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Regulation of Growth, PSA/PAP and Androgen Receptor Expression by 1α,25-Dihydroxyvitamin D_3 in the Androgen-Dependent LNCaP Cells

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The involvement of vitamin D in prostate carcinogenesis was investigated using the human prostatic LNCaP cells. Incubation of the LNCaP with 100 nM 1α,25-dihydroxyvitamin D_3 for 2 days resulted in a 30–40% suppression of cell growth, which was accompanied by a greater than 70% down-regulated expression of the proliferating cell nuclear antigen (PCNA). The intracellular and secreted forms of PSA showed a 2-fold increase following a 48 h culture in the presence of vitamin D_3. The vitamin D_3-elicited PSA increases were preceded by an induction of androgen receptor (AR) expression, as measured by Western blot analysis and by binding assays using [^{3}H]R1881 as the ligand. These results are consistent with the hypothesis that the growth inhibitory effects of vitamin D_3 is partially mediated through its ability to modulate PCNA expression. Moreover, vitamin D_3 may effect increases in PSA expression indirectly by up-regulating androgen receptors.

Since the early 1980s, the classical role of vitamin D as a regulator of bone and mineral metabolism has been expanded to include control of cell growth and differentiation. The pioneering observation by Abe et al. (1) that 1α, 25-dihydrovitamin D_3, the active metabolite of vitamin D, suppresses growth and induces differentiation of myeloid M1 leukaemia cells has since been extended to both normal and abnormal human myeloid cells and a variety of nonleukemic cancer cells (2, 3). More recently, data from a number of epidemiological and laboratory studies have provided support for the involvement of vitamin D in prostate carcinogenesis (4–8). The effects of vitamin D is presumably mediated through interaction with vitamin D receptors. Consistent with such a mechanism, vitamin D receptors have been demonstrated in both primary and established prostate cells (8–10).

Prostate specific antigen (PSA), a 34-kDa tissue-specific glycoprotein, is a kallikrein-like serine protease produced exclusively by epithelial cells lining the acini and ducts of the prostate gland (11–13). In recent years much emphasis has been given to understanding the ways in which PSA is controlled at the genetic level (14–18), in large part due to the general acceptance that PSA levels appear to be important for monitoring responses of prostate cancer patients to different therapies (19–23). By contrast, much more limited information is currently available with regard to the pathways controlling PSA biosynthesis and processing. In a recent report the synthetic androgens were found to regulate the synthesis and secretion of PSA in the LNCaP cells (24), the only androgen-responsive prostatic cell line capable of expressing PSA (25).

Although several studies have reported on changes in secreted PSA in LNCaP cells treated with vitamin D_3 (7–10), the mechanism for the elicited PSA increase, however, has not been completely elucidated. Because PSA gene is controlled by androgens acting via interaction with androgen receptors (AR), we reasoned that the effects of vitamin D_3 on PSA may be coupled with changes in AR expression. Results supporting such a hypothesis are presented in this communication.

MATERIALS AND METHODS

RPMI 1640, fetal bovine sera (FBS), and antibiotics were purchased from Gibco. Charcoal-stripped fetal bovine sera (CS-FBS) were obtained from HyClone Lab. Both 5α-dihydrotestosterone (DHT) and [^{3}H]-R1881 (methyltrienolone) were

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from NEN-DuPont. The Tandem-E immunoenzymatic kit was purchased from Hybritech, San Diego. Antibodies against proliferating cell nuclear antigen (PCNA) and PSA were obtained from Santa Cruz and Dako, respectively.

Cell cultures and proliferation assay. LNCaP cells from the ATCC were maintained in RPMI 1640 culture media supplemented with 10% heat-inactivated FBS, 5 mM glutamine, 50 units/ml of penicillin G and 50 μg/ml of streptomycin as described previously (18, 24, 25). The cells were routinely seeded at 1×10^5 cells/ml in T-75 flasks, allowed to attach overnight, then treated with 10 or 100 nM 1,25-dihydroxyvitamin D_3. After 24 and 48 h, cells were harvested by trypsinization. Media from control and treated cells were saved for PSA analysis. LNCaP cell proliferation was assessed by counting control and treated cells. Cell viability was determined by trypan blue dye exclusion.

