Pergamon

0960-0760(94)E0057-R

J. Steroid Biochem. Molec. Biol. Vol. 49, No. 4–6, pp. 347–350, 1994

0960-0760/94 $7.00 + 0.00

Androgen-dependent Prostatic Tumors: Biosynthesis and Possible Actions of LHRH

Patrizia Limonta,* Roberta M. Moretti, Donatella Dondi, Marina Montagnani Marelli and Marcella Motta

Center for Endocrinological Oncology, Department of Endocrinology, University of Milano, Via Balzaretti 9, 20133 Milano, Italy

Testosterone (T) is the major exogenous stimulus for the growth of prostatic carcinoma. It is believed that the proliferative action of T may be mediated by locally expressed growth modulatory factors. Recent evidence from our laboratory suggests that a LHRH (or a LHRH-like) loop might be expressed in human prostatic tumor cells. To verify this hypothesis, we have studied whether a mRNA for LHRH is expressed in the human androgen-responsive prostatic cancer cell line LNCaP, using the reverse transcription-polymerase chain reaction technique in the presence of a pair of specific oligonucleotide primers. A cDNA band of the expected size was obtained from LNCaP cells; this band hybridized with a ^{32}P-labeled LHRH oligonucleotide probe and its sequence showed a complete match with the reported sequence of the human placental LHRH cDNA. These observations indicate that the mRNA coding for LHRH is expressed in LNCaP cells and suggest that a LHRH (or a LHRH-like) peptide might be produced by these cells. To clarify the possible action of this peptide, LNCaP cells were grown in a steroid-free medium and treated with a LHRH antagonist. The treatment resulted in a significant increase of tumor cell growth. These data clearly indicate that the LHRH system expressed in LNCaP cells plays an inhibitory role on cell proliferation, and that this system seems to be regulated in a negative way by steroids. An EGF/TGFα autocrine stimulatory loop (peptides, receptors, intracellular signals) is also functional in these cells. Treatment of LNCaP cells grown in serum-free conditions (i.e. in the absence of exogenous growth factors) with a monoclonal antibody against the EGF receptor, or with immunoneutralizing antibodies against EGF or TGFα, resulted in a significant decrease of cell proliferation. T positively regulates this EGF/TGFα system by increasing the concentration of EGF binding sites. The present data indicate that an inhibitory LHRH (or LHRH-like) system is expressed in LNCaP cells and participates in the local mechanisms regulating tumor cell proliferation together with an EGF/TGFα stimulatory loop. Both systems appear to be modulated by T.

J. Steroid Biochem. Molec. Biol., Vol. 49, No. 4–6, pp. 347–350, 1994

INTRODUCTION

It is now well established that the growth of prostatic carcinoma is, at least in its early stages, largely dependent on androgens [1–5]. However, the molecular basis of the proliferative action testosterone (T) exerts at the level of the tumor is still not fully understood. It has been proposed that T might stimulate the growth of prostatic cancer through the modulation of the activity of locally produced growth regulatory factors [6–10]. Growth modulatory polypeptides, mainly endowed with stimulatory activity (e.g. EGF, TGFα, bFGF, NGF, etc.) have actually been reported to be produced by prostatic tumor cells [7–9, 11–14].

The human prostate cancer cell line LNCaP (Lymph Node Carcinoma of the Prostate) represents a very useful *in vitro* model for the study of androgen-dependent carcinoma, since it maintains the peculiar characteristics of the original tumor, particularly the androgen-responsiveness and the expression of androgen receptors [15–17]. Recently, we have shown that LHRH agonists (Zoladex and Buserelin) exert a direct and dose-dependent inhibitory action on the proliferation of LNCaP cells; this effect was found to be specific, since it was completely counteracted by the simultaneous treatment of the cells with a potent LHRH antagonist [18]. Moreover, we have reported

Proceedings of the XVI Meeting of the International Study Group for Steroid Hormones, Vienna, Austria, 28 Nov.–1 Dec. 1993.
*Correspondence to P. Limonta.

that binding sites for LHRH analogs are present on LNCaP cell membranes, particularly when the cells are cultured in the absence of steroids (RPMI-1640 medium supplemented with 5% charcoal stripped fetal calf serum, FCS-C) [18]. These data seem to indicate that, when utilized for the treatment of prostatic carcinoma, LHRH agonists might inhibit tumor growth not only by suppressing the activity of the pituitary–testicular axis, but also by exerting a direct and specific antiproliferative action at the level of the tumor.

