Pergamon

J. Steroid Biochem. Molec. Biol. Vol. 53, No. 1–6, pp. 401–405, 1995
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0960-0760/95 \$9.50 + 0.00

0960-0760(95)00086-0

Growth of the Androgen-dependent Tumor of the Prostate: Role of Androgens and of Locally Expressed Growth Modulatory Factors

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The crucial role played by androgens in the growth of prostatic carcinoma is now well established. However, the mechanisms of this proliferative action are still poorly understood. Experiments have been performed to clarify: (1) the metabolism of androgens in prostatic tumor cells; and (2) the role played by locally produced growth factors in the autocrine regulation of prostatic tumor cell proliferation and the possible regulation exerted by testosterone (T) on the activity of these factors. These studies have been performed by utilizing the human androgen-responsive prostatic cancer LNCaP cell line. (1) By incubating LNCaP cells with different ^{14}C-labeled androgenic precursors, it has been shown that all the major key enzymes involved in the metabolism of androgens (5α-reductase, 17β-hydroxysteroid-oxidoreductase, 3α- and 3β-hydroxysteroid-oxidoreductases) are present and active in these cells. In particular, the 5α-reductase, which converts T and Δ_4 to DHT and 5α-A respectively, seems to be more active when Δ_4 is the substrate, suggesting a preference for this precursor. (2) The hypothesis that LNCaP cells might produce LHRH (or a LHRH-like peptide) has been verified by RT-PCR, performed in the presence of a pair of specific oligonucleotide primers. A cDNA band of the expected size (228 bp), which specifically hybridized with a ^{32}P-labeled LHRH oligonucleotide probe, has been obtained in LNCaP cells. To clarify the possible role played by this factor in the regulation of tumor growth, LNCaP cells, cultured in steroid-free conditions, have been treated with a LHRH antagonist; the treatment resulted in a significant increase of cell proliferation. Taken together, these data indicate that a LHRH (or LHRH-like) growth modulatory system is expressed in LNCaP cells and plays an inhibitory role in the regulation of tumor cell proliferation. This system seems to be regulated in a negative way by steroids. Growth factors endowed with stimulatory activity, such as EGF and TGFα, have also been shown to be produced by LNCaP cells. The present studies show that the immunoprecipitation of the EGF receptor with a specific monoclonal antibody (Ab225) reveals a protein band of the expected size (170 kDa) which is phosphorylated even in basal conditions. Moreover, the treatment of LNCaP cells, cultured in serum-free conditions, either with a monoclonal antibody against the EGF receptor, or with immunoneutralizing antibodies against EGF and TGFα, results in a significant decrease of cell proliferation. These observations clearly confirm the expression, in prostatic tumor cells, of an EGF/TGFα loop which exerts a stimulatory action on cell proliferation. T seems to exert a positive regulation on this loop, at least in terms of EGF receptor concentration.

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Proceedings of the IX International Congress on Hormonal Steroids, Dallas, Texas, U.S.A., 24–29 September 1994.
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INTRODUCTION

Since the pioneer work by Huggins and Hodges in 1941 [1], the crucial role played by androgens in the development and progression of prostatic carcinoma has been well established [2–5]. The major steps proposed in the mechanism of action of testosterone (T) include: conversion into active metabolites through the effect of specific enzymes [6–11], binding to the androgenic receptor (either wild type or mutated) [12–14], transcription of genes involved in the regulation of the expression and/or activity of growth modulatory factors, and of their receptors [5, 15–18]. It is known that growth factors, endowed with either stimulatory or inhibitory activity on cell proliferation, have actually been suggested to be involved in the control of the growth of prostatic cancer [19–24]. However, most of these mechanisms are still poorly understood.

The present experiments have been performed to get further information on: (1) the metabolism of androgens in prostatic tumor cells; and (2) the role played by growth factors, endowed with either inhibitory or stimulatory activities, in the control of prostatic tumor cell growth and the possible regulation exerted by T on the activity of these growth factors.

For these experiments, the human prostatic cancer cell line LNCaP (Lymph Node Carcinoma of the Prostate) has been utilized. This cell line represents a very useful *in vitro* model, since it retains the most peculiar characteristics of the original tumor such as the responsiveness to androgens, the expression of the androgen receptor, etc. [12, 13, 25–28].

ANDROGEN METABOLISM IN LNCaP CELLS

The ability of LNCaP cells to respond to androgens with an increased growth rate has been widely reported [17, 25–28]. In the culture conditions adopted in the authors' laboratory, T stimulates LNCaP cell proliferation following a biphasic dose–response curve, maximal stimulation occurring at the dose of 10^{-10} M.

