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Effect of Tumour Progression on the Androgenic Regulation of the Androgen Receptor, TRPM-2 and YPT1 Genes in the Shionogi Carcinoma

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Progression of an androgen-dependent tumour to an androgen-independent state is characterized by the loss of apoptotic potential, a property of cells which have differentiated under the influence of androgens. In an attempt to relate progression to mechanisms of apoptotic failure, we compared the relative levels of expression of androgen receptor and TRPM-2 (clusterin) genes in androgen-dependent and -independent tumours derived from the Shionogi carcinoma. The amount of 10 kb androgen receptor mRNA in androgen-dependent and -independent cells was similar thus showing no relationship to progression. Owing to cross-hybridization of androgen receptor cDNA with non-receptor transcripts, two new androgen-repressed mRNAs (ADS31 and ADS39) were cloned. Each was found to have a 20/21 bp GC-rich region of sequence homology with the androgen receptor, implying selective conservation of a domain whose function is unknown. Sequencing results also revealed that ADS31 cDNA encodes a polypeptide identical to mouse YPT1, a ras-related GTP-binding protein. Expression of the ADS31/YPT1, ADS39 and TRPM-2 genes was sensitive to androgen withdrawal and replacement both in the parent androgen-dependent and the recurrent androgen-independent carcinomas. The uncoupling of TRPM-2 expression and apoptosis observed in androgen-independent tumour cells implies that the function of androgen receptor becomes more restricted with tumour progression. Furthermore, the fact that the expression of ADS31/YPT1 transcript becomes dominant in the advanced stages of androgen-independent growth, suggests that the mechanism of progression is subserved by duplication and possibly redundancy of alternative (signal transduction) pathways mediating tumour cell survival and growth.

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INTRODUCTION

Androgens control the expression of three types of regulatory genes during the growth and differentiation of normal stem cells in the prostate and other androgen-dependent tissues [1]. Initiation of DNA synthesis and cell proliferation (i.e. androgen sensitivity) is an example of positive gene regulation by androgens. Inhibition of these processes in the presence of a rising titre of hormone typifies negative gene regulation by androgens. Withdrawal of androgen induces autophagic lysis (apoptosis) which accounts for androgen-dependence and involves the expression of a number of androgen-repressed genes [1–8]. Both positive and androgen-repressed types of gene regulation are manifested in androgen-dependent tumours; hence optimum androgen withdrawal therapy will have the double effect of arresting the initiation of DNA synthesis and inducing apoptotic cell death [9]. In the absence of androgen replacement, surviving tumourigenic stem cells irreversibly progress to an androgen-independent condition and give rise to a recurrent

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autonomous tumour with a greatly amplified population of androgen-independent stem cells [10]. Since there is no evidence of selective survival of androgen-independent stem cells in regressing tumours, the question arises whether such cells already co-exist with androgen-dependent cells in the original population of cells or whether progression to androgen-independence involves the nascent synthesis of transcripts coding for growth factors that substitute for androgens in preventing or reversing apoptosis in a small fraction of the cell population [10]. The resultant inconsequential role of androgens implies that the androgen receptor ceases to function in the normal way in regulating the expression of genes, especially those involved in apoptosis. In an attempt to relate progression to changes in the functional capacity of androgen receptor, we first compared the amount of receptor mRNA in parent, regressed and recurrent tumours derived from the androgen-dependent Shionogi mouse mammary carcinoma. We then attempted to determine whether androgen-independence develops in association with any changes in the level of expression of TRPM-2 (clusterin), a gene implicated in the apoptotic process [3, 11, 12]. During the course of these experiments, it was found that the androgen receptor cDNA shares partial sequence homology with two previously unrecognized androgen-repressed genes, one of which codes for the ras-related GTP-binding protein, YPT1.

EXPERIMENTAL

Shionogi mammary carcinoma

The Toronto subline [13] of the transplantable SC-115 androgen-dependent mouse mammary carcinoma [14] was used in the following experiments. Approximately 5×10^6 cells of the Shionogi carcinoma were injected s.c. into male DDS strain mice. When the tumours reached a mean weight of 4–5 g, the animals were castrated through an abdominal incision.

Preparation and screening of cDNA libraries

Tumour bearing mice were castrated and 3 days later poly(A^+) RNA was isolated from the regressing cells as described below. This was used in a cDNA cloning system (Amersham Canada Ltd, Oakville, Ontario, Canada) with oligo (dT)-primers to synthesize cDNA which was ligated into phage λgt10 after addition of *Eco*R1 linkers. The library contained 1.4×10^6 original recombinants half of which were amplified once before use. Approximately 200,000 recombinants were screened using ^{32}P-labelled androgen receptor cDNA; 15 positive clones were detected, plaque purified and subcloned into the *Eco*R1 site of pUC19. Of the 4 unique clones found, ADS31 (600 bp) and ADS39 (1.5 kb) were studied further.

Probe preparation

The full length rat androgen receptor cDNA (from E. M. Wilson, University of North Carolina, Chapel Hill, NC, U.S.A.) was digested with *Eco*R1 and *Nae*1 and a 527 bp sequence from the 5′-end subcloned into the *Eco*R1–*Sma*1 site of PTZ vector. This probe was isolated using *Eco*R1 and *Bam*H1 restriction enzyme digest and subsequently used to screen the cDNA library.

Glucocorticoid receptor poly(A^+) RNA was detected with a human glucocorticoid receptor probe (from P. Chambon, Institut de Chimie Biologique, Faculte de Medicine, Strasbourg, France) consisting of a 1.5 kb 5′-fragment of cDNA in the plasmid Bluescribe M13+. The insert was excised with *Eco*R1.

