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Control of Tumor Progression by Maintenance of Apoptosis

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ABSTRACT: The ability to induce multiple apoptotic regressions of an androgen-dependent tumor cell population by repeated cycles of androgen withdrawal and replacement may be advantageous in therapeutic strategies aimed at delaying or preventing tumor progression. With greater insight into factors that either initiate or limit apoptosis, more efficient application of intermittent therapy might be achieved, especially if methods could be devised to increase the length or number of treatment cycles. Both calreticulin and clusterin represent proteins with a potential role in the regulation of apoptosis. Calreticulin may inhibit target gene transcription by interacting with steroid hormone receptors, thereby masking their DNA-binding sites and triggering the onset of the apoptotic process. Clusterin, on the other hand, is a membrane-stabilizing protein that appears to be involved in limiting the autophagic lysis of epithelial cells during apoptosis. Also, the increasing tendency for nuclear localization of clusterin after androgen withdrawal may preserve the nuclear environment, limiting the lethal effect of treatment. Thus, tumor progression, characterized by the loss of apoptotic potential, appears to be linked in part to the inappropriate activation of the *TRPM-2* gene, which accounts for the constitutive expression of clusterin.

KEY WORDS: **prostate cancer, androgen deprivation, apoptosis, calreticulin, clusterin**

INTRODUCTION

The use of androgen ablation as a means of shrinking prostate tumor mass did not begin until the late nineteenth century. In 1895 William White compiled case records on 111 men who had been treated for senile enlargement of the prostate by double castration, a time when the chemical factor elaborated by the testis was still a matter of speculation [1]. Rapid atrophy of prostatic enlargement was reported in 87% of the cases with a mortality rate of 18%. The procedure was sometimes associated with psychic disturbances and shock out of proportion to the extent of mutilation [2]. Interest in therapeutic applications of castration waned over the next 40 years, until David and colleagues, in 1935, isolated a potent androgenic compound from fresh testicular tissue, which they named testosterone. This and other developments directed attention again to potential use of castration as a therapeutic modality. Radio-orchiectomy for the treatment of metastatic prostate cancer was first described by Keys and Ferguson in 1936 and surgical orchiectomy by Huggins and Hodges in 1941. The terms *autophagic lysis* and *apoptosis* were adopted in the 1970s to describe the mechanism of active cell death in the prostate and prostate cancer in response to castration [3,4].

ANDROGEN DEPENDENCE AND INDEPENDENCE

Normal conditions for prostatic growth are established by three levels of androgen-mediated regula-

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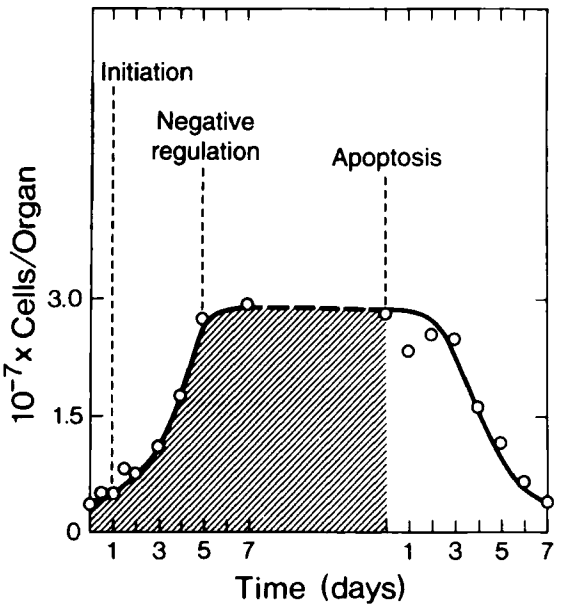

Fig. 1. Androgenic regulation of the prostate: three essential mechanisms: (1) initiation of DNA synthesis and cell proliferation by androgens; (2) negative regulation; and (3) apoptosis (autophagic lysis). Modified from Bruchovsky et al. [5].

tion (Fig. 1): first, positive effects on initiation of DNA synthesis and cell proliferation; second, negative or inhibitory effects that limit the number of cells in the prostate; and third, apoptosis, a form of controlled cell death that occurs in the prostate when androgens are withdrawn. Androgen dependence is the clinical manifestation of apoptosis after androgen withdrawal in both normal and malignant tissues [5]. In the early stages of prostate cancer, only the form of androgen-mediated regulation that limits the number of cells in the prostate is missing [5]. Because the other two mechanisms are still functional, androgen ablation has the double effect of triggering apoptosis and inhibiting DNA synthesis and cell proliferation. Even in malignancy, the ability to undergo apoptosis is acquired as a feature of differentiation under the influence of androgens. Therefore, in the absence of androgens, it is impossible for dividing cells to differentiate and become pre-apoptotic again [5,6]. This explains why recurrent tumor growth is characterized by androgen independence. In attempting to avert or delay progression to the androgen-independent state, it has been hypothesized that if malignant cells that survive androgen withdrawal are forced into a normal pathway of differentiation by androgen replacement [6] (Fig. 2), apoptotic potential might be restored, hence, setting the stage for another response to androgen withdrawal.

