Gene Expression during the Early Phases of Regression of the Androgen-dependent Shionogi Mouse Mammary Carcinoma

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ABSTRACT

The effects of castration on gene expression were measured in the androgen-dependent Shionogi mouse mammary carcinoma. From 0 to 144 h after castration, polyadenylated RNA from the tumors was analyzed by Northern blotting for the expression of genes associated with cell maintenance (β-actin and α-tubulin), cell growth and differentiation (c-fos and c-myc), and cell stress and death (heat shock 70 and TRPM-2). During the first 48–72 h after castration, the tumor continued to increase its mass but thereafter (72–144 h) began to regress. Throughout, the concentration of polyadenylated RNA recovered and the expression of both β-actin and α-tubulin remained relatively constant, implying that in the surviving cells there are no major decreases in RNA synthesis. By comparison, c-myc exhibited a small increase in relative expression during the entire period examined, whereas c-fos displayed a transient peak at 12 h after castration, suggesting that this gene is acutely sensitive to androgen withdrawal. Heat shock protein 70 displayed a pattern similar to that of c-fos; however, the transient rise in the level of heat shock protein 70 expression could be induced by sham operations alone. The concentration of the transcripts encoding TRPM-2 rose only when tumor regression was most evident (72–144 h after castration). Thus, gene expression in the Shionogi carcinoma following castration can be grouped according to those genes which show little or no response, those which are acutely sensitive, and those which show a late effect and are more closely affiliated with cell death.

INTRODUCTION

The Shionogi mouse mammary carcinoma (1) has been studied by many groups (2–5) to delineate the mechanisms by which androgens control cell proliferation in hormone-responsive tumors. For the most part, these investigations have been limited to observations of overall changes in cell activity, such as the ability of cloned cells to grow under defined conditions (4), and of changes in morphology resulting from a drift from androgen dependence to androgen independence (5, 6). While it has been shown that differences exist in the cytoplasmic and nuclear binding components for androgens between dependent and independent variants (6–9), little work has been done describing the molecular and biochemical properties associated with androgen withdrawal and with progression to androgen independence. Previous studies on RNA metabolism in the Shionogi carcinoma have been limited largely to measuring changes in rRNA and total mRNA metabolism resulting from androgen stimulation (3, 10).

In this paper, we report on studies concerning early changes in gene expression resulting from androgen withdrawal and subsequent regression of the Shionogi carcinoma. The polyadenylated mRNA isolated from androgen-dependent tumors growing in noncastrated mice was compared to that obtained from tumors in mice castrated for 12 to 144 h with respect to the expression of mRNA transcripts for genes related to cell maintenance (β-actin and α-tubulin), growth and differentiation (c-fos and c-myc), and cell stress and death (heat shock 70 and TRPM-2). While the cell maintenance genes were expressed at relatively constant levels, there was a transient peak in the amount of poly(A)+ RNA3 coding for both c-fos and hsp 70 within 12 h after castration. By comparison, there was a gradual increase in transcript levels encoding the TRPM-2 gene beginning 72 h after castration.

MATERIALS AND METHODS

Experimental Animals. Following the injection of 2 × 107 androgen-dependent Shionogi carcinoma cells into adult male DDS mice, tumor weight reaches 3–6 g by 15 days. Castrations were performed via the abdominal route while the animals were under ether anesthesia. Details on the maintenance of mice, tumor stocks, and operative procedures are given in earlier publications (9, 11).

Extraction of RNA. Tumors were excised, immediately frozen in liquid N2, and pulverized with a mortar and pestle. The pulverized tissue was suspended in 9 ml (4.5 ml/g tissue) of 50 mM Tris-HCl (pH 7.5), 5 mM guanidium thiocyanate, and 10 mM disodium EDTA, containing 5% (v/v) β-mercaptoethanol, and homogenized at maximum speed with a polytron homogenizer. RNA was extracted using the LiCl procedure essentially as described by Cathala et al. (12). The concentration of RNA was estimated using the formula 1 A260 = 50 μg/ml.