Maintenance of LNCaP cells in CS-FBS. Two ml of cells maintained in FBS were added to 6-well plates at a density of 1×10^5 cells/ml. Cells were kept in FBS or switched to 10% CS-FBS for 24 h to reduce steroids present in FBS, before treatment with 10 or 100 nM vitamin D_3. Binding to labelled R1881 was assayed 24 h post vitamin D_3 treatment, as described below.

AR binding assays. Control and vitamin D_3-treated cells maintained in FBS or CS-FBS were incubated with plain media or with media supplemented with 1.25 μM unlabeled R1881, for 15 min. They were labelled with 5 nM [^{3}H]-R1881 (87 Ci/mmol, NEN) for 2 h at 37°C. Labeled cells were washed with PBS to remove unbound ligand, then solubilized in 0.3 N NaOH-ethanol (4:1) for 5–10 min, after which the incorporated radioactivity was determined by scintillation counting. Specific binding (cpm/10^6 cells) is defined as the difference in counts between samples incubated with [^{3}H]-R1881 only and those that had the label with a 500-fold excess "cold" R1881. Typically a 5-fold difference in counts was found between the two conditions.

Measurement of intracellular and secreted PSA and PAP. The assay was based on the quantitative binding of PSA or PAP to the alkaline phosphatase-conjugated anti-PSA or anti-PAP monoclonal IgG and the cleavage of p-nitrophenyl phosphate by the IgG-tagged alkaline phosphatase, yielding colored products that can be quantified by measuring absorbance at 405 and 450 nm. Postmitochondrial extracts were prepared from FBS- or CS-FBS-treated cells using buffers supplemented with multiple protease inhibitors as described previously (26–28).

Western blot analysis. Postmitochondrial supernatants (5 μg) from control and vitamin D_3-treated cells were separated on 10% SDS-PAGE. The presence of PCNA and PSA was scored using monospecific antibodies directed against PCNA and PSA. Immunoreactivity was demonstrated by chemiluminescence (ECL) or by color reaction.

RESULTS

Growth parameters. Figure 1 shows that growth of LNCaP cells was significantly inhibited (25–30%) by a 24 h or 48 h exposure to vitamin D_3. Suppression of cellular proliferation was correlated with a 50–65% reduction in expression of PCNA, which is commonly used as a mitotic index to evaluate proliferative activity in mammalian cells (29, 30).

Induction of intracellular and secreted PSA and PAP by vitamin D_3. The ability of vitamin D_3 to regulate prostate-specific genes was investigated. Both intracellular and secreted PSA and PAP

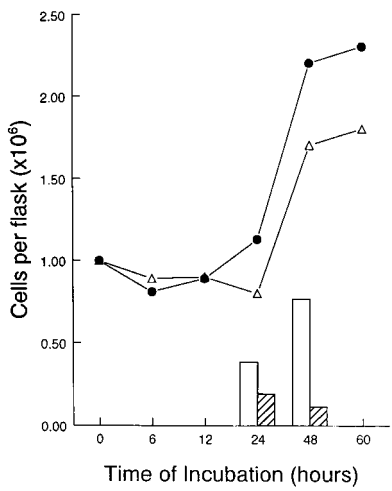

FIG. 1. Growth of LNCaP cells with (△) or without (●) the addition of 100 nM 1,25-dihydroxyvitamin D_3. PCNA expression was evaluated by Western blot analysis and appeared as bar graphs. Open bars, control samples; hatched bars, vitamin D_3-treated samples.

were elevated following a 24 h and 48 h treatment with vitamin D_3 (Figure 2A and 2B). PAP was induced earlier and to a greater degree than was PSA; a 4-fold increase in PAP was typically seen at 24 h whereas PSA was only elevated 2-fold at 48 h. PAP but not PSA measurements in both control and treated cells exhibited large fluctuations between experiments.