Under experimental conditions, apoptosis indeed can be induced multiple times in a tumor cell population by repeated cycles of androgen withdrawal and replacement [6]. The results of preliminary clinical studies suggest that the repetitive induction of apoptosis is also achievable in prostate cancer with periodic interruption of androgen blockade therapy [7].

THERAPEUTIC MAINTENANCE OF ANDROGEN DEPENDENCE

With the introduction of anti-androgens such as cyproterone acetate and flutamide, and luteinizing hormone-releasing hormone (LHRH) agonists such as leuprolide acetate and goserelin acetate, new methods of androgen suppression have become available. These mimic the effects of orchiectomy by lowering the intranuclear concentration of dihydrotestosterone by 80% or more. Emphasis has been placed on the combined use of such agents [8], with little attention given to their reversibility of action, the significance of which is far-reaching. The potential for a full recovery from therapy makes it possible to alternate a patient between periods of treatment and no treatment. When the patient is off treatment, the function of the testes and the concentration of serum testosterone return to normal slowly over a period of 8–14 weeks [6,7]. In response to this incremental androgenic stimulus, atrophic cells are recruited into a normal pathway of differentiation, where the risk of progression is small. With the associated movement through the division cycle, the cells become pre-apoptotic again, making it possible to repeat therapy (Fig. 3).

INDUCTION OF MULTIPLE APOPTOTIC RESPONSES

Experimental

The idea of inducing more than one regression of androgen-dependent malignancy arose from the observation that the involution of prostate brought on by castration is an active process involving the rapid elimination of large number of epithelial cells [4,9]. It was first postulated that the replacement of androgens even in small amounts would have a conditioning effect on surviving cells, allowing them to conserve or regain desirable traits of differentiation [9]. In the case of tumors, several lines of evidence obtained by Foulds [10], Noble [11], and others [9] implied that long periods of hormone deprivation simply accelerated progression toward autonomous growth and thus were better avoided [9]. Although no practical method of countering progression by hormonal means emerged from this work, the subsequent demonstration that consecutive episodes of testosterone-induced regeneration of involuted pros-