TRPM-2 cDNA, available as a 1.3 kb fragment in pGEM, was excised using *Eco*R1 and *Bam*H1.

Following agarose gel electrophoresis, all DNA inserts were purified by Geneclean (Bio/Can Scientific Inc., Mississauga, Ontario, Canada) and labelled by nick translation [15] using [α-^{32}P]dCTP and a nick translation kit (Amersham). The labelled probes were purified using Sephadex G-50 (Pharmacia Fine Chemicals, Montreal, Quebec, Canada) and spin column (Isolab, Akron, OH, U.S.A.) chromatography [16] and had specific activities of $5–7 \times 10^8$ dpm/mg of DNA.

RNA isolation and Northern blot analysis

The frozen tumours were pulverized with a mortar and pestle yielding a powder which was suspended in 50 mM Tris–HCl buffer (4.5 ml/g tissue), pH 7.5 containing 5 M guanidium thiocynate, 10 mM Na_2EDTA and 5% β-mercaptoethanol and then homogenized with a polytron homogenizer. RNA was extracted using the LiCl method [17], dissolved in 20 mM Tris–HCl (pH 7.5), containing 0.1% sodium dodecyl sulphate (SDS), 5 mM Na_2EDTA, and 0.5 M NaCl. The RNA was subjected to repeated oligo(dT)-cellulose (Boehringer Mannheim GmbH, Mannheim, Germany) chromatography for isolation of poly(A^+) RNA [18]. 10 mg of poly(A^+) RNA were heat denatured (incubated at 60°C for 15 min and cooled on ice for 5 min) in MOPS buffer [40 mM 3-(*N*-morpholino)-propanesulphonic acid, pH 7.0, 10 mM sodium acetate, 1 mM Na_2EDTA], containing 2.2 M deionized formaldehyde and 50% deionized formamide, and applied to 1.2% agarose, 2.2 M formaldehyde gels made in MOPS buffer. After electrophoresis at 60 V for 2.5 h, the RNA was blotted onto Nytran membranes (Schleicher and Schuell Inc., Keene, NH, U.S.A.) and fixed by baking under vacuum at 80°C for 2 h [19]. Prehybridizations of blots were carried out with 6 × SSC (15 mM sodium citrate, 150 mM NaCl, pH 8.0), 10 mM phosphate buffer, 0.1% SDS, 5 mM Na_2EDTA, 5 × Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidine, 0.1% bovine serum albumin), 50% deionized formamide, and 200 mg/ml denatured salmon testis DNA

for 4 h at 42°C. After the addition of heat denatured ^{32}P-labelled probe, hybridizations were performed at 42°C for 18 h. The blots were washed twice at 20°C for 15 min with 2 × SSC, 0.1% SDS and twice at 60°C for 30 min with 0.1 × SSC, 0.1% SDS. When ^{32}P-labelled probe for steroid receptor was used, the second cycle of washes was performed at 50°C. For repeat Northern blot analysis, filters were stripped in 6 × SSC and 50% deionized formamide at 65°C for 30 min.

Autoradiography was carried out by exposing the blots to Kodak/k XAR-5 film (Eastman Kodak, Rochester, NY, U.S.A.) with intensifier screens at −70°C. Quantitation of the relative expression of various transcripts was determined relative to 18S RNA by densitometry [6]. All values were standardized relative to the expression of various transcripts in the parent tumour by arbitrarily setting the value for parent tumour as 1 unit.

DNA sequencing

For DNA sequencing of ADS31 and ADS39, the dideoxy-termination method [20] was used with the Sequenase Version 2.0 kit (United States Biochemical Corporation, Cleveland, OH, U.S.A.) with 5′[α-^{35}S]dATP-sequencing grade for Sequenase (New England Nuclear-Dupont Canada Inc., Mississauga, Ontario, Canada). For DNA sequencing of the androgen receptor, the dsDNA Cycle Sequencing kit (Gibco-BRL, Gaithersburg, MD, U.S.A.) was used to end label a primer with [γ-^{32}P]dATP (> 5000 Ci/mmol) (Amersham). Thirty cycles of denaturing, primer annealing, and extension with deoxy/dideoxy oligonucleotides were performed using a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT, U.S.A.) [21]. Electrophoresis was performed on a 21 × 40 cm Sequi-Gen Cell sequencing apparatus (Bio-Rad Labs Ltd, Mississauga, Ontario, Canada) at 40 watts constant power per gel, on 8 and 6% polyacrylamide-8.3 M urea gels, in 89 mM Tris–borate buffer, pH 8.3, containing 2 mM EDTA. Gels were dried and used to expose Kodak X-OMAT XRP-1 film (MedTec Marketing, Burnaby, British Columbia, Canada).

RESULTS

Progression of the Shionogi carcinoma

The characteristics of progression are well demonstrated by the Shionogi carcinoma as shown by the results in Fig. 1. After the initial implant into a male host animal, the parent tumour becomes palpable after an interval of about 10 days. Following castration of the host, the tumour continues to grow for 1–2 days before the onset of apoptosis is evident with the appearance of autophagic vacuoles and apoptotic bodies [22]. Over the next 6–7 days the tumour regresses rapidly and in some mice becomes non-palpable. After 40–50 days recurrent growth begins and gradually accelerates. If the recurrent tumour is subsequently transplanted into a non-castrated male animal, it also becomes palpable after about 10 days and grows with a doubling time similar to that of the parent tumour. However, castration fails to induce apoptosis or alter the growth rate (Fig. 1); demonstrating that the tumour is androgen-independent. In the following experiments, the androgen regulation of several genes in the parent androgen-dependent and recurrent androgen-independent tumours was compared to determine whether progression was associated with any distinct changes in the expression of genes linked to the programming of cell death.

