deprived of this critical interaction and are unable to express their potential *in vitro*, whereas cells isolated and immediately reimplanted express this alteration by interaction with host tissue. This idea is supported by the finding that cells allowed to remain *in situ* for 1 to 4 weeks following exposure show an increasing ability to exhibit their altered growth potential by forming EF *in vitro*. (*b*) A small fraction (<10%) of the outgrowths in the CD assay expressed persistent dysplastic lesions during the first 16 weeks, whereas injections of EF_s derived from these same cell preparations resulted only in normal ductal outgrowths. This suggests that altered proliferative potential *in vitro* may be different from altered morphogenic potential *in vivo*. However, in addition to a possible early requirement for host interaction discussed previously, it is possible that more than one carcinogen exposed cell type contributes to the expression of persistent dysplastic lesions *in vivo* (22). If this is true, the mixed cell population injected with no intervening *in vitro* culture is capable of expressing the persistent dysplastic phenotype in the CD assay; however, the focus derived EF_s populations, although obviously pluripotent but tumorigenic only after repeated subculturing, might be unable to express their altered morphogenic phenotype at low *in vitro* passages. In addition, other investigators have reported that normal appearing ductal growth sometimes results from injection of overtly tumorigenic cell lines in rats (23, 24).

Second, although carcinogen altered cells can be detected in up to 100% of all exposed mice by 4 weeks in both the *in vivo* and *in vitro* assays, the lifetime incidence of mammary tumors is only 14% for 100-rad γ-radiation (25) and only 22% for 75 μg DMBA.[5] Moreover, in these studies, ductal dysplasias *in situ* did not appear until 64 weeks and tumors not until 15–18 months following carcinogen exposure. These data are quite similar to those obtained for the rat tracheal epithelial system in which, although 80% of tracheas contain cells with neoplastic potential (assessed by growth in soft agar), only 9% give rise to invasive carcinomas (20). In both mouse mammary gland and rat trachea *in vivo* carcinogen exposure elicits a large number of cells with altered growth potential very soon after exposure. These data are consistent with data on cells isolated from other organ systems (22, 26–28) which suggest that the process of initiation is essentially complete with the end of carcinogen exposure. Initiation would also appear to be a relatively common event. Since the frequency of EF peaks early and continues to decrease with time, the probability of tumor formation does not appear to depend only upon the quantitative amplification of a finite population of initiated cells. The actual fate of cells *in vivo* which can be detected as EF *in vitro* is unknown. These data suggest several possibilities. Some initiated cells may still possess differentiative capability and are thereby lost from the proliferative compartment. Carcinogen induced alterations may not be irreversible, especially at low levels of carcinogen exposure. In addition, the data are consistent with a mechanism in which EF are converted *in vivo* to EF_s, although the selection of a preexisting population of EF_s cannot be ruled out. Either process might be facilitated by promoting effects of hormones, growth factors, and prostaglandins, or by loss of immune competence possibly required for full expression of tumorigenic potential (29–31). These factors *in vivo* which either enhance or inhibit the expression of these initiated cells and their capacity to escape the process of differentiation then become of paramount importance to ultimate tumor incidence. Evidence for this multistage process has previously been described in the liver (32, 33), lung (34, 35), trachea (20, 36), kidney (26), and skin (37, 38). Perhaps cell lines derived from the EF assay will be useful in determining which of these factors or the sequence

[5] Unpublished observations.

of which events are crucial to mammary carcinogenesis.

Third, both the EF and CD data indicate that cells continue to acquire new phenotypes with time *in vivo* and *in vitro* following carcinogen treatment. EF_s derived 1–16 weeks posttreatment required many *in vitro* passages to yield neoplastic growth *in vivo*, while those derived 52 weeks posttreatment required only a relatively few *in vitro* passages. The continued acquisition of new phenotypes is further supported by the results at the 52-week time point. With the CD technique, a new cell population was detected in cells disassociated 52 weeks postcarcinogen treatment which had acquired the ability to elicit a host response *in vivo*. The ability to elicit such a host response following injection was also detected in the EF_s derived from the same cells using the EF assay. These observations coupled with the fact that such responses are also associated with many of the preneoplastic ductal dysplasias which develop in the intact mammary gland 12–14 months after carcinogen treatment, suggest that the ability to elicit such a host response including angiogenesis as postulated by Folkman (39) may be an important characteristic in the process of neoplastic development in the mouse mammary gland.

Finally, in addition to the study of the dynamics of neoplastic progression, the present studies also compared these dynamics following the qualitatively different carcinogens DMBA and/or radiation. The major difference between the two was time related, with DMBA induced EF appearing sooner and persisting longer than radiation induced EF. However, the frequency of EF_s was approximately equal by 52 weeks and there was no significant difference between the two in either the expression or persistence of ductal dysplasias at the 52-week time point. Because of data (not shown) demonstrating a synergistic effect of DMBA and radiation for tumor induction, mice exposed to these same combined doses were tested in the EF assay. Although fewer EF were observed than after DMBA alone, higher frequencies of cells which had acquired the ability to maintain the altered proliferative phenotype (EF_s) were observed at all time points examined. These results suggest that radiation exposure influenced the probability of acquiring this second alteration. Dual carcinogen exposure could directly affect target mammary cells or could exert its effect on stroma or mesenchymal cells in the fat pad microenvironment. Based on these results, further studies examining the interactions of radiation and DMBA seem warranted. The release of growth facilitating factors such as prostaglandins or the release of oxygen free radicals by mesenchymal cells or via lipid peroxidation in fat cells could alter cell/cell infrastructure, communication, and control (22, 31, 40).

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