Preparation of 32P-Labeled Probes. The DNA probes used in these experiments were kindly provided by the following individuals: β-actin (PA-1) and α-tubulin (PT-1) (14) from D. Cleveland (John Hopkins University, Baltimore, MD); murine c-myc (third exon) (15) from Frederick Alt (Columbia University, New York, NY); c-fos (16) from T. Curran (Roche Institute of Molecular Biology, Nutley, NJ); mouse heat shock 68 (17) from L. Moran (University of Toronto, Toronto, Ontario, Canada); and TRPM-2 (18) from M. Tenniswood (University of Ottawa, Ottawa, Ontario, Canada). The DNA inserts were purified by electrophoresis and labeled by nick translation (19) using [32P]dCTP and a nick translation kit purchased from Amersham (Amersham Corp., Arlington Heights, IL). The labeled probes were purified using Sephadex G-50 (Pharmacia Fine Chemicals, Montreal, Quebec, Canada) and spin column (Isolab, Akron, OH) chromatography (20) and had specific activities of 5–7 × 106 dpm/μg of DNA.

Electrophoretic and Hybridization Procedures. Samples (5 μg) of poly(A)+ RNA were made to 40 mM MOPS, 10 mM sodium acetate, and 1 mM disodium EDTA (MOPS buffer), containing 2.2 mM denized formaldehyde and 50% (v/v) deionized formamide, and were denatured by heating at 60°C for 15 min and cooling on ice for 5 min. After electrophoresis at 60 V for 2.5 h at 20°C on 1.2% agarose gels with MOPS as running buffer, the RNA was blotted (21) onto Nytran membranes (Schleicher & Schuell, Inc., Keene, NH) and fixed by baking at 80°C for 2 h under vacuum. Prehybridizations were performed with 6 × SSC (15 mM sodium citrate-150 mM NaCl, pH 8.0, 0.1 M phosphate, 0.1% SDS, 5 mM disodium EDTA, 5 × Denhardt’s solution (1 × = 0.02% Ficoll-0.02% polyvinylpyrrolidone-0.02% bovine serum albumin)) and烘热至80°C后于真空下预变性。预杂交溶液为6× SSC (15 mM sodium citrate-150 mM NaCl, pH 8.0, 0.1 M phosphate, 0.1% SDS, 5 mM disodium EDTA). After electrophoresis at 60 V for 2.5 h at 20°C on 1.2% agarose gels with MOPS as running buffer, the RNA was blotted (21) onto Nytran membranes (Schleicher & Schuell, Inc., Keene, NH) and fixed by baking at 80°C for 2 h under vacuum. Prehybridizations were performed with 6 × SSC (15 mM sodium citrate-150 mM NaCl, pH 8.0, 0.1 M phosphate, 0.1% SDS, 5 mM disodium EDTA, 5 × Denhardt’s solution (1 × = 0.02% Ficoll-0.02% polyvinylpyrrolidone-0.02% bovine serum albumin)) and

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3 The abbreviations used are: poly(A)+ RNA, polyadenylated RNA; hsp 70, heat shock protein 70; SDS, sodium dodecyl sulfate; SSC, 15 mM sodium citrate-150 mM NaCl, pH 8.0; MOPS, morpholinopropanesulfonic acid, pH 7.0.
are expressed as the mean ± SE (n > 3).

Effects of Castration on Tumor Weight and Concentration of RNA Recovered. In agreement with previous observations (11), the mass of the androgen-dependent Shionogi tumor continues to increase slightly during the first 72 h following castration but undergoes a significant decrease (t test, P < 0.05) when this interval is extended to 144 h (Table 1). However, even at the longest time period, there is no significant effect of castration on the yield (per g of tissue) of either total RNA or poly(A)+ RNA (Table 1). Thus while there is a measurable decrease in tumor mass by 144 h following castration, the concentration of RNA extracted from the surviving cells remains relatively constant.