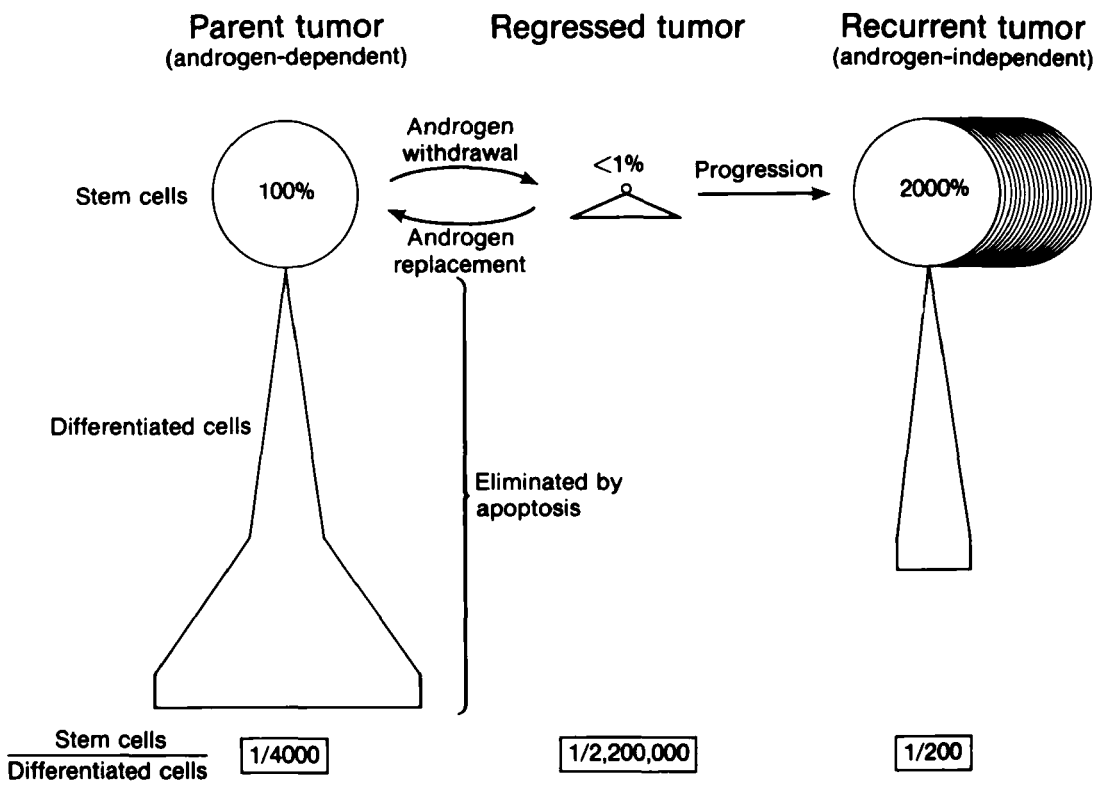

Fig. 2. Model of stem cell composition of the androgen-dependent Shionogi carcinoma. The change in the ratio of stem cells/differentiated cells, which occurs after androgen withdrawal, is shown. The effect of androgen replacement in stimulating the differentiation of stem cells in the regressed tumor and the recovery of apoptotic potential also is illustrated. Reproduced from Bruchovsky et al., 1990, [13] with permission from the publisher.

tate completely restored the susceptibility of the epithelium to further androgen withdrawal [12] was an incentive to try similar experiments on tumors. The underlying assumption was that the maintenance of apoptotic potential by successive rounds of androgen withdrawal and replacement might forestall tumor progression [6].

Unfortunately, there are no animal models of prostate cancer that regress predictably after castration and that can be correctly termed androgen dependent. The tumor best suited for studies on castration-induced cell death is the Shionogi carcinoma, a transplantable mouse mammary tumor that is androgen dependent and that closely mimics the clinical course of prostate cancer in response to treatment. It grows more rapidly in the presence of androgens, undergoes apoptotic regression if androgens are ablated, and ultimately progresses to a hormone-refractory state [13]. In studying the possibility of inducing multiple apoptotic responses, we transplanted the parent androgen-dependent tumor into a succession of male mice, each of which was castrated when the estimated tumor weight became about 3 g [6]. After the tumor had regressed to 30% of the original weight, it was transplanted into another noncastrated male. This cycle of transplantation and castration-induced apoptosis was repeated successfully four times before growth became androgen independent during the fifth cycle. With this regimen of intermittent androgen suppression, the time for a tumor to progress to androgen independence was about 150 days. By comparison, if the tumor was treated by one-time castration only, androgen-independent recurrence followed after an interval of approximately 50 days. These results demonstrated that apoptotic potential could be reinduced in a tumor cell population several times by replacement and withdrawal of endogenous testosterone. They also raised the possibility that progression to androgen-independent malignancy might be delayed by intermittent therapy.

The relative size of the stem cell compartment in Shionogi carcinoma undergoes a large increase as the tumor progresses to an androgen-independent state after one-time castration (Fig. 2). We therefore performed experiments to determine whether the compartment of stem cells was also affected by the intermittent regimen [14]. Tumor regression was induced three times before the carcinoma became refractory

