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Growth of Human Nondiploid Primary Prostate Tumor Epithelial Cells in Vitro

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Abstract

Research into molecular and cellular defects underlying prostate cancer would be advanced by in vitro models of prostate tumor cells representing patient tumors. We have propagated, in serum-free medium, epithelial cell cultures derived from nondiploid prostate tumors and normal human prostate. The serial passage tumor cells exhibited nondiploid karyotype and transformed phenotypes of focus formation and anchorage-independent growth. In contrast, the normal prostate cells showed diploid karyotype and lacked transformed phenotypes. Both the tumor and normal cells were positive for prostate-specific antigen and cytokeratins 18 and 19 and negative for keratin 15. These results demonstrate that the nondiploid prostate tumors and normal prostate epithelial cell cultures retained their respective in vivo properties and should allow studies to elucidate molecular alterations involved in human prostate cancer.

Introduction

Prostate cancer is the most common malignancy in men over 40 years of age, and its incidence continues to increase due to the rising life expectancy of American men. During 1996, approximately 317,000 men were expected to be diagnosed with prostate cancer and 41,400 to die from the disease (1). Despite the magnitude of the problem, our understanding of the cellular and molecular mechanisms involved in prostate carcinogenesis remains limited, particularly when compared with other major cancers i.e., lung, urothelial, and breast. The major obstacle for this lack of progress has been the inability to provide optimum nutritional conditions for the culturing of well-characterized primary prostate tumor cells that continue to reflect in situ characteristics. Although immortalized cell lines from normal human prostate and prostate tumors have been described (2), these models are of limited value because the immortalized cells frequently contain viral oncogenic DNA and accompany major cytogenetic alterations and growth dysregulation. In addition, attempts to culture primary tumor cells reportedly produce cultures that invariably exhibit a normal karyotype and lack transformed phenotypes (3, 4). Because almost 70% of primary prostate tumors are diploid (5), it is not clear whether the production of diploid cells from primary tumors represents the original tumor or is a function of culture conditions that select and promote growth of the diploid cells. Alternatively, it is possible that the diploid cells may represent an overgrowth of normal stromal cells. This is a critical issue because an availability of cells reflecting primary prostate tumors and normal prostate will be a major advance and allow studies of genetic and molecular aberrations involved in malignant transformation and of the genetic analysis of cells derived from patients at high risk of developing prostate tumors. For many years, our laboratory has focused on efforts to develop well-defined in vitro conditions for human epithelial cells derived from normal and tumor tissues and to elucidate molecular alterations involved in the dysregulation of tumor cell growth. In this study, we describe the culturing of epithelial cells derived from human nondiploid prostate cancers and normal prostate. The ploidy of the tumors was determined by flow cytometric analysis prior to culture. Our results demonstrate that the cells derived from the nondiploid tumors continue to show a nondiploid karyotype, epithelial phenotype, and in addition express transformed phenotypes of focus formation and AIG. In contrast, the normal prostate epithelial cells show a diploid karyotype, epithelial phenotype, and lack the transformed phenotypes. We also demonstrate that as compared to normal prostate epithelial cells, prostate tumor cells exhibit important alterations in growth regulation and their responses to the growth factors.

Materials and Methods

Prostate Tissues. Primary prostate tumors and normal prostate tissues were obtained as described previously (4) through the Tissue Procurement Service, Karmanos Cancer Institute, Wayne State University, Detroit, Michigan. For nondiploid prostate cancer cell cultures, cases with grossly apparent cancerous areas were selected for immediate flow cytometric evaluation (see below). A mirror image sample of each tissue procured was subsequently processed for routine histology. Two nondiploid tumors and two normal prostate tissues obtained from different patients were studied.

Flow Cytometry. Because our goal was to culture nondiploid prostate tumor cells, it was essential to determine the ploidy of tumors prior to cell culture. The tumors were analyzed for DNA content by flow cytometry as described previously (6). Tumor cell suspensions were analyzed using fluorescence activated cell-sorting Flow Cytometer and Cell Fit Software (Becton Dickinson, San Jose, CA) with a pulse height aggregate discrimination module elongated system. Histograms were generated by gating on the cytokeratin-positive cell population. Histograms were considered aneuploid when a second distinct cell peak having a DNA index of 1.1–1.9 was identified. Tumors with a DNA index of 1.9–2.1 were considered tetraploid.

