Radiation-induced Neoplastic Transformation of Human Prostate Epithelial Cells

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Abstract

We report the malignant transformation of human prostate epithelial cells (267B1) after multiple exposures to ionizing radiation. Carcinogenic progression of cells from immortal growth to anchorage-independent growth in soft agar to tumorigenicity in athymic mice resulted after a cumulative X-ray dose of 30 Gy. The tumors were characterized histologically as poorly differentiated adenocarcinomas, expressed prostate-specific antigen, and stained positive for keratin. No p53 or ras mutations were observed. Numerous chromosomal defects were noted on karyotypes after radiation exposure. However, chromosomes 3 and 8 translocations were observed predominantly in the tumor outgrowths. These findings provide the first evidence of malignant transformation of human prostate epithelial cells exposed to ionizing radiation.

Introduction

Prostate cancer is the most common neoplasm in men and a leading cause of male deaths in the United States (1). In 1995, 244,000 new cases will be diagnosed, with over 40,000 deaths (1). It is estimated that 1 of every 11 American men will eventually develop prostate cancer. Compared with all other cancers, the incidence of prostate cancer increases most rapidly with age (2, 3). The genetic and environmental factors responsible for the high incidence of prostate cancer are largely unknown. In addition, unlike other major cancers such as breast, lung, and colon, little is known at the molecular level about prostate cancer (4). In vitro models of human prostate epithelial cells provide a practical approach to analyze the molecular mechanisms of prostate tumorigenesis. To our knowledge, radiation-induced neoplastic transformation of human prostate epithelial cell culture has not been described previously.

We have shown previously that the immortal human prostate epithelial cell line 267B1 is capable of neoplastic transformation by the v-Ki-ras oncogene (5, 6). In the present study, we have demonstrated the capability of X-ray irradiation to transform 267B1 cells to a malignant phenotype.

Materials and Methods

Cell Culture and Media. 267B1 cells were contributed by M. E. Kaighn (Biological Research Faculty and Facility, Inc., Ijamsville, MD). These cells were derived from human fetal prostate epithelium and immortalized by the SV40 T antigen (5). Cells were used at passage 19 for these transformation studies. Growth and maintenance media for these cells consisted of P4-8F (Biogenics), PAP (DAKO), and PSA (DAKO).

Transformation of 267B1 Cells by Ionizing Radiation. Altered epithelial cells (267B1) after multiple exposures to ionizing radiation. Carcinogenic progression of cells from immortal growth to anchorage-independent growth in soft agar to tumorigenicity in athymic mice resulted after a cumulative X-ray dose of 30 Gy. The tumors were characterized histologically as poorly differentiated adenocarcinomas, expressed prostate-specific antigen, and stained positive for keratin. No p53 or ras mutations were observed. Numerous chromosomal defects were noted on karyotypes after radiation exposure. However, chromosomes 3 and 8 translocations were observed predominantly in the tumor outgrowths. These findings provide the first evidence of malignant transformation of human prostate epithelial cells exposed to ionizing radiation.

Radiation Transformation Assay. Exponentially dividing cells, inoculated at 5 × 10^5 cells/cm^2-flask, were irradiated daily with 2 Gy of X-rays from a Siemens 6 MeV linear accelerator at a distance of 100 cm and at a dose rate of 2 Gy/min. After receiving a cumulative dose of radiation ranging from 2 to 30 Gy, cells were allowed to grow to confluence, with changes of media every 3 days. Cells were inspected daily for changes in morphology or growth pattern.

Anchorage-independent Growth. Control and irradiated cultures were trypsinized and resuspended at densities of 5 × 10^3 cells/ml in 2 ml of 0.35% Noble agar and overlaid in 60-mm dishes containing 0.6% agarose base. Anchorage-independent growth assays were performed in triplicate. Media were replenished every 7 days, and viable anchorage-independent macroscopic colonies containing >50 cells (>0.2 mm) were scored at 21 days (7).

Tumorigenicity in Athymic Mice. Three to four-week-old BALB nu/nu male mice were given injections s.c. at the mid-dorsal interscapular region with 1 × 10^5 cells in 0.2 ml Matrigel matrix (Collaborative Biomedical Products, Bedford, MA). Mice were screened twice weekly for tumor formation for a minimum of 120 days. Tumors were excised, divided, and reestablished as cultures. A portion of each tumor was immunostained, using monoclonal antibodies to cytokeratins, namely, AE1/AE3, (Boehinger Mannheim); MAK-6, (Triton Biosciences); and CAM 5.2, (Becton Dickenson), vimentin (Biogenics), PAP (DAKO), and PSA (DAKO).

