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Hormone Action and Apoptosis in Human Prostate Cancer Models

Abstract

Androgen ablation therapy aiming at reducing tumor burden by inhibition of proliferative activity and inducing programmed cell death in the tumor tissue, is the current front-line therapy for advanced prostate cancer. A panel of human prostatic xenograft models, representing various stages of clinical prostate cancer, was used to study the effect of hormonal manipulation on tumor cell proliferation, occurrence of apoptotic cell death, and on some functional markers. High-dose androgen-induced cell loss in the androgen sensitive LNCaP cell line in vitro was demonstrated to be apoptotic cell death. The results obtained with this cell line subtantiated the dual action of androgens in this target cell: depending on the concentration applied, proliferation can be stimulated, and apoptotic cell death may be induced.

Introduction

Prostate Cancer

The occurrence of prostate cancer has increased dramatically over the past decade (Severson *et al*, 1995). It is at present the second leading cause of cancer death in the Western male population. On average, patients are now diagnosed at an earlier stage of the disease. Most patients with tumors that are clinically confined to the prostate are treated with curative intention, either by radical prostatectomy or radiotherapy (Chodak *et al*, 1994). Patients with advanced (metastatic) disease are usually hormonally treated, i.e. by medical or surgical castration, leading to a reponse in about 40-50% of the patients. This androgen ablation therapy aims at reducing tumor burden by inhibition of proliferative activity (Walsh, 1975) and by inducing programmed cell death (apoptosis) in the tumor tissue (Szende *et al*, 1993; Denmeande *et al*, 1996). After an initial response, however, tumor relapse occurs due to the growth of androgen-independent prostate cancer cells. This relapse towards androgen refractory disease develops even if complete androgen blockade is used, and as a consequence androgen ablation is rarely curative. Prostate cancer is resistant to most cytotoxic drugs (Manni and Vogelzang, 1996), which makes advanced disease hardly treatable.

Xenograft Models of Human Prostate Cancer

The transition of androgen-dependent prostate cancer to an androgen-independent state is a process which is still poorly understood and which can only adequately be studied in experimental model systems which can easily be (hormonally) manipulated (Isaacs and Coffey, 1981). Much of the knowledge of the cellular aspects of androgen regulated growth and progression of prostatic cancer stems from investigations with the normal rat (ventral) prostate and the Dunning R3327 rat prostate cancer system (Isaacs *et al*, 1978). Extrapolation of the results obtained with such systems in the rat is, however, limited by their non-human origin and restricts the direct applicability of such models to the study of human prostate cancer.

Establishment of human prostate cancer cell lines in culture and *in vivo*, as heterotransplant in athymic nude mice, is difficult and generally a very low rate of success has been recognized (Otto *et al*, 1988). All in all, no more than ten *in vitro* cell lines, including the androgen responsive LNCaP model, and approx. 15 *in vivo* xenograft models of human prostate cancer have been described. In our institution the hormone-dependent PC-82 and two independent tumor lines, PC-133 and PC-135 were established more than ten years ago. In particular the PC-82 has been used for many studies focussing on various aspects related to endocrine dependence and androgen-regulated functions of human prostatic carcinoma (Van Steenbrugge *et al*, 1984a).

More recently, our institution was successful in developing a new series of seven permanent prostate cancer models *in vivo*. By using athymic nude mice of the NMRI strain in stead of the Balb/c strain, a substantial increase in the take rate of human prostate cancer tissues was achieved. Accordingly, during the last three years seven new prostatic xenograft models were established, originating from primary tumors (prostatectomy and transurethral resection material) as well as metastatic lesions (Van Weerden *et al*, 1996). These tumors represent various stages of clinical prostate cancer, as appears from differences in the pattern of androgen responsiveness, histological grade of differentiation, expression of the androgen receptor (AR) and of prostate specific markers, prostatic acid phosphatase (PAP) and prostate specific antigen (PSA).

