Stroma is not a major target in DMBA-mediated tumorigenesis of mouse mammary preneoplasia

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Summary

Carcinogenesis in the mammary gland is thought to involve carcinogen-induced initiation in mammary epithelial cells. Recent experiments have demonstrated that the mammary stroma can be a target of carcinogenic agents, which results in the stroma positively affecting carcinogenesis. To determine whether the stroma or epithelium is the primary target in chemically induced mouse mammary tumorigenesis, we used transplantation of untreated or 7,12-dimethylbenzanthracene (DMBA)-treated immortalized preneoplastic mammary cells into untreated or DMBA-treated stroma. The results demonstrate that the chemical-carcinogen treated stroma did not enhance mammary tumorigenesis in this model and that carcinogen treatment of the mammary epithelium was essential for tumorigenesis.

Key words: Stroma, Chemical carcinogens, Mammary cancer

Introduction

Mouse mammary tumorigenesis occurs within the context of the intact organ. The epithelial cells give rise to the invasive cancer cells and the mesenchymal stroma supports, and is essential for, normal epithelial growth and morphogenesis (Medina, 1996). Preneoplastic development is also dependent upon the adipose stroma, as neither normal nor preneoplastic cells exhibit growth when implanted in subcutaneous sites, in stark contrast to the expansive growth of invasive cancer (DeOme et al., 1959; Cardiff et al., 2000).

The importance of mesenchymal stroma for normal mammary growth is illustrated by the role of estrogen receptors in the stroma and estrogen- and progesterone-induced paracrine factors from the stromal cells (Haslam and Woodward, 2003; Parmar et al., 2002). Additionally, the experiments of several investigators (Emerman and Pitelka, 1977; Radisky et al., 2003; Bissell et al., 2003) demonstrate convincingly the necessity of specific extracellular matrix (ECM) molecules for correct epithelial cell polarity, morphogenesis and functional differentiation.

Recently, several experiments have demonstrated the important role of the mammary mesenchymal stroma in epithelial tumorigenesis. Mammary epithelial cells that overexpress matrix metalloproteases (MMP) 3 or Mt-1 result in tumorigenesis (Sternlicht et al., 1999; Ha et al., 2001), albeit with a long latency, which suggests secondary events. Of more interest, irradiation (4 Gy) of the stroma alone can promote tumorigenesis in unirradiated immortalized COMMA-D cells and with a short latency (Barcellos-Hoff and Ravani, 2000). Of interest, transforming growth factor β (TGF-β) signaling is markedly altered by irradiation of the stroma. In three-dimensional cultures, but not two-dimensional cultures, modifying specific signaling pathways of β1 integrins, MAPK or phosphoinositide-3-kinase (PI3K) in human breast tumor cell lines (i.e., MCF7, MDA-MB-231) can induce phenotypic revision or death (Wang et al., 2002). The interpretation of most of these experiments focuses on the capability of stromal cells or stromal ECM molecules to modulate preneoplastic progression.

Recently, an experiment was published that purported to show that the crucial obligatory target in chemical carcinogenesis of the rat mammary gland was not the mammary epithelial cell but the mammary stromal cell (Maffini et al., 2004). Untreated mammary epithelial cells injected into stroma previously exposed to the chemical carcinogen methylnitrosourea (MNU) gave rise to mammary tumors. During the past year, we have also tested the role of carcinogen-treated stroma in mouse mammary tumorigenesis. The results of our experiments could not demonstrate a tumorigenic effect of carcinogen-treated stroma in the mouse model. The different results in the two models illustrate important lessons for interpreting and understanding the biology of mammary tumorigenesis.

Materials and Methods

Mice

BALB/c female mice were bred and maintained at Baylor College of Medicine. All mice were maintained in a conventional mouse facility with food and water provided ad libitum, and the room temperature was set at 70°F. The animal facility is accredited by the American Association of Laboratory Animal Care.

Experimental design

The transplantable immortalized, preneoplastic mammary outgrowth line TM10 was used in all experiments (Medina et al., 1993). The TM10 outgrowth line was derived from a passage 2 in-vitro cell culture of normal mouse mammary epithelial cells. The resulting outgrowth in the syngeneic BALB/c mammary fat pad is a ductular-

Table 1. Experimental protocol for different groups

Group 1	TM10 transplanted at 3 weeks of age. No DMBA.
Group 2	TM10 transplanted at 3 weeks of age and DMBA given at 8 weeks of host age.
Group 3	TM10 transplanted at 11 weeks of host age into untreated cleared fat pads.
Group 4	TM10 transplanted at 11 weeks of age into DMBA-treated cleared fat pads.
Group 5	TM10 transplants that had been treated with DMBA (group 2) transplanted at 11 weeks of age into untreated cleared fat pads.
Group 6	TM10 transplants that had been treated with DMBA (group 2) transplanted at 11 weeks into DMBA-treated cleared fat pads.
Group 7	TM10 transplanted at 11 weeks of age (untreated fat pad – group 3), then transplanted as a whole gland into an age-matched untreated host at 12 weeks.
Group 8	TM10 transplanted at 11 weeks (DMBA-treated fat pad – group 4), then transplanted as a whole gland into an age-matched untreated host at 12 weeks.