Ras and p53 Analysis. Exponentially dividing cells were labeled with [35S]methionine at 100 μCi/ml for 4 h. The cell lysates were immunoprecipitated with anti-p21 antibody (Y13-259; Oncogene Science, Uniondale, NY) and analyzed by SDS-PAGE. Analyses of cellular ras and p53 proteins were performed by Western blotting using antisera obtained from Oncogene Science. Total genomic DNA was isolated (8), and SSCP analysis was performed (9). DNA sequencing was performed as described previously (10). The oligonucleotide primers were used to determine point mutations of the cellular ras genes (H-, K-, and N-ras), and the p53 genes (exons 2–11) were obtained from Clontech (Palo Alto, CA).

Chromosomal Analysis. Karyotypic analysis were carried out by Drs. J. Kaplan and B. Hukku. Evidence for the human origin of cells included the presence of membrane-specific antigen; the isozyme phenotype confirmed that these cells were derivatives of 267B1. Exact chromosome counts of 100 metaphases were made for ploidy determination.

Results

Transformation of 267B1 Cells by Ionizing Radiation. Altered morphology was observed in the 267B1 cells following two X-ray...
exposures of 2 Gy. The cells began to pile up in focal areas, formed small projections, and released small cells from the foci (Fig. 1b). Expanded individual colonies exhibited the typical polygonal arrangement of epithelial cells by phase-contrast microscopy, even after repeated subcultivation, but were smaller in size than unirradiated 267B1 cells. Isolated irradiated cell foci were further selected by growth in soft agar (Table 1 and Fig 1c), resulting in anchorage-independent clones. Additional irradiation of these cells, for a total of 30 Gy, resulted in limited survival. The few surviving cells were maintained without subculture and were allowed to grow within flasks over 1 month, resulting in radiation-transformed colonies. Subcultivation of these colonies produced densely packed polygonal cells,

Fig. 1. Transformation of the human prostate epithelial cell line (267B1) with X-rays. a. unirradiated 267B1 cells, X40; b, irradiated 267B1 cells (2 Gy × 2), showing focus of anchorage independent growth, X40; c, soft agar colonies of plated cells (2 Gy × 2); d, soft agar of unirradiated 267B1 cells; e, poorly differentiated epithelial cells obtained from irradiating 267B1 cells (2 Gy × 15), X40; f, tumor explant cell line after s.c. injection of irradiated cells (2 Gy × 15), X40.
The anchorage-independent and radiation-transformed clones were differentiated, exhibited many mitotic figures, and required more frequent subcultivation compared to the unirradiated 267B1 cells. In addition, all of the established clones appeared poorly differentiated, exhibited many mitotic figures, and required more frequent subcultivation compared to the unirradiated 267B1 cells.

<table>
<thead>
<tr>
<th>Characterization of Radiation-transformed 267B1 Cell Line.</th>
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| The anchorage-independent and radiation-transformed clones were further characterized by quantitative differences in growth properties, such as saturation densities and soft agar colony forming efficiency. Saturation densities of the anchorage-independent and radiation-transformed cells were 7 and 23 times that of the parental 267B1 cells, respectively (Table 1). Moreover, the anchorage-independent and radiation-transformed cells grew in soft agar with colony-forming efficiencies of 0.26 and 0.30, respectively, whereas the unirradiated cells did not grow in soft agar (Table 1).

As reported previously, the immortal 267B1 cells were nontumorigenic for up to 6 months after s.c. inoculation in athymic mice (5). The 267B1 cells irradiated to cumulative doses of 2-14 Gy all exhibited anchorage-independent growth but were nontumorigenic in athymic mice as well. Further irradiation to 30 Gy, however, resulted in transformed cells that developed tumors within 4 weeks after s.c. inoculation in four of five athymic mice (Table 1). Microscopic examination of tumor explants revealed poorly differentiated adenocarcinoma consistent with prostate cancer. Injecting these cells into different strains of athymic mice resulted in no significant difference in tumor formation (data not shown).

Cytokeratins were detected in the parental 267B1, anchorage-independent, and radiation-transformed cells by immunoperoxidase staining consistent with epithelial origin (Table 1). Immunostaining of cells and Western analysis of cell lysates with antisera specific to prostate-specific antigen and prostatic acid phosphatase were positive (Table 1).

