Induction of Terminal Differentiation-resistant Epidermal Cells in Mouse Skin and in Papillomas by Different Initiators during Two-Stage Carcinogenesis

Don R. Miller, Aurora Viaje, Joel Rotstein, Claudio M. Aldaz, Claudio J. Conti, and Thomas J. Slaga

ABSTRACT

Carcinogen treatment of normal mouse epidermal cells causes some cells, if cultured under the appropriate conditions, to continue to proliferate instead of terminally differentiate, forming foci at 37°C in medium with a calcium level above 0.1 mM. We have examined these Calcium(Ca)-resistant cells formed in the skin of SENCAR mice after treatment with the carcinogen initiator 7,12-dimethylbenz[a]anthracene (DMBA) followed by tumor promotion with 12-O-tetradecanoylphorbol-13-acetate (TPA). Although in our previous studies TPA promotion initially increased the size but reduced the number of foci caused by the carcinogen initiator N-methyl-N-nitro-N-nitrosoguanidine (MNNG), TPA promotion of DMBA-treated mice increased the size but had no effect on the number of foci. Papillomas resulting from DMBA plus TPA treatment contained many rapidly growing Ca-resistant cells, corroborating our earlier results with MNNG. Permanent cell lines prepared from papilloma-derived foci formed squamous cell carcinomas in nude mice after relatively short periods in culture. These data provide further evidence that Ca-resistant cells may be papilloma (and perhaps carcinoma) precursors in vivo. In addition, since TPA tends to reduce the number of early Ca-resistant cells caused by MNNG but not by DMBA, this may at least partially explain why treatment with DMBA plus TPA is much more effective in producing papillomas in SENCAR mice than is treatment with MNNG plus TPA.

INTRODUCTION

Culture media with calcium concentrations of 0.3-1.8 mM are commonly used for growing attached mammalian cells. Normal mouse epidermal cells proliferate very well if cultured in vitro at 37°C in low levels of calcium (around 0.05 mM), but cease proliferating and terminally differentiate in calcium concentrations above 0.1 mM (1). Carcinogen treatment of epidermal cells grown in low calcium and then switched to high calcium causes some cells to continue to proliferate under high calcium conditions, forming foci of calcium (Ca)-resistant cells on a deteriorating lawn of apparently normal cells (2). It has been suggested that such Ca-resistant cells, since they can also be isolated from "initiated" skin in two-stage carcinogenesis experiments, may in fact be initiated cells (3). Permanent lines of Ca-resistant cells eventually become tumorigenic (4).

Recently, we examined Ca-resistant cells formed in the skins of adult SENCAR mice treated with the direct-acting carcinogen initiator MNNG followed by tumor promotion with TPA (5). Epidermal cells from these mice were dispersed and assayed in vitro for their ability to form foci at 37°C in 0.6 mM calcium. In these experiments, TPA treatment reduced the number but increased the size of early foci caused by MNNG, suggesting that TPA in vivo had preferentially inhibited those Ca-resistant cells less capable of rapid growth. Skins with very early (developing) papillomas produced many rapidly growing foci, consistent with the idea that papillomas comprised Ca-resistant cells and that these cells were initiated.

In order to investigate in more detail the relationship of Ca-resistant cells to papillomas, we chose for the present experiments the carcinogen initiator DMBA, a polycyclic aromatic hydrocarbon requiring metabolic activation that is also a much better inducer of papillomas in SENCAR mice than is MNNG. Interestingly, although we found that in vivo TPA treatment increased the size of early DMBA foci as it had done for MNNG, it did not correspondingly reduce focus number, indicating that the biological effects of the two initiators during two-stage carcinogenesis were apparently different. On the other hand, cell suspensions prepared directly from papillomas induced by DMBA plus TPA produced many rapidly growing foci, corroborating our previous results with MNNG and further indicating that Ca-resistant cells were possibly initiated cells. The relative abilities of MNNG and DMBA to induce Ca-resistant foci did not parallel their abilities to induce papillomas, indicating that the two endpoints were not directly comparable.