Coordinated induction in AR and PSA. Since PSA gene responds to androgens, presumably via interaction with androgen receptors, changes in AR were also measured by Western blot analysis in both control and treated cell extracts. Results in Figure 3 show that AR expression was increased several-fold after a 24 h exposure to vitamin D_3 and preceded the 2- to 3-fold 48 h increase in PSA. As a control for accuracy of protein loading and efficiency of transfer, expression of actin was also quantified by Western blot analysis and found to remain essentially unchanged. To further check the validity of the observed PSA increase, different amounts of lysates and media from control and treated cells were assayed for PSA; the PSA increase was directly proportional to the amount of lysates added (data not shown).

Measurement of AR expression by binding to [^{3}H]R1881. The binding of the control and treated cells to [^{3}H]R1881 was also studied, using cells cultured with FBS or with CS-FBS. Results in Table 1 show that vitamin D_3 increased the binding of radioactive R1881 by 2- to 3-fold, which was in close agreement with the Western blot data.

DISCUSSION

Results of these experiments show that vitamin D_3 suppresses prostate cell growth and effects increases in both intracellular and secreted PSA and PAP expression. The growth modulatory effects of vitamin D_3 have been reported previously (6–10). Using LNCaP cells cultured for prolonged period of time in 5% CS-FBS, Miller et al. (8) reported that vitamin D_3 stimulated the growth of LNCaP cells in a concentration-dependent manner. In contrast, Skowronski et al. (7)

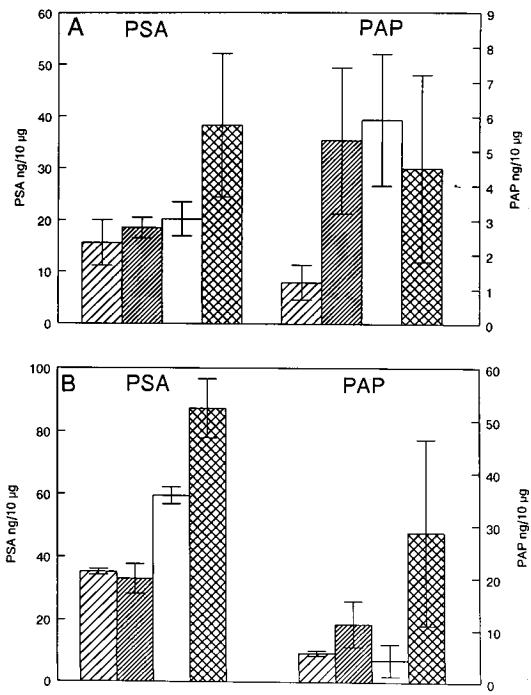

FIG. 2. Effects of 1,25-dihydroxyvitamin D_3 on intracellular (panel A) and secreted (panel B) PSA and PAP in LNCaP cells. Broad hatched bars, control, 24 h; fine hatched bars, 100 nM vitamin D_3-treated samples, 24 h; open bars, control, 48 h; crossed bars, vitamin D_3-treated samples, 48 h. Each point represents the mean of 3 experiments.

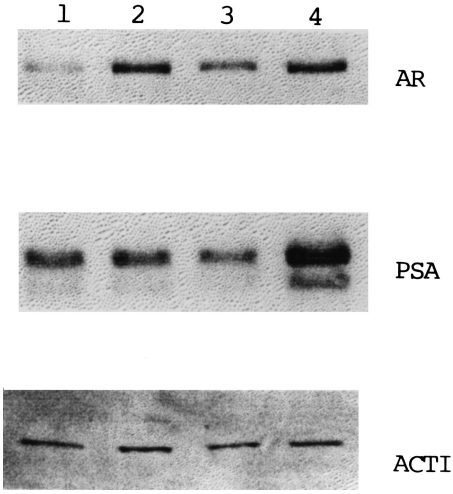

FIG. 3. Relative changes in the expression of the androgen receptor (AR), PSA, and actin in control and 100 nM vitamin D_3-treated LNCaP cells. Western blot analysis was performed by ECL on control and treated postmitochondrial cell extracts. The figures came from Hewlett Packard scans of the original exposed films. Lanes 1 and 2, 24 h samples; lanes 3 and 4, 48 h samples. Lanes 1 and 3, controls; lanes 2 and 4, vitamin D_3-treated samples.

