Mesenchyme-induced Changes in the Neoplastic Characteristics of the Dunning Prostatic Adenocarcinoma

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ABSTRACT

To investigate the possibility that mesenchyme can alter the neoplastic properties of an established carcinoma, small (0.5-mm cubes) pieces of the Dunning prostatic adenocarcinoma (DT) were grown in association with seminal vesicle mesenchyme (SVM) for 1 mo. Differentiated DT epithelial cells harvested from the resultant tissue recombinants (1°SVM+DT) were recombined with fresh SVM to generate 2°SVM+DT recombinants which were grafted to secondary male hosts. After 3 additional mo of in vivo growth, grafts of (a) 2°SVM+DT recombinants, (b) DT epithelial cells derived from 1°SVM+DT recombinants, or (c) DT by itself were examined for growth rate and tumorigenicity.

Grafts of DT by itself formed large tumorous masses that completely overgrew the host's kidney, while 2°SVM+DT recombinants and differentiated DT epithelial cells from 1°SVM+DT recombinants exhibited only modest growth during a 3-mo period. The loss of tumorigenicity was associated with a striking reduction in [3H]thymidine labeling index in epithelial cells of 2°SVM+DT recombinants. DT graft by itself maintained its typical histopathological characteristics containing small ducts lined with undifferentiated squamous to cuboidal epithelial cells. Grafts of 2°SVM+DT recombinants contained large ducts lined by epithelial cells exhibiting three different patterns of histodifferentiation: (a) basophilic tall columnar epithelial cells with a clear supranuclear cytoplasm and basally located oval nuclei; (b) heterogenous epithelium containing large clear cells with pale cytoplasm interspersed among dark staining tall columnar epithelial cells; and (c) undifferentiated squamous to cuboidal epithelial cells. The first two epithelial types were the predominant cell types. Grafts of differentiated DT epithelial cells derived from 1°SVM+DT recombinants formed medium-sized ducts lined with cuboidal to low columnar epithelial cells. These results demonstrate a continued responsiveness of carcinoma cells to mesenchymal inducers which can induce secretory cytodifferentiation and elicit a reduction in growth rate and loss of tumorigenesis.

INTRODUCTION

Normal development of male accessory sexual glands involves an interaction between mesenchyme and epithelium in which the mesenchyme induces epithelial morphogenesis, regulates epithelial proliferation, and elicits the expression of specific secretory proteins (1-3). Not only do these cell-cell interactions play important roles in development, but they also continue to be of importance postnatally as adult epithelial cells can be induced by mesenchyme to express completely new morphological and functional phenotypes (4-7). The continued responsiveness of adult epithelial cells to mesenchymal induction raises the possibility that emerging or established carcinomas might be also be influenced in biologically important ways by inductive mesenchymes. Earlier studies suggest that this is indeed the case, although a systemic examination of the influence of embryonic mesenchyme on carcinoma cells is not available. Nevertheless, embryonic mammary mesenchyme has been reported to induce mammary carcinoma cells to express a more orderly histodifferentiation and a lowered growth rate (8, 9). In addition, Cooper and Pinkus (10) reported that, when basal cell carcinomas are grown in association with normal stroma, the malignant epithelial cells differentiated with an apparent loss of their malignant properties. Similarly, human colon carcinoma cells differentiated and formed glandular structures in response to embryonic rat intestinal mesenchyme (11, 12). Mesenchymes from the male urogenital tract (UGM) and SVM) have been shown to have profound effects upon certain normal embryonic and adult epithelia as well as their neoplastic counterparts. UGM has been shown to be able to induce prostatic differentiation from adult urinary bladder epithelial cells (4, 7, 13) and to elicit adenocarcinomatous differentiation from transitional carcinoma cells of the urinary bladder (14). For example, SVM induced the expression of seminal vesicle differentiation from the embryonic Wolffian duct and adult epithelial cells of the ureter, ductus deferens, and epididymis (1, 2, 6, 15). Extending these concepts to prostatic carcinoma, we have recently shown that UGM, SVM, and BUG-M induced the undifferentiated epithelial cells of the DT to differentiate into tall columnar epithelial cells which secreted material into the ductal lumina (16, 17). Associated with these mesenchyme-induced changes in epithelial differentiation, SVM+DT and UGM+DT recombinants had an increased DNA content following 1 mo of growth, which suggested that these mesenchymes have a growth-promoting effect on the DT. However, such tissue recombinants were heterogeneous, being composed of areas of parental DT as well as large fluid-filled ducts lined by tall columnar epithelial cells. These results suggested that only those DT cells in intimate contact with the mesenchyme were induced by the mesenchymes. Even though the SVM+DT and UGM+DT recombinants gave a mixed response, we have now determined whether this striking change in epithelial cytodifferentiation of the Dunning adenocarcinoma is associated with a change in epithelial growth rate and tumorigenic potential. Data presented herein demonstrate a reduction in epithelial growth rate and an apparent reduction or loss of tumorigenesis in DT epithelial cells induced to differentiate by SVM.