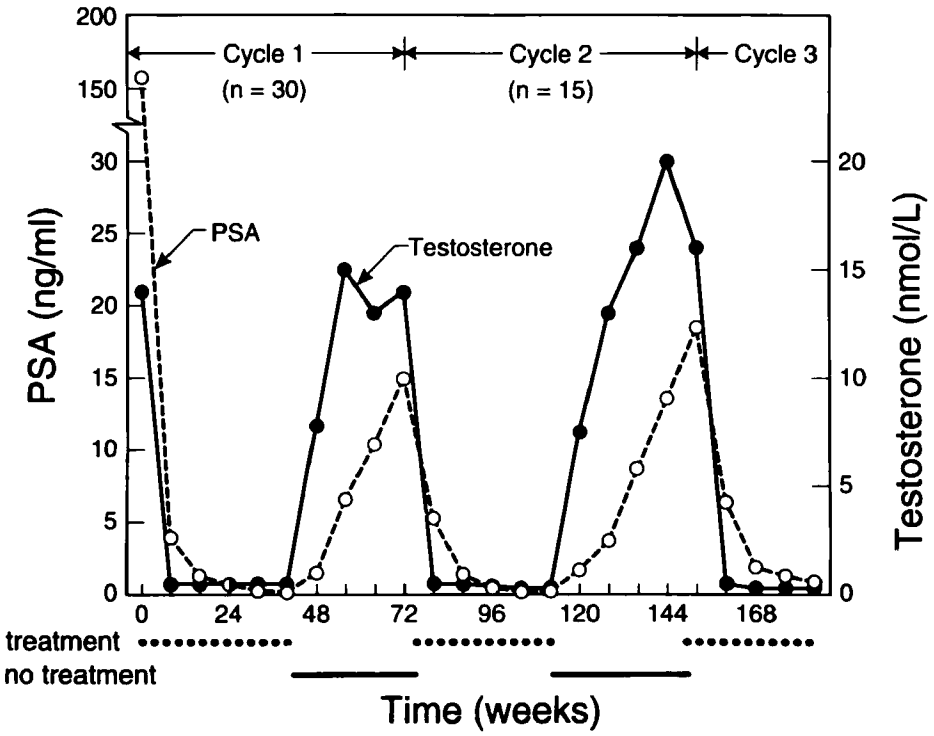

Fig. 3. Intermittent androgen blockade. Composite results for 47 patients with prostate cancer, 30 of whom have completed one cycle of treatment, 15 two cycles, and two are still on the first cycle. (Modified from Bruchovsky et al. [9].)

after a partial fourth response to castration. It was found that the proportion of total stem cells (androgen-dependent and -independent) in each cycle-specific tumor was fairly constant at about one stem cell per 4,000 tumor cells in cycle 1, cycle 2, and cycle 3 tumors. In the cycle 4 tumor, the proportion of total stem cells increased to one stem cell per 310 tumor cells, which was virtually identical to the proportion in a recurrent androgen-independent tumor after one-time castration.

The proportion of the androgen-independent stem cells was found to increase with each cycle of therapy resulting in a composition similar to that in the recurrent tumor. In the parent androgen-dependent tumor before androgen ablation, the androgen-independent stem cell population formed 0.8% of the total stem cell compartment. This population increased to 33% in the recurrent tumor after one-time castration and to a statistically similar ($P = 0.8$) 47% after the fourth and final cycle of intermittent androgen suppression. Thus, the replacement of one-third or more of the total stem cell compartment by androgen-independent stem cells probably represents a sufficient condition for the establishment of androgen independence. This end result occurs more quickly in a single step after one-time castration; with intermittent androgen suppression, the same end result develops, but over a longer time frame.

During cyclic therapy, the lag period between tumor regression and regeneration is likely determined by the variably reduced number of stem cells in the regressing tumor at the time when androgen replacement begins. The cumulative effect of a series of therapy-induced lag periods, especially if lengthy in duration, would be expected to result in a delay in progression. If a tumor lacks the androgen-dependent phenotype and does not undergo apoptotic regression when androgens are withdrawn, intermittent therapy is ineffective [15,16]. Thus, cyclic androgen deprivation should be used only for the treatment of androgen-dependent cancers.

Clinical

Over the past 50 years, the treatment of advanced prostate cancer has adhered quite strictly to the principle of irreversible one-time castration, with one or two exceptions. Early attempts to minimize side effects of therapy with estramustine phosphate and diethylstilbestrol (DES) led to the administration of these estrogenic drugs on an intermittent basis with no apparent risk to the patient [17,18]. With the recognition of the reversibility of new types of combination therapy employing LHRH agonists and antiandrogens, it became clear that it was relatively easy to alternate a patient between periods on and off andro-

gen withdrawal. Also, with sequential measurements of serum testosterone and prostate-specific antigen (PSA), successive periods of tumor response and regrowth could be monitored with considerable precision. In extending our preliminary experience with this approach [6], we investigated the effects of cyclic androgen withdrawal and replacement therapy in a group of 47 patients with prostate cancer (clinical stages 14 D2, 10 D1, 19 C, 2 B2, and 2 A2), with a mean follow-up time of 125 weeks [7]. Treatment was initiated with combined androgen blockade and continued for at least 6 months until a serum PSA nadir was observed. Medication was then withheld until the serum PSA increased to a mean value of 10–20 ng/ml. This cycle of treatment and no treatment was repeated until the regulation of serum PSA became androgen independent.