MATERIALS AND METHODS

Animal Treatment. Female SENCAR mice (Frederick Cancer Research Facility, Frederick, MD), 7-9 weeks old, were treated in a standard two-stage carcinogenesis protocol (5) with an initiating dose (30 nmol) of DMBA (Sigma Chemical Co., St. Louis, MO) in acetone followed by continuous promotion with TPA (LC Services Corp., Woburn, MA) beginning 1 week later (2 µg in acetone twice per week). These mice were part of a larger, very long-term study. We used less DMBA than for the short-term study below in order to reduce early papilloma burden and premature death in these animals. At various times, TPA treatment was stopped on some of the mice, and after a week without TPA, epithelial cells were prepared from these mice and from mice treated only with DMBA (acetone in lieu of TPA). One cell preparation was made from each group of mice at each time point. Because of the tedious nature of the cell preparations and because our earlier controls (untreated or TPA only) had produced very few spontaneous foci (5), we did not routinely prepare cells from control animals at every time point. We instead made several cell preparations from untreated (i.e., acetone only) or TPA-treated controls over the course of these experiments to establish a maximum level of spontaneous foci. The large, pendulous papillomas (2-10 mm diameter) that eventually developed following treatment with DMBA plus TPA were removed before cell preparation. Cell suspensions were then made from both the isolated papillomas and the surrounding skin, which at that time usually also contained numerous small, nonpendulous papillomas of 1 mm diameter or less.

In another experiment designed to directly compare the production of Ca-resistant cells by DMBA and MNNG, mice were treated with maximal initiating doses of either 100 nmol DMBA or 4 µmol MNNG (Sigma), followed 1 week later by 2 weeks of promotion with TPA. Other groups of mice received DMBA or MNNG without TPA. Negative controls received TPA only. After a further week without treatment, cell preparations were made from all groups, none of which then had papillomas.

Cell Culture. Separate cell preparations were made from each treat-
EPIDERMAL CELLS DURING TWO-STAGE CARCINOGENESIS

RESULTS

Induction of Focus-forming Cells by DMBA. Upon the switch from LoCa to HiCa MEM, epidermal cells from control mice terminally differentiated, rarely forming expanding foci after 1 month in vitro. Numerous foci [similar in appearance to the foci induced by MNNG (5)] formed in cultures from DMBA-initiated mice. The Ca-resistant cells in these foci continued to proliferate as well as terminally differentiate, forming an overlaying keratinization typical of cultured epithelial cells. The number of Ca-resistant cells in the skin of DMBA-treated mice (as reflected by focus number) remained remarkably constant with time after initiation (Fig. 1). Since the epidermal cells were dispersed before plating, we assume that each focus arose from a single Ca-resistant cell, although the plating density was too high to justify the use of a term other than “focus.” TPA had little effect on the number of Ca-resistant cells in the skin prior to the formation of large papillomas, even though in some cases developing papillomas were present (DTpap). Although at 9 weeks the three initiated groups (D, DT, and DTpap) were significantly different, these values were still in line with cor-

4 S. H. Yuspa, National Cancer Institute, Bethesda, MD, personal communication.
accumulations of altered cells. These changes may be qualitative (as our evidence seems to indicate) with quantitative changes only becoming apparent at about the time of papilloma formation (see Fig. 1).

Comparison of Focus Production by DMBA and MNNG. As we noted previously (5), absolute focus production tends to vary from experiment to experiment. We therefore set up a single, short-term experiment including both DMBA and MNNG at maximal initiating doses. The proliferative abilities of Ca-resistant cells were similar with both initiators (Fig. 3A). The real difference (significant at the 0.001 level of probability) was in the number of foci (Fig. 3B): 0.34 foci per million cells (or 4.4 foci per mouse) for 100 nmol DMBA, 3.9 foci per million (28 per mouse) for 4 µmol MNNG. MNNG was a much better inducer of Ca-resistant cells at initiating doses than was DMBA, although this effect was not apparent if the data were adjusted for concentration (3.4 foci per million cells per µmol DMBA, 0.98 foci per million per µmol MNNG).