MATERIALS AND METHODS

Preparation of Tissue Combinations. SVM+DT tissue combinations were prepared by associating a 0.5-mm3 fragment of the R3327 Dunning prostatic adenocarcinoma (obtained from Dr. Altman, University of Miami) with SVM from 0-day-old Fisher 344 rats (Simonson, Gilroy, CA) and were grown overnight in vitro to allow adhesion of the tissues as described by Hayashi and coworkers (16, 17). Such SVM+DT tissue recombinants were grown in male nude mouse hosts for 1 mo to induce differentiation of the DT epithelium and were designated as 1°SVM+DT recombinants. At harvest, the large fluid-filled ducts of the 1°SVM+DT recombinants were excised and cleaned of surrounding parental DT tissue with watchmaker's forceps under observation with...
cystic ducts of differentiated DT were cut into 1-mm segments and placed under a dissecting microscope to yield differentiated DT. These large cystic ducts of differentiated DT were usually lined by a columnar epithelium (16, 17) but could not be entirely cleaned of adherent DT cells. The cystic ducts of differentiated DT were cut into 1-mm segments and either grafted directly to new hosts or combined with fresh neonatal SVM to generate 2'SVM+DT recombinants, which were grafted under the renal capsule of male athymic mice. The rationale of preparing 2'SVM+DT recombinants is that any residual parental DT cells would be induced to differentiate while the differentiated DT would be maintained as such. By this method, we hoped to achieve regulation of all or nearly all DT cells by a two-step induction system. Generally, a given host was grafted with three to four 1-mm³ pieces of the original R3327 DT on one kidney and an equal number of either 2'SVM+DT recombinants or differentiated DT ducts from 1'SVM+DT recombinants on the contralateral kidney. All hosts were treated with a 20 to 30-mg s.c. pellet of testosterone propionate (Sigma Chemical Co., St. Louis, MO) as described earlier (16, 17). For all 3 types of grafts, the starting size was about the same (1 to 2 mm³) with the 2'SVM+DT recombinants being slightly larger than the other 2 specimens. Secondary hosts were maintained for 3 mo.

Histology. After harvest, specimens were fixed with 4% paraformaldehyde and embedded in paraffin, and 6-µm serial sections were stained with hematoxylin and eosin.

Labeling Index. [3H]Thymidine autoradiography was performed on 2'SVM+DT recombinants and parental DT after 3 mo of in vivo growth and on 1'SVM+DT recombinants at harvest after 1 mo of in vivo growth under the renal capsule of male athymic mice (10 specimens per group). For this purpose, hosts were given injections of [3H]thymidine (1.5 µCi/g of body weight; specific activity, 78 Ci/mM; Amersham, Chicago, IL) 1 h before sacrifice. Tissues were harvested, fixed with 4% paraformaldehyde, and embedded in paraffin. Six-µm serial sections were mounted on glass slides, deparaffinized, and dipped in photographic emulsion (NTB-2; Kodak, Rochester, NY). After 1 mo of exposure, the autoradiograms were developed with Kodak D-19 developer and stained with hematoxylin and eosin. Labeling index was obtained by counting the number of labeled epithelial cells (600 cells counted randomly per specimen) and calculated as the percentage of labeled cells per total epithelial cells encountered.