The first two treatment cycles lasted 73 and 75 weeks, with a mean time off therapy of 30 and 33 weeks and an overall mean percentage time off therapy of 41% and 45% (Fig. 3). Serum testosterone returned to the normal range within 8 weeks (range: 1–26 weeks) of stopping treatment. The off-treatment period in both cycles was associated with an improvement in sense of well-being, and the recovery of libido and potency in the men who reported normal or near-normal sexual function before the start of therapy. In 7 patients with stage D2 disease, the cancer progressed to an androgen-independent state. The mean and median times to progression were 128 weeks and 108 weeks. Seven patients have died, one from a non-cancer-related illness, with mean and median overall survival times of 210 weeks and 166 weeks, respectively.

These observations confirmed that prostate cancer is amenable to control by intermittent androgen suppression. However, the long-term safety of cyclic therapy and its value in extending either progression free or overall survival are unproven and will require randomized studies.

AUGMENTATION OF THERAPEUTIC APOPTOSIS

With a better understanding of what triggers and limits apoptosis, it is possible that intermittent therapy could be improved by adding to the number of cycles or their length. In searching for factors that might be involved in apoptosis, we focused on two proteins with possible nuclear functions: calreticulin, a calcium-binding protein implicated in the regulation of the early stages of apoptosis; and TRPM-2(clusterin), an androgen-repressed gene associated with active cell death and implicated in the late stages of apoptosis.

TABLE I. Comparison of the Amino Acid Sequence in the α-Integrins and the Nuclear Steroid Hormones

Protein	Sequence
α-Subunit of integrins	**KXGFFKR**
Steroid nuclear receptors	
Retinoic receptors-α	ACEGC**KGFFRR**SIQK
Thyroid hormone receptor-β	TCEGC**KGFFRR**TIQK
Vitamin D receptor	TCEGC**KGFFRR**SMKR
Glucocorticoid receptor	TCGSC**KVFFKR**AVEG
Mineralocorticoid receptor	TCGSC**KVFFKR**AVEG
Androgen receptor	TCGSC**KVFFKR**AAAG
Progesterone receptor	TCGSC**KVFFKR**AMEG
Estrogen receptor	SCEGC**KAFFKR**SIQG

Calreticulin

Calreticulin, a 60-kDa calcium-binding protein of the endoplasmic reticulum [19], can bind to an amino acid sequence motif, KXGFFKR, found in the cytoplasmic domain of all integrin α-subunits [20]. A highly homologous amino acid sequence, KXFFKR (where X is either G, A, or V) is also present in the DNA-binding domain of all known members of the steroid hormone receptor family. A comparison of this conserved amino acid sequence motif between integrins and steroid nuclear receptors is shown in Table I.

Furthermore, in steroid hormone receptors, the amino acids in the conserved sequence reside in the P-box of the DNA-binding domain and are crucial for DNA binding, making direct contact with nucleotides in the hormone-responsive elements [21]. Using gel-mobility shift assays, we have demonstrated that calreticulin can inhibit the binding of androgen receptor (AR) to an androgen-responsive element (ARE) in a sequence-specific manner. The results illustrated in Figure 4 depict a gel-mobility assay of AR binding to a ^{32}P-labeled ARE. The migration of ARE by itself is shown in lane 1. Addition of AR (lane 2) results in a shift in the band to a position of slower mobility due to the binding of AR to the ARE and the formation of a higher-molecular-weight complex. This AR–ARE interaction is inhibited by the addition of calreticulin (lane 3). Peptides specific to calreticulin binding to α-integrins (lanes 4 and 5) or to the AR (lanes 8 and 9) reverse the calreticulin inhibition of AR binding to ARE. Calreticulin inhibition is sequence specific because a scrambled peptide (lanes 6 and 7) does not reverse the inhibition by calreticulin of the AR–ARE interaction.

Calreticulin has also been shown to inhibit androgen receptor transcriptional activity in vivo, suggesting the existence of a nuclear form of the protein [22].

