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ANDROGEN SENSITIVITY AND GENE EXPRESSION IN *ras* + *myc*-INDUCED MOUSE PROSTATE CARCINOMAS

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Summary—We established an androgen-sensitive cell line (BR31-5) from a *ras* + *myc*-induced mouse prostate carcinoma and used this cell line together with a previously reported transplantable androgen-independent mouse prostate carcinoma to investigate patterns of expression for apoptosis-related genes in an androgen-deprived environment. Single cell suspensions derived from the BR31-5 cell line were inoculated into the flank of intact or castrated adult male C57BL/6 mice and tumors were harvested 12 days post-inoculation for Northern blotting. A transplantable androgen-independent prostate cancer was also inoculated into intact or castrated mice and tumors harvested 21 days later. Tumor volume analyses showed that BR31-5 carcinomas were androgen-sensitive. Northern blotting showed that mRNA levels for two apoptosis-related genes, transforming growth factor-beta 1 and c-*myc*, were significantly elevated to a similar extent in carcinomas grown in castrated hosts compared to intact hosts for both the androgen-sensitive BR31-5 and androgen-independent carcinomas. Levels of mRNA for tissue type plasminogen activator, shown previously to be elevated in androgen-independent carcinomas following growth in castrates, were also increased in BR31-5 carcinomas under similar androgen-deprived conditions but to a lesser extent. Interestingly, testosterone repressed prostate mRNA No. 2 levels shown previously to be similar in both the intact and castrated groups for androgen-independent carcinomas were significantly increased in the castrated group compared to the intact group for BR31-5 carcinomas. Therefore, specific patterns of expression for apoptosis-related genes may be able to discriminate androgen-sensitive and androgen-independent prostate cancer under androgen-deprived conditions.

INTRODUCTION

The prostate is distinct from other major sites of cancer in that it is exquisitely sensitive to the growth-promoting effects of androgens and is dependent on androgens for the maintenance of strucural integrity and function. Because this sensitivity to androgens is also characteristic of prostate cancer cells, medical and surgical therapy for prostate cancer has been directed toward blocking the effects of androgens pharmacologically or eliminating the source of androgens altogether. Since the biological effects of androgens in normal prostate are exceedingly complex and poorly understood at the molecular level, it follows that the mechanisms underlying the sensitivity of prostate cancer to androgens are also equally complex and largely unknown.

The study of castrated adult male rats has provided important information about androgen-regulated DNA synthesis in the ventral prostate [1] and, more recently, about genes under androgenic control. Some of these genes are growth factor genes and others are non-growth factor proto-oncogenes that may be relevant to prostate cancer. Androgen ablation leads to an active process of cell death specific to luminal epithelial cells of the ventral prostate [2, 3]. Steady-state levels of mRNAs coding for transforming growth factor-β1 (TGF-β1) [4], c-*myc* [3, 5], c-*fos* [3], and testosterone repressed prostate mRNA No. 2 (TRPM-2) [6] are markedly increased in the ventral prostate following castration. Increased enzymatic activity for tissue type plasminogen activator (tPA) was also demonstrated in normal rat ventral prostate 1–5 days following castration [7]. Accumulation of these specific mRNAs and enzymatic activity is transient and either precedes or directly coincides with the loss of luminal epithelial cells. In the case of c-*myc*, elevated levels of mRNA were localized by *in*

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situ hybridization to residual epithelial cells 4 days post-castration [5]. Castration induced expression of TRPM-2 has also been localized by *in situ* hybridization to the luminal epithelial cells in the distal regions of prostate ducts and may be influenced by direct interaction with adjacent stromal cells [8]. In addition to increased levels of TGF-β1 mRNA, the number of receptor binding sites for TGF-β1 [9] and epidermal growth factor [10, 11] is increased in rat ventral prostate following castration. These increases also correlate with the loss of luminal epithelial cells.

Gene expression has also been analyzed in rat prostate following the administration of testosterone to animals several days post-castration. Under these circumstances, there is a rapid mitogenic response of the prostatic epithelium to the androgenic stimulus [1, 3]. Transient increases in the steady-state mRNA levels of several genes including c-*fos*, c-*myc*, c-Ki-*ras* and basic-fibroblast growth factor have been observed during this period of androgen-induced cell proliferation [12].