provided evidence showing that physiological levels of vitamin D_3 markedly reduced LNCaP cell proliferation. Furthermore, it was suggested that the growth modulatory effects of vitamin D_3 were significantly dependent on the culture media and conditions (8). In this study, we show that 10–100 nM vitamin D_3 consistently produced a 30–40% inhibition of LNCaP cell growth, irrespective of whether the cells were maintained in FBS (Figure 1) or in CS-FBS for up to 2 days (data not shown). The vitamin D_3-elicited growth suppression was correlated with the down regulation of PCNA (Figure 1).

The ability of vitamin D_3 to regulate changes in secreted PSA has previously been reported by other laboratories (8–10), using culture conditions different from the ones described in this report. For example, Miller et al. (8) cultured LNCaP cells in 5% CS-FBS and observed 1–10 nM vitamin D_3 to change secreted PSA only after 3 days of treatment. No significant effects of vitamin D_3 could be demonstrated after a 24 h and 48 h treatment. Furthermore, 100 nM vitamin D_3 was largely ineffective in inducing PSA change. In the studies reported by Feldman and coworkers (7,9), cells were maintained in culture for at least 48–72 h before treatment with vitamin D_3 was

TABLE 1
AR Binding Activities in Control and Vitamin D_3-Treated LNCaP cells

Culture condition		Specific AR binding (cpm/10^6 cells)[a]
Experiment One: cells cultured with FBS		
Control		16,780
Vitamin D_3-treated	10 nM	26,550
	100 nM	37,750
Experiment Two: cells cultured with CS-FBS		
Control		18,890
Vitamin D_3-treated	10 nM	34,190
	100 nM	52,490

[a] Nonspecific binding amounting to 10.7–16.2% of total ligand bound were subtracted. The values were also normalized for cell numbers.

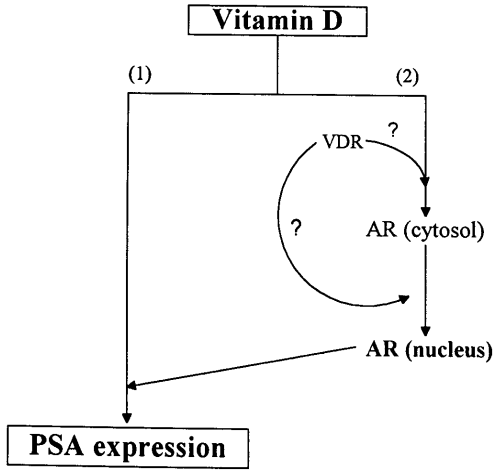

FIG. 4. Proposed pathways for PSA regulation by vitamin D_3. Pathway 1 may involve the phosphoinositide-mediated signalling reaction sequences. Pathway 2 may involve the increased translocation of the AR from the cytosol to the nucleus.

initiated (as opposed to 24 h in our case). Neither published reports measured possible changes in AR as a result of exposure to vitamin D_3.

To our knowledge results reported here is the first time showing an effect of vitamin D_3 on increasing both intracellular and secreted PSA and PAP, as well as AR. Vitamin D_3 may act by activating PKC (pathway 1, Figure 4), as has been reported for muscle, bone, and human leukaemia HL-60 cells (31–33). Alternatively, vitamin D_3 could facilitate increased translocation of protein molecules, e.g., the AR, from the cytosol to the nucleus, either singly or via cooperative interaction with PKC- or vitamin D receptor (VDR)-mediated steps (pathway 2). These and other possible mechanism of action of vitamin D_3 are currently being investigated.

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