All mice had both inguinal fat pads cleared of epithelial rudiments at 3 weeks of age. Donor transplants were from TM10 hyperplastic mammary outgrowth line. Mice were palpated weekly for tumors for 52 weeks post transplantation.

alveolar outgrowth that fills the fat pad by 8 weeks after transplantation. The outgrowth represents an immortalized cell population that gives rise to mammary adenocarcinomas at a moderate frequency (Medina et al., 1993; Kittrell et al., 1992). The outgrowth is serially transplanted in the mammary fat pad of syngeneic mice every 8-10 weeks. Transplant generation 61 was used in these experiments. The TM10 preneoplastic cells used in the experiments reported herein were never grown in in-vitro cell culture after the initial passage 2.

Both inguinal mammary fat pads were cleared at 3 weeks of age (Medina, 1996). Mice were divided into eight groups and the treatment summarized in Table 1 and outlined in Fig. 1. 7,12-dimethylbenzanthracene (DMBA) was administered to 8-week-old mice by oral gavage at a dose of 1 mg/0.2 ml cottonseed oil. Groups 1 and 2 represented the traditional carcinogenesis protocol, in which the hosts received TM10 transplants at 3 weeks of age followed by either no DMBA or 1 mg DMBA. At 5 weeks post-transplantation, the mammary transplants have a high rate of DNA synthesis. In groups 3 and 4, the mice received the transplants at 3 weeks *after* DMBA treatment of the host; thus, in this group, the TM10 epithelial cells were not exposed to DMBA. In groups 5 and 6, TM10 transplants that had been treated with DMBA (group 2 above) served as donors for transplants into 11-week-old mice that had been untreated or treated with DMBA, respectively, at 8 weeks of age. In groups 7 and 8, whole mammary fat pads from groups 3 and 4, respectively, were transplanted onto the abdomens of untreated 12-week-old mice. Thus, this group represented the situation where *only* the mammary fat pad (group 8) received DMBA treatment. The recipient host and the TM10 epithelial cells were not exposed to DMBA. The procedure of whole gland transplant is not often used, but the mammary fat pad is readily visualized when implanted on the abdomen and both epithelial and stromal cells remain viable for up to one year. All transplants were evaluated for tumor development by weekly palpation for 50-52 weeks. All tumors were evaluated histologically and all whole mounts that did not produce tumors were evaluated as whole mounts (Medina, 1996). The tumor incidences were statistically evaluated using Fisher's exact test. The TE_{50} refers to the time for 50% of the TM10 preneoplastic outgrowths to produce palpable tumors.

Results

The results of the eight groups are shown in Fig. 2A-D and summarized in Table 2. The figures show the comparisons in groups of two. Fig. 2A shows the results of groups 1 and 2. The exposure to a single dose of DMBA caused a marked tumorigenic response in the immortalized preneoplastic outgrowth line TM10, with the 50% tumor endpoint reduced from 44 weeks to 17 weeks after transplantation ($P<0.05$). Fig. 2B shows the results of groups 3 and 4, in which the TM10 epithelial cells were transplanted into untreated or DMBA-treated stroma, respectively. The tumorigenic capability was unaffected by DMBA treatment of the stroma (TE_{50}=41 and 45 weeks in groups 3 and 4, respectively) and was comparable to control group 1 (TE_{50}=44 weeks) ($P>0.05$). Fig. 2C shows the results of groups 5 and 6 and demonstrates that DMBA treatment of the stroma did not enhance tumorigenicity in DMBA-treated epithelial cells (TE_{50}=35 and 32 weeks in groups 5 and 6, respectively) ($P>0.05$). Fig. 2D shows the results of groups 7 and 8 and shows that transplantation of whole glands of untreated and treated stroma into untreated mice also did not affect tumorigenic response (TE_{50}=38 and 38 weeks for groups 7 and 8, respectively) ($P>0.05$).

