The Expression of Inducible cAMP Early Repressor (ICER) Is Altered in Prostate Cancer Cells and Reverses the Transformed Phenotype of the LNCaP Prostate Tumor Cell Line

Ghassan Yehia, Reza Razavi, Elisabeth Memin, Florence Schlotter, and Carlos A. Molina

Abstract

Inducible cAMP early repressor (ICER) has been shown to be an important mediator of cAMP antiproliferative activity. In this report, it was found that cAMP retards LNCaP cell growth; in contrast, cAMP inhibits the growth of PC-3 and DU-145 cells. ICER protein levels were markedly reduced in prostate cancer epithelial cells and undetectable and uninducible by cAMP in LNCaP and DU 145 cells. Forced expression of ICER in LNCaP cells caused inhibition of cell growth and thymidine incorporation and halted cells at the G1 phase of the cell cycle. These ICER-bearing LNCaP cells were rendered unable to grow in soft agar and unable to form tumors in nude mice. These results suggest that deregulation of ICER expression may be related to carcinogenesis of the prostate gland.

Introduction

The prostate gland requires androgens to sustain cellular growth. Seventy to 80% of prostate cancers are initially responsive to androgen deprivation therapies. Progression to a more malignant, androgen-independent (also termed androgen-resistant, -insensitive, or -refractory or hormone-escaped) state occurs, and the tumor becomes refractory to treatment (1, 2). Androgen insensitivity represents a poorly understood dilemma to the treatment of prostate cancer. The elucidation of the molecular mechanism leading to androgen insensitivity may lead to alternative cures for prostate tumors. Deregulation of the transcriptional components of the cAMP/PKA signal transduction pathway might be involved in the acquisition of the androgen-independent phenotype.

A large family of transcription factors mediates the nuclear response to the cAMP pathway (3). The best characterized of these factors are the CRE-binding protein and CREM proteins. These factors bind to CREs, regulating transcription from the promoters of cAMP-responsive genes. CRE-binding and CREM genes encode several isoforms that can act as activators or repressors of cAMP-induced transcription. The CREM gene contains two alternative promoters termed P1 and P2 (4). P1 is a housekeeping promoter that directs the expression of the several transcriptional activators (CREMα, CREMβ, CREMγ, CREMδ) as well as the expression of several transcriptional repressor (CREMα-, β-, γ-, etc.). Most of the CREM isoforms emanating from P1 are regulated by PKA phosphorylation (Ref. 3 and references therein). The P2 promoter is strongly induced by cAMP by virtue of four CREs in tandem (4). The induced isoform, termed ICER, essentially consists of a DNA-binding domain, functions as a powerful repressor of cAMP-mediated gene expression, and is not directly regulated by PKA phosphorylation (4). ICER constitutes a negative autoregulatory control mechanism by repressing its own production. A family of four isoforms named ICER-I, ICER-Iγ, ICER-II, and ICER-IIγ are collectively referred to as ICER.

ICER-Iγ inhibits the growth and DNA synthesis of murine pituitary tumor cells and human choriocarcinoma cells (5, 6). This alteration in cell growth is coupled with the reduced ability of ICER-Iγ-expressing cells to grow in an anchorage-independent manner and to form tumors in nude mice. Under these criteria, ICER-Iγ has the characteristic of a tumor suppressor gene product that mediates the antiproliferative activity of cAMP. In this report, we studied the effect of activation of the cAMP pathway on the growth of the prostate cancer cell lines LNCaP, DU 145, and PC-3, the expression, and cAMP-mediated inducibility of the CREM gene in all three cell lines, and the effect of ICER-forced expression on the growth and tumorigenicity of LNCaP cells. We found that cAMP differentially affects the growth of LNCaP, PC-3, and DU-145 cells. We propose that these differences might be related to the observed reduction in ICER protein levels in prostate cancer epithelial cells and to the lack of ICER inducibility in LNCaP and DU 145 cells. Finally, we showed that forced expression of ICER in LNCaP cells inhibited cell growth and reversed their transformed phenotype. These results suggest that ICER might be acting as a novel tumor suppressor gene product in certain prostate cancers.