It is well known that hypothalamic LHRH is rapidly degraded at the pituitary level [19] and that, consequently, the circulatory levels of the hormone are very low [20]. Therefore, the observation that LHRH receptors are expressed on LNCaP cells seems to suggest that LHRH (or a LHRH-like material) might be synthesized at the level of the prostate, and that this locally produced peptide might participate, with its receptors, in a growth modulatory loop, possibly endowed with inhibitory activity. The present experiments have been performed to verify: (a) the presence and the role of a LHRH (or LHRH-like) autocrine system in the androgen-dependent prostate tumor cell line LNCaP; (b) the role played by the EGF/TGFα growth factor system in the control of LNCaP cell growth; and (c) the possible effects exerted by androgens on the activity of these two peptidergic systems.

LHRH AS AN AUTOCRINE INHIBITORY LOOP IN LNCaP CELLS: NEGATIVE REGULATION BY ANDROGENS

The presence of a LHRH (or LHRH-like) autocrine loop in LNCaP cells has been investigated by studying whether the mRNA coding for LHRH is expressed in these cells. For these experiments, we have used the RT-PCR (reverse transcription-polymerase chain reaction) technique which allows the determination of specific mRNAs, even when they are present in low amounts. Total RNA was extracted, according to a modification of the guanidium thiocyanate/cesium chloride method [21], from LNCaP cells, from rat hypothalami (positive control), and from rat pituitaries (negative control). The RNAs were reverse transcribed by reverse transcriptase in the presence of an oligo$(dT)_{16}$ primer. The cDNA fragments were then amplified by PCR using two pairs of oligonucleotide primers, one pair for LNCaP cells, and the other pair for rat hypothalami and pituitaries. The sequence of the primers has been designed according to the reported sequence of the human and rat LHRH cDNAs, respectively [22]. According to the conditions adopted, a PCR product of 228 base pairs was expected. After the PCR reaction, the amplified cDNAs were electrophoresed on a 1.5% agarose gel stained with ethidium bromide. A cDNA fragment of the predicted size was obtained from LNCaP cells as well as from rat hypothalami; no band of this size could be obtained from rat pituitaries [Fig. 1(a)]. The cDNAs were then Southern blotted onto a nylon membrane [23] and hybridized with a ^{32}P-labeled LHRH oligonucleotide (17-mer) probe. Both cDNA bands obtained from LNCaP cells and from the rat hypothalami specifically hybridized with the ^{32}P-labeled probe [Fig. 1(b)]. Moreover, the analysis of the sequence of the cDNA obtained from LNCaP cells showed a complete match with the human placental LHRH cDNA sequence [22]. These results unequivocably demonstrate that a mRNA for LHRH is expressed in human prostatic tumor cells. In line with these data, Qayum *et al.* [24, 25] have reported the presence of a LHRH-like immunoreactive material in the culture media of LNCaP cells, particularly when grown in FCS-C supplemented medium. Taken together with our previous observations, these data indicate that a LHRH or LHRH-like system (mRNA, peptide, receptors) is expressed in LNCaP cells, and suggest that this system might participate in the local regulation of tumor cell growth. To verify the role of the locally produced LHRH, we have studied the effects of treatment with a LHRH antagonist ("Nal-Arg-LHRH", ANT, kindly provided by Dr W. Vale, The Salk Institute, La Jolla, CA) on LNCaP cell proliferation. These experiments have been performed using LNCaP cells cultured in FCS-C supplemented medium, since both LHRH receptors [18, 24] and LHRH-immunoreactivity [24, 25] have been found in cells grown in these culture conditions. The treatment with ANT results in a significant stimulation of prostatic tumor cell proliferation. It is interesting to note that a similar treatment with ANT did not modify the proliferation of LNCaP cells grown in the presence of steroids.

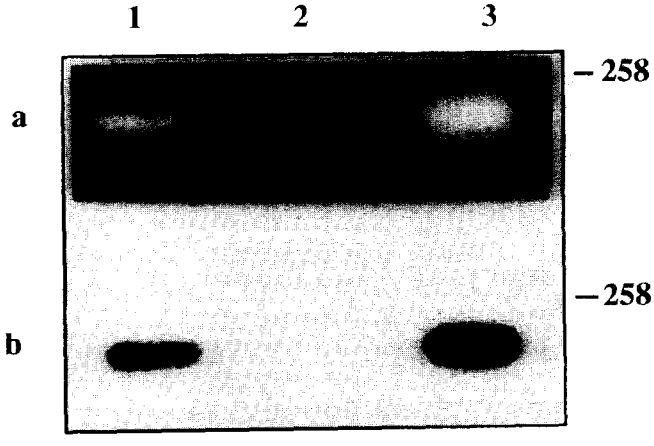

Fig. 1. Amplification of LHRH cDNA transcript from rat hypothalamus (lane 1), rat pituitary (lane 2) and LNCaP cells (lane 3) by RT-PCR. (a) Ethidium bromide stained agarose gel of the amplified cDNAs. (b) Autoradiography of the Southern blot obtained from the gel shown in (a) after the hybridization with a ^{32}P-labeled oligonucleotide LHRH probe.