Apoptosis in the Prostate

Growth rate of tumors in general is related not only to cell proliferation but also to the rate of (apoptotic) cell death (Dive and Wyllie, 1993). Apoptosis or programmed cell death (Wyllie, 1980) is a process of major interest and a subject of an increasing number of studies in a variety of normal and malignant tissues, including (hormone-responsive) prostate cancer (Howell, 1997). Androgens, besides having the well-established agonistic ability to stimulate prostate cell proliferation, also have an antagonistic ability to inhibit prostatic cell death (Isaacs, 1984). Apoptosis is important with regard to prostate cancer because this is the mechanism by which prostate cancer cells die in response to androgen withdrawal, irradiation, or chemotherapy. Following castration-induced androgen deprivation the rat ventral prostate rapidly involutes with as many as 80 percent of cells being lost within the first 10 days after castration (Lee, 1981). Androgen ablation also inhibits cell proliferation and promotes apoptotic

death in experimental human prostate cancer (Van Weerden *et al.*, 1993), and increases the apoptotic index in clinical prostate cancer (Westin *et al.*, 1995).

The death of androgen-dependent ventral prostatic glandular epithelial cells involves a cascade of biochemical changes characteristic for apoptosis (Kyprianou and Isaacs, 1988). A similar series of events, leading to (programmed) cell death in human prostate cancer tissue, has been described for the human prostate cancer xenograft, PC-82 (Kyprianou *et al.*, 1990). The molecular mechanism(s) for androgen ablation-induced glandular epithelial cell death is not very clear, but cell proliferation, DNA repair, and p53 function do not appear to be involved (Berges *et al.*, 1993). Although conflicting data were presented by Colombel (1992) with respect to the role of p53.

Bcl-2 and Prostate Cancer

Among the varies genes that are demonstrated to be implicated in the apoptotic program, also termed as the 'reactive cascade' (Buttyan *et al*, 1988) is also bcl-2 known to be a inhibitor of apoptosis (Korsmeyer, 1992). Bcl-2 is expressed in approximately 1 of 4 primary prostate cancers (McDonell et al., 1992; Bauer et al., 1996), although higher figures (up to 60% of positive cancers) have been reported by Colombel (1993). Increased expression of bcl-2 was found in androgen independent disease, implying that prostate cancers that express bcl-2 may be less responsive to endocrine treatment. It is tempting to speculate that the resistance of prostate cancers to undergo apoptosis and to be treated effectively by cytostatic drugs is determined either by increased expression of cell death blocking factors like bcl-2, or by decreased expression of death-promoting gene products.

Scope of this Chapter

This paper describes the some functional characteristics of a series of androgen-dependent and independent human prostate xenografts, and it provides information on the effect of androgen manipulation on proliferation, the occurrence of apoptosis models and on the expression of bcl-2 in these model systems.

In addition, data are presented about androgen-induced cell proliferation and cell death in the hormone-sensitive human prostatic tumor cell line LNCaP in vitro. We previously showed that androgens exert a biphasic response on growth of the LNCaP cell line and that relatively high dosages of androgen inhibited growth of LNCaP cells (Langeler *et al*, 1993). The present in vitro study showed that this androgen-induced growth inhibition has the characteristics of apoptosis. The origin of an 'apoptosis-resistant' subline of the parental LNCaP cell line is described.

Materials and Methods

In vivo Studies with Prostatic Xenografts

Transplantation of tumor tissue is routinely carried out by implanting of small tissue fragments derived from freshly obtained prostate tumor specimens into athymic nude mice (Van Steenbrugge *et al*, 1984). Tumor tissue was grafted subcutaneously

in athymic nude mice of the Balb/c or NMRI strain (derived from the breeding colony of the central animal facilities of Erasmus University). Details about the technique of transplantation, performed under light ether anesthesia, and the way tumor growth was monitored were described previously (Van Steenbrugge *et al*, 1984), as were the properties of the extensively studied PC-82 and PC-EW tumors (Hoehn *et al*, 1980; Hoehn *et al*, 1984; Van Steenbrugge *et al*, 1984a).