Materials and Methods

Cell Culture and Generation of Cell Clones. The human prostate cancer cell lines LNCaP, PC-3, and DU 145; the nonneoplastic adult human prostatic epithelial cells PWR-1E; and the human choriocarcinoma IEG-3 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured as recommended. Several cell clones were generated from the LNCaP cells. The cell clones were established by cotransfection of a neomycin-resistant gene and ICER-II cDNA under the human metallothionein promoter (4–7). The anti-tubulin monoclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and used as suggested by the supplier.

Received 2/7/01; accepted 6/21/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 USC Section 1734 solely to indicate this fact.

This work was in part supported by NIH Grant R29 CA69316 and by a New Jersey Cancer Commission Research Award (to C. A. M.).

1 The first two authors contributed equally to this report.

2 To whom requests for reprints should be addressed, at UMDNJ-New Jersey Medical School, Obstetrics/Gynecology & Women’s Health, 185 S. Orange Avenue, Newark, NJ 07103. Phone: (973) 972-4197. Fax: (973) 972-4574; E-mail: molinaca@umdnj.edu.

3 The abbreviations used are: PKA, cAMP-dependent protein kinase or protein kinase A; CRE, cAMP-response element; CREM, CRE-modulator; ICER, inducible cAMP early repressor; BPH, benign prostatic hyperplasia.

4 The abbreviations used are: PKA, cAMP-dependent protein kinase or protein kinase A; CRE, cAMP-response element; CREM, CRE-modulator; ICER, inducible cAMP early repressor; BPH, benign prostatic hyperplasia.

5 The abbreviations used are: PKA, cAMP-dependent protein kinase or protein kinase A; CRE, cAMP-response element; CREM, CRE-modulator; ICER, inducible cAMP early repressor; BPH, benign prostatic hyperplasia.
The human prostate tissues used in this study were formalin-fixed, paraffin-embedded archival blocks donated by two regional hospitals (University Hospital at Newark, NJ and East Orange Veterans Administration Hospital, NJ). The tissues had been removed at surgery, fixed in 10% neutral buffered formalin for 16–24 h, processed, and embedded in paraffin. Serial 4-μm sections were then stained with H&E or used in this study. Two pathologists independently evaluated the samples for diagnostic verification. Immunohistochemical staining for ICER protein on paraffin-embedded specimens was performed with a peroxidase-labeled streptavidin-biotin technique using the Zymed Histostain SP Kit following the recommended procedure. Before immunostaining, the samples were boiled in a microwave oven for 10 min to achieve complete denaturation of mouse ICER riboprobe (4). The oligonucleotides used to amplify human full-length ICER cDNA were CGG GAT CCA ACA TGG CTG TAA CTG GAGA and CGG AAT TCA TGC TGT AAT CAG TTT ATAG. All sequences were 5′-3′. ICER cDNA was inserted into pBluescriptIIsk to create the DNA binding domain was subcloned into pBluescriptIIsk to create the DNA binding domain was subcloned into pBluescriptIIsk. The antisense riboprobe was generated using T3 polymerase after digestion with BamHI to linearize the plasmid. For loading control, a human glyceraldehyde-3-phosphate dehydrogenase riboprobe was purchased from Ambion (Austin, TX) and used as directed by the supplier. This riboprobe was linearized by XbaI and used as a loading control. For loading control, a human glyceraldehyde-3-phosphate dehydrogenase riboprobe was purchased from Ambion (Austin, TX) and used as directed by the supplier. This riboprobe was linearized by XbaI and used as a loading control.

To determine the effect of cAMP on the growth of LNCaP, PC-3, and DU 145 cells, cells were treated for 5 days with 8-Br-cAMP (Fig. 1B). The number of cells was determined each day. We found that cAMP retarded the growth of LNCaP, and it completely inhibited the growth of PC-3 and DU 145 cells (Fig. 1B). These differences in cell growth were reflected in differences in DNA synthesis of the cAMP-treated cells as shown in Fig. 1A. cAMP-treated cells had a 25% reduction in DNA synthesis compared with 50 and 70% reduction in PC-3 and DU 145 cells, respectively, was observed (Fig. 1C).