The parental 267B1 cell line is aneuploid human male with near diploid counts (82%). No structural alterations were detected in both early or late passage 267B1 cells. The anchorage-independent cells had chromosome counts in the near diploid range. Normal chromosome 18 was missing but was observed as a marker (Fig. 2; Table 2). One copy of chromosome 10 and both normal X and Y were missing but were present as markers 2, 4, and 7, respectively. Besides these above mentioned markers, other markers were observed as shown in Table 2.

The radiation-transformed cells were also aneuploid human male with most chromosome counts in the near diploid range. Three new markers, M8, M9, and M10 (Table 2) were observed. Markers M1 and M8 were observed in a few metaphases (<50%). Chromosome 18 was observed in very few metaphases as M1 but it was observed in seven of nine karyotypes as M9, t(8q;18q). The marker M10, t(3qter>3p24:8q9q21>qter) was first observed in the radiation-transformed cells in 2 of 9 karyotypes. Karyotypes from tumor explants reestablished in culture were performed. In these tumor cells, the M9 marker remained stable (six of eight karyotypes), whereas M10 increased to six of eight karyotypes. Representative karyotypes of these three cell lines are shown in Fig. 2.

### Table 1 Properties of 267B1 human prostate epithelial cell lines by X-ray irradiation

<table>
<thead>
<tr>
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<th>267B1 immortal cells</th>
<th>Anchorage-independent cells</th>
<th>Tumor cells</th>
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<tbody>
<tr>
<td>Saturation density</td>
<td>2.5 × 10⁴</td>
<td>1.7 × 10⁹</td>
<td>5.8 × 10⁵</td>
</tr>
<tr>
<td>Soft agar colony</td>
<td>&lt;0.01</td>
<td>0.26</td>
<td>0.30</td>
</tr>
<tr>
<td>formation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumorigenicity</td>
<td>0/4</td>
<td>0/4</td>
<td>4/5</td>
</tr>
<tr>
<td>in nude mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSA/PAP</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Keratin</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Karyotype</td>
<td>Near diploid</td>
<td>Near diploid, hypotetraploid</td>
<td>Near diploid, hypotetraploid</td>
</tr>
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</table>

*Colonies were >2 mm. The mean of three determinations is reported.

**Ras and p53 Analysis.** Western blot analyses with antisera specific for cellular ras and p53 genes indicated no change in the level of expression of these genes in the 267B1 parental, anchorage-independent or radiation-transformed cells (data not shown). Radioimmuno-precipitation analysis of the cellular ras genes with anti-p21 antisera revealed no differences in migration patterns or band intensities in the unirradiated and X-ray-treated 267B1 cells. SSCP analysis of the cellular ras genes (Ki-, Ha-, and N-ras) displayed identical banding patterns in both tumorigenic and nontumorigenic prostate cells. These observations were verified by DNA sequencing of the PCR-generated products of each sample tested. Only wild-type sequences in the exons of Ki-, Ha-, and N-ras genes were seen.

A similar PCR-SSCP analysis for exons 2-11 of the p53 gene revealed no mutations in the 267B1 parental, anchorage-independent, radiation-transformed, or tumorigenic cells. DNA sequencing of both strands of each exon indicated that no deletions, point mutations, or rearrangements were present.

### Discussion

Malignant transformation of human prostate epithelial cells was achieved by the cumulative action of a DNA tumor virus and X-ray irradiation. At least two and possibly more alterations in cell growth properties seem to be required. The measurable event was the development of apparently unlimited growth potential as a result of SV40 immortalization (5). Treatment of nontumorigenic early passage SV40 immortalized prostate cells with ionizing radiation resulted in additional changes in their growth properties. Concomitantly acquired properties of the radiation-transformed cells included morphological alterations, the ability to grow in soft agar, and the formation of rapidly growing adenocarcinomas in athymic mice.

In this model system, X-ray irradiation is similar to the v-Ki-ras oncogene in its ability to complement SV40 virus in fully transforming human prostate epithelial cells. However, unlike the rapid transformation of 267B1 cells observed after v-Ki-ras transfection (6), the alterations in the growth patterns after irradiation were delayed in their appearance and required several subcultures for visualization. These findings suggest that multiple cell divisions are required for fixation and expression of the transformed phenotype in response to radiation. It is possible that more than one genetic lesion may be required as well. Cooperating cellular or viral oncogenes have been shown to induce neoplastic transformation of embryonic rodent fibroblasts (11, 12). In addition, the combined action of tumor virus and X-ray irradiation has been demonstrated to produce neoplastic transformation of human epithelial cells (13, 14). Our data on neoplastic transformation of SV40 immortalized human prostate epithelial cells by exposure to ionizing radiation provide additional support for the theory that neoplastic conversion is a multistep process.