Hormonally manipulated mice received Silastic implants (Talas, Zwolle, The Netherlands) filled with crystalline steroid, providing constant levels of hormone for longer periods of time (Van Steenbrugge *et al*, 1984b). This method also facilitates hormonal withdrawal and (re)substitution of androgens in tumor-bearing castrated male or female mice. Castration was carried out via the scrotal route under anesthesia with tribromoethanol (Aldrich, Beerse, Belgium).

Immunohistochemistry

Tissue sections of formalin-fixed paraffin-embedded tumor specimens were used for routine histological examination as well as for immunohistochemical staining using an indirect peroxidase-antiperoxidase method with the following monoclonal antibodies, p53 (antibody DO-7 reactive with wild-type and mutant protein) and bcl-2 (clone 124), both purchased from Dakopatts, Denmark; F39.4.9 an anti-human androgen receptor (AR) monoclonal antibody provided by the Dept. of Pathology, Rotterdam. For staining of p53, bcl-2 and AR the technique of antigen retrieval using a microwave equipment was applied. The frequency of apoptotic events occurring in xenografts was estimated in tissue sections using the TUNEL method described by Wijsman et al. (1993).

The LNCaP Cell Line and Apoptosis Detection in vitro

The androgen dependent human prostate cell line LNCaP-FGC, which was derived from an early passage of the LNCaP cultures (Horoszewicz *et al*, 1980), was obtained from Dr. Julius Horoszewicz. The cells were routinely cultured in RPMI-160 medium (Life Technologies, Breda, The Netherlands) supplemented with 7.5% fetal bovine serum (Hyclone, Logan, Utah, U.S.A.) and glutamine, penicillin and streptomycin (Van Steenbrugge *et al*, 1991). Androgen-depleted medium contained 5% dextran-coated charcoal (DCC)-treated serum. For the present experiments LNCaP cells of passage 70-80 were used. The growth characteristics of these (p70) cells under various hormonal conditions have previously been described (Langeler *et al*, 1993). Androgenic effects on LNCaP growth were tested with the synthetic, non-metabolizable androgen R1881 (methyltrienolon; New England Nuclear, Boston, MA, U.S.A.).

Apoptosis in LNCaP cell cultures was determined by application of the assay of cell viability based on simultaneous staining of LNCaP cell cultures *in situ* with propidium iodide (PI) and Hoechst 33342 (Polysciences, Warrington, U.S.A.). This method provides a means to discriminate between live, necrotic, early- and late apoptotic cells (Pollack and Ciancio, 1991). In addition, a 'Cell Death Detection Elisa' (Boehringer, Mannheim, Germany) was applied in an attempt to quantify the occurrence

of apoptosis in LNCaP cell cultures. This assay is based on the quantitative *in vitro* determination of cytoplasmatic histone-associated DNA fragments (mono- and oligonucleosomes). For both methods, camptothesin (CAM) treated cells of the human myelogenous leukemic cell line HL-60 were used (Del Bino *et al*, 1990) as control, apoptotic cells. Finally, LNCaP cell cultures treated with high dosages of R1881 were processed for transmission microscopy studies.

Results and Discussion

A Panel of Human Prostate Tumor Xenograft Models

In our institution during a period of more than ten years (1977-1990) almost 200 clinical specimens were transplanted in Balb/c nude mice, resulting in a very low number of permanent tumor models: the hormone-dependent PC-82 model, two hormone-independent tumors: PC-133 and PC-135 and more recently, the hormone dependent PC-295 tumor. The PC-82 was established about 20 years ago, and was the first hormone-dependent xenograft model described in literature. Another hormone-dependent model, PC-EW, was founded by Hoehn and coworkers (Hoehn *et al*, 1984) and was also included in the panel of models used.