Although the relationship between androgen-regulated gene expression and the therapeutic effects of androgen ablation on human prostate cancer is not clear, it is of interest that some of the same genes found to be overexpressed in prostate cancer are regulated by testosterone (e.g. c-*myc* [13, 14] and TGF-β1 [15]). Interestingly as discussed above, overexpression of these genes under normal conditions occurs during acute periods of epithelial cell death and proliferation. Whether these gene activities are directly affected by androgenic hormones or are coincidental with the degenerative and proliferative biological processes triggered by changes in androgen levels remains to be established. In either case, it is conceivable that inappropriate androgenic regulation of growth factor and proto-oncogene activities could contribute to the progression of prostate cancer.

Multistep carcinogenesis in prostate cancer can be mimicked experimentally to a large extent using the mouse prostate reconstitution (MPR) model system [15–17]. The MPR model exploits the capacity of the fetal mouse urogenital sinus (UGS) to undergo morphogenesis and differentiation into mature prostate after grafting under the renal capsule of adult isogenic male hosts [18–21]. Exogenous genes are introduced into dissociated UGS cells prior to grafting under the renal capsule. Morphogenesis and functional differentiation are achieved in both mock-infected and non-oncogenic virus-infected MPRs after 4 weeks growth. Initial studies using recombinant retroviruses that contain either the v-Ha-*ras* or the v-*gagmyc* (MC29) oncogenes alone showed that the *ras* oncogene induces a grossly dysplastic phenotype with a strong angiogenesis component in the mesenchymal compartment and a focally hyperplastic phenotype in the epithelial compartment. The *myc* oncogene alone induced predominantly focal epithelial hyperplasia. The *ras* and *myc* oncogenes introduced in combination via a single retrovirus, Zipras/myc 9, induced predominantly carcinomas [16]. We further demonstrated that the transition from benign hyperplasia to malignant cancer induced by Zipras/myc 9 is associated with overexpression of specific growth factors including TGF-β1 [15].

The properties of the MPR model are well suited to address important questions in prostate cancer. Organ-specific localization of cancer-inducing oncogenes and the potential for multiple independent experiments over relatively short time periods offer distinct advantages for experimental studies concerning genetic differences in the progression of prostate cancer. In addition, the ability to create primary prostate cancers, determine their clonal status by the analysis of unique virus–cell DNA junction fragments and subsequently transplant primary cancer-derived cell lines into both normal intact and castrated hosts presents a novel approach for studying the response of prostate cancer to androgen deprivation.

Recently, we analyzed gene expression in a transplantable androgen-independent carcinoma produced by total UGS infection with Zipras/myc 9 using the MPR protocol. We observed increased mRNA levels for a panel of genes shown previously to be associated with castration-induced apoptosis when this tumor was grown in castrated male hosts compared to those grown in intact males [22]. Here we compare the pattern of apoptosis-related gene expression demonstrated in the androgen-independent carcinoma with that of androgen-sensitive carcinomas under similar conditions.

In these initial studies we show that differences in apoptosis-related gene activities exist for androgen-sensitive versus independent mouse prostate carcinomas grown in castrated males. We speculate that progressive loss of selected gene activities related to apoptosis coincides with the development of androgen-independent growth.

MATERIALS AND METHODS

Carcinomas

MPRs were produced as described previously [16]. Day 17 fetal UGS tissue from C57BL/6 mice was dissociated with trypsin and collagenase then infected with either Zipras/myc 9 [16] or BAGα [23] which was devoid of detectable amounts of replication-competent helper virus. Following transfer of the cells into a collagen matrix and overnight incubation *in vitro*, MPRs were grafted under the renal capsule. An androgen-sensitive cell line designated, BR31-5 was derived from an MPR infected with Zipras/myc 9 after 4 weeks growth in an isogenic host. The transplantable androgen-independent carcinoma was derived from a Zipras/myc 9-induced 8 week MPR as described previously [22]. BR31-5 cells and early passage carcinomas were reduced to single cell suspensions and 4×10^6 cells were inoculated subcutaneously into the flank region of normal intact or castrated (7 days post-surgery) isogenic male hosts.