On the other hand, androgen metabolism in prostatic tumor cells has been less extensively studied so far. To better characterize the metabolic pathways through which T and other androgens are metabolized in prostatic carcinoma, LNCaP cells have been incubated, in steroid-free medium, in the presence of different ^{14}C-labeled androgenic precursors. The quantification of the formation of the respective metabolites, as indicators of the specific activities of the enzymes involved in such conversions has followed. The precursors utilized were: T, androst-4-en-3,17-dione (Δ_4), 17β-hydroxy-5α-androstan-3-one (DHT), and 5α-androstan-3,17-dione (5α-A). The incubation was performed for 2 h at 37°C in a thermostatic room; dishes containing the medium and the labeled steroid, but without cells, were processed as blanks. At the end of the incubation period, media were removed and stored at −20°C until extraction; the determination of the androgen metabolites was performed by means of an *in vitro* radiometric method [29, 30].

The results obtained show that, when [^{14}C]T is used as substrate, both DHT and Δ_4 are formed, with a prevalence of the latter steroid. When [^{14}C]Δ_4 is the substrate, cells form both T and 5α-A, which represents the major product. In additions, diols (3α, 17β-dihydroxy-5α-androstane and 3β, 17β-dihydroxy-5α-androstane) and 5α-A are formed from [^{14}C]DHT, while A and DHT are formed from [^{14}C]5α-A; a prevalence of the diols in the former case and of A in the latter one has been observed.

These data show that LNCaP cells possess all the major key enzymes involved in the metabolism of T (5α-reductase, 17β-hydroxysteroid-oxidoreductase, 3α- and 3β-hydroxysteroid-oxidoreductase). In particular, the enzyme 5α-reductase, which converts T and Δ_4, respectively, to DHT and 5α-A, appears to be more active when Δ_4 is the substrate, suggesting a preference for this precursor. The pattern of androgen metabolism described here for prostatic tumor cells in culture appears to be similar to that previously found in human prostatic cancer tissues [6–11]; all the metabolic steps which contribute to DHT degradation exceed the ones leading to its accumulation.

It is known that 5α-reduced isozymes may be present in different tissues (prostate, epidydimis, etc.) [29–31], and that two 5α-reductases have been cloned [32, 33]. It is then possible that one 5α-reductase isozyme, which shows a greater affinity for Δ_4 than for T, might be preferentially expressed in LNCaP cells. However, further studies are required to confirm this hypothesis.

INHIBITORY AND STIMULATORY GROWTH FACTORS IN LNCaP CELLS: POSSIBLE REGULATION BY ANDROGENS

Inhibitory growth factors

In a previous work performed in the authors' laboratory it has been shown that LHRH agonists exert a dose-dependent antiproliferative action on LNCaP cells; moreover, binding sites for these compounds have been found on these cells, particularly when cultured in steroid-free conditions [34]. Since hypothalamic LHRH is rapidly degraded at the pituitary level [35], these data suggest that LHRH, or a LHRH-like peptide might be produced by prostatic tumor cells, possibly to act locally as a growth modulatory factor. To verify this hypothesis, the presence of a mRNA coding for LHRH has been investigated. These experiments have been performed using the RT-PCR (reverse transcription-polymerase chain reaction) technique. Total RNA was extracted, according to a modification of the guanidium thiocyanate/cesium chloride method [36] from LNCaP cells, from rat hypothalami and pituitaries. The RNAs were first reverse transcribed in the presence of an oligo(dT)$_{16}$

primer, and the cDNAs were then amplified in the presence of a pair of specific oligonucleotide primers. The sequences of these primers have been designed according to the reported sequence of the human and rat LHRH cDNAs, for LNCaP cells and rat tissues respectively [37]. After the RT-PCR reaction, a cDNA fragment of 228 bp was expected. The amplified cDNAs were electrophoresed on a 1.5% agarose gel stained with ethidium bromide, then Southern blotted onto a nylon membrane [38], and hybridized with a ^{32}P-labeled LHRH oligonucleotide probe. The results obtained show that a cDNA band of the expected size, which specifically hybridizes with the LHRH probe, can be obtained from LNCaP cells, as well as from the rat hypothalamus; no band could be detected in the case of the pituitary tissue. Moreover, the sequence of the cDNA obtained from LNCaP cells was found to be identical to that of the human placental LHRH cDNA [37]. In line with this observation, it has recently been shown in the authors' laboratory that a mRNA for LHRH is also expressed in specimens of human prostatic cancer tissues as well as in samples of the Dunning R3327 rat prostate tumor, an experimental model of prostatic cancer. These results show that a mRNA for LHRH is expressed in human prostatic tumor cells. The possibility that this mRNA could be translated into LHRH (or a LHRH-like peptide) is supported by the observations reported by Qayum and coworkers [39, 40]. These authors have found the presence of LHRH-like immunoreactivity in the culture media of LNCaP cells, particularly when grown in steroid-free conditions.