Cell cycle analysis of LNCaP cells revealed that 10–15% more cAMP-treated cells were G1 when compared with the untreated control, accounting for the same reduction in the number of cells in the S phase (Fig. 1D). Approximately 50% more cAMP-treated PC-3 cells were in the S phase when compared with the untreated control, accounting for the same reduction in the number of cells in the G1 phase. The cell cycle profile of DU 145 cells was minimally affected by treatment with cAMP. These data demonstrate that cAMP differentially affects the growth and cell cycle of LNCaP, PC-3, and DU 145 cells.

Anchorage-independent Cell Growth and tumorigenicity Studies.

Twenty thousand cells of each indicated LNCaP cell line were seeded on soft agar, and colony formation was assessed after 14 days as described elsewhere (6). Nude mice were injected s.c. with 4 × 10^6 of the indicated LNCaP cell line clone suspended in 0.5 ml of a 50% solution of complete media and Matrigel (Becton Dickinson, Bedford, MA). The animals were studied for 3–5 weeks and scored for tumor formation as described elsewhere (6). Animals were cared for according to the New Jersey Medical School guidelines.

Results

cAMP Affects the Growth of LNCaP, PC-3, and DU 145 Cells.

The effect of cAMP on the morphology of LNCaP, PC-3, and DU 145 cells was determined after 16 h of treatment with 8-Br-cAMP. The photomicrographs shown in Fig. 1A demonstrate that cAMP altered the morphology of all three cell lines. LNCaP and PC-3 cells developed neuroendocrine-like differentiation of these cells (10–12). DU 145 cells did not develop neuroendocrine processes, but many cells that are more refractory were observed. These changes in cell morphology may be related to the effect of cAMP on cell growth.

To determine the effect of cAMP on the growth of LNCaP, PC-3, and DU 145 cells, cells were treated for 5 days with 8-Br-cAMP (Fig. 1B). The number of cells was determined each day. We found that cAMP retarded the growth of LNCaP, and it completely inhibited the growth of PC-3 and DU 145 cells (Fig. 1B). These differences in cell growth were reflected in differences in DNA synthesis of the cAMP-treated cells as shown in Fig. 1A. cAMP-treated cells had a 25% reduction in DNA synthesis compared with 50 and 70% reduction in PC-3 and DU 145 cells, respectively, was observed (Fig. 1C).

Cell cycle analysis of LNCaP cells revealed that 10–15% more cAMP-treated cells were G1 when compared with the untreated control, accounting for the same reduction in the number of cells in the S phase (Fig. 1D). Approximately 50% more cAMP-treated PC-3 cells were in the S phase when compared with the untreated control, accounting for the same reduction in the number of cells in the G1 phase. The cell cycle profile of DU 145 cells was minimally affected by treatment with cAMP. These data demonstrate that cAMP differentially affects the growth and cell cycle of LNCaP, PC-3, and DU 145 cells.

Altered Expression of ICER in Primary Prostate Tumors and in LNCaP, PC-3, and DU 145 Cells.

We hypothesized that this previously observed differential response to cAMP was related to the difference in cell cycle composition of the tumor cell lines. The rate of DNA synthesis was assessed by incorporation of [3H]thymidine (1 μCi/ml) into DNA, as described before (6). Cells were fixed with 5% trichloroacetic acid, and trichloroacetic acid-insoluble radioactivity was isolated and determined by scintillation counting.

The number of cells in each stage of the cell cycle was determined by flow cytometric analysis of cellular DNA content after staining ethanol-fixed and 1 mg/ml of RNase A (Sigma Chemical Co.)-treated cells in a solution of 50 μg/ml propidium iodide. The obtained data were analyzed to determine the percentage of cells in each stage of the cell cycle (G1, S, and G2-M) using ModFit V3.0 (PMac) software.
expression of the transcription factors responsible for the nuclear response to cAMP in LNCaP, DU 145, and PC-3 cells. The expression of CREM protein was determined in nonneoplastic adult human prostatic epithelial PWR-1E cells and compared with LNCaP, PC-3, and DU 145 cells after treatment with 8-Br-cAMP (Fig. 2, A and B).