About three years ago, for technical reasons, we started to use the NMRI strain of nude mice as host animal for heterotransplanting prostatic carcinomas. Remarkably enough, this resulted in a considerably increased take rate: within a period of two years 6 out of 19 transplants had a positive take, leading to the development of permanent tumor lines. These models originated from primary prostatic carcinomas (derived from prostatectomy specimens), from progressive, hormone refractory tumors (tissues derived from transurethral resection material) and from metastatic lesions (lymph nodes as well as skin).

Among this group of seven (including PC-295) newly established xenografts, clearly two subgroups of models can be recognized: differentiated, androgen-dependent tumors which express the androgen receptor (AR) and which express and secrete Prostate Specific Antigen (PSA) (PC-295, PC-310 and PC-295) and two undifferentiated, androgen-independent tumors which are devoid of androgen receptors and did not express or secrete PSA. The newly established androgen dependent tumor models PC-295, PC-310 and PC-329 all showed a reduction in tumor volume after androgen ablation. The hormone independent PC-324 and PC-339 models grow equally well in male and female mice. Although not applied in the present study, tumor PC-346 is a very interesting model as it continued to grow after an initial period of tumor regression following androgen withdrawal. Therefore, it is the first human xenograft model showing the clinically so important relapse phenomenon.

Cell Death in the Androgen Dependent PC-82 and PC-EW Models

Volumes of PC-82 and PC-EW tumors declined after castration of tumor-bearing mice, the regression of the PC-EW tumor being faster than that of the PC-82 tumor (half-life of 6 and 18 days, respectively (Figure 1). The resultant decline of the

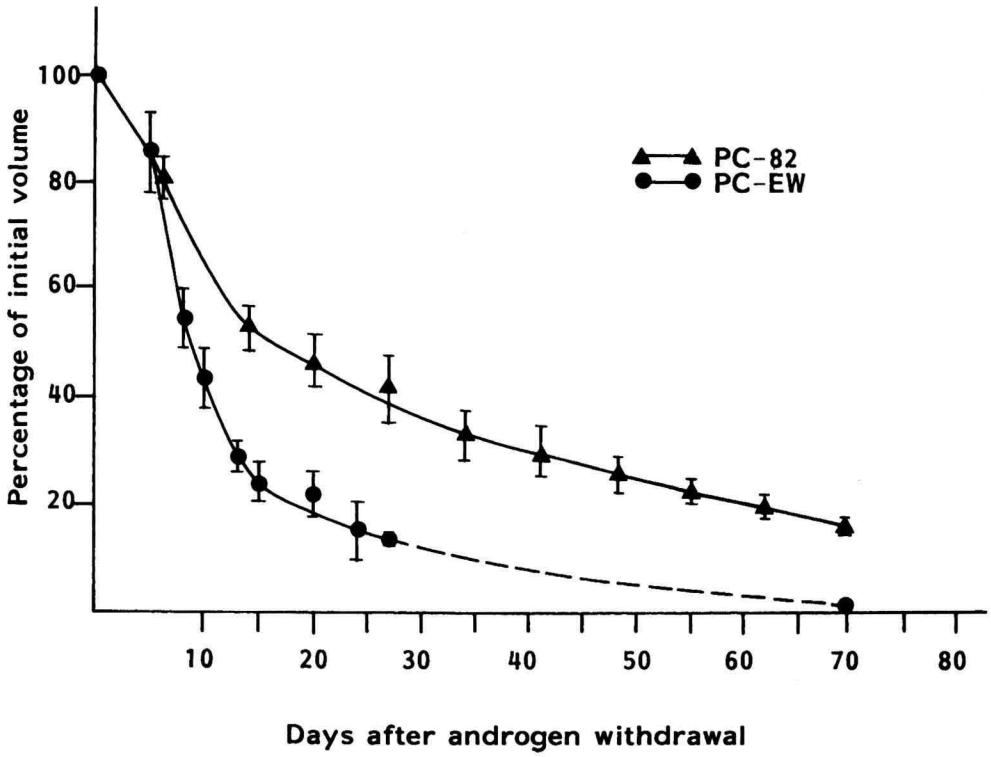

Fig. 1. Patterns of regression after androgen withdrawal from PC-82 (n=8) and PC-EW (n=7) tumor-bearing nude mice.