Papilloma Cells and Lines. Foci that arose in cultures of cells isolated directly from papillomas (Fig. 4A) were identical in appearance to those that arose from either DMBA- or MNNG-treated skin. These cells could be readily subcultured in HiCa MEM to form permanent cell lines. We examined two such lines derived from papillomas for growth in nude mice. One line, comprising two different cell morphologies, is shown in Fig. 4B. There were isolated pockets of cells more cobblestone-like in appearance, strongly resembling normal epidermal cells grown in LoCa MEM (5). The predominant morphology, however, was a type more characteristic of epidermal cells in HiCa MEM, but with little overlying keratinization. This uncinned line at the 30th population doubling (as well as the other line injected at the 11th doubling) formed squamous cell carcinomas in nude mice (Fig. 4C), with some areas well differentiated and other areas only poorly to moderately differentiated. We purposely avoided cloning these lines because of the arbitrary cell selection and further proliferation this procedure requires.

DISCUSSION

It was reported that, on an equimolar basis, DMBA was a much more potent inducer of Ca-resistant cells than was MNNG, whether these chemicals were applied in vitro (10) or in vivo (11). The difference in absolute focus number in those experiments was not so pronounced; indeed, there were generally more foci in cultures treated with MNNG than in cultures treated with DMBA (10). We chose to compare DMBA and MNNG at maximal initiating concentrations (100 nmol and 4 µmol, respectively), subcutaneous doses that would generate the maximum number of initiated cells in SENCAR mice. We wanted to relate the production of initiated cells (as indicated by tumor number after subsequent promotion) to the production of Ca-resistant cells in these same animals.

At these concentrations, MNNG produced 11 times as many foci as did DMBA. From earlier studies, 100 nmol DMBA followed by TPA would be expected to produce just under 30 papillomas and about 1 carcinoma° per mouse (9). For 4 µmol

° M. W. Ewing and J. DiGiovanni. The University of Texas M. D. Anderson Cancer Center, Smithville, TX, personal communication concerning an experiment with 80 nmol DMBA.
Fig. 4. Morphology of papilloma cells in vitro and after injection into nude mice (bars, 0.1 mm). A, edge of focus after 5 weeks in HiCa MEM (phase contrast). B, 36 population doublings later, cell line that arose from above culture (phase contrast). Two cell types persisted, both epithelial, presumably having originated from different primary foci. The more cobblestone-like morphology (left side) covered only about 5% of the culture surface. C, s.c. squamous cell carcinoma in nude mouse from above cell line injected at the 30th population doubling (H & E stained).

MNNG plus TPA, there should be under three papillomas and less than 0.3 carcinomas per mouse (12). Clearly, the relative production of Ca-resistant cells by DMBA and by MNNG did not reflect either tumor endpoint. On the other hand, Ca-resistant cells did accumulate in papillomas [in fact, a permanent such cell line prepared from treated skin prior to papilloma development later formed papillomas in vivo (5)], and permanent lines prepared directly from papillomas subsequently formed squamous cell carcinomas in nude mice [also see similar results (13)]. These data thus argue for a developmental progression (14–17) extending from Ca-resistant cells through papillomas and on to carcinomas, but they also demonstrate that production of Ca-resistant cells is not a direct indicator of initiating activity.

This discrepancy may perhaps be explained by differential toxicity. Initiating doses of MNNG are generally considered to be much more toxic than corresponding doses of DMBA. SENCAR skin treated with 4 μmol MNNG was thicker, with more edema and erythema, than skin treated with 100 nmol DMBA. In other studies, DNA synthesis in SENCAR mice was inhibited for a much longer period of time by MNNG than by DMBA. Thus, MNNG toxicity may have stimulated compensatory, focal accumulations of Ca-resistant cells prior to our sampling that would not have arisen with DMBA treatment, although we now have preliminary data that argue against this. As another possibility, perhaps recently initiated epidermal cells become Ca-resistant only after several subsequent cell divisions. MNNG-initiated cells would then have undergone more mitoses (in response to toxicity) than cells initiated by DMBA, thereby becoming detectable without necessarily forming focal accumulations (assuming one daughter of each pair terminally differentiated). An interesting corollary of this would be that without focal accumulations, there were evidently more MNNG-initiated cells per mouse than would later develop into papillomas, a concept not without precedent (18). As a final possibility, there may not be a one-to-one relationship between Ca-resistant cells (capable of forming foci in vitro) and initiated cells (capable of forming papillomas after TPA treatment in vivo), possibly leading to different effects if the initiators function in different ways.