In our opinion, two major considerations can be drawn from these data. First, the fact that the antagonist stimulates cell proliferation indicates that the LHRH (or LHRH-like) loop expressed in LNCaP cells plays an inhibitory role in the local regulation of tumor cell proliferation. As mentioned in the Introduction, several growth stimulatory peptides have been shown to be produced by prostatic tumor cells [7–9, 11–14]. The present data also point to the local expression of an inhibitory system. Second, the data which show that the treatment with the LHRH antagonist is effective in promoting cell growth only in cells cultured in the absence of steroids suggest that the production of LHRH, or the activity of the local LHRH system, may be particularly relevant in these culture conditions. This observation, when taken together with the finding that the receptors for LHRH agonists [18, 24] and the LHRH-like immunoreactivity [24, 25] are expressed only on LNCaP cell cultured in the absence of steroids, clearly points to the conclusion that the prostatic LHRH-like system may be negatively regulated by steroids, and possibly by testicular androgens. Finally, the fact that LHRH antagonists may exert, in given experimental conditions, proliferative effects on LNCaP cells, may suggest a word of caution in their clinical utilization. The authors do not want to overemphasize their results, which might only be due to the utilization of cell cultures grown in particular conditions; however, the present data suggest that the relationship between LHRH antagonistic analogs and normal and pathological cell proliferation should be given particular attention in the future, especially in connection with prostatic pathology.

EGF/TGFα AS AN AUTOCRINE STIMULATORY LOOP IN LNCaP CELLS: POSITIVE REGULATION BY ANDROGENS

The LHRH system present in LNCaP cells might inhibit cell proliferation by interfering with the activity of locally expressed growth stimulatory factors. The following studies have been performed to verify the role of the EGT/TGFα growth factor system in the control of the growth of prostatic tumor cells. The presence of an EGF dominated loop is suggested by the observation that LNCaP cells respond to EGF and TGFα with an increased growth rate [7, 10, 26–28], produce both EGF and TGFα polypeptides [7, 9, 11, 28] and express the EGF receptor [26–28]. Moreover, we have recently shown that, in LNCaP cells, the binding of EGF to its receptor is followed by an activation of intracellular signalling mechanisms, as indicated by the increased expression of the *c-fos* protooncogene.

To verify the functional significance of the EGF/TGFα loop, LNCaP cells were grown in serum-free conditions (RPMI-1640 medium containing 6.25 μg/ml of insulin and transferrin, 6.25 ng/ml of selenous acid, 1.25 mg/ml of bovine serum albumin), i.e. in the absence of exogenous growth factors, and treated either with a monoclonal antibody against the EGF receptor (Ab225, kindly donated by Dr J. Mendelsohn, Memorial Sloan-Kettering Cancer Center, New York, NY) or with antibodies able to immunoneutralize EGF and TGFα. A 4-day treatment with Ab225 (1 μg/ml) resulted in a significant decrease of LNCaP cell proliferation; this effect was completely reversed by the addition to the culture medium of EGF (0.5 μg/ml) (Fig. 2). Similar observations have recently been reported for the human androgen-independent prostate cancer cell lines DU 145 and PC3 [8, 12]. A 4-day treatment of LNCaP cells with either the anti-EGF or -TGFα antibodies, at the dose of 5 μg/ml, induced a significant inhibition of cell growth; the treatments resulted respectively, in a 59.4 and 58.8% inhibition of cell proliferation. Taken together, these data clearly confirm that an autocrine EGF/TGFα growth stimulatory system is functionally expressed in LNCaP cells.