PC-82 tumor nodules after castration was associated with a decrease in cells incorporating BrdU (Van Weerden *et al*, 1993). Concomitantly, an increase in the number of apoptotic bodies (scored on basis of their morphological appearance) could be observed, which reached its maximum at 4 days post-castration (Table I). These observations are in agreement with previously reported results of the PC-82 tumor (Kyprianou *et al.*, 1990). The regressing PC-82 tumors did not show any sign of necrotic death, whereas androgen depletion of the androgen-dependent PC-EW tumor induced both apoptosis and necrosis. Regressing PC-EW tumors showed widespread necrosis with clusters of apoptotic cells (Table I). As a consequence of the necrotic cell death, the PC-EW tumor regressed completely (within 3-4 weeks), leaving small nodules consisting of fibrotic tissue. Whether androgen depletion-induced regression of PC-EW represents a type of secondary necrosis following an apoptotic response could not be assessed as yet. Regressing PC-EW tumors could not be restimulated by androgens even after a relatively short period (2 weeks) of androgen depletion. By contrast, in regressing PC-82 tumors viable cells remain present, which could be restimulated to grow by androgens even after long-term (over 6 months) androgen depletion (Van Weerden *et al*, 1993). Spontaneous, i.e. androgen-independent, regrowth

Table I. Cell death in regressing PC-82 and PC-EW tumors.

Time after castration	PC-82 % apoptosis	PC-82 % necrosis	PC-EW % apoptosis	PC-EW % necrosis
0 (days)	1.3 ± 0.6 (5)	< 10	1.6 ± 0.9 (5)	10
4	10.8 ± 3.0 (5)	< 10	5.4 ± 2.6 (5)	30
7	7.2 ± 3.0 (4)	< 10	2.9 ± 1.1 (4)	30
15	2.8 ± 0.2 (3)	< 10	2.4 ± 1.0 (5)	50
30	1.9 ± 1.0 (5)	< 10	2.2 ± 1.3 (4)	80

Data are expressed as mean ± SD, with number of samples in parentheses.

of these long-term androgen deprived PC-82 tumors has never been observed, however. This androgen sensitive but 'apoptosis-resistant' PC-82 tumor cell population possibly represents an intermediate step between androgen-dependence and independence.

Effects of Hormonal Manipulation on Proliferation and Cell Death in Prostate Xenografts

An androgen depletion-repletion protocol (i.e. castration for 14 days and testosterone resubstitution for 7 days) was applied to two androgen dependent (PC-295 and PC-310) and two androgen independent (PC-324 and PC-339) xenografts. In tumors PC-295/310 a clear apoptotic response (detected by the TUNEL method) was seen, concomitantly with diminished proliferative activity (table II). In these models the expression of immunohistochemically determined prostatic markers, AR and PSA was completely absent at 14 days after androgen withdrawal, whereas re-expression was seen after 7 days of androgen resubstitution. In contrast to tumor PC-295, irrespective the hormonal status of the host animal, no bcl-2 expression was observed in PC-310 (table II). Androgen withdrawal in PC-295 resulted in a increased overall expression of bcl-2. Neither the proliferative activity nor occurrence of apoptotis were significantly affected in the androgen independent PC-324 and PC-339 tumors (table II). These tumors were devoid of AR and PSA expression. In addition, these tumors demonstrated overexpression of *p53* at the immunohistochemical level, that is nuclei of over 90% of the cells were intensely stained. Interestingly enough, the same two hormone-independent tumors with *p53* overexpression were demonstrated to be also strongly immunoreactive for *bcl-2*. Although the *bcl-2* expression may be a differentiation related phenomenon, in these tumors with *p53* overexpression the intense *bcl-2* staining is most likely the result of a constitutive *bcl-2* expression. Based on these results, which were in agreement with literature data, it was concluded that tumors PC-324 and PC-339 represent late stage, hormone refractory disease.