We found that the transcriptional activator CREM\(\text{\textregistered}\) was strongly expressed in all of the four cell lines, and its expression was not affected by cAMP. ICER protein was detected in nontreated PWR-1E cells and maximally induced after 1 h of cAMP treatment, returning to basal levels 5 h after treatment. On the contrary, ICER protein was barely detectable in LNCaP and DU 145 cells, and 8-Br-cAMP failed to induce ICER protein in both cell lines. In PC-3 cells, ICER protein was detected and induced after 12 h of cAMP treatment with sustained induction after 24 h of treatment. These results demonstrate that the expression of ICER protein is altered in certain prostate cancer cells in culture.

On the basis of these results we hypothesized that ICER protein expression would be altered in prostate cancer. The expression of CREM and ICER protein was determined by immunohistochemistry in several prostatic lesions and compared with normal prostate tissue (Fig. 2C). We found that in normal prostate tissue, CREM/ICER was predominantly expressed in the nucleus of luminal epithelial cells and not in stromal cells (not shown). Similarly, CREM/ICER was highly expressed in the nucleus of luminal epithelial cells in BPH tissue (not shown). As demonstrated before (4) CREM/ICER expression was strictly nuclear (not shown). The number of positive-stain nuclei was determined in three samples with normal prostate tissue, in seven samples with BPH, and in seven samples with prostate cancer. We found that the number of ICER/CREM-expressing nuclei was reduced by at least 50% in all of the tested tissues with prostate cancer when compared with normal or BPH tissue. In conjunction, all these results suggested that CREM/ICER expression might be altered in some types of human prostatic lesions.

The observed altered expression and induction of ICER protein in prostate cancer cells might be related to the lack of expression and induction of ICER RNA. To test this assumption, the expression of ICER RNA was determined in LNCaP, PC-3, and DU 145 cells and compared with CREM RNA expression in the human choriocarcinoma JEG-3 cells (Fig. 3). As shown previously in JEG-3 cells (4, 6), ICER RNA is strongly induced after 1 h of treatment with 8-Br-cAMP. Both LNCaP and DU 145 cells showed no or insignificant induction of ICER RNA. These data indicate that the previously observed (Fig. 2B) lack of ICER protein induction in these two cell lines might be attributable primarily to the lack of RNA induction. Compared with the JEG-3 cells, PC-3 cells showed a delayed induction of ICER RNA after 5 h of treatment, in accordance with the previously observed delay in ICER protein induction in Fig. 2B. These data demonstrate that the observed lack of ICER expression and induction in LNCaP and DU 145 cells is attributable to the lack of expression of ICER RNA. In addition, these data suggest that deregulation of ICER in some prostate tumors might be at the level of ICER gene expression.

**Forced Expression of ICER Affects the Growth and Tumorigenicity of LNCaP Cells.** We next explored the effect of forced expression of ICER on the growth and tumorigenicity of LNCaP cells. LNCaP cell clones expressing ICER-II\(\text{\textregistered}\) under the control of an inducible metallothionein promoter (5, 6) were generated. Fig. 4A shows a representative Western blot of three independent ICER-expressing clones (LNICER 14, 16 and 30) and a control neomycin-resistant clone (LNeo). All of the clones showed low expression of ICER protein and a robust augmentation upon induction with CdCl\(\text{\textregistered}\). Basal expression of ectopic ICER caused an altered cellular phenotype that is not neuroendocrine-like (Fig. 4B). The most obvious difference was that the nucleus of ICER-expressing cells was easily discernible and contained, in most cases, fragmented nucleoli when compared with control cells. This phenotype was observed in all ICER-expressing clones studied. The physiological consequences of this observation may be related to the fact that several nucleoli are formed in early G\(\text{\textregistered}\) phase of the cell cycle before fusing into a single nucleolus in late G\(\text{\textregistered}\) (13). This observation suggests that ICER-expressing LNCaP cells have an altered G\(\text{\textregistered}\) phase.