PAGE. The secretory products of 1° and 2'SVM+DT recombinants and parental DT were collected by slicing the grafts and gently squeezing them with forceps in calcium-magnesium-free-PBS (University of California Cell Culture Facility) containing the protease inhibitors 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetic acid, 1 mM N-ethylmaleimide, 100 µM leupeptin, 100 µM bestatin, and 100 µM pepstatin A (all from Sigma). An equal volume of 10% (w/v) SDS Bio-Rad, Richmond, CA) was added to the above, heated at 100°C for 5 min, and stored at −20°C.

Protein samples prepared in PBS-SDS were mixed with 0.2 volume of 5x sample loading buffer (final concentrations, 50 mM Tris (Sigma), 1.2% SDS, 0.04 mg/ml of bromophenol blue (Sigma), and 4% glycerol (Fisher Scientific, Fair Lawn, NJ) with 5% β-mercaptoethanol (Bio-Rad) and heated at 100°C for 5 min before analysis on a one-dimensional polyacrylamide gradient gel system (7.5 to 15% acrylamide and 2.5% bis) containing 1% SDS. A discontinuous buffer system was used (18). Proteins (30 µg per lane) resolved by this method (SDS-PAGE) were visualized by staining overnight in 0.1% Coomassie Blue R250 (Sigma) in acetic acid:methanol:H₂O (1:3:6, v/v) followed by destaining in the same solution without the dye.

RESULTS

Morphological Analysis. Following 3 mo of in vivo growth under the renal capsule of a male host, the three or four 1-mm³ grafts of the parental DT (n = 21) completely overgrew the host’s kidney, forming a single large tumorous mass weighing 5 to 7 g (Fig. 1a). Conversely, 2'SVM+DT recombinants (n = 45) prepared with differentiated DT epithelial cells derived from 1'SVM+DT recombinants grew little, forming spherical masses 2 to 4 mm in diameter and weighing 5 to 10 mg (Fig. 1b). Grafts of cystic ducts (n = 19) of differentiated DT epithelial cells derived from 1'SVM+DT recombinants also exhibited modest growth but were larger than 2'SVM+DT recombinants (Fig. 1c). The take rate (number of recovered grafts/total number of grafts) for differentiated DT epithelial cells from 1'SVM+DT recombinants was lower (11 of 19, 58%) than for 2'SVM+DT recombinants (35 of 45, 78%); all recovered specimens contained epithelial ductal structures. Since the 3 to 4 grafts of the parental DT per kidney grew into a single tumorous mass, it was not possible to calculate take rate for the DT, although all kidneys grafted with parental DT displayed tumorous growth. Grafts of SVM by itself (n = 22) gave rise to fibrous tissue only.

Histology. 1'SVM+DT tissue recombinants grown for 1 mo contained large fluid-filled cystic ducts lined with squamous to tall columnar epithelial cells (Fig. 2) as well as areas of parental DT, which are thought to represent DT cells too far from the SVM to be influenced by short-range paracrine influences as described earlier (16, 17). Grafts of these mesenchyme-induced, differentiated DT epithelial cells recovered from 1'SVM+DT recombinants, when grown for an additional 3 mo in secondary hosts, contained medium-sized to large ducts lined with a cuboidal to low columnar epithelium as well as smaller ducts indicative of the parental DT (Fig. 3). Since small amounts of residual DT were apparently carried over in grafts of the large ducts dissected from 1'SVM+DT recombinants, parental DT was consistently found in all 3-mo grafts of differentiated DT epithelial cells (Fig. 3).