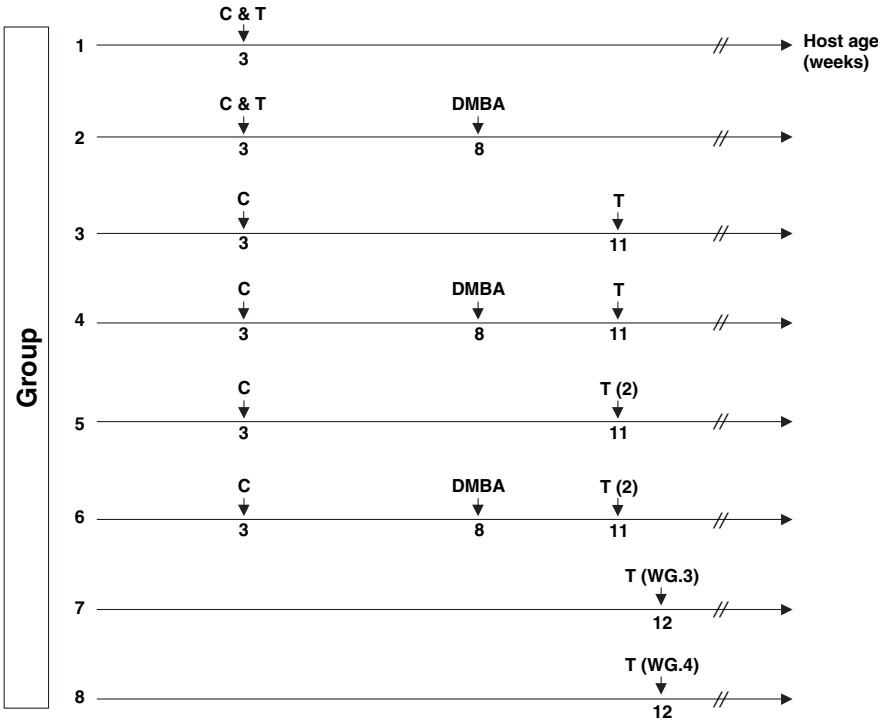

Fig. 1. Experimental protocol for transplant experiments. The numbers under the lines represent the host age when animals were experimentally manipulated. c, clear the fat pad; t, transplant of TM10 fragments; DMBA, administration of DMBA; t(2), transplant from group 2; t(WG.3,4), transplant whole gland from group 3 or 4.