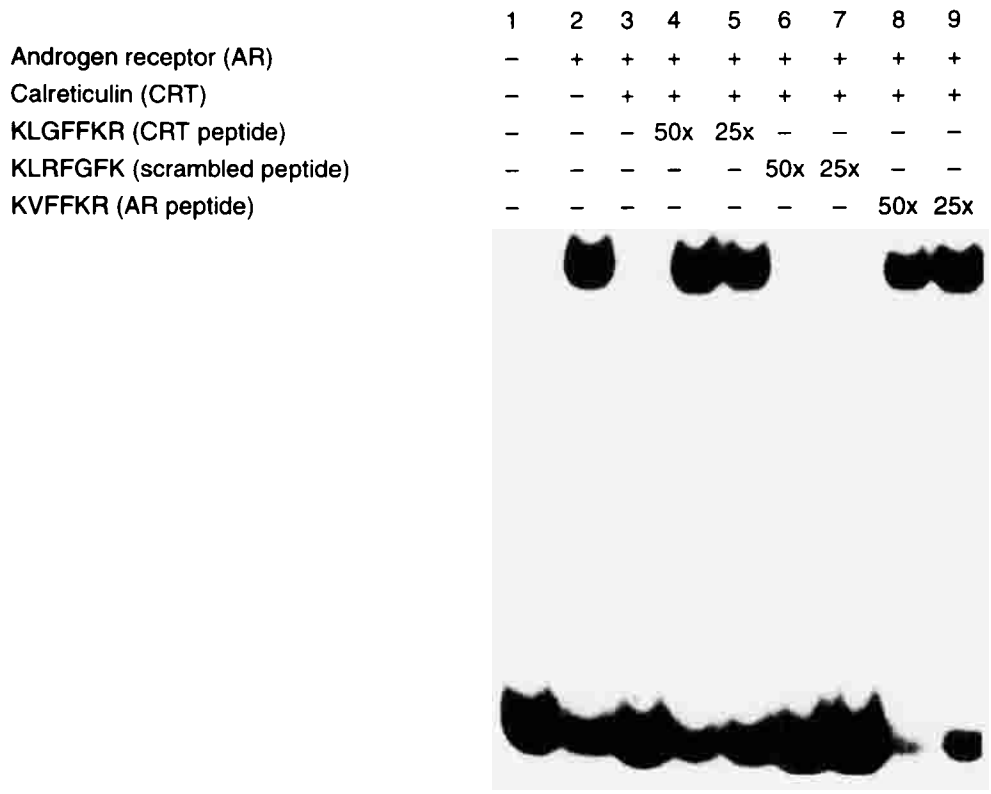

Fig. 4. Interactions between the DNA-binding domain of androgen receptor, calreticulin, and DNA. Recombinant rat AR was preincubated with or without purified calreticulin (CRT), incubated with ^{32}P-labeled ARE and analyzed by gel-retardation assay. Lane 1, ^{32}P-labeled ARE by itself; lane 2, with AR; lane 3, with AR and CRT; lanes 4,5, with AR, CRT and CRT peptide; lanes 6,7, with AR, CRT, and scrambled peptide; lanes 8,9, with AR, CRT, and AR peptide.

This is supported by the presence of a putative nuclear localization signal, PPKKIKDPD (residues 187–195), and the detection of calreticulin in the nucleus of certain cell types, including the rat prostate. When cytosol of rat prostate was examined by Western blot analysis and probed with anti-calreticulin antibody, the results shown in Figure 5 were obtained. The 60-kDa calreticulin band is prominent on day 0 but declines after castration, is barely detectable by day 7, and disappears by day 14. The administration of testosterone to 7-day castrated rats results in an increase in the level of calreticulin within 1 day and a further increase over the next 6 days restoring it to normal. The results of a similar analysis of nuclear extracts are presented in Figure 6. The 60-kDa band corresponding to calreticulin is clearly evident on day 0 and undergoes a gradual decline which is most pronounced by day 14 after castration.

Although the functional significance of the cytoplasmic and nuclear localization of calreticulin is unknown, we infer that calreticulin interferes with nuclear receptor action as depicted in Figure 7.

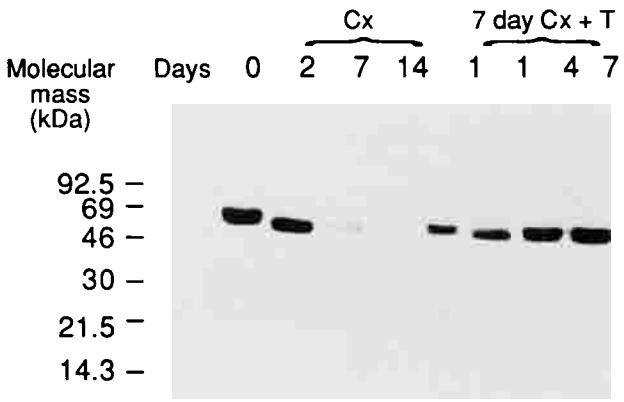

Fig. 5. Analysis of calreticulin in cytosol of rat ventral prostate. Prostate tissue was recovered from noncastrated rats (day 0) and from rats castrated (Cx) 2, 7, and 14 days previously. Seven-day castrated rats were treated with testosterone (T) for 1, 4, and 7 days. Cytosolic protein (50 μg) was subjected to denaturing SDS-PAGE, blotted onto PVDF membranes, and probed with anti-calreticulin antibody.

