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Progestins and Androgens Increase Expression of Spot 14 in T47-D Breast Tumor Cells

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Enhanced expression of fatty acid synthase and other lipogenic enzymes has been observed in a subset of breast cancers with poor prognosis. This phenomenon has been related to amplification of a gene on chromosome region 11q13 encoding Spot 14, a putative regulator of lipogenic enzyme expression. In this paper we demonstrate that the induction of lipogenesis by progestins and androgens in the breast cancer cell line T47-D is accompanied by a marked increase in the expression of Spot 14. These data corroborate the correlation between Spot 14 expression and increased lipogenesis. Moreover they show that apart from gene amplification there is another steroid-regulated pathway that may enhance Spot 14 expression and lipogenesis in tumor cells. © 2000 Academic Press

Key Words: **breast cancer; steroid receptors; lipids; gene expression.**

The "Spot14" (S14) gene encodes a small (≈17 kDa) acidic (pI 4.9) protein that is predominantly expressed in tissues producing lipids such as the liver, white and brown adipose tissue and the lactating mammary gland (1). Its expression is rapidly induced by hormonal, metabolic and developmental stimuli that induce lipid synthesis such as thyroid hormone, carbohydrate intake, adipose tissue differentiation and lactation (1, 2). The immunohistochemical finding that S14 is located in the cell nucleus (3) and the observation that S14 antisense oligonucleotides disrupt the lipogenic effects of glucose and thyroid hormone in hepatocytes (4) suggest that S14 may act as a transcriptional regulator responsible for the induction of lipogenic enzymes.

Aberrant expression of S14 may be clinically relevant under conditions where lipid metabolism is markedly disturbed. This is for instance the case in human tumors overexpressing fatty acid synthase (FAS) and other lipogenic enzymes such as acetyl-CoA carboxylase (ACC). Overexpression of lipogenic enzymes has been observed in subsets of many cancers including breast, prostate, ovarian and colon tumors, and it has been demonstrated that this phenomenon is accompanied by a poor prognosis. Moreover, growth of these tumors seems to be critically dependent on increased lipogenesis as pharmacological inhibition of FAS results in apoptosis (5–9).

Recent evidence indicates that S14 resides on the telomeric end of a chromosome region (11q13) that is frequently amplified in breast tumors and that signals a poor prognosis. In a panel of primary breast tumors, amplification of this region has been shown to be accompanied by expression of S14 and of a key lipogenic enzyme: ACC, suggesting that S14 may provide a pathophysiological link between two prognostic indicators in breast cancer: enhanced lipogenesis and 11q13 amplification (10).

In some human breast tumor cell lines such as T47-D, progestins and androgens markedly stimulate lipid synthesis and accumulation (11). In the present paper we examined whether this steroid-induced lipid synthesis is accompanied by induction of S14 expression.

MATERIALS AND METHODS

Cell culture. The human breast cancer cell line T47-D was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cultures were trypsinized twice weekly and were maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 10 mM Hepes buffer pH 7.4 (Life Technologies, Paisley, Scotland), 4.5 g/L glucose (Merck, Darmstadt, Germany), 0.6 μg/ml insulin (Sigma Chemical Co, St. Louis, MO), 1.0 mM sodium pyruvate (Life Technologies), 100 μg/ml streptomycin and 100 U/ml penicillin (Life Technologies). To study the effects of steroids, cells were plated in dishes and cultured in medium containing 5% dextran-coated charcoal-stripped FCS (CT-FCS), prepared as described by Leake *et al.* (12). Steroids were dissolved in ethanol and added to the cultures. Final ethanol concentrations did not exceed 0.1%. Control cultures received similar amounts of ethanol only. Natural steroids were obtained from Sigma Chemical Co. R1881 (methyltrienolone) was purchased from Dupont-New England Nuclear (Boston, MA). Org 2058 was obtained from Amersham (Buckinghamshire, United Kingdom). Casodex (bicalutamide) was kindly provided by

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