Growth and phenotypic characterization

Tissues from MPRs were harvested after 4 or 8 weeks growth as subcapsular renal grafts and wet weights determined. Portions were either frozen in liquid N_2 or fixed and paraffin embedded for sectioning and hematoxylin and eosin (H&E) or immunohistochemical staining.

Tumor growth in intact and castrated hosts was monitored over time by measuring the dimensions of each tumor with vernier calipers and calculating the volume [24]. Tumor samples were harvested and processed as described for MPRs above.

Northern blot analyses

RNA from frozen tissues was isolated by the guanidinium isothiocyanate method, electrophoresed through 1% agarose–formaldehyde gels and transferred to Hybond N (Amersham) by capillary transfer. Filters were sequentially hybridized with random-primed ^{32}P-labeled cDNA probes. Probes used were: a 1.6 kb EcoRI fragment containing the entire murine TGF-β1 cDNA [25]; a 2.65 kb XbaI–Hind III fragment containing exons 2 and 3 of the mouse c-*myc* gene [26]; a 2.5 kb Bam HI–Hind III full-length cDNA for murine tPA [27]; a 1.7 kb EcoRI fragment of the rat TRPM-2 cDNA [6] and a 1.5 kb fragment of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA [28]. The blots were stripped with boiling 0.1% SDS between probes. Audioradiographs were scanned with a BioRad Model 620 densitometer and results normalized for RNA loading as estimated by the signal for GAPDH RNA.

RESULTS

Growth and phenotypic alterations in ras + myc-induced carcinomas

The initial studies which demonstrated *ras* + *myc*-induced carcinogenesis using the MPR model system were carried out with tissues obtained from inbred C57BL/tan mice [16]. This strain is a coat color variant of C57BL/6 which was isolated in London in 1958 and subsequently maintained as an inbred mouse strain [29]. These studies as in others [15, 17] were carried out using inbred C57BL/6 mice. Total UGS cells were infected with high titer ($>10^5$ colony or focus-forming U/ml) BAGα [23] or Zipras/myc 9 [16] supernatants, reconstituted together in collagen, grafted into isogenic adult male hosts, and allowed to grow *in vivo* for a period of 4 or 8 weeks. The phenotypic alterations induced by the *ras* and *myc* oncogenes were examined by H&E and immunohistochemical staining of sections from fixed, paraffin-embedded tissues (Fig. 1). BAGα-infected MPRs demonstrated normal prostate morphology [Fig. 1(A)]. Zipras/myc 9-infected MPRs produced rapidly growing, poorly differentiated carcinomas >90% of individual experiments [Fig. 1(B) and Table 1].

Androgen sensitivity in vivo of a cell line derived from a ras + myc-induced carcinoma

Our initial approach to elucidating the androgen sensitivity status of 4-week *ras* + *myc*-induced carcinomas was to test cell lines derived from these tumors for androgen-regulated growth *in vivo*. One cell line BR31-5 was used in a study in which cell inoculums were introduced under the flank skin of normal intact or castrated (7 days post-castration) male mice. A significant lag period was observed for the growth of tumors in the castrated group (Fig. 2) indicating that the tumors produced from this cell line were sensitive to hormones with respect to growth under these conditions. Further immunohistochemical analysis demonstrated the presence of cytokeratin in the tumor cells (not shown) indicating their epithelial derivation and consistent with their outgrowth from a 4-week *ras* + *myc*-induced carcinoma.