On the basis of the data so far reported, it appears that a LHRH or LHRH-like system (mRNA, peptide, receptors) is expressed in LNCaP cells; this system seems to be negatively regulated by steroids, since both the peptide [39, 40] and its receptors [34] are particularly produced and expressed, respectively, when steroids are not present in the culture medium. To verify the possible role played by this intrinsic LHRH-like system, LNCaP cells have been grown in steroid-free conditions (in order to have the system hyperexpressed and/or hyperactive) and treated with a LHRH antagonist (LHRH-ANT, "Nal-Arg-LHRH", 10^{-8} M) for 7, 9, 12 or 15 days. The results obtained show that after 7 days of treatment, LNCaP cell number is only slightly increased; however, cell proliferation was found to be significantly increased after 9, 12 and 15 days of treatment (Fig. 1). It is interesting to note that a similar treatment with the antagonist does not modify the proliferation of cells grown in the presence of steroids.

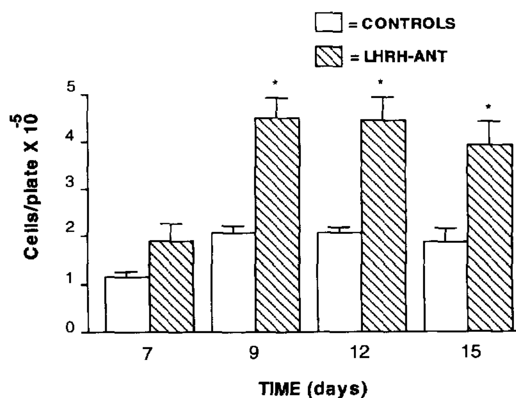

Fig. 1. Effect of a LHRH antagonist (LHRH-ANT, 10^{-8} M) on the proliferation of LNCaP cells. Results are expressed as the mean cell number per plate ± SE. Controls are without drug. $^*P < 0.05$ vs controls.

These data indicate that, in LNCaP cells, the locally produced LHRH or LHRH-like peptide may participate in the local regulation of cell proliferation by acting as a growth inhibitory factor. Growth factors endowed with inhibitory activity have previously been reported to be produced by prostatic tumor cells [5, 24]. The present data also suggest that the LHRH-like system might be particularly expressed and/or active when steroids are not present in the culture medium, i.e. this system seems to be negatively regulated by steroids, possibly by androgens, since these cells are clearly androgen-dependent [17, 18, 25–28]. It is then possible to postulate that T might stimulate LNCaP cell proliferation, at least partially, by decreasing the activity of this local LHRH-like inhibitory loop.

Stimulatory growth factors

LNCaP cells have been shown to respond to EGF and TGFα with an increased growth rate [15–17, 41], to produce both EGF and TGFα polypeptides [15, 19, 26, 42], and to express the EGF receptor [15–17]. Moreover, the intracellular signalling mechanisms which are usually associated with the activation of the EGF receptor are also functional in these cells. In particular, previous work of this laboratory has shown that treatment of LNCaP cells with EGF (5 ng/ml) for 15, 30 and 60 min results in a significant increase of *c*-fos mRNA levels, which appear to peak at 30 min after the initiation of the stimulus. Finally, it has also been shown that, in LNCaP cells previously labeled with [^{32}P]orthophosphate, the immunoprecipitation of the EGF receptor by means of the specific monoclonal antibody Ab225 (kindly donated by Dr J. Mendelsohn, Memorial Sloan-Kettering Cancer Center, NY) reveals a band of the size (170 kDa) expected for the EGF receptor which is phosphorylated even in basal conditions (i.e. in the absence of exogenous EGF) (Fig. 2). Taken together, all these observations point to the presence, in prostatic tumor cells, of a functional EGF/TGFα growth stimulatory system. To further verify this hypothesis, LNCaP cells were cultured in serum-free conditions and treated for 4 days with two antibodies able to immunoneutralize respectively EGF or TGFα (5 μg/ml); the treatment results in a significant decrease of cell proliferation. Similar observations have been obtained by treating