Cell Culture. Eight primary tumors, obtained from different patients, were analyzed by flow cytometry, and two of these were found to have a nondiploid DNA content. Primary cultures of these two cases were initiated by the explant-outgrowth method as described previously (4, 7, 8). The culture medium was keratinocyte basal medium (Clonetics, San Diego, CA) supplemented with 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 10 ng/ml EGF, 50 μg/ml BPE, 10 ng/ml cholera toxin, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cultures were maintained at 37°C in an atmosphere of 95% air and 5% CO2, and the medium was changed three times per week. Primary cultures of normal prostate tissues were prepared in a similar manner. The primary cultures were passaged as described previously (4). The serial passage cultures were maintained in collagen-coated (Vitrogen, Celtrix, Santa Clara, CA) culture dishes.

Characterization of Cell Cultures. Immunocytochemical procedures were used as described previously to determine the presence of PSA and cytokeratins (4, 7). The PSA antibody was obtained from Becton Dickinson (San Jose, CA); antikeratin antibodies for cytokeratins 15, 18, and 19 were 3

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3 The abbreviations used are: AIG, anchorage-independent growth; PSA, prostate-specific antigen; BPE, bovine pituitary extract; CPDT, cell population-doubling time; EGF, epidermal growth factor.
obtained from Sigma Chemical Co. (St. Louis, MO). Chromosome harvesting for cytogenetic analysis followed the method described previously (9). Twenty to thirty trypsin/Giemsa-banded metaphases were analyzed from each normal and tumor cell culture. Growth rates were determined as described previously (4, 7). To determine whether the cells exhibit the transformation phenotype of AIG, their growth in semisolid medium was examined (4, 7). The presence of gene mutations in c-K-ras and c-Ha-ras and p53 genes were conducted at passage 2. Briefly, 1 μg of DNA extracted from cultured cells was used as a template for the PCR amplification for c-K-ras and c-Ha-ras codon 12. The amplicons were subjected to nonradioactive single-strand conformation polymorphism analysis. The amplicons were also subjected to Southern slot blot hybridization using allele-specific oligonucleotide probes (32P-labeled probes) to identify codon 12-specific mutations (10, 11). For p53 mutation analysis, PCR amplification of exons 2–11 was performed using their respective exon-specific primers (11, 12). The amplicons were subjected to single-strand conformation polymorphism analysis.

Effects of Growth Factors on Proliferation. To determine which of the supplemental factors in the medium are essential for growth of the tumor and normal cells, we examined the effects of deleting the individual factors while maintaining the remaining factors at concentrations given above. For these experiments, groups of cultures (three per group) were plated in the appropriate medium, and number of cells were determined at 7 days after plating. The growth of the cultures was expressed as a percentage of control i.e., cells maintained in the complete medium.

Results

Prostate Tissues. Prostate tumor and normal prostate specimens were obtained from radical prostatectomy specimens. Two of eight grossly visible primary tumors were found to be nondiploid, and these were used for the cell culture. In one case, the tumor histogram was interpreted as DNA aneuploid with a DNA index of 1.8. Histologically, the tumor was composed predominantly of solid sheets of fused glands corresponding to a primary Gleason grade 4 pattern (Fig. 1A); the overall Gleason score for the tumor was 7 (4 + 3). The tumor invaded through the prostatic capsule into periprostatic adipose tissue and extended to involve the linked surgical resection margin (stage T3aN0Mx). The second case was DNA tetraploid with a DNA index of 2. Pathologically, the tumor in this case also was composed predominantly of fused glands (Gleason grade 4) with an overall Gleason score of 7 (4 + 3). There was extension of the tumor through the prostatic capsule into periprostatic adipose tissue and into the wall of one seminal vesicle; metastatic adenocarcinoma was present in one pelvic lymph node (stage T3aN1Mx). Two other patients provided the normal prostate specimens.

Cell Culture. Outgrowth cultures derived from the nondiploid tumors were relatively heterogeneous. Frequently, portions of the outgrowths consisted of sheets of compact squamous cells whereas other areas showed actively proliferating polygonal epithelioid cells. The serial passage tumor cell cultures consisted mostly of epithelioid cells that proliferated actively as mitotic cells were present throughout the cultures (Fig. 1B). At confluence density, the tumor cells continued to proliferate and formed foci typical of transformed cells (see below).

Primary outgrowths of normal prostate consisted of relatively homogeneous cells with polygonal epithelioid morphology (Fig. 1C). The serial-passage cultures were similar to the primary cultures and frequently exhibited a columnar shape typical of glandular cells in vivo. At confluence, the normal cells reflected contact inhibition of growth and ceased to proliferate until splitting.