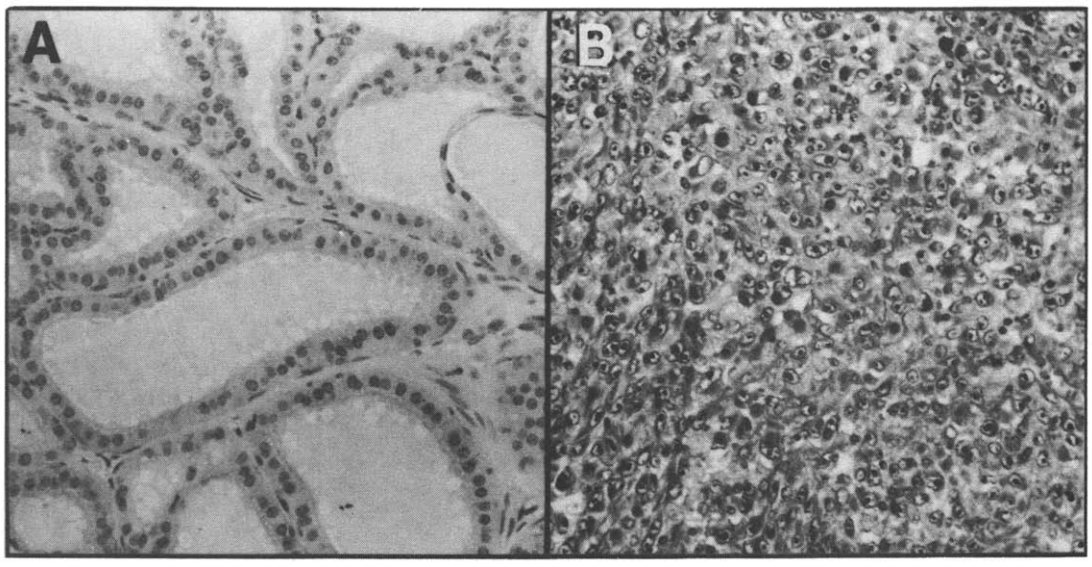

Fig 1 Phenotypic alterations in *ras* + *myc*-induced carcinomas H&E stained tissue sections of. (A) BAGα infection of C57BL/6 and (B) Zipras/myc 9 infection of C57BL/6.

Androgen independence of a transplantable ras + myc-induced carcinoma

We used the *in vivo* growth assay to test the androgen sensitivity of a transplantable, clonal *ras* + *myc*-induced mouse prostate carcinoma [22]. Following inoculation of monocell suspensions into both intact and previously castrated male hosts, growth was monitored as above. Tumor volumes in the intact and castrated groups did not achieve statistically significant differences at any time point (Fig. 3). Examination of cell type and morphology of these carcinomas by H&E staining and immunohistochemistry showed that tumors from both the intact and castrate groups were highly malignant, pleomorphic anaplastic carcinomas [22]. Interestingly, when the extent of apoptosis was evaluated in both groups by counting apoptotic cells, there were no significant differences between the transplantable, clonal carcinoma growth in intact or castrated animals [22].

Table 1 Summary of *ras* + *myc* activities in MPR derived from C57BL/6 tissues

Virus	*n*	Phenotype
BAGα	36	36 Normal
Zipras/myc 9	31	29 Carcinomas
		1 Focal epithelial hyperplasia
		1 Normal

For each organ reconstitution 1 5 × 10^6 UGS cells were infected with high titer retrovirus stocks (1 × 10^6–3 × 10^6 CFU or FFU/ml) at a multiplicity which did not exceed 1 Phenotypic alterations were evaluated on H&E stained tissue sections and carcinomas were verified by positive cytokeratin staining in >95% of the cell population

Differential gene expression in androgen-sensitive versus androgen-independent mouse prostate carcinomas

To further compare androgen-sensitive versus androgen-independent carcinomas with respect to their response to conditions of androgen deprivation, we analyzed mRNAs extracted from androgen-sensitive and androgen-independent mouse prostate carcinomas grown in both intact and castrated hosts. We showed previously that mRNA levels for four growth related genes TGF-β1, TGF-β3, tPA and c-*myc* were significantly elevated and androgen receptor (AR) mRNA levels significantly reduced in androgen-independent mouse prostate carcinomas grown in castrated hosts compared to