2'SVM+DT recombinants were heterogeneous histologically, although in all cases large cystic ducts were present. Three different patterns of epithelial histodifferentiation were observed in 2'SVM+DT recombinants (Fig. 4a). Most 2'SVM+DT recombinants contained large cystic ducts lined by secretory epithelial cells of the following two types: (a) tall basophilic columnar epithelial cells with a clear supranuclear cytoplasm and an oval nucleus basally (Fig. 4b), which was the most commonly observed phenotype; and (b) a heterogeneous epithelium of large clear cells with pale cytoplasm interspersed among dark staining tall columnar epithelial cells (Fig. 4c). These highly differentiated secretory epithelial cell types were recognized in thirty-five of forty-five 2'SVM+DT tissue recombinants. A small percentage of these thirty-five 2'SVM+DT recombinants also contained large cystic ducts lined with undifferentiated squamous to cuboidal epithelial cells (Fig. 4a).
MESENCHYME-INDUCED CHANGE IN TUMOR GROWTH

Fig. 2. SVM+DT recombinants grown for 1 mo in male hosts. Large fluid-filled ducts develop (a and b), but areas of residual DT (arrowheads) can always be found in a given recombinant (b). The epithelium lining the large ducts varies from simple squamous to tall columnar (c). a and b, × 50; c, × 400.

Very few small tubules were found lined with undifferentiated epithelial cells characteristic of the DT. Ten of the 45 specimens consisted solely of the parental DT. Grafts of the parental DT by itself contained numerous small and medium-sized ducts lined with an undifferentiated squamous to cuboidal epithelium (Fig. 5).

Labeling Index. The [³H]thymidine labeling index of epithelial cells of DT grown for 3 mo as renal capsular grafts was 8.31 ± 0.36 SD (n = 10), while that of 2°SVM+DT tissue recombinants (n = 10) was 1.35 ± 0.10. The epithelial labeling index of 1°SVM+DT recombinants at 1 mo after grafting was reduced to a value intermediate between these extremes (5.48 ± 0.24). Labeling indices of both the 2°SVM+DT recombinants and the DT were statistically different (P < 0.001) by the Student test.

Polyacrylamide Gel Electrophoresis. In SVM+DT recombinants, it was possible to monitor the possibility of contamination of the SVM with its own homologous epithelium, which, if present, would have given rise to SV tissue and the production of...

Fig. 3. Histological sections of grafts of differentiated DT epithelial cells derived from 1°SVM+DT recombinants grown for 1 mo, harvested, and grown for an additional 3 mo in a second host. Note the large ducts lined with a cuboidal to low columnar epithelium as well as smaller ducts indicative of the parental DT (arrowheads). a, × 80; b, × 320.
of SV secretory proteins. Rat SV secretion contains 5 major proteins. Grafs of rat SVM, 1°SVM+DT, and 2°SVM+DT recombinants did not contain SV secretory proteins, which indicates that the SVM was free of homologous epithelial cells (not illustrated).

**DISCUSSION**

In this paper we examined the effect of SVM on DT. Earlier we showed that SVM, UGM, and BUG-M elicited secretory cytodifferentiation of DT epithelial cells (16, 17). The SVM+DT tissue recombinant has been selected for further analysis because of several inherent advantages of SVM+DT recombinants over UGM+DT or BUG-M+DT recombinants. Controls (grafts of mesenchyme only) are essentially not required, since epithelial contamination of the SVM with normal epithelium would give rise to rat SV tissue which would be easily detected due to its characteristic histology and its unique five major tissue-specific secretory proteins detectable by SDS-PAGE and monospecific antibodies (2, 19). The absence of both SV tissue and SV secretory proteins in grafts of SVM, 1°, or 2°SVM+DT recombinants in conjunction with the fact that grafts of SVM by itself gave rise to fibrous tissue devoid of epithelium demonstrates conclusively that the differentiated epithelial cells within SVM+DT recombinants are derived from the DT and not epithelial contamination of the SVM.

The rationale for this study comes from the pioneering studies of Pierce and coworkers (20–22), who propounded the idea that cancer is an aberration or caricature of the process of differentiation. According to these researchers the type of neoplasm is related to the differentiation state of the target cell at the time of initiation of carcinogenesis. Moreover, for any given cancer an embryonic environment may exist that is capable of exerting profound regulatory influences on the tumor. In support of this concept is the finding that embryonal carcinoma cells, when injected into the mouse blastocyst, are induced to participate in normal development with complete loss of tumorigenesis (23). Whether this idea is also applicable to the more common carcinomas afflicting humans and animals is unclear, since earlier reports of mesenchyme-induced regulation of epithelial neoplasia have not been pursued adequately (8–10, 24). The Dunning tumor model reported herein and earlier (16, 17) and recently confirmed by Chung et al. (25) now provides a suitable model for the continued exploration of the role of mesenchyme in epithelial neoplasia.