Expression of androgen and glucocorticoid receptor genes in parent, regressed and recurrent tumours

The fact that androgen withdrawal has no effect on the growth rate of the recurrent Shionogi carcinoma (Fig. 1), confirms that the tumour has progressed to an androgen-independent state. The basis for loss of apoptotic potential may be linked to the androgen receptor in one of two ways. First, since apoptotic potential is induced by androgens via the androgen receptor during cell differentiation, a reduced amount of functional receptor in the recurrent tumour would eliminate any possibility of expressing this mechanism. Alternatively, a mutational change in the androgen receptor might alter its binding characteristics such that androgen-repression of apoptosis by the receptor mechanism is irreversible. However, when the

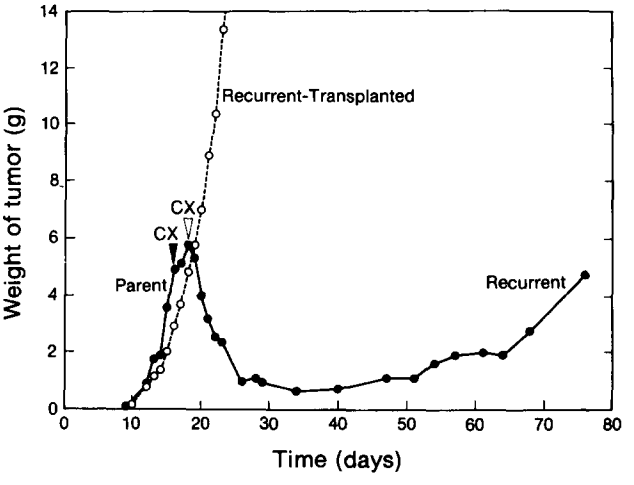

Fig. 1. Progression of androgen-dependent Shionogi carcinoma cells. About 5 × 10^6 cells of the parent Shionogi carcinoma were injected s.c. into male DDS strain mice. A similar number of recurrent tumour cells were also injected s.c. into male mice. When the tumours reached a mean weight of about 5 g the animals were castrated (CX). Each animal had only one tumour mass, and tumour diameters were measured in mm with calipers. The formula (length × width^2)/2 = mass (mg) was used to estimate the mean weight of the tumours. Failure of the recurrent-transplanted tumour to regress after castration indicates that the parent tumour cells have progressed to an androgen-independent state. Each point represents the mean from 7 to 10 tumours.

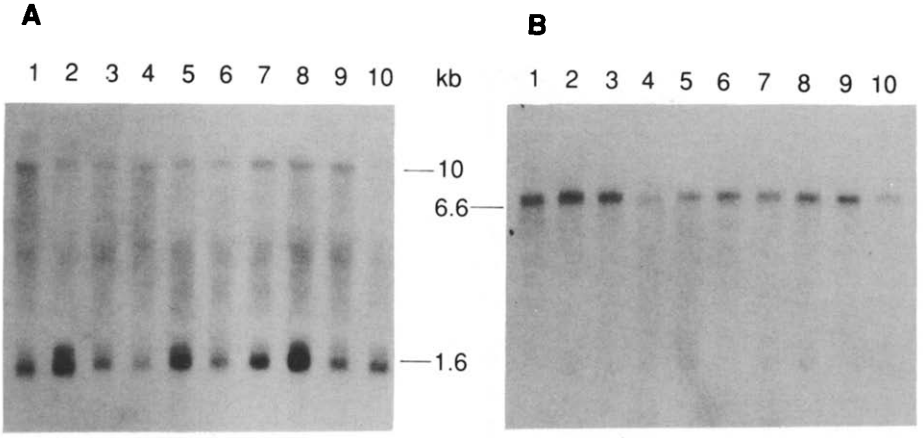

Fig. 2. Expression of androgen and glucocorticoid receptor genes in parent, regressed and recurrent tumours. Poly(A$^+$) RNAs from parent, regressed and recurrent tumours were purified, separated by gel electrophoresis and transferred to Nytran membrane by blotting. The blots were hybridized with ^{32}P-labelled cDNA probe. Northern analysis was performed using a 527 bp 5′-fragment of rat androgen receptor cDNA, and a 1.5 kb 5′-fragment of the human glucocorticoid receptor. Northern analysis with androgen receptor cDNA (A) and glucocorticoid receptor cDNA (B). Poly(A$^+$) RNA (10 mg/lane) from parent tumour before castration (lane 1), parent tumour 6 days after castration (lane 2), recurrent tumour (lane 3), recurrent transplanted tumour before castration (lane 4) and 6 h (lane 5), 12 h (lane 6), 1 day (lane 7), 2 days (lane 8), 4 days (lane 9) and 6 days (lane 10) after castration.