Although the activation of cellular ras has been demonstrated in...
Fig. 2. Representative karyotypes of 267B1-derived cell lines. A, normal male karyotype from SV40 immortalized 267B1 cell line. B, 267B1 cells exhibiting anchorage-independent growth after 2 Gy × 2. Marker chromosomes are listed in Table 2. C, tumor cell lines obtained from tumor explants after 2 Gy × 15. Translocations of chromosomes 3 and 8 were observed frequently. Fewer marker chromosomes were present, suggesting that tumorigenic progression is a result of clonal events.
rodent tumors induced by ionizing radiation (15–17), the activation of unique non-ras oncogene has been shown in malignant radiogenic transformed rodent cells (18). The neoplastic transformation of the 267B1 human prostate epithelial cell line by X-ray irradiation suggests that other cellular oncogenes may be activated as part of the process. Our findings further indicate that ras oncogenes were not activated in this radiation-induced transformation, even though ras oncogenes have been implicated in radiation-induced animal tumors (15–17) and in some human prostate carcinomas (19, 20). This system may be useful in efforts to detect and characterize other cellular genes contributing to the neoplastic phenotype of human prostate epithelial cells.

Ionizing radiation is known to cause DNA double-strand breaks, which can lead to deletions and rearrangements within cellular DNA and to modulate gene expression. A potential target for this action is the tumor suppressor gene p53. This gene is susceptible to small rearrangements, deletions, and point mutations, and thus is a potential site of action for the mutagenic effects of ionizing radiation. Mutations in this gene have been associated with malignant transformation in human and animal systems (21, 22) and implicated in the pathogenesis of prostate carcinoma (23, 24). Loss of p53 function after radiation transformation of primary human mammary epithelial cells has been reported recently (25). Our analysis of exons 2–11 of the p53 gene in the parental 267B1, anchorage-independent clones and tumorigenic clones, indicates an absence of point mutations, deletions, or rearrangements in this tumor suppressor gene. This finding suggests a different pathway involved in the carcinogenic progression of these cells.

Numerous genetic changes have been reported in prostate cancer, including allelic loss, point mutations, and DNA methylation. However, the most frequent changes are allelic loss from at least one chromosome arm and the short arm of chromosome 8 appears to be the most frequent allele lost (26–33). Karyotypic analysis of the radiation-transformed 267B1 cells suggests that both chromosome 3 and 8 may be important in the transformation process. In both the radiation-transformed cells and tumor explants, duplication of one allele of chromosome 8 and loss of 8p in the other allele was seen in the majority of karyotypes, even though allelic changes were not present in

<table>
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<th>Table 2</th>
<th>Marker chromosome distribution in the 267B1 human prostate epithelial cell lines by X-ray irradiation</th>
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<tbody>
<tr>
<td>Cell lines</td>
<td>M1</td>
</tr>
<tr>
<td>267B1</td>
<td>–</td>
</tr>
<tr>
<td>Anchorage-independent cells</td>
<td>+</td>
</tr>
<tr>
<td>Tumor cells</td>
<td>–</td>
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</tbody>
</table>

a M1, (18q?); M2, 10q+; M3, t(1q19); M4, t(Xqter>Xp22:7q); M5, t(20q); M6, t(1p:4p); M7, del.?; M8, i(8q); M9, t(8:18) (qq); M10, t(3qter>3p24::8q21>qter).
b –, absent; +, present in >50% metaphases; *, present in <50% metaphases.
any of the precursor cells. In addition, a translocation of chromosome 3 was observed for the first time in the radiation-transformed cells, followed by an increased selection of this marker in the tumor explants. The number of marker chromosomes present in tumor cells is less than in the anchorage-independent cells, which is consistent with the hypothesis that tumorigenic progression is a result of clonal events.

The carcinogenic action of ionizing radiation in humans has been well recognized from epidemiological data. The human prostate epithelial cell system described here may be a useful in vitro tool for dissecting the process of radiation-induced malignant transformation in human prostate epithelial cells. Additional molecular analysis of these cells will be needed to determine the specific events that are responsible for radiation-induced neoplastic transformation.

Acknowledgments

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References

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