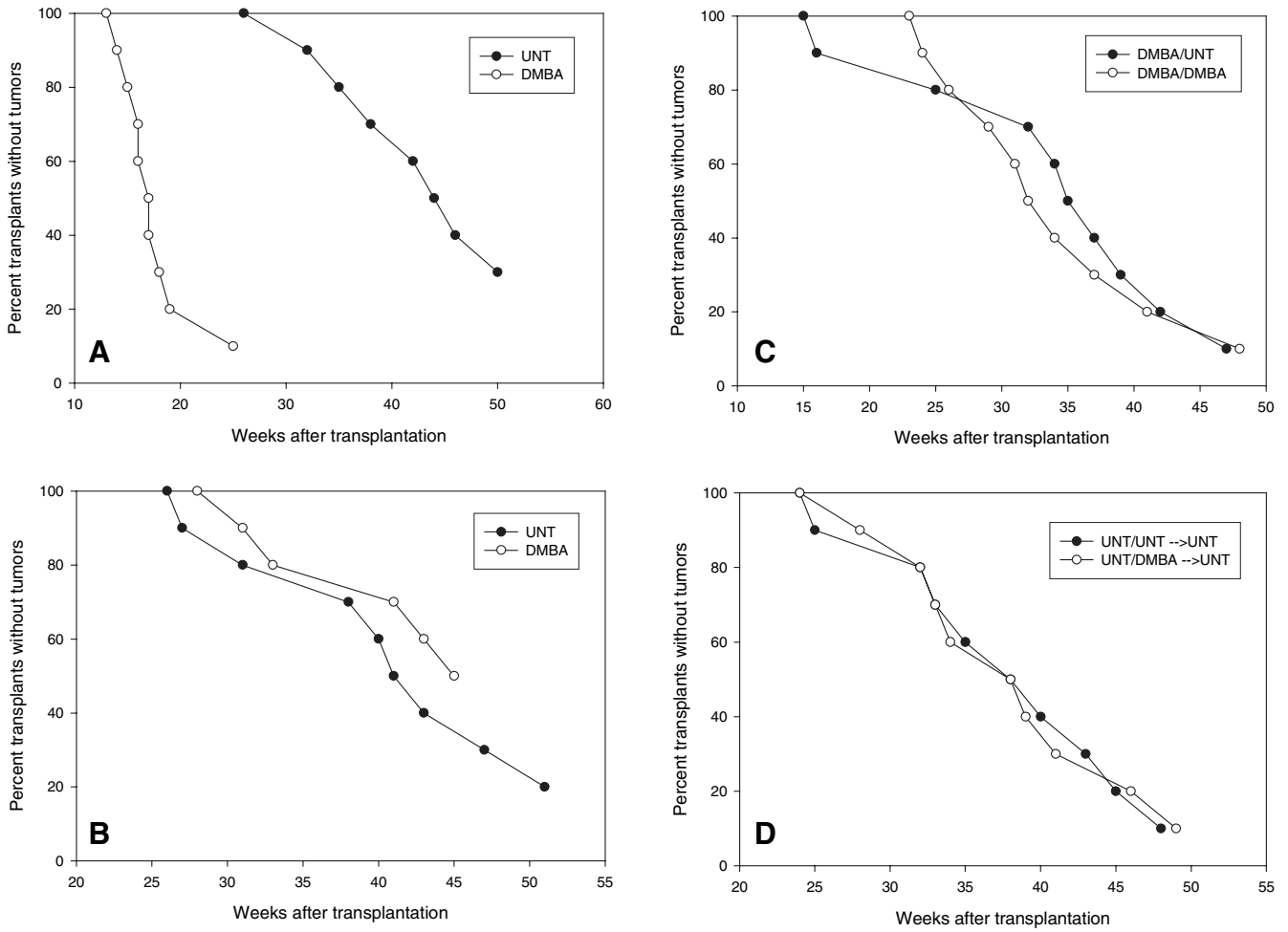

Fig. 2. The timecourse of tumorigenesis from transplanted TM10 mammary preneoplastic outgrowths. The mice were palpated weekly for tumors. (A) Groups 1 and 2; shows the effect of a single dose of DMBA on mammary tumorigenesis in TM10 mouse mammary outgrowth line. (B) Groups 3 and 4; shows the effect of DMBA treatment of stroma on mammary tumorigenesis in TM10 mouse mammary outgrowth line. UNT, untreated; DMBA, DMBA-treated; (C) Groups 5 and 6; shows mammary tumorigenesis of DMBA-treated TM10 mammary outgrowths in untreated and DMBA-treated stroma. DMBA/UNT, DMBA-treated epithelial cells/untreated host; DMBA/DMBA, DMBA-treated epithelial cells/DMBA-treated host. (D) Groups 7 and 8; shows tumorigenesis in the whole mammary gland transplants into untreated hosts. UNT/UNT → UNT; untreated epithelial cells/untreated host and transplanted as whole mammary gland into untreated host; UNT/DMBA → UNT, untreated epithelial cells/DMBA-treated host and transplanted as whole mammary gland into untreated host.

All tumors were mammary adenocarcinomas with no unusual pathologies. There was no unusual stromal response in any of the groups. In all whole mounts examined, the percentage of successful transplants was 100%. The only group to experience a significant loss of animals due to tumors in other organs was group 4, in which ovarian, stomach and lymphosarcomas caused death in mice between weeks 46 and 52. In those mice, the whole mounts showed nonpalpable mammary tumors in four of six transplants. Tumor development in these other organs was commonly seen in mice treated with DMBA and occurred later than tumors in preneoplastic mammary cells. The rapid tumor development in TM10 is demonstrated in Fig. 1A. Tumor development in these other organs also excludes the possibility that these mice did not receive a tumorigenic dose of DMBA.

Table 2. Mammary tumorigenesis in TM10 transplants

Group	DMBA treatment		Host age at transplant (weeks)	No. tumors/ No. transplants	TE_{50} (weeks)
	Host	Epithelium			
1	–	–	3	11/17	44
2	+	+	3	16/17	17†
3	–	–	11	13/16	41
4	+	–	11	8/16	45
5	–	+	11	9/10	35
6	+	+	11	11/12	32
7	–	–	12	14/16	38
8	+(MFP)‡	–	12	13/16	38

†$P<0.05$ compared with group 1. ‡MFP, mammary fat pad.

Discussion

The principal conclusion drawn from these experiments is that a tumorigenic dose of DMBA does not induce changes in the mouse stroma that results in enhancement of the carcinogenic process. How then does one interpret these results in light of existing experiments in the mouse and the rat? The simplest

explanation is that the mouse and rat exhibit completely different dependencies for chemically induced carcinogenesis. It is well established that the rat mammary gland is much more susceptible to chemical carcinogenesis than the mouse mammary gland (Medina and Thompson, 2000; Swanson et al., 1994). Although it has been difficult to determine the molecular basis for this strong difference in carcinogen-induced susceptibility between the two model systems, it is possible that carcinogen-induced changes in the stroma of the rat mammary gland might provide one explanation.