When Northern blot analysis is performed using probes for cell maintenance genes such as β-actin and α-tubulin, the pattern seen in Fig. 1 is observed. The expression of the 2.2-kilobase β-actin and 2.1-kilobase α-tubulin transcripts appears relatively uniform in the tumors from the growing and castrated animals (Fig. 1, A and B). Densitometric analysis (Fig. 1C) confirms this impression and indicates that there are only small oscillations in the concentration of these transcripts in the poly(A)+ RNA preparations in relation to the almost constant amount of residual 18S RNA present in these samples. While these changes may reflect continued growth of the tumors during the first 72–96 h after castration, there is no apparent direct response to androgen withdrawal.

Effects of Castration on the Expression of the Protooncogenes c-fos and c-myc. In various types of cells the expression of the protooncogenes c-fos and c-myc is rapidly induced by several mitogens and growth factors (22–26). Accordingly, it was of interest to determine whether removal of a potent growth stimulator, testosterone, would influence the level of expression of these genes. The results in Fig. 2 indicate that both of these protooncogenes are expressed at very low levels in the tumors growing in noncastrated mice. However, within 12 h after castration there is approximately a 10-fold increase in the induction of a 2.6-kilobase c-fos transcript; followed by an almost equally rapid decline (Fig. 2, A and C). This early effect of castration was not observed in sham-operated controls (data not shown). A more gradual increase in the concentration of c-fos transcripts is seen during the period (72–144 h) when a significant regression of the tumor is observed. By comparison, there is a slight yet relatively constant increase in the 2.7-kilobase c-myc transcript following castration (Fig. 2, B and C). Thus in agreement with that recently found for chronic lymphocytic leukemia cells (27), increased expression of c-fos and c-myc is not directly correlated with cell proliferation in the androgen-dependent Shionogi carcinoma.
Effects of Castration on the Expression of Stress- and Cell Death-associated Genes. While the precise molecular functions of heat shock proteins have not been fully elucidated, it is clear that they play an important role in stress physiology (28, 29). Since abnormal proteins result when cells are induced to synthesize heat shock proteins (30), and since the hsp 70 class of these proteins have some proteolytic activity (31), the heat shock proteins may be involved in the destructive processes associated with cell death and tumor regression. To test for this, the poly(A)+ RNA samples were probed with complementary DNA for heat shock protein 68, a member of the hsp 70 family of genes.

The results, shown in Fig. 3A, indicate that the tumors express 2 transcripts homologous to hsp 70, a major one of 2.4 kilobases and a minor one of 3.2 kilobases. The temporal pattern of induction of these transcripts is qualitatively similar in each case. As with c-fos, there is greater than a 10-fold rise in transcript levels within 12 h after castration, followed by a subsequent decline during the next 12 h (Fig. 3C). However, 12 h after sham castration a nearly identical increase in hsp 70 transcripts is observed (data not shown). Thus, the expression of the hsp 70-related genes in the Shionogi carcinoma is probably more closely related to surgical stress than to androgen withdrawal.

Monpetit et al. (32) have reported that the rat prostate contains 2-4 androgen-repressed poly(A)+ RNA sequences that are induced during prostatic involution. One of these, designated TRPM-2 (18), shows a 400-fold increase 4 days after castration, a time when the rate of cell death in this system is maximal (33). To determine whether this gene is also expressed in the regressing Shionogi carcinoma, complementary DNA for TRPM-2 was used to probe the tumor poly(A)+ RNA after castration.

As can be seen in Fig. 3B, a 2.3-kilobase transcript hybridizes with the TRPM-2 probe. Although a trace amount of a larger RNA species (5.8 kilobases) is occasionally observed, the most prominent form (2.3 kilobases) corresponds closely to the size of the transcript seen in the rat prostate (32). While constitutively expressed at a low level in the growing tumors (Fig. 3C), the timing of its increased expression is associated with the period when the tumor mass is decreasing (Table 1). Thus whereas c-fos is rapidly induced by androgen withdrawal, the expression of the TRPM-2 gene is more directly associated with cell death.