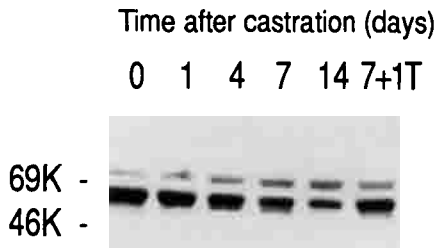

Fig. 6. Analysis of calreticulin in nuclei of rat ventral prostate. Prostate tissue was recovered from noncastrated rats (day 0) and from rats castrated 1, 4, 7, and 14 days previously. Seven-day castrated rats were treated with testosterone (T) for 1 day. Nuclear protein (100 μg) was subjected to denaturing SDS–PAGE, blotted onto PVDF membranes, and probed with anti-calreticulin antibody.

According to this model, calreticulin binds to the androgen receptor at the DNA-binding domain; this prevents the receptor from interacting with promotor DNA and thus inhibits transcription.

It is of interest that calreticulin may serve as the link between integrin receptors on the cell surface and the nucleus, acting as a mediator of signal transduction and modulating the role of androgen receptor in gene activation. This is supported by the observation by Boudreau et al. [23] that the addition of antibody specific to the exterior domain of an integrin induces apoptosis in CID-9 mammary epithelial cells. Conceivably, apoptosis is initiated in the prostate when calreticulin enters the nucleus and binds to the androgen receptor, an effect that might be magnified by castration.

Clusterin

Clusterin, an anti-cytolytic protein encoded by the gene *TRPM-2* (testosterone repressed prostatic message-2), is highly expressed in cells undergoing programmed cell death [24–26]. The mature protein is a glycosylated dimer that results from cleavage of its 70-kDa precursor, yielding 43-kDa and 35-kDa subunits. Unlike calreticulin, clusterin levels increase after androgen ablation in the rat prostate as shown in Figure 8. Before castration (day 0) the bands corresponding to the clusterin subunits, demonstrated by Western blot analysis and staining with anti-clusterin antibody, are very faint. The bands become more prominent on day 1 after castration, reach maximum intensity on day 4, and taper slightly by day 7 and further by day 14. The amount of clusterin is downregulated by the administration of testosterone to 7-day castrated rats; this effect is already apparent on the first day of treatment but is more marked after 7 days.

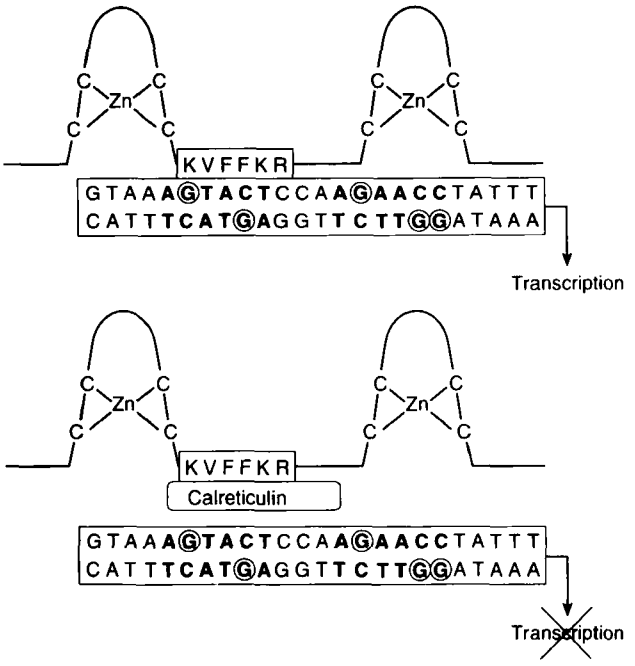

Fig. 7. The DNA-binding domain of the AR showing the two zinc fingers and the binding of the KVFFKR sequence to an ARE. Calreticulin binding to the DNA-binding domain of the AR (at the KVFFKR amino acid sequence) inhibits ARE-dependent gene transcription.