One interpretation of the experiment published by Maffini and colleagues (Maffini et al., 2004) suggests that the 'primary target of MNU is the stroma.' However, there are alternative interpretations. Previous reports have demonstrated that in-vitro exposure of normal mouse mammary epithelial cells in short-term culture can induce changes in the cells that result in tumor development when the cells are implanted in the mammary fat pad (Guzman et al., 1987). In the experiments by Maffini et al. (Maffini et al., 2004), the authors wanted to avoid any possibility that carcinogen-altered fibroblasts were inadvertently carried along with mammary epithelial cells in the injections, so the authors 'repeatedly trypsinized and subcultured the mammary epithelial cells' (Maffini et al., 2004). The number of subcultures and the exact number of days the cells were in culture are not specified, but it is possible that this procedure led to 'spontaneous initiation of transformation' in culture and the effect of methylnitrosourea (MNU)-treated stroma was on progression, not initiation. It is well established in the scientific literature that there is a strong correlation between chromosomal instability and cell culture of epithelial cells of diverse epithelia (reviewed by Jones et al., 1985). Without an examination of the presence and extent of chromosomal damage in the cultured epithelial cells, this possibility cannot be excluded. This interpretation does not abrogate the importance of the results by Maffini et al. (Maffini et al., 2004), but puts the results in a different perspective. In effect, the results confirm the experiments and interpretation of Barcellos-Hoff and Ravani (Barcellos-Hoff and Ravani, 2000), who demonstrated that irradiation of only the stromal compartment causes changes in stroma that affect preneoplastic progression in non-irradiated mouse mammary cells but do not induce initiation. Irradiation of the stromal compartment did not cause tumorigenesis in non-irradiated normal mouse mammary cells by 15 months after transplantation (M. H. Barcellos-Hoff, personal communication). Thus, the experiments of Maffini et al. (Maffini et al., 2004) are consistent with those of Barcellos-Hoff and Ravani (Barcellos-Hoff and Ravani, 2000) in this interpretation.

How then to explain the differences between our results and those of Barcellos-Hoff and Ravani? Both lots of experiments involved immortalized BALB/c mouse mammary cells. One possible explanation is the marked difference in tumorigenic potential between COMMA-D cells and TM10 cells, with the former being more tumorigenic than the latter. The experiments of Barcellos-Hoff and Ravani were carried out for 6-10 weeks after transplantation, and a low frequency of tumors arose in COMMA-D cells transplanted into sham-irradiated hosts (Barcellos-Hoff and Ravani, 2000). In our hands, COMMA-D cells can give rise to a low incidence of spontaneous tumors by 14 weeks after transplantation (Medina et al., 1986). A second explanation is that irradiation is much more effective than DMBA in causing alterations in stromal cells that can result in promoting activities. Reciprocal experiments on the two mouse cell populations need to be performed to address this question.

It is possible, but unlikely, that the different results between those reported herein and those reported in Maffini et al. (Maffini et al., 2004) can be explained by differences in carcinogenic action. MNU and DMBA are both potent mammary carcinogens in the rat and evoke some common mutagenic alterations. However, until a comparative study is performed in both species using both carcinogens and modern assays evaluating genomic changes, one has to entertain the possibility of carcinogen- and species-specific effects that might explain the observed differences discussed in the preceding paragraphs (Medina and Thompson, 2000). Furthermore, MNU can transform mouse mammary epithelial cells in vitro, and these cells produce tumors when transplanted back in vivo (Swanson et al., 1994). The likelihood that fibroblasts inadvertently contaminated the epithelial cells in vitro and were carried over upon injection of cells is not consistent with the observation that mammary fibroblasts do not expand when transplanted into the cleared fat pad (Kittrell et al., 1992; Guzman et al., 1987; Elmann et al., 1987). Furthermore, fibrosarcomas are not a consequence in either the in-vitro experiments (Kittrell et al., 1992; Guzman et al., 1987) or the in-vivo experiments (Medina and Thompson, 2000).

In summary, it is clear that carcinogen-induced alterations in stroma can influence tumorigenesis in some, but not all, mammary systems. The evidence is persuasive that this effect is achieved by inducing events that promote or progress initiated cells. The evidence is also persuasive that mammary epithelial cells are targets for chemical carcinogens and that effects on the stroma alone are not the key factors in some mammary tumor models.

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