sequences of the androgen receptor cDNA from parent and recurrent tumours were compared (data not shown) no difference from the wild-type sequence [23] was found; in agreement with the finding of Furuya *et al.* [24]. Since the recurrent tumour cells have low levels of nuclear androgen receptor as measured by dihydrotestosterone-binding [10, 22], we looked for evidence of reduced expression of androgen-receptor transcript. Accordingly, poly(A$^+$) RNA was isolated from the Shionogi carcinoma at various stages of progression and analyzed by Northern hybridization using a 527 bp sequence from the 5′-end of rat androgen receptor cDNA. The results are shown in Fig. 2(A). A 10 kb transcript representing full-length androgen receptor mRNA is observed in the parent tumour both before castration (lane 1) and 6 days after castration (lane 2), the recurrent tumour (lane 3), the recurrent transplanted tumour before castration (lane 4) and at several time points after castration (lanes 5–10). The slightly greater intensity of the 10 kb band in the recurrent tumours in lanes 7, 8 and 9 suggests that up-regulation of androgen receptor mRNA takes place in the recurrent transplanted tumour between 1 and 4 days after castration. A second smaller transcript of 1.6 kb is also detected by Northern blot analysis with the 5′-527 bp hybridization probe. The expression of the 1.6 kb mRNA increases in the parent tumour after castration (lane 2), and also in the recurrent transplanted tumour at 6 h (lane 5), 1 day (lane 7) and 2 days (lane 8) after castration. Hybridization with full-length androgen receptor cDNA gives rise to virtually identical results; in contrast, a cDNA probe consisting of an 800 bp 3′-sequence hybridizes only with the 10 kb mRNA (data not shown). This observation indicates that the 1.6 kb fragment shares homology with the 5′-region of the androgen receptor.

The growth of the Shionogi carcinoma is stimulated by pharmacological doses of glucocorticoids but this is not accompanied by an associated induction of apoptotic potential [25]. Thus, it was of interest to compare the expression of the glucocorticoid receptor gene to that of androgen receptor during tumour progression. As shown in Fig. 2(B), a 6.6 kb transcript representing the glucocorticoid receptor is expressed in parent and recurrent tumours (lanes 1–3); in the recurrent transplanted tumour, the expression, initially low (lane 4), increases after castration (lanes 5–9) and is low again on the 6th day (lane 10). No band corresponding to 1.6 kb mRNA is observed.

The foregoing results indicate that despite progression to an androgen-independent condition, the Shionogi carcinoma continues to express transcripts of the expected size for both the androgen and glucocorticoid receptors.

Cloning of the androgen-related 1.6 kb transcript and relative levels of expression

In order to determine if the up-regulated 1.6 kb mRNA (Fig. 2) represented expression of a truncated form of the androgen receptor, a cDNA library was constructed using poly(A$^+$) RNA from regressing tumours. The library was then screened with hybridization probe consisting of the 527 bp 5′-sequence of androgen receptor cDNA. Of the 15 unique clones selected, 2 were subcloned and used to probe poly(A$^+$) RNA from parent, regressed and recurrent tumours. Levels of expression were compared to those of TRPM-2 and 18S rRNA giving rise to the results shown in Fig. 3. The first of the two subclones,

ADS31, detects 2 transcripts [Fig. 3(A)], one corresponding to a size of 1.6 kb and the other, 3.2 kb. Both are detected at low levels in the parent tumour (lane 1) and on the basis of comparative densitometry [Fig. 4(A)], increase approx. 2- to 3-fold over the next 4 days after castration (lanes 2–5) relative to the amount of 18S RNA in the samples [Fig. 3(D)]. By day 6 (lane 6), the relative amounts of both transcripts decline to about 2-fold the parent tumour levels. In the recurrent tumour (lane 7), little change from the level in the parent tumour is detected.

When ADS39 cDNA is used as hybridization probe [Fig. 3(B)], a transcript of 2.0 kb is detected and follows a time course of expression similar to that of the 3.2 kb mRNA detected by the ADS31 cDNA. As measured by relative densitometry units [Fig. 4(B)], expression is low in the parent tumour (lane 1) and then increases approx. 2-fold during days 1 to 4 following castration (lanes 2–5). By the sixth day (lane 6), the expression is only slightly above (1.3-fold) the parent level. The level of expression observed in the recurrent tumour (lane 7) is similar in magnitude to that seen in the parent.