Fig. 1. A, histological section of the nondiploid prostatic carcinoma used for cell culture. The section shows a tumor characterized by complex fused glands (Gleason grade 4) with individual cells having large vesicular nuclei and macronucleoli (×250). B, low-passage (passage 2) epithelial cell culture derived from a nondiploid prostatic carcinoma. The culture consists of actively proliferating mostly epithelioid cells. ×350; C, primary culture derived from normal prostate. The cells exhibit typical polygonal epithelioid morphology (×750).
Growth Characteristics. The cells derived from the tumors proliferated actively with CPDT of approximately 36–42 h. The tumor cells formed numerous foci of transformed cells (Fig. 2A). More importantly, the tumor cells showed AIG as they formed colonies in semisolid medium (Fig. 2B). The cells derived from both the tumors have been serially passaged six to eight times. In the present culture conditions, their growth subsequently slowed, and most cells senesced between passages eight and ten.

The normal prostate cells proliferated at a relatively lower rate with a CPDT of about 50 h. In contrast to the tumor cells, the normal prostate cells exhibited contact inhibition of growth, did not form foci, and also lacked AIG. The normal cells were serially passaged at least 10 times; between passage 11 and 14, their growth slowed, and the cells senesced.

Karyotype and Mutation Analyses. The cytogenetic analysis of the cultured cells was performed independently of any knowledge of either the histological diagnosis or DNA content parameters. Chromosome analysis was conducted on cells at the second-third passage. Both the tumors with nondiploid DNA content revealed two cell populations; a tetraploid population with 92,XXYY karyotype (Fig. 2C) and a diploid with 46,XY karyotype. In both the tumors, no structural aberrations were found. In contrast, the metaphases derived from both the normal prostate cell cultures yielded only a normal diploid karyotype (Fig. 2D).

Mutation analysis of c-K-ras, c-Ha-ras, and p53 genes reflected no alterations in the tumor cells. Similarly, the cells derived from the normal prostate also lacked mutations in c-K-ras, c-Ha-ras, and p53 genes.

Expression of PSA and Cytokeratins. These studies were conducted mostly on low-passage cultures to avoid any alteration that may occur as a result of serial passaging of the cells. Cultures growing on collagen-coated coverslips were used for these experiments. Both the normal and tumor cells showed positive staining for PSA (Fig. 3A). The tumor and normal cells also showed strong titers for cytokeratins 18 and 19 (Fig. 3B). However, both the cell types were negative for keratin 15 (Fig. 3C). Negative controls, i.e., cultures in which the treatment with a primary antibody was omitted, showed no reaction (data not shown).

Effects of Medium Supplements on Growth. Because the tumor and normal cells had variable CPDT, it was of interest to determine whether the medium supplements have variable effects on their growth. To avoid variations due to the passaging of the cells, these experiments were done on the third-fourth passage cultures. For the tumor cells, deletion of EGF, hydrocortisone, and insulin stimulated growth by approximately 79, 45, and 27%, respectively (Fig. 4). In contrast, the deletion of BPE inhibited the growth of the cells by 63%, whereas the deletion of cholera toxin had no significant effect. Deletion of all of the supplements simultaneously inhibited growth of the tumor cells by almost 95%.

Growth of normal diploid cells required EGF, hydrocortisone,
insulin, BPE, and cholera toxin, as individual deletion of these factors inhibited growth by 53, 83, 84, 70, and 70%, respectively. Deletion of all of the factors inhibited growth of the normal cells by 98%.

Discussion

Prostate cancer is the most common malignancy in men over 45 years of age and yet, it is among the least investigated and most poorly understood of human malignancies. One major obstacle for the lack of progress has been the difficulty of growing sufficient amounts of cells that reflect in situ characteristics of prostate tumors and normal prostate in order to conduct molecular and genetic studies. In particular, technical problems have precluded the propagation of cells exhibiting transformed phenotypes from primary prostate tumors. An availability of in vitro prostate tumor cell models would greatly facilitate direct comparison between the normal and tumor cells and also provide important tools for the elucidation of specific phenotypes of the transformed cells. Previously, we have described the culturing of human primary prostate tumor cells (4). However, the tumor cells had a diploid karyotype and lacked transformed phenotypes, although their growth requirements varied considerably from the normal prostate cells derived from the same patient. Because a majority of human primary prostate tumors are diploid (5) and we had not determined the ploidy of the tumor prior to culturing the cells, it was not clear whether the cultured diploid cells originated from the diploid tumor or whether our culture conditions promoted selective growth of the diploid cells and caused degeneration of aneuploid cells. To address this crucial issue, we propagated epithelial cells from prostate tumors that were determined to be nondiploid by flow cytometry prior to culture. We now report that the two nondiploid tumors that we cultured produced aneuploid and tetraploid epithelial cells that exhibited transformed phenotypes, i.e., focus formation and AIG. In contrast, the cultures derived from the normal prostate consisted of diploid cells that lacked the transformed phenotypes.