The exact nature of Ca-resistant cells remains unclear. Further characterization with respect to neoplastic markers such as α-glutamyltransferase (19) and aneuploidy (15) may provide additional insight. These markers, although useful with more established papillomas, were shown to be much less effective with papillomas of less than 12-mm diameter (19) or with papillomas promoted less than 20 weeks (15). We did not include these markers in this study since we wanted to investigate early papillomas as well as initiated skin prior to papilloma development.

TPA did uncover certain differences in the production of Ca-resistant cells by DMBA and MNNG. Although TPA treatment of MNNG-treated mice reduced the number of Ca-resistant cells prior to papilloma formation (5), it had no similar effect on DMBA-treated mice. Assuming Ca-resistant cells are in some way related to initiated cells, this may at least partially explain why MNNG routinely produces fewer papillomas than does DMBA under optimal conditions. Papillomas initiated by DMBA and MNNG, while in both cases accumulating rapidly growing Ca-resistant cells, also differed, in that skin with developing papillomas following MNNG plus TPA treatment had more Ca-resistant cells than skin without these papillomas, whereas with DMBA there were no differences until the papillomas were large enough for direct cell preparation (perhaps more mitoses were required to effect Ca-resistance, see previous paragraph).

TPA had similar effects on the proliferative abilities of Ca-resistant cells by DMBA and MNNG. Although TPA treatment of MNNG-treated mice reduced the number of Ca-resistant cells prior to papilloma formation (5), it had no similar effect on DMBA-treated mice. Assuming Ca-resistant cells are in some way related to initiated cells, this may at least partially explain why MNNG routinely produces fewer papillomas than does DMBA under optimal conditions. Papillomas initiated by DMBA and MNNG, while in both cases accumulating rapidly growing Ca-resistant cells, also differed, in that skin with developing papillomas following MNNG plus TPA treatment had more Ca-resistant cells than skin without these papillomas, whereas with DMBA there were no differences until the papillomas were large enough for direct cell preparation (perhaps more mitoses were required to effect Ca-resistance, see previous paragraph).


d and TPA, there should be under three papillomas and less than 0.3 carcinomas per mouse (12). Clearly, the relative production of Ca-resistant cells by DMBA and by MNNG did not reflect either tumor endpoint. On the other hand, Ca-resistant cells did accumulate in papillomas [in fact, a permanent such cell line prepared from treated skin prior to papilloma development later formed papillomas \textit{in vivo} (5)], and permanent lines prepared directly from papillomas subsequently formed squamous cell carcinomas in nude mice [also see similar results (13)]. These data thus argue for a developmental progression (14–17) extending from Ca-resistant cells through papillomas and on to carcinomas, but they also demonstrate that production of Ca-resistant cells is not a direct indicator of initiating activity.

This discrepancy may perhaps be explained by differential toxicity. Initiating doses of MNNG are generally considered to be much more toxic than corresponding doses of DMBA. SENCAR skin treated with 4 μmol MNNG was thicker, with more edema and erythema, than skin treated with 100 nmol DMBA. In other studies, DNA synthesis in SENCAR mice was inhibited for a much longer period of time by MNNG than by DMBA. Thus, MNNG toxicity may have stimulated compensatory, focal accumulations of Ca-resistant cells prior to our sampling that would not have arisen with DMBA treatment, although we now have preliminary data that argue against this. As another possibility, perhaps recently initiated epidermal cells become Ca-resistant only after several subsequent cell divisions. MNNG-initiated cells would then have undergone more mitoses (in response to toxicity) than cells initiated by DMBA, thereby becoming detectable without necessarily forming focal accumulations (assuming one daughter of each pair terminally differentiated). An interesting corollary of this would be that without focal accumulations, there were evidently more MNNG-initiated cells per mouse than would later develop into papillomas, a concept not without precedent (18). As a final possibility, there may not be a one-to-one relationship between Ca-resistant cells (capable of forming foci \textit{in vitro}) and initiated cells (capable of forming papillomas after TPA treatment \textit{in vivo}), possibly leading to different effects if the initiators function in different ways.