The expected embryonic environment capable of regulating carcinoma cells is mesenchyme. Embryonic (or neonatal) mesenchyme induces epithelial morphogenesis, specifies patterns of epithelial cytodifferentiation, is involved in regulating epithelial growth, and elicits the expression of specific genes that account for functional cytodifferentiation (1–3, 26–29). Such effects of mesenchyme are not restricted to undifferentiated embryonic epithelia, since recently we have shown that SVM can induce epithelium of the adult ureter, ductus deferens, and epididymis to express SV differentiation, morphologically and functionally (6, 15). Our studies on the DT clearly indicate that various mesenchymes have profound regulative effects on prostatic carcinoma cells as well as on normal adult prostatic epithelial cells (16, 17, 30). Effects of SVM on the DT included the induction of larger more normal ducts, the differentiation of tall columnar secretory epithelial cells, the accumulation of putative secretory product in the ductal lumina, a lowering of epithelial labeling index, and an apparent loss in tumorigenesis. Whether the observed results are due to selection versus induction of DT cells is not clear at the moment, but modifications in technique will allow us to address this issue in the future.

One technical problem with the current model is that after 1 mo of growth of 1°SVM+DT recombinants, the grafts contain differentiated epithelial cells organized into large tubules and associated uninuclear parental DT. To prepare the 2°SVM+DT recombinants, the large differentiated ducts were "cleaned" as well as possible of surrounding tissue and either regrafted directly or combined with fresh SVM to form 2°SVM+DT recombinants. Because the large differentiated DT ducts were apparently contaminated by small amounts of residual DT, these specimens consistently showed regions of parental DT.
after 3 mo of growth, and after longer periods they might have formed tumors. 2°SVM+DT recombinants showed less parental DT than grafts of differentiated DT by itself after 3 mo of growth, and in many cases DT was not detected. We believe that residual DT in association with the differentiated DT ducts was induced to differentiate by the fresh SVM. Thus, after 2 rounds of induction, the tumorigenic property of DT was virtually eliminated concomitant with a reduction in growth rate. Whether this change is stable after even longer periods of growth remains to be examined. Histologically, the larger, more normal ducts lined by tall columnar epithelial cells in both 1° and 2°SVM+DT recombinants were in striking contrast to the narrow tubules of the DT which are normally lined by undifferentiated squamous to cuboidal epithelial cells (31). The induced tall columnar cells of 1° and 2°SVM+DT recombinants are clearly secretory by both light and electron microscopic criteria. Accumulated secretory proteins within the lumina of 2°SVM+DT recombinants are currently under analysis.