Epithelial cell cultures derived from normal prostate, benign prostatic hyperplasia, and primary prostate carcinoma have been reported (4); all three cell types had diploid karyotypes and lacked focus...
formation and AIG, although their nutritional requirements varied. These results seemed consistent with those reported for human breast tumors in which the low-passage cultures were frequently diploid and lacked AIG (3). It was suggested that the culture conditions used for the breast tumors promoted growth of the diploid cells and caused degeneration of the aneuploid cells (3). However, more recently, serial propagation of aneuploid breast tumor epithelial cells has been reported (13, 14). Our results reported in this study show that the epithelial cells derived from the primary nondiploid prostate tumors were hyperdiploid and also showed transformed phenotypes of focus formation and AIG. In contrast, the normal prostate cells were diploid and lacked the transformed phenotypes. Furthermore, the tumor cells grew at a much higher rate than the normal prostate cells. Additionally, our cultures derived from the prostate tissues did not appear to contain any fibroblasts, as all of the cells examined showed positive staining for cytokeratins. These results clearly demonstrate that the cells derived from the nondiploid tumors and normal prostate represent the in situ characteristics and do not involve any apparent selection of a particular phenotype caused by the culture conditions. Our previous observations (4) on the production of diploid epithelial cells from one primary prostate tumor of an African-American patient were most likely related to the diploid nature of the tumor, as almost 70% of the prostate tumors are diploid (5) and we had not prescreened the tumor. Our results further demonstrate that we can routinely culture epithelial cells from primary prostate tumors and normal prostate. This is a major accomplishment because it will now be possible to determine molecular and/or genetic parameters associated with the neoplastic prostate cells and also those parameters that may be associated with the ethnic differences in the incidence of prostate cancer.

Immunocytochemical analysis showed that both the normal prostate and tumor cells expressed PSA. Cytokeratin markers were applied to determine the cell type of origin of the normal and prostate tumor cells. Cytokeratin 15 is expressed mainly in basal cells and 18 in luminal cells of the prostate (15, 16); most prostate tumors express keratin 18 (16), indicating the luminal cell phenotype of the tumors. Breast tumor cells also express keratins 18 and 19 suggesting the luminal cell phenotype (17, 18). In the present study, both the prostate tumor and normal cells expressed very high titers of keratins 18 and 19, whereas no expression of keratin 15 was observed. These results strongly indicate that the cultures derived from the normal prostate and prostate tumors are of luminal cell origin.

Different cell types may have variable growth factor requirements in vitro. In addition, the growth factor requirement of cells may change resulting from genetic changes associated with preneoplastic and malignant-cell transformation. For instance, in low-passage cultures, human breast tumor epithelial cells were independent of insulin and EGF, whereas these factors were essential for the growth of normal mammary epithelial cells (19). Epithelial cells derived from benign prostatic hyperplasia had a reduced requirement for EGF, whereas the diploid prostate tumor cells were completely independent of EGF and hydrocortisone; in fact, the deletion of EGF and hydrocortisone stimulated the growth of diploid prostate tumor cells (4). Our present results show that nondiploid prostate tumor cells, in addition to being independent of EGF and hydrocortisone, did not require insulin and cholaera toxin for their growth. BPE was the major supplement required for the growth of the prostate tumor cells, the deletion of which inhibited growth by almost 65%. In contrast to the prostate tumor cells, normal prostate cells required EGF, hydrocortisone, insulin, cholaera toxin, and BPE, as deletion of any one of these factors strongly inhibited their growth. The results indicate that the prostate epithelial cells progressively achieve growth-factor independence as they advance through the multistep process of malignant transformation and ultimately achieving almost complete independence of the exogenous growth factors. In this regard, it is noteworthy that PC-3 cells, a line derived from a metastatic prostate tumor proliferated actively without the above growth factors (4).

In summary, our results demonstrate that the cell cultures derived from the nondiploid prostate tumors and normal prostate continue to reflect their in situ characteristics. The tumor cells showed nondiploid karyotype and transformed phenotypes, including focus formation and AIG. The tumor cell growth was independent of EGF, hydrocortisone, insulin, and cholaera toxin. In contrast, the normal prostate cells showed diploid karyotype, lacked transformed phenotypes, and their growth was dependent on EGF, hydrocortisone, insulin, and cholaera toxin.

References

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