The exact nature of Ca-resistant cells remains unclear. Further characterization with respect to neoplastic markers such as α-glutamyltransferase (19) and aneuploidy (15) may provide additional insight. These markers, although useful with more established papillomas, were shown to be much less effective with papillomas of less than 12-mm diameter (19) or with papillomas promoted less than 20 weeks (15). We did not include these markers in this study since we wanted to investigate early papillomas as well as initiated skin prior to papilloma development.

TPA did uncover certain differences in the production of Ca-resistant cells by DMBA and MNNG. Although TPA treatment of MNNG-treated mice reduced the number of Ca-resistant cells prior to papilloma formation (5), it had no similar effect on DMBA-treated mice. Assuming Ca-resistant cells are in some way related to initiated cells, this may at least partially explain why MNNG routinely produces fewer papillomas than does DMBA under optimal conditions. Papillomas initiated by DMBA and MNNG, while in both cases accumulating rapidly growing Ca-resistant cells, also differed, in that skin with developing papillomas following MNNG plus TPA treatment had more Ca-resistant cells than skin without these papillomas, whereas with DMBA there were no differences until the papillomas were large enough for direct cell preparation (perhaps more mitoses were required to effect Ca-resistance, see previous paragraph).

TPA had similar effects on the proliferative abilities of Ca-resistant cells by DMBA and MNNG. Although TPA treatment of MNNG-treated mice reduced the number of Ca-resistant cells prior to papilloma formation (5), it had no similar effect on DMBA-treated mice. Assuming Ca-resistant cells are in some way related to initiated cells, this may at least partially explain why MNNG routinely produces fewer papillomas than does DMBA under optimal conditions. Papillomas initiated by DMBA and MNNG, while in both cases accumulating rapidly growing Ca-resistant cells, also differed, in that skin with developing papillomas following MNNG plus TPA treatment had more Ca-resistant cells than skin without these papillomas, whereas with DMBA there were no differences until the papillomas were large enough for direct cell preparation (perhaps more mitoses were required to effect Ca-resistance, see previous paragraph).

\(4\) J. DiGiovanni, personal communication.
resistant cells produced by both DMBA (Fig. 2) and MNNG (5). There was an initial, transient increase in focus size with TPA treatment that subsequently returned to normal prior to papilloma development (in spite of continuous TPA treatment).

A similar return to normal occurred in our previous study with MNNG (5). We speculated at that time that the effect, because of its time course, may be an in vitro biological manifestation of something that normally occurs in vivo during Stage 1 of promotion (20) (e.g., an early induction of cells capable of more rapid proliferation). Why this effect would be only temporary is unclear (it may not, however, be temporary in that very small minority of altered cells destined to form papillomas), but at the very least it may be of sufficient duration to allow in situ accumulations of altered cells, which conceivably would then enlarge and reorganize to form papillomas under the generalized hyperplasia associated with Stage 2 of promotion.

We found large numbers of rapidly proliferating cells in papillomas after DMBA plus TPA treatment. From our earlier study with MNNG, we could only conclude that the early, transient effect of TPA reflected an in vivo inhibition of the more slowly proliferating Ca-resistant cells that later appeared in vitro, since there were fewer but larger foci with TPA. From our present study with DMBA, we can now conclude that TPA also caused an induced induction of the more rapidly proliferating Ca-resistant cells, since there were larger but no fewer foci with TPA.

In summary, our data provide further evidence that Ca-resistant cells may indeed be initiated cells, although there does not appear to be a direct relationship between the two. We found that at initiating doses in vivo, DMBA and MNNG produced cells that responded differently to TPA treatment, although this was not too surprising since the papilloma/carcinoma ratio indicated certain differences between the two initiators. Finally, TPA induced in vivo a more rapidly growing population of Ca-resistant cells that later accumulated in papillomas, in a sequence we feel may be related to its mechanism of tumor promotion.

REFERENCES


Induction of Terminal Differentiation-resistant Epidermal Cells in Mouse Skin and in Papillomas by Different Initiators during Two-Stage Carcinogenesis

Don R. Miller, Aurora Viaje, Joel Rotstein, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/49/2/410

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.