The reduction in epithelial growth rate ([3H]thymidine labeling index) and the apparent loss in tumorigenesis of the SVM-induced DT epithelial cells appear at first sight to contradict our earlier report that UGM+DT and SVM+DT recombinants grown for 1 mo were larger and had 75% to 100% higher DNA contents than expected from analysis of grafts of the DT, SVM, or UGM by themselves (16, 17). Our newer data now indicate that the increases in the DNA contents of 1-mo grafts of UGM+DT and SVM+DT recombinants versus grafts of UGM, SVM, or DT grown alone probably reflect short-term survival in the immediate postgrafting period of the DT cells combined with mesenchyme and not a mesenchymal-induced increase in DT proliferation as was originally suggested (16, 17). It is now evident that the proliferative rate (labeling index) of parental DT epithelial cells is considerably higher than that of epithelial cells of 2°SVM+DT recombinants (8.31% versus 1.35%, respectively). The low level of epithelial labeling index of 2°SVM+DT recombinants compares favorably with that of normal prostate in situ which has a labeling index of <1% (32, 33). Following an extended (3 to 5 mo) growing period, this differential growth rate between the parental DT and 2°SVM+DT recombinants is manifested in the production of huge tumorous masses in grafts of DT by itself and very little growth in 2°SVM+DT recombinants and differentiated DT epithelial cells derived from 1°SVM+DT recombinants. Thus, our original demonstration of increased DNA contents of SVM+DT recombinants over that of isolated SVM and DT grown for 1 mo by themselves must be attributed to something other than a growth enhancement of the DT by SVM as originally suggested. Our interpretation is that, following grafting of small (0.5 mm³) cubes of DT as reported earlier (16, 17), considerable cell death may occur immediately following grafting due to interruption of the blood supply, an effect which in one way or another is ameliorated by combination with SVM. This interpretation is supported by preliminary studies which show that, when 1-mm³ fragments of DT were grafted under the renal capsule, DNA contents at 1, 2, and 3 mo of in vivo growth represent 12-, 55-, and 545-fold increases, respectively, over the initial DNA content of the DT graft. Thus, by preserving a larger seed population of DT stem cells during the first few days postgrafting through association with mesenchyme, the size and DNA content of SVM+DT recombinants would be expected to be larger than that of DT by itself in the short term (1 mo of growth), even though the proliferation rate is higher in the DT than in epithelial cells of 1°SVM+DT recombinants (5.48 ± 0.29); this intermediate labeling index represents an average labeling index between the faster growing uninduced DT cells and SVM-induced differentiated DT epithelial cells. In time, however, the faster growing parental DT may ultimately outstrip the growth of the slower growing differentiated epithelial cells of the 1° or 2°SVM+DT recombinants. Thus, it appears that the predominant mode of the parental R3327 DT is proliferation, while differentiation and function are the dominant features of 1° and especially 2°SVM+DT recombinants with a concomitant reduction in growth rate and an apparent loss of tumorigenesis. This mesenchyme-induced change in the DT implies that cells within the DT retain a responsiveness to mesenchymal inductors as do normal adult prostatic epithelial cells (30).

Whether all DT epithelial cells are responsive to SVM is unclear at the moment. The androgen-dependent R3327 DT is known to contain both androgen-dependent and androgen-independent cells (31). Additional heterogeneity within these two populations is likely. Thus, it is possible that SVM may select for particular cell populations with the potential to differentiate with a concomitant loss in neoplastic growth potential. Indeed, in the experiments reported herein in which a 0.5-mm³ piece of DT is grown in association with SVM, we have...
noted that the grafts after 1 mo of growth have regions containing highly differentiated epithelial cells as well as regions of parental DT. This heterogeneity we attribute to the fact that the effect of the SVM is restricted spatially to only those DT cells in intimate contact with the SVM. As an experimental model capable of differentiating. These results, therefore, favor the view that a large proportion of DT epithelial cells are responsive to the differentiating effect of SVM.

Epithelial growth and ductal morphogenesis are complex processes regulated in part by cell-cell interactions which may be mediated by growth factors (34-37). In the male genital tract a variety of growth factors (epidermal growth factor, fibroblast growth factor, and transforming growth factor β) and their receptors have been detected in adult organs (38-41), although the expression of such growth factors has not been examined in the developing prostate or seminal vesicle. The effect of SVM on the DT could be mediated by short-range paracrine effectors such as growth factors, or alternatively could require direct cell contact as appears to be the case for the developing kidney (42, 43). Serum-free culture systems now available for growing the neonatal mouse seminal vesicle and bulbourethral gland (44, 45) should facilitate future analysis of paracrine effects on the DT.

That certain carcinoma cells are responsive to their connective tissue microenvironment has been established by several groups (8-12, 14, 24, 46-49), although rarely has this led to a reduction in tumorigenic potential. This mesenchyme/DT model provides a flexible system for the continued analysis of the regulative role of mesenchyme on a hormonally responsive carcinoma. The chemical mediators of these cell-cell interactions may provide new therapeutic modalities in the future.

REFERENCES

MESENCHYME-INDUCED CHANGE IN TUMOR GROWTH


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