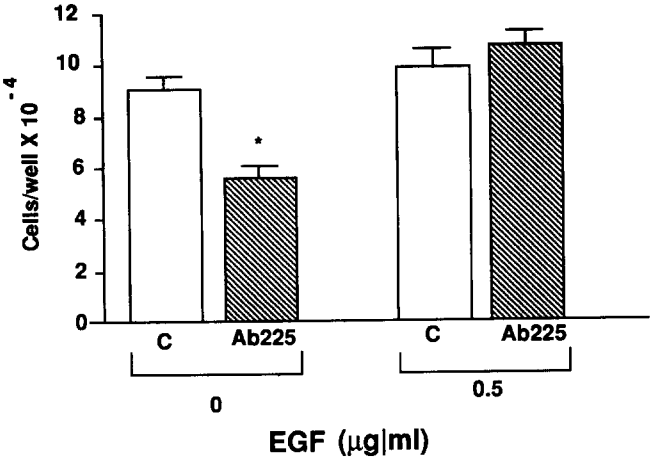

Fig. 2. Effects of a monoclonal antibody to the EGF receptor (Ab225, 1 μg/ml) on the proliferation of LNCaP cells cultured in serum-free conditions, either in the absence or in the presence of exogenous EGF. Results are expressed as mean cell number per well ± SE. C, Controls without Ab225. *$P < 0.05$ vs C.

To clarify whether the proliferative action of testicular steroids on prostate cancer might be mediated by the EGF/TGFα stimulatory loop, the effects of T on the binding parameters of the EGF receptor have been studied. LNCaP cells were grown in FCS-C supplemented medium and treated for 3 days with T at the dose of 10^{-10} M. This dose of the androgen has previously been shown to be effective in stimulating LNCaP cell growth. The treatment with T significantly increased the concentration of EGF receptors on LNCaP cells; the K_d values of EGF binding sites were not modified by the treatment. These observations are clearly in line with those previously reported by Schuurmans *et al.* [26, 28] and by Wilding *et al.* [7], but are in contrast with the data obtained by MacDonald

and Habib [10]. Differences in the experimental conditions adopted (cell culture media, length of the treatment, androgen used, etc.) may be responsible for the discrepancy. In conclusion, the present data suggest that the proliferative action of T on prostatic carcinoma might be mediated by an increase of the activity of the EGF/TGFα stimulatory loop, at least in terms of EGF binding sites.

CONCLUSIONS

The present data show that an inhibitory LHRH (or LHRH-like) system is functionally expressed in LNCaP cells; this system participates in the local regulation of tumor cell proliferation together with growth stimulatory systems, like those represented by T and by the growth factors of the EGF/TGFα family. It appears from the data that testicular steroids might stimulate prostate tumor growth by inhibiting the activity of the LHRH-like inhibitory loop and by increasing simultaneously the efficacy of the EGF/TGFα stimulatory loop via the increase of the EGF receptors.

Acknowledgements—This study was 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.