Androgen-induced Cell Death an in vitro Prostatic Cell Line

In steroid-depleted (DCC) medium androgen dependent LNCaP-FGC (FGC) cells continued to grow at a decreased rate. Maximal growth was induced with the addition of 0.1 nM of the synthetic, non-metabolizable androgen R1881, whereas the

Table II. Effect of androgen manipulation on proliferation, apoptosis, and some functional markers in human prostate cancer xenografts

Tumor model	AD	STATUS	AR (+/-)	PSA (+/-)	BCL-2 (+/-)	APOP (%)	BrdU (%)
PC-295	+	control:	+	+	+/-	5	17
		castr:	-	-	+	25	0
		castr+T:	+	+	+	13	14
PC-310	+	control:	+	+	-	9	13
		castr:	-	-	-	16	0
		castr+T:	+	+	-	8	13
PC-324	-	control:	(+)	-	+	9	48
		castr:	-	-	+	7	42
		castr+T:	(+)	-	+	7	41
PC-339	-	control:	-	-	+	6	23
		castr:	-	-	+	11	20
		castr+T:	-	-	+	8	46

The post-castration period was 14 days (PC-295: 4 days), whereafter androgen (testosterone (T)) was resubstituted for 7 days. Data were based on 4 animals per treatment group. Immunohistochemical scores were semiequantitative estimates. Proliferative (BrdU) and apoptotic (APOP) indices were based on scores of 1000 cell count in random high power field.
AD: androgen dependence; AR: androgen receptor; PSA: prostate specific antigen.

addition of 100-fold (10 nM) of R1881 to medium with DCC led to a decrease in cell number (Langeler *et al*, 1993). In these high dose R1881-treated cultures a high rate of cell death was found. It appeared that all dying cells were detached and that attached cells were vital and continued to grow. Staining of such cultures *in situ* with a combination of the DNA binding dye, Hoechst 33324, and propidium iodide (PI), clearly demonstrated that the detached cells had features indicative for apoptosis. Specifically, cytoplasmic contraction, condensation of nuclear chromatin and the formation of membrane-bound 'apoptotic bodies' were observed. Apoptotic events were also confirmed by electron- microscopical examination of high dose R1881-treated cells. Interestingly, in the R1881-treated cultures initially blue (i.e. intact), PI excluding apoptotic cells were found, whereas gradually cultures also showed increasing amounts of cells with red luminescence that lost their membrane integrity, but which also had apoptotic features. Apparently, with this method early and late apoptotic cells could be detected.

Estimates were made of the percentage LNCaP cells that were recognized as being apoptotic based upon their pattern of DNA Hoechst stain and the number of death cells that had a positive stain with PI. From the time-course experiment shown in Figure 2, it can be seen that androgen-induced apoptosis is a relatively slow process. The peak of 18 percent apoptotic cells in 10 nM R1881-treated cells was reached about 6 days after start of treatment (Figure 2A). From this graph it can be read that in cultures grown with 0.1 nM R1881, a dosage which optimally stimulates growth of LNCaP cells, also a relatively high percentage of apoptotic cells was found. By