The growth of ICER-expressing cells was compared with the growth of LNCaP parental cells and Neo-resistant control LNCaP clones. Fig. 4C shows that basal levels of ectopic ICER severely retards LNCaP cell growth, and that cell growth was completely inhibited in the presence of high levels of ICER expression. ICER expression minimally affected the cell cycle profile of these cells, demonstrating that most cells (70\%–75\%) are arrested in G\(\text{\textregistered}\) (Fig. 4D). Indeed, thymidine incorporation in ICER-expressing cells was found to be severely diminished when compared with Neo-resistant control clones (Fig. 4E). These data demonstrate that ICER blocks the growth of LNCaP cells by inhibiting DNA synthesis.

The ability of cells to grow in an anchorage-independent manner and to form tumors in athymic nude mice are hallmarks of cellular transformation and are considered indispensable to determine tumor-
igenicity of a given cell line. To this end, the ability of LNCaP clones to grow in an anchorage-independent manner and to form tumors in nude mice was assessed (Fig. 5, A and B). We found that the ICER-expressing LNCaP cells formed 80–90% fewer colonies than both the parental LNCaP and LNeo cells (Fig. 5A). These results demonstrate that ICER inhibits the ability of LNCaP cells to grow in an anchorage-independent manner. We then injected nude mice with LNeo or LNICER cell lines, and tumor formation was assessed after 3 weeks (Fig. 5B). We observed no or very small tumor formation in all of the ICER-expressing LNCaP cell clones tested. Conversely, LNeo cells developed large tumors, as originally shown for the parental LNCaP cell line (14). These data show that ICER inhibits the formation of tumors in nude mice.

Discussion

About 60% of advanced prostate cancers contain loss of heterozygosity at chromosome 10q23, resulting in the lack of expression of the tumor suppressor PTEN (15). It has been found that mutations other than loss of heterozygosity at 10q23 are also related to prostate cancer (16–22). These results suggested that inactivation of one or more tumor suppressor genes on the short arm of chromosome 10 play an important role in the late stages of prostate cancer. Our results suggest that the expression of ICER protein in some primary prostate tumors might be significantly lower than in normal prostate (Fig. 2C). ICER expression and inducibility was found to be abrogated in LNCaP and DU 145 cells and abnormal in PC-3 cells. Reintroduction of ICER in...
LNCaP cells totally reversed their neoplastic characteristics. These results suggest that ICER may be one of these other putative tumor suppressors residing in chromosome 10p, because the human ICER-coding region is mapped to chromosome 10p11 (9). The determination of ICER on human prostate primary tumors and in prostate tumor cell lines will ultimately determine whether the observed alteration in ICER expression is related to prostate cancer.

Other than mutations, other molecular mechanisms may explain the fact that ICER protein and RNA expression and inducibility were found abnormal in prostate cancer cells. For example, the RNA of other members of the CREM family has been found to be regulated by alternative polyadenylation sites (8). It is possible that the lack of ICER inducibility is the result of an unstable ICER mRNA. Another possibility is abnormal regulation of ICER promoter (P2) by DNA methylation, histone acetylation, or transcription factors. These other possibilities and the relationship between this phenomenon and its occurrence in human prostate cancer are presently being investigated.

CREM-mutant mice have been generated by homologous recombination resulting in the sterility of male mice attributable to a postmeiotic arrest at the first step of spermatogenesis (23, 24). Judging by the apparent pleiotropic function of the CREM gene products, such specific phenotype was surprising. Nevertheless, it must be noted that the CREM homozygous mutant mice lacked activators and repressors such as CREM+ and ICER. Therefore, this phenotype cannot be attributed to the lack of expression of a specific CREM activator or repressor. We hypothesized that the balance mechanism controlling CREM-mediated gene expression in these mice might be affected in two opposite directions, resulting in mutual cancellation without other apparent phenotypic differences. This might explain why the CREM-null mice have not been reported to be tumor prone. In the future, the generation of ICER-specific null mice could help clarify these discrepancies and shed light on the role of ICER in tumorigenesis.

Acknowledgments

We thank Nuris E. Portuondo for her assistance in the preparation of this manuscript.

References

The Expression of Inducible cAMP Early Repressor (ICER) Is Altered in Prostate Cancer Cells and Reverses the Transformed Phenotype of the LNCaP Prostate Tumor Cell Line

Ghassan Yehia, Reza Razavi, Elisabeth Memin, et al.