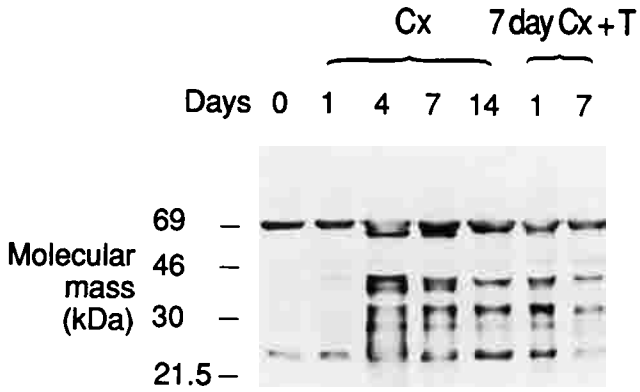

Fig. 8. Analysis of clusterin in cytosol of rat ventral prostate. Prostate tissue was recovered from noncastrated rats (day 0) and from rats castrated (Cx) 1, 4, 7, and 14 days previously. Seven-day castrated rats were treated with testosterone (T) for 1 and 7 days. Cytosolic protein (150 μg) was subjected to denaturing SDS–PAGE, blotted onto PVDF membranes, and probed with anti-clusterin antibody.

TRPM-2 (clusterin) gene expression is also upregulated in the Shionogi carcinoma after castration and is maintained at a high level in androgen-independent cells that result from tumor progression [27] (Fig. 2). Immunohistochemical staining with anti-clusterin antibody has shown that clusterin is located

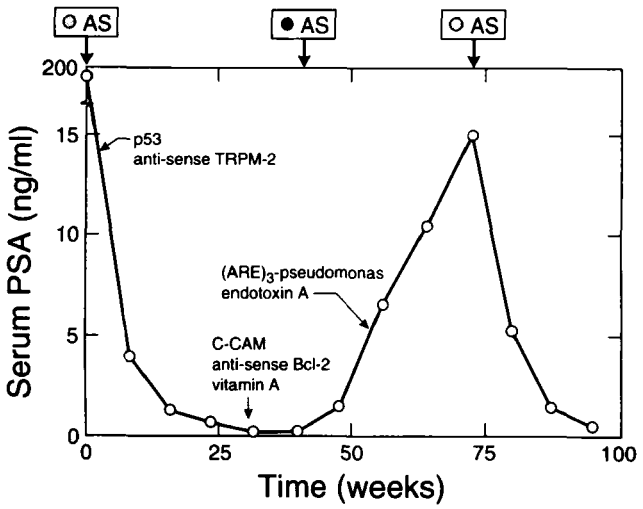

Fig. 9. Potential methods for augmenting intermittent androgen suppression (AS) therapy. Start of therapy is indicated by [O AS], interruption by [● AS]. Serum PSA indicates periods of response and regrowth of clinical prostate cancer. Windows of opportunity for augmentative therapies with p53, antisense *TRPM*-2, C-CAM, antisense Bcl-2, vitamin A, and $(ARE)_3$-*Pseudomonas* endotoxin A are shown.

predominantly in the cytoplasm of cells that are undergoing castration-induced apoptosis [14]. By contrast, in recurrent androgen-independent tumor cells, intense staining of nuclei is observed [14]. Thus, it appears that androgen ablation promotes the deregulation of *TRPM*-2 gene expression, as well as the nuclear localization of its encoded protein. Like calreticulin, the clusterin protein also contains a nuclear localization signal, with the consensus sequence (n3)RK(n9)KKKK (residues 63–80). The presence of clusterin in the nucleus may serve to inhibit early events in the apoptotic process and thereby foster the generation and outgrowth of androgen-independent stem cells in an androgen-depleted environment.

FUTURE DIRECTIONS

Intermittent therapy with reversible modalities based on antiandrogens and LHRH agonists offers potential for long-term control of prostate cancer, while minimizing side effects, especially the suppression of libido and potency, in the younger male patient. Conceivably, the intermittent therapy option will become an alternative to radical prostatectomy or irradiation for the primary treatment of localized prostate in older men with a life expectancy of less than 10 years and might be considered for the prevention of disease in high-risk populations. Augmentation of intermittent therapy to increase the length and number of cycles might be accomplished by administration of cytotoxic drugs, irradiation, differentiation agents, or gene therapy at specific times during a cycle of treatment when the modality of choice would have its maximum effect. There are at least three different times in the cycle when augmentation of intermittent therapy is theoretically possible (Fig. 9). The first arises during the initial period of androgen withdrawal when the apoptotic response might be magnified to encompass a greater number of cells. Methods would include gene therapy to enhance apoptosis (p53) or prevent cell survival (anti-*TRPM*-2). The second opportunity would occur when the tumor reaches its nadir, and a further cell kill (anti-Bcl-2), a differentiation stimulus (C-CAM and vitamin A), might result in prolongation of the cycle. The third favorable time is during the period of tumor regrowth, when the rising titre of testosterone could be used to activate an ARE-regulated gene encoding a lethal protein, such as *Pseudomonas* endotoxin A.

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