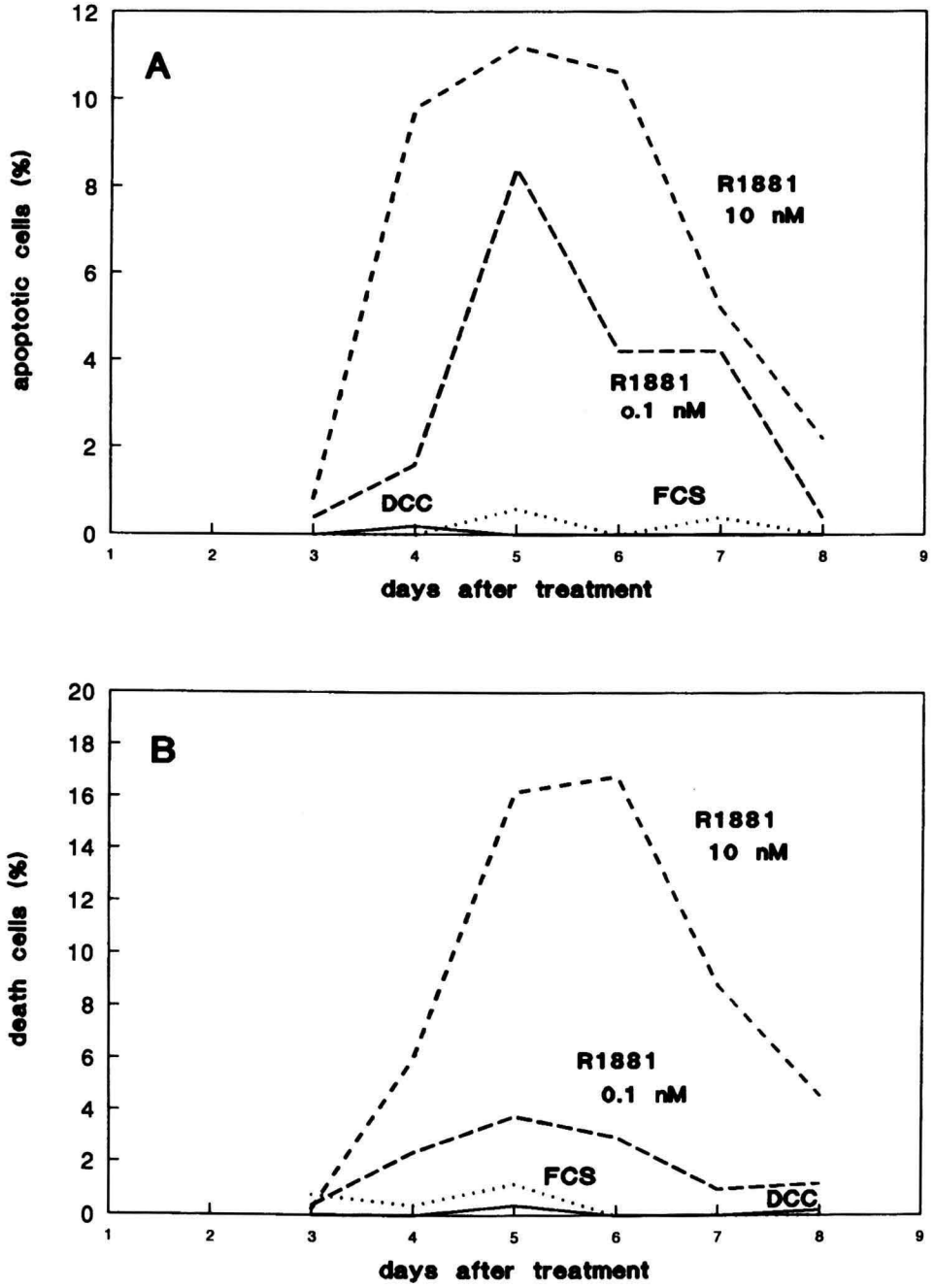

Fig. 2. Apoptotic cell death in LNCaP cells cultured in medium with complete serum (FCS), androgen-depleted serum (DCC) and DCC supplemented with 0.1 and 10 nM of the synthetic androgen R1881. Cultures were stained with propidium iodide (A) and the DNA binding dye, Hoechst 33324 (B), respectively.