DISCUSSION

In the present investigation using the androgen-dependent Shionogi carcinoma as a model system, we measured the early effects of castration on the expression of representatives of three general categories of cellular genes, those related to (a) cell maintenance, (b) cell growth and differentiation, and (c) cell stress and death, as an approach to determine the sequence of molecular events leading to tumor regression. As examples of cell maintenance genes, both β-actin and α-tubulin displayed only relatively minor fluctuations in their expression over the period of 0 to 144 h after castration (Fig. 1). Whether the small, transient decrease in the expression of β-actin and α-tubulin at 48 h is indicative of a switch from growth to regression is uncertain. During the first 48-72 h the mass of the tumor continued to expand, but thereafter tumor regression was observed (Table 1). Thus despite the differences in growth phases, the surviving cells maintain nearly constant levels of these transcripts. This finding supports the notion that tumor regression in the Shionogi carcinoma is not due principally to a decline in cellular renewal and synthetic processes but rather to an active autophagic mechanism which increases the rate of cell death, as has been proposed previously for prostatic involution (34, 35).

The protooncogenes c-fos and c-myc, as representatives of genes involved in growth and differentiation, displayed different patterns of induction in response to castration (Fig. 2). The levels of c-myc transcripts expressed by the tumors, although very low, increased in an almost linear fashion with time following castration. Since the tumor growth does not parallel the expression of this gene, a direct association between cell proliferation and c-myc induction is not evident. An alternative explanation is that c-myc is being expressed in the small number of cells of hormone-resistant clones which are also present in these tumors and the growth of which is not impeded by castration (11). These cells eventually lead to recurrence and regrowth of the tumor in an androgen-depleted environment. It should be possible to test this hypothesis either by measuring the levels of expression of c-myc in recurrent tumors or by in situ analysis of c-myc mRNA expression in regressing tumors.

Compared to c-myc, c-fos has a very different pattern of induction (Fig. 2C). Within 12 h after castration, the expression of c-fos peaks but subsequently undergoes a rapid decline during the next 12-h period. However, between 96 and 144 h, a gradual increase in the expression of this gene is observed. While this secondary rise may, as is proposed for c-myc, be related to clonal expansion, the initial peak at 12 h indicates that the expression of c-fos is acutely affected by androgen withdrawal.

The two genes examined in this system as representative of gene activities related to cell stress and cell death were hsp 70 and TRPM-2 (Fig. 3). The induction of hsp 70 transcripts (Fig. 3C) resembles that measured for c-fos; both show a rapid induction and a rapid falloff in the level of expression. While in the former, the rise may be attributed to the stress associated with surgical trauma as demonstrated in the sham-operated controls, with the latter an induction may occur in response to elevations in intracellular calcium (36, 37). Since changes in intracellular calcium have been shown to precede the death of...
cells in other systems (38), it is reasonable to expect that a similar mechanism is operational during the programmed cell death which follows androgen deprivation.

While transient induction of c-fos is an early, acute response to castration, the TRPM-2 gene(s) may be more directly involved in the mediation of the autophagic mechanism. In the involuting rat prostate, maximum expression of this gene (32) coincides with the time at which the maximal rate of cell death is occurring (33). A similar temporal relationship is observed in the Shionogi carcinoma. During the first 48–72 h after castration, the tumor mass continues to expand (Table 1) and coincides with the time at which the maximal rate of cell death which follows androgen deprivation.

In conclusion, gene expression during the early phases of regression of the Shionogi carcinoma can be grouped on the basis of genes that show: (a) minimal or no response to castration; (b) a very rapid response to castration (c-fos); and (c) a response to cell death (TRPM-2).

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