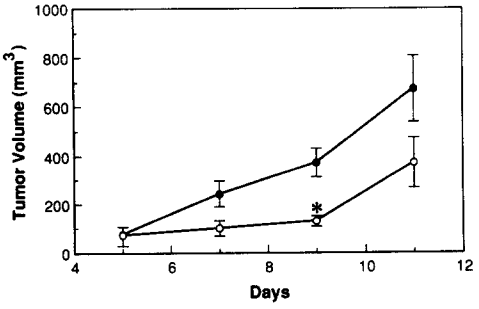

Fig. 2. Growth of BR31-5 mouse prostate cancer cell line in intact and castrated male hosts Tumor growth was monitored over time and the volume calculated The values obtained were averaged from five independent tumors for both the intact (closed circles) and castrated (open circles) groups ±SEM. Significant differences between carcinomas in the intact and castrated groups are indicated: *$P < 0.05$

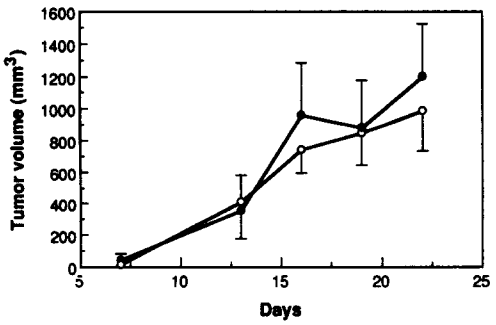

Fig. 3. Growth of transplantable androgen independent prostate carcinomas in intact and castrated male hosts. Tumor growth was monitored as described (see Fig. 2). The values obtained were averaged from seven independent tumors for the intact group (closed circles) and 10 independent tumors for the castrated group (open circles) ±SEM.

those grown in intact hosts [22]. In contrast, TRPM-2 mRNA levels, which have been reported to be elevated following castration in normal rat ventral prostate [6], were not increased in the castrated group. Therefore, the androgen-independent mouse prostate cancer demonstrates a unique pattern of expression for a set of growth related genes in an androgen-deprived environment. Some of the alterations (e.g. elevated TGF-β1 and c-*myc* mRNAs) are consistent with those seen following castration in normal rat prostate. However, in two cases (unchanged TRPM-2 mRNA and reduced AR mRNA) a notable deviation from the apoptosis-related genetic program was observed.

We analyzed mRNA levels by Northern blotting for TGF-β1, tPA, c-*myc*, TRPM-2 and GAPDH in the androgen-sensitive BR31-5 carcinomas recovered after 12 days growth in intact or castrated male hosts. As reported for the transplantable androgen-independent carcinoma, relative quantitation of mRNA levels was achieved by densitometric scanning and normalization with respect to levels of the GAPDH transcripts. These data were compared to similarly derived data for the transplantable hormone-independent carcinoma [22] and the results are presented in Fig. 4. Increased mRNA levels for TGF-β1 and c-*myc* were demonstrated in the castrated group compared to the intact group for both the androgen-sensitive, BR31-5 carcinoma and the transplantable androgen-independent carcinoma. Interestingly, the fold increase is remarkably similar for both tumors. Levels of tPA mRNA shown previously to be markedly increased in androgen-independent carcinoma grown in castrates were significantly increased in the androgen-sensitive BR31-5 carcinoma castrated group, but the fold change was significantly reduced compared to the androgen-independent carcinomas. In contrast, marked differences in TRPM-2 mRNA levels were seen in the androgen-sensitive BR31-5 carcinoma versus the androgen-independent carcinoma after growth in castrates. Whereas similar mRNA levels for TRPM-2 were shown previously for androgen-independent carcinomas following a growth period in intact and castrated hosts, androgen-sensitive BR31-5 carcinomas grown in castrates produced significantly higher TRPM-2 mRNA levels compared to those grown in intact males.

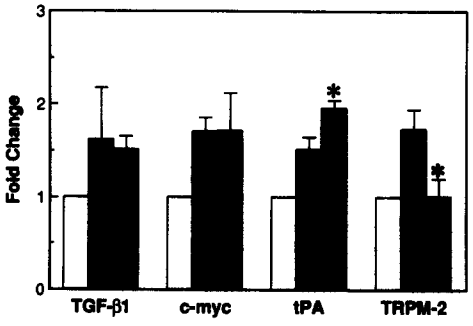

Fig. 4. Quantitation of relative gene expression by densitometry in androgen-sensitive carcinomas derived from cell line, BR31-5 and transplantable androgen-independent carcinomas grown in intact or castrated male hosts. Autoradiographs were scanned using a Bio-Rad Model 620 video densitometer and mean values of relative intensities ±SEM for gene expression in the BR31-5 castrated groups (cross-hatched bars) and the androgen-independent castrated group (solid bars) were plotted relative to that of the intact groups (open bars) which were arbitrarily designated as one. Therefore, expression in the castrated groups is expressed as the fold change relative to expression in the intact groups. Significant differences between carcinomas in the castrated groups are indicated: $^*P < 0.05$.