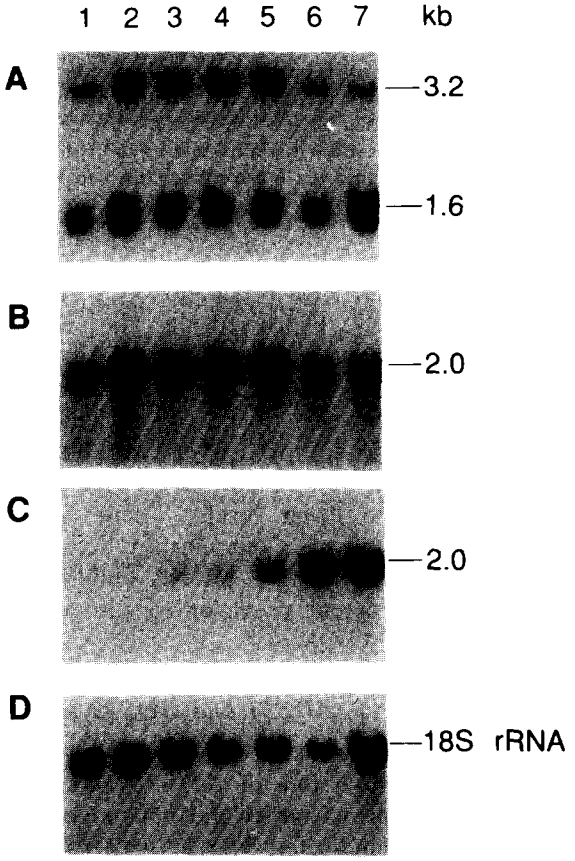

Fig. 3. Expression of ADS31, ADS39 and TRPM-2 genes in parent, regressed and recurrent tumours. ADS31 and ADS39 cDNAs isolated by screening of a Shionogi carcinoma cDNA library were subcloned and subsequently used as probes for Northern blot analysis of poly(A$^+$) RNA from parent, regressed and recurrent tumours. Similar hybridizations were carried out with a 1.3 kb fragment of TRPM-2 cDNA. DNA inserts were purified and labelled with [^{32}P]dCTP prior to Northern blotting and autoradiography. Northern analysis with ADS31 (A), ADS39 (B), TRPM-2 (C) cDNAs and 18S rRNA (D). Poly(A$^+$) RNA (10 mg/lane) from parent tumour before (lane 1), 1 day (lane 2), 2 days (lane 3), 3 days (lane 4), 4 days (lane 5), 6 days (lane 6) after castration; and from recurrent tumour before transplantation (lane 7).

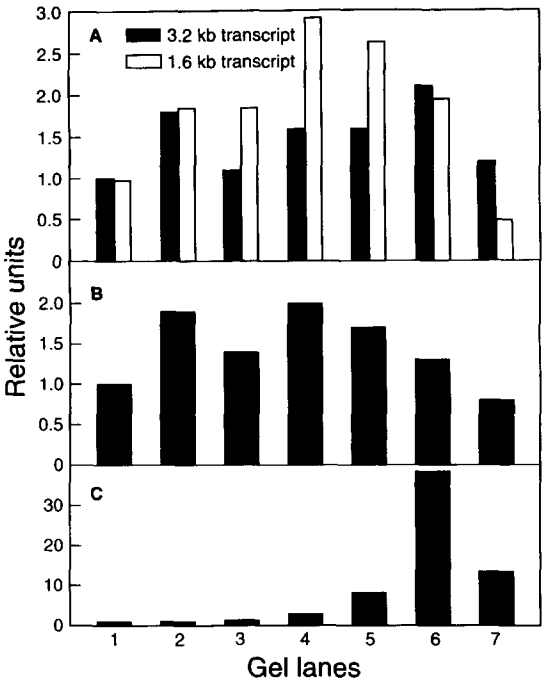

Fig. 4. Quantitation of relative expression of ADS31, ADS39 and TRPM-2 genes in parent, regressed and recurrent tumours. Quantitation of the transcripts in the Northern blots shown in Fig. 3 was determined relative to 18S RNA by densitometry. The values were standardized by setting the value for the parent tumour to 1.

Activity of TRPM-2, an androgen-repressed gene closely associated with programmed cell death, is characterized by very different kinetics as shown in Figs 3(C) and 4(C). Detected as a 2.0 kb band, TRPM-2 mRNA is present at a very low level in the parent tumour (lane 1) which increases gradually after castration to a 38-fold greater level at 6 days (lane 6). Unexpectedly, a high level of expression (14-fold) is observed in the recurrent tumour (lane 7).

Androgen regulation of ADS31, ADS39 and TRPM-2 expression in recurrent transplanted tumours

Since ADS31, ADS39 and TRPM-2 transcripts were up-regulated after androgen withdrawal and continued to be expressed in recurrent tumours, further passage of the recurrent tumour into an intact male host was performed to determine whether any residual regulation by hormones could be demonstrated in an otherwise androgen-independent tumour. As shown in Figs 5(A) and 6(A), ADS31 cDNA again detects transcripts having a size of 1.6 and 3.2 kb. As found before [Fig. 3(A), lane 7], both transcripts are observed in the recurrent tumour (lane 1). Down-regulation occurs in the 1.6 kb transcript when the recurrent tumour is

transplanted into a non-castrated male animal (lane 2). By 6 h after castration, there is a marked elevation of both the 1.6 and 3.2 kb mRNAs (lane 3) which persists through the 12 h, 1 day, 2 day and 4 day postcastration intervals (lanes 4–7).

ADS39 cDNA detects a 2.0 kb transcript [Figs 5(B) and 6(B)], the expression of which shows a time course similar to that obtained with the ADS31 hybridization probe [Fig. 6(A)]. Expression of the ADS39 transcript is low in the recurrent tumour (lane 1), further decreases when the recurrent tumour is transplanted into an intact male animal (lane 2), but recovers to a much higher level of expression from 6 h after castration onwards (lanes 3–7); with a slight decrease seen 6 days after castration (lane 8).

The pattern of expression of TRPM-2 mRNA contrasts with the ADS31 and ADS39 profiles as described above. As shown in Figs 5(C) and 6(C), TRPM-2 mRNA is strongly expressed in the recurrent tumour before transplantation (lane 1) and is sharply reduced after the recurrent tumour is transplanted into a non-castrated male (lane 2). The 2.0 kb transcript increases at 6 and 12 h after castration (lanes 3 and 4) but on subsequent days 1 through 6 (lanes 5–8) falls to lower levels. There is a tendency towards an inverse relationship between the levels of expression of ADS mRNA and TRPM-2 mRNA in the recurrent transplanted tumours after castration.