REFERENCES

1. Hodges C. V.: Hormone therapy of prostatic cancer. In *Endocrinology of Cancer* (Edited by D. P. Rose). C.R.C. Press, Boca Raton, Vol. II (1979) pp. 57–67.
2. Motta M. and Serio M.: *Hormonal Therapy of Prostatic Diseases: Basic and Clinical Aspects*. Medicom Europe, Amsterdam (1988).
3. Gittes R. F.: Carcinoma of the prostate. *New Eng. J. Med.* **324** (1991) 236–245.
4. Schroder F. H.: Endocrine therapy: where do we stand and where are we going? *Cancer Surv.* **11** (1991) 177–194.
5. Wilding G.: The importance of steroid hormones in prostate cancer. *Cancer Surv.* **14P** (1992) 113–130.
6. Sonnenschein C., Olea N., Pasanen M. E. and Soto A. M.: Negative controls of cell proliferation: human prostate cancer cells and androgens. *Cancer Res.* **49** (1989) 3474–3481.
7. Wilding G., Valverius E., Knabbe C. and Gelmann E. P.: Role of transforming growth factor-α in human prostate cancer cell growth. *The Prostate* **15** (1989) 1–12.
8. Hofer D. R., Sherwood E. R., Bromberg W. D., Mendelsohn J., Lee C. and Kozlowski J. M.: Autonomous growth of androgen-independent human prostatic carcinoma cells: role of transforming growth factor α. *Cancer Res.* **51** (1991) 2780–2785.
9. Knabbe C., Kellner U., Schmahl M. and Voigt K.-D.: Growth factors in human prostate cancer cells: implications for an improved treatment of prostate cancer. *J. Steroid Biochem. Molec. Biol.* **40** (1991) 185–192.
10. MacDonald A. and Habib F. K.: Divergent responses to epidermal growth factor in hormone sensitive and insensitive human prostate cancer cell lines. *Br. J. Cancer* **65** (1992) 177–182.
11. Connolly J. M. and Rose D. P.: Production of epidermal growth factor and transforming growth factor-α by the androgen-responsive LNCaP human cancer cell line. *The Prostate* **16** (1990) 209–218.
12. Connolly J. M. and Rose D. P.: Autocrine regulation of DU 145 human prostate cancer cell growth by epidermal growth factor-related polypeptides. *The Prostate* **19** (1991) 173–180.
13. MacGrogan D., Saint-Andre J. P. and Dicou E.: Expression of nerve growth factor and nerve growth factor receptor genes in human tissues and in prostatic adenocarcinoma cell lines. *J. Neurochem.* **59** (1992) 1381–1391.
14. Zuck B., Goepfert C., Nedlin-Chittka A., Sohrt K., Voigt K.-D. and Knabbe C.: Regulation of fibroblast growth factor-like protein(s) in the androgen-responsive human prostate cancer cell line LNCaP. *J. Steroid Biochem. Molec. Biol.* **41** (1992) 659–663.
15. Horoszewicz J. S., Leong S. S., Kawinski E., Karr J. P., Rosenthal H., Ming Chu T., Mirand E. A. and Murphy G. P.: LNCaP model of human prostatic carcinoma. *Cancer Res.* **43** (1983) 1809–1818.
16. Veldscholte J., Ris-Stalper C., Kuiper G. G. J. M., Jenster G., Berrevoets C., Claassen E., van Rooij H. C. J., Trapman J. and Brinkmann A. O.: A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. *Biochem. Biophys. Res. Commun.* **17** (1990) 534–540.
17. Culig Z., Klocker H., Eberle J., Kaspar F., Hobisch A., Cronauer M. V. and Bartsch G.: DNA sequence of the androgen receptor in prostatic tumor cell lines and tissue specimens assessed by means of the polymerase chain reaction. *The Prostate* **22** (1993) 11–22.
18. Limonta P., Dondi D., Moretti R. M., Maggi R. and Motta M.: Antiproliferative effects of luteinizing hormone-releasing hormone agonists on the human prostatic cancer cell line LNCaP. *J. Clin. Endocr. Metab.* **75** (1992) 207–212.
19. Horsthemke B., Knisatschek H., Rivier J., Sandow J. and Bauer K.: Degradation of luteinizing hormone-releasing hormone analogues by adenohypophyseal peptidases. *Biochem. Biophys. Res. Commun.* **100** (1981) 753–759.
20. Nett T. M., Akbar A. M. and Niswender G. D.: Serum levels of luteinizing hormone and gonadotropin-releasing hormone in cycling, castrated and anestrous ewes. *Endocrinology* **94** (1974) 713–718.
21. Rambaldi A., Young D. C. and Griffin J. D.: Expression of the M-CSF (CSF-1) gene by human monocytes. *Blood* **69** (1987) 1409–1413.
22. Adelman J., Mason A. J., Hayflyck J. S. and Seeburg P. H.: Isolation of the gene and hypothalamic cDNA for the common precursor of gonadotropin-releasing hormone and prolactin release-inhibiting factor in human and rat. *Proc. Natn. Acad. Sci. U.S.A.* **83** (1986) 179–183.
23. Wood W. I., Gitschier J., Lasky L. A. and Lawn R. M.: Base composition-independent hybridization in tetramethylammonium chloride: a method for oligonucleotide screening of highly complex gene libraries. *Proc. Natn. Acad. Sci. U.S.A.* **82** (1985) 1585–1588.
24. Qayum A., Gullick W. J., Clayton R. C., Sikora K. and Waxman J.: The effects of gonadotrophin releasing hormone analogues in prostatic cancer cell lines. *Br. J. Cancer* **62** (1990) 96–99.
25. Qayum A., Gullick W. J., Mellon K., Krausz T., Neal D., Sikora K. and Waxman J.: The partial purification and characterization of GnRH-like activity from prostatic biopsy specimens and prostatic cancer cell lines. *J. Steroid Biochem. Molec. Biol.* **37** (1990) 899–902.
26. Schuurmans A. L. G., Bolt J. and Mulder E.: Androgen receptor-mediated growth and epidermal growth factor induction in the human prostate cell line LNCaP. *Urol. Int.* **44** (1989) 71–76.
27. Eaton C. L., Davies P., Harper M., France T., Rushmere N. and Griffiths K.: Steroids and the prostate. *J. Steroid Biochem. Molec. Biol.* **40** (1991) 175–183.
28. Schuurmans A. L. G., Bolt J., Veldscholte J. and Mulder E.: Regulation of growth of LNCaP human prostate tumor cells by growth factors and steroid hormones. *J. Steroid Biochem. Molec. Biol.* **40** (1991) 193–197.