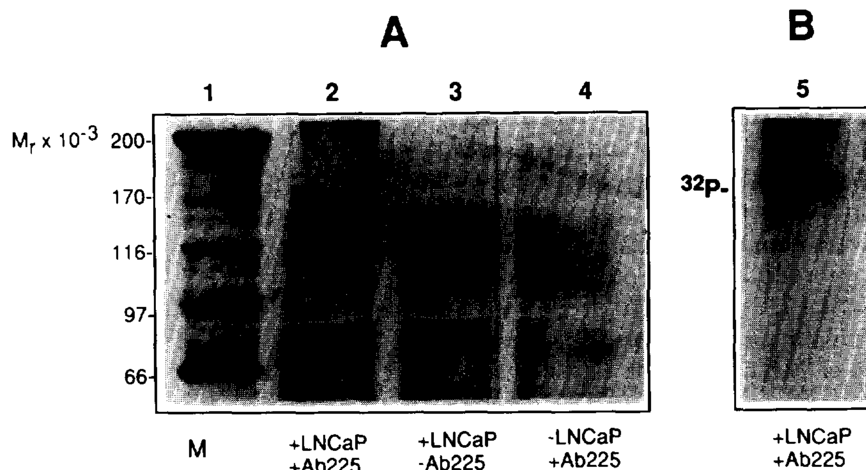

Fig. 2. Immunoprecipitation (A) and phosphorylation (B) of the EGF receptor in lysates from LNCaP cells. (A) Lysates from LNCaP cells were incubated with the Ab225 monoclonal antibody to the EGF receptor and the immunoprecipitated EGF receptor was then bound to protein A-Sepharose beads. After washing the immunoprecipitates, proteins were extracted from the beads with SDS–PAGE sample buffer, resolved by 7.5% SDS–PAGE and stained with silver. Lane 1, marker; Lane 2, sample containing both LNCaP cell lysate and Ab225; Lane 3, sample containing LNCaP cell lysate without Ab225 (negative control); Lane 4, sample containing Ab225 without LNCaP cell lysate (negative control). (B) Autoradiogram of SDS–PAGE of the immunoprecipitated EGF receptor from LNCaP cells prelabeled (18 h) with [^{32}P]orthophosphate.

LNCaP cells with the monoclonal antibody raised against the EGF receptor (Ab225, 1 μg/ml). The data confirm that both EGF and TGFα are produced by LNCaP cells, and that they both participate as growth stimulatory factors in the autocrine regulation of cell proliferation, through the activation of the same receptor. Data similar to those presented here have also been reported for androgen-independent prostatic tumor cell lines [20, 43].

The possible interaction between T and the autocrine EGF/TGFα loop is still controversial. To get further information on this issue, the effects of a treatment with T (10^{-10} M, the most effective dose in stimulating prostatic cell proliferation) on the binding characteristics of the EGF receptors have been studied. It has been found that, after 3 days of treatment, the concentration of EGF binding sites was significantly increased; the K_d values of these receptors for the ligand used ([^{125}I]EGF) were not modified. These observations are in line with those reported by some authors [15–17] but are in contrast with those published by another group [41]. The reason for these discrepancies may reside in the different experimental conditions adopted, namely the length of the experiments, the culture media used, the dose and/or the type of androgen, etc. Data similar to those presented here have recently been obtained by Liu and coworkers [18] by using another androgen-responsive human prostatic cancer cell line (ALVA101). These authors have shown that the treatment of ALVA101 cells with either T or DHT (10^{-8} M) results in a significant increase of EGF receptor mRNA levels. The last result suggests that T might act, at least partially, by increasing the activity of the EGF/TGFα system, possibly through an up-regulation of the EGF receptors.

GENERAL CONCLUSIONS

The present studies, aimed at investigating the mechanisms through which T exerts its action at the level of the tumor of the prostate, indicate that: (1) all the key enzymes involved in the metabolism of androgens are present in the human hormone-responsive prostatic cancer cell line, LNCaP; and (2) locally produced growth modulatory factors, both with inhibitory (LHRH-like) and with stimulatory (EGF/TGFα) activity, are expressed and active in LNCaP cells; both these systems may mediate the stimulatory action of T on prostatic tumor cell growth.

Acknowledgements—These studies were supported by A.I.R.C., by Consiglio Nazionale delle Ricerche through the special projects 'B.T.B.S.' contract No. 93.01103.PF70, 'A.C.R.O.' contract No. 93.02197.PF39, 'Aging' contract No. 93.00438.PF40, 'F.A.T.M.A.' contract No. 93.007008.PF41 and by MURST.

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