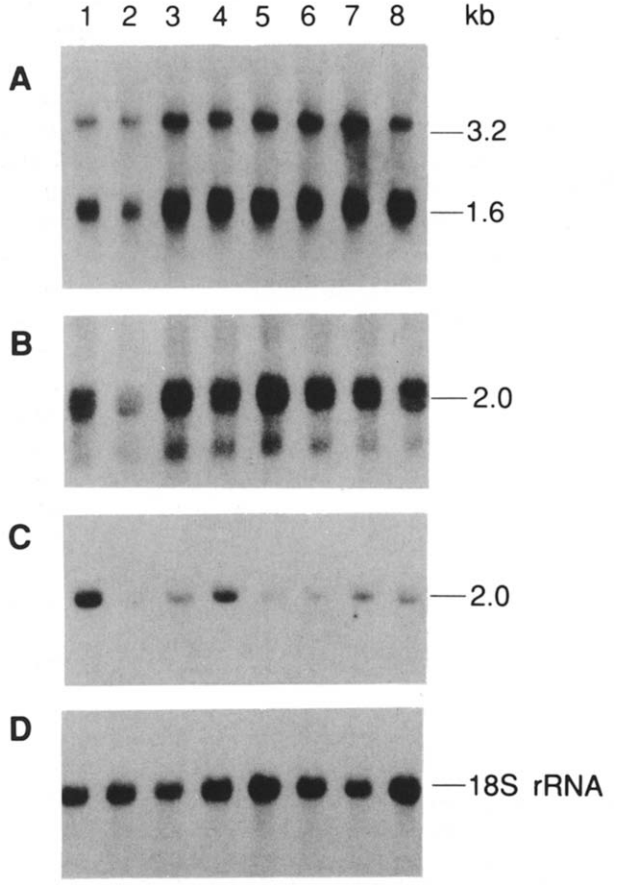

Fig. 5. Expression of ADS31, ADS39 and TRPM-2 genes in recurrent, transplanted tumours. Recurrent tumour (Fig. 1) was transplanted into non-castrated male mice and allowed to grow to a size of 3–4 g. The mice were castrated and the tumours harvested at various times after castration. mRNA was isolated and analyzed by hybridization using [^{32}P]dCTP labelled ADS31, ADS39 and TRPM-2 cDNA probes. Northern analysis using ADS31 (A), ADS39 (B), TRPM-2 (C) cDNAs and 18S rRNA (D). Poly(A$^+$) RNA (10 mg/lane) from recurrent tumour before transplantation (lane 1), recurrent transplanted tumour before castration (lane 2), and 6 h (lane 3), 12 h (lane 4), 1 day (lane 5), 2 days (lane 6), 4 days (lane 7) and 6 days (lane 8) after castration.

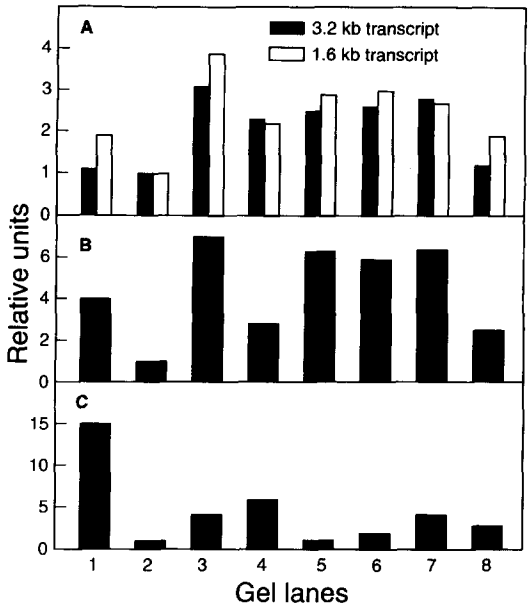

Fig. 6. Quantitation of relative expression of ADS31, ADS39 and TRPM-2 genes in recurrent, transplanted tumours. Quantitation of the transcripts in the Northern blots shown in Fig. 5 was determined relative to 18S RNA by densitometry. The values were standardized by setting the value for the transplanted tumour before castration to 1.

Relative expression of ADS31 and ADS39 in normal tissues

The relative expression of ADS31 and ADS39 were studied in normal target and non-target tissues for androgens. As indicated by relative densitometry units, which are corrected for loading (18S RNA) and expressed relative to the content in parent tumour on the same blot, both ADS31 and ADS39 transcripts are present in very low levels in mouse liver but are highly expressed in the kidney (Fig. 7). In the rat prostate, ADS31 mRNA decreases after castration in contrast to ADS39 transcript which increases. The somewhat different levels of expression of ADS39 in liver and prostate from non-castrated animals and the inverse effects of androgen withdrawal on the levels of expression of ADS31 and ADS39 suggest that the two genes are differentially regulated.

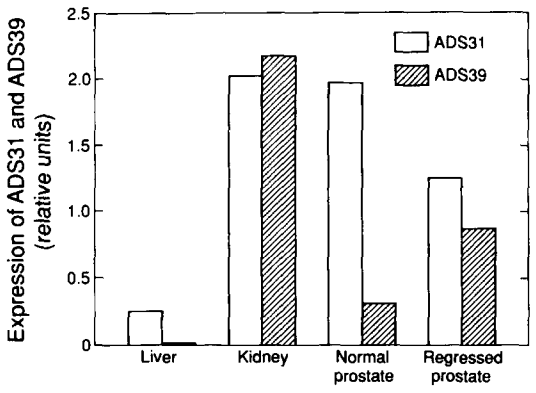

Fig. 7. Relative expression of ADS31 and ADS39 in normal tissues. Poly(A$^+$) RNA was isolated from mouse liver and kidney and rat prostate before and 3 days after castration. Expression of ADS31 and ADS39 was examined by Northern hybridization analysis as described in the legend to Fig. 2. Quantitation of relative expression of transcript was determined by densitometry.

cDNA sequences of ADS31 and ADS39

Sequencing of the 5′-region of the cloned ADS31 cDNA revealed complete sequence homology with the ras-related mouse YPT1 gene [26] between nucleotides −57 and +568. On this basis it was concluded that ADS31 and YPT1 are identical genes.