DISCUSSION

The clinical relapse demonstrated by the majority of patients who initially respond to hormone ablation can be explained by two possible mechanisms: (1) the selection and expansion of preexisting androgen-insensitive clones, and (2) the adaptation of androgen-sensitive cells to an androgen depleted environment [30, 31]. Evidence for clonal selection is compelling, but indirect, involving the use of fluctuation analysis [30]. Part of the difficulty in addressing the mechanism(s) involved in the continuation of prostate cancer growth following androgen ablation is the exclusive use of growth kinetics in defining hormone sensitivity and the possibility that both clonal selection and adaptive changes occur simultaneously. Furthermore, the preexisting capacity to respond to

androgen deprivation by activating compensatory pathways might represent an adaptive mechanism(s) whereby the growth of androgen-independent clones occurs and thus represents a condition where selection and adaptation are closely related. Since it is these cancer cells which ultimately threaten the lives of prostate cancer patients who demonstrate recurrence post-castration therapy, it is of major importance to define the mechanism(s) responsible.

We showed previously that the androgen-independent carcinomas described in this paper demonstrated a unique pattern of expression for a set of apoptosis-related genes following a period of growth in castrated hosts [22]. Elevated mRNA levels for TGF-β1, c-*myc* and tPA were consistent with a similar pattern seen in normal rat ventral prostate following castration. However, mRNA levels for TRPM-2, normally elevated following castration and directly associated with apoptosis [6], were not increased. It remains to be established whether these altered gene activities were preexisting in a minor population of cancer cells prior to the growth period in castrates or whether these cells were endowed with the capacity to develop these properties in response to castration. We feel that because clonality with respect to the initiating recombinant retrovirus, Zipras/myc 9, was demonstrated [22] and previous studies have shown remarkable homogeneity among a large number of *ras* + *myc*-induced carcinomas; and because growth kinetics for both the intact and castrated groups were similar indicating that the tumor was androgen-independent prior to inoculation into castrates; that the majority of the cancer cells were clonal at the time of inoculation, not only for virus-integration, but for the capacity to respond to the androgen depleted environment via specific alterations in gene expression.

The initial results of similar studies reported here for the androgen-sensitive BR31-5 prostate cancer cell line demonstrates that mRNA levels for TGF-β1 and c-*myc* were elevated in castrates to the same extent as seen in the androgen-independent carcinomas. Interestingly, levels of tPA mRNA were also elevated in androgen-sensitive BR31-5 carcinomas following growth in castrates but to a lesser extent than in the androgen-independent carcinomas. However, unlike the androgen-independent cancer, apoptosis-related TRPM-2 mRNA levels were elevated. In androgen-sensitive PC-82 human prostate adenocarcinoma xenografts grown in castrated nude mice as well as normal rat ventral prostate following castration, increased mRNA levels for TGF-β1 and TRPM-2 are seen and are accompanied by widespread apoptosis [4, 6, 8, 32]. Thus as in normal rat ventral prostate, growth of both human and mouse androgen-sensitive prostate cancer in an androgen-deprived environment results in a genetic program that includes elevated expression of TGF-β1 and TRPM-2.

The molecular events associated with the selection of androgen-independent cells may involve alterations in the pattern of expression for apoptosis-related genes including TRPM-2 as observed in the androgen-independent carcinomas. Further studies that involve the assessment of apoptosis and additional apoptosis-related gene activities during progression to androgen independence may establish a common pattern associated with castration-induced selection in prostate cancer. This information may prove useful for the development of molecular markers for recurrent prostate cancer in man and possibly provide insight into additional secondary therapies for this important malignancy.

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