Table III. Androgen (R1881)-induced cell death in the LNCaP prostatic cell line.

Conc. R1881	Enrichment Factor* (DCC = 0)		
	Day 5	Day 6	Day 7
100 nM	12.9	37.0	37.7
10 nM	2.7	59.7	73.1
1 nM	1.3	29.7	21.9
0.1 nM	2.9	43.0	19.3

* Enrichment factor of nucleosomes in the cytoplasma of LNCaP cell treated with different concentrations of the synthetic androgen R1881.

contrast, cultures grown in medium with either complete serum or androgen-depleted (DCC) serum had a very low frequency of apoptotic cells. This should be considered as spontaneous cell death occurring in all cell cultures. As described in one of the previous sections, withdrawal of androgens from the rat ventral prostate or from the androgen-dependent human PC-82 tumor in vivo, resulted in the induction of apoptosis (Kyprianou and Isaacs, 1988; Kyprianou *et al*, 1990). This apparently does not apply to the androgen- sensitive human LNCaP cell line in vitro, as in androgen-depleted medium, growth of the cells was only retarded and no increase of apoptosis was observed (figure 2).

The patterns of occurrence of PI positive cells under the different hormonal conditions (Figure 2B) paralleled those found for apoptotic cell with yet intact membranes (Figure 2A), except that the level of death cells found in cultures treated with 0.1 nM R1881 was considerably lower compared to that observed in cells cultured in the high concentration of 10 nM R1881.

The occurrence of apoptosis in the LNCaP cell cultures could also be quantified by the use of a photometric enzyme immunoassay (Cell Death Detection Elisa). This test is based upon the determination of cytoplasmatic histone-associated DNA fragments present in cells that undergo the process of apoptosis. Samples of the time course experiment with LNCaP cultures were measured in this Elisa. The nucleosome enrichment factor in androgen treated cell cultures, relative to that found for the DCC cultured cells, clearly increased in time and in a dose dependent manner (Table III). Although also in the cultures with low androgen concentrations, increased nucleosome concentrations, i.e. DNA fragmentation, was found, also with this method the highest rate of apoptotic cell death was observed with 10 nM R1881.

The observed effect of high androgen-induced apoptosis was not restricted to the synthetic androgen R1881. Although the natural androgen Dihydrotestosterone (DHT) was rapidly metabolized by the LNCaP cells, daily exposure of high dosages (1uM) of DHT resulted in similar patterns of cell death as observed for R1881. DHT-induced cell death was clearly observed after 14 days, which was even later when compared to R1881-treated cell cutures. Apparently, the cell death promoting effect of high dose androgens is a real androgenic effect, which is not restricted to synthetic

androgens. It is not yet known whether this phenomenon is mediated by the androgen receptor, or that the membrane is involved.

Not the entire culture of high dose R1881-treated cells died, and a small surviving fraction of cells continued to growth after 3 weeks of exposure. The resulting cell culture developed into a fast growing cell line with a distinct cellular morphology compared to the parental line. The cells of this subline could not be driven into apoptose by high dosages of androgen, and with this regard were considered 'apoptosis-resistant'. In contrast to the original LNCaP cells, which are poorly transplantable in athymic nude mice, cell of the developed subline were highly tumorigenic.

Conclusions

Further studies of this panel of prostatic xenograft models may result in a better understanding of the biochemical pathway involved in programmed cell death in prostate cancer. In addition it may yield information about the importance of apoptosis as a mechanism implicated in the progression of clinical prostate cancer, i.e. the transition of androgen-dependent towards androgen-resistant prostate cancer.

Androgen-induced cell death in the LNCaP prostatic carcinoma cell line was demonstrated to be apoptotic cell death. The present results obtained with LNCaP cultures subtantiate the proposed dual action of androgen: depending on the concentration applied, cell proliferation can be stimulated and apoptotic cell death may be induced.

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