Partial sequencing of cloned ADS39 cDNA yielded the results shown in Fig. 8. No homologies with sequences in the GENBANK or EMBL Databases were found.

Homologous sequences in mouse androgen receptor cDNA, ADS cDNA and YPT1 cDNA

In comparing the sequences of androgen receptor cDNA and ADS31 cDNA, a 15-bp segment of the 5′-region extending from nucleotides +188 to +202 is completely homologous with a 5′-untranslated section of ADS31 cDNA, from nucleotides −30 to −16 [Fig. 9(A)]. After a 1-bp mismatch, there is a further 5-bp homology between mouse androgen receptor cDNA (+204 to +208) and ADS31 cDNA (−14 to −10). These regions of homology explain why the 527 bp sequence from the 5′-end of androgen receptor cDNA hybridizes with ADS31 mRNA [Fig. 2(A)]. Whereas ADS31 and YPT1 cDNAs are completely homologous, ADS39 [Fig. 9(B)] contains an 18-bp GC-rich segment which is homologous to nucleotides −27 to −10 in YPT1 cDNA and to the sequence in androgen receptor DNA comprised of nucleotides +188 to +208. The glucocorticoid receptor cDNA is devoid of homologous sequence and therefore does not hybridize with ADS transcripts.

DISCUSSION

The principal effect of tumour progression is the loss of apoptotic potential which is clearly demonstrated by the difference in the responses of parent and recurrent forms of the Shionogi carcinoma to androgen withdrawal (Fig. 1). Since apoptosis may be considered an androgen-induced marker of stem cell differentiation, the potential to express apoptosis is not apparent in the recurrent Shionogi carcinoma owing to the prior depletion of androgens and the resultant altered differentiation of surviving stem cells. Once the tumour has progressed to the stage of recurrent growth, further transplantation of the tumour into an intact male host (i.e. androgen replacement) is no longer effective in re-inducing apoptotic potential (Fig. 1). In previous studies on the basis of androgen independence of the Shionogi carcinoma, we found that recurrent tumour cells only had low levels of nuclear androgen receptor [10, 22]. Furthermore, in contrast to the effect of androgen replacement on the androgen-depleted parent tumour, similar treatment had no effect on restoring the nuclear androgen receptor levels in the recurrent malignancy. Thus, the finding of persistent expression of the wild-type 10 kb mRNA in the recurrent and recurrent transplanted tumours [Fig. 2(A)] was unexpected and in the face of a normal DNA

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5′...CGTACAGGTTCCTAGTATTCCCCAGCCTGGGGAGGAGCCTTCAGTCAGCCCTGGATGACA
ACCCAAAGCATGTGGTATCAGAGAGATGTGTGCTTCAGGTGGCCTGCAGGCTGCTGGATGCTCTG
GAGTATCTCCATGAAAATGAGTATGTTCACGGGAACCTGACAGCTGAGAATGTCTTTGTGAATCC
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GACCTGCACAAGGGATGCGGACCCTCCCGCCGCAGCGATCTCCAGACCTTGGGCTACTGTATGCT
CAAGTGGCTTTATGGGTCCCTGCCATGGACAAATTGCCTTCCCAACACCGAAAAGATAACTAGGC
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CACCCTATGACCCTCTGGACCTCCAGATGGTGCCTTAGATGGAATCCAGAGCTTCCGACTTGCAG
CTTGAAGTAGAACATGAAGTAGTGTGACTGGAGGCCTGTTTGAACTCATAGCTCCTAAAAGAATC
CCTTGAATGTGCATTCTCACCGCTCCCTTAGGACATATGAATCAGCACTTGTGTTGGGGAACCTG
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GGATGCTGGCGGCGGCGGCGGCAGCAGCCACTCCACTCCCTATGGCATTTCTGTGATGGCATAAT
AAACTGTTTTTTAATCAAAAAAAAAAAAAAA
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Fig. 8. Partial nucleotide sequence of ADS39 cDNA. cDNA sequence homologous to androgen receptor is underlined.

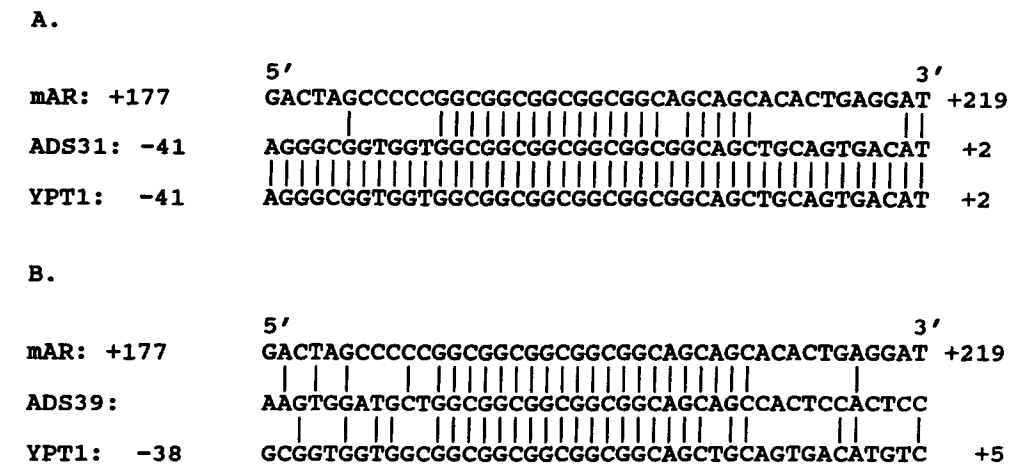

Fig. 9. Homologous nucleotide sequences in mouse androgen receptor. YPT1, ADS31 (A) and ADS39 (B) cDNAs.

sequence, implies uncoupling of gene transcription and synthesis of androgen receptor protein. It remains possible that the glucocorticoid receptor assumes some of the functions of the androgen receptor since the synthesis of glucocorticoid receptor transcript is relatively constant in parent, regressed and recurrent Shionogi carcinoma cells [Fig. 2(B)]. However, the glucocorticoid sensitivity of the recurrent tumour has not been studied extensively and it is not known whether glucocorticoid binding is affected by progression.

TRPM-2 (clusterin) is perhaps the gene most closely identified with apoptotic cell death [27]. A marked increase in the level of expression of TRPM-2 mRNA occurs in the parent androgen-dependent Shionogi carcinoma after castration [Figs 3(C) and 4(C)]. Although it was originally inferred that the TRPM-2 gene product was a cell-death promoting factor, it was subsequently found that the TRPM-2 gene encodes a polypeptide which is almost identical to sulphated glycoprotein 2 (clusterin), a Sertoli cell-derived protein that elicits aggregation of erythrocytes and Sertoli cells *in vitro* [28, 29]. The encoded amino acid sequence of the TRPM-2 gene shares significant homology with plasma complement cytolysis inhibitor, a factor which may restrict cytolytic attack on target cell membranes [30]. Our finding of a very high level of expression of TRPM-2 transcript in the recurrent tumour [Figs 3(C) and 4(C), lane 7] is more consistent with TRPM-2 functioning as a survival mechanism rather than a trigger of autophagic regression of tumour cells. The expression of TRPM-2 was markedly down-regulated in the recurrent tumour after transplantation into an intact male host [Figs 5(C) and 6(C), lane 2] but increased again transiently following castration of the host animal [Figs 5(C) and 6(C), lanes 3 and 4]. These observations demonstrate that the TRPM-2 gene remains sensitive to changes in the androgen environment of the tumour despite progression to an overall androgen-independent condition. How androgen-repression is achieved in the absence of functional androgen receptor is not clear. In view of the relatively abundant level of mRNA for androgen receptor in the recurrent tumour [Fig. 2(A)] it remains possible that very low levels of androgen receptor are synthesized in tumour cells but the amounts are below the level of detection.

Northern blot analysis of tumour mRNA with a 527 bp 5′-fragment of androgen receptor cDNA unexpectedly revealed a 1.6 kb transcript in addition to the 10 kb full-length copy of androgen receptor mRNA [Fig. 2(A)]. The 1.6 kb mRNA was up-regulated by androgen withdrawal lending support to the tentative conclusion that it might be a truncated form of the native androgen receptor. However, subsequent isolation and sequence analysis of cloned 1.6 kb cDNA indicated that the encoded polypeptide was not related to the androgen receptor; rather, the ADS31 clone was derived from the mouse YPT1 gene which codes for a ras-related GTP-binding protein [26]. Similar characterization of a second cDNA clone, ADS39, revealed no similarity with the YPT1 gene. However, it was found that ADS31/YPT1, ADS39 and mouse androgen receptor cDNAs all share a common 20 or 21 bp GC-rich region of homology which accounts for the cross-hybridization results observed in Fig. 2. Whether this homology is of any functional significance is unknown. The ADS31/YPT1 1.6 kb and 3.2 kb [Fig. 3(A)] transcripts which are found in the Shionogi carcinoma are identical to the forms of YPT1 mRNA observed in a variety of normal mouse tissues [26]. The androgen-repressed state of the YPT1 gene has not been reported previously but it is evident from the data presented in Figs 4(A) and 6(A) that the level of expression of this gene in the Shionogi carcinoma may increase after androgen withdrawal. Confirming this finding, the 1.6 kb transcript of the YPT1 gene is down-regulated in recurrent tumour cells transplanted

into a non-castrated male animal [Figs 5(A) and 6(A)]. Sustained rebound to the higher level of expression is observed after androgen withdrawal [Figs 5(A) and 6(A), lanes 3–7]. As with regulation of TRPM-2 (clusterin) gene expression, whether there is sufficient androgen receptor to account for androgenic regulation of the YPT1 gene in androgen-independent tumour cells is unknown. Owing to GTP-binding ability, YPT1 encoded protein may be part of a signal transduction pathway [31]; thus, the augmented expression of YPT1 in the late stages of tumour progression is in keeping with regulatory dominance of alternative growth factors with survival enhancing properties [32]. One such candidate is basic fibroblast growth factor-like peptide which is synthesized by androgen-independent Shionogi carcinoma cells in tissue culture [33]. This growth factor may also play a role *in vivo* since both the parent and recurrent tumours described in Fig. 1 contain abundant transcript for FGF receptor (K. Akakura, unpublished data).

In summary, our results indicate that tumour progression results in a complete loss of apoptotic potential despite persistence of the expression of androgen receptor mRNA. Androgen-independence of tumour growth is not paralleled by resistance to androgen regulation at the genetic level. The expression of both TRPM-2 and YPT1 genes is repressed by androgens in both androgen-dependent parent and androgen-independent recurrent Shionogi carcinoma cells and up-regulation occurs in both cell types after castration. The presence of abundant YPT1 transcript in androgen-independent recurrent tumours is in keeping with the concept that alternative signal-transduction pathways activated by autocrine or paracrine growth factors enhance the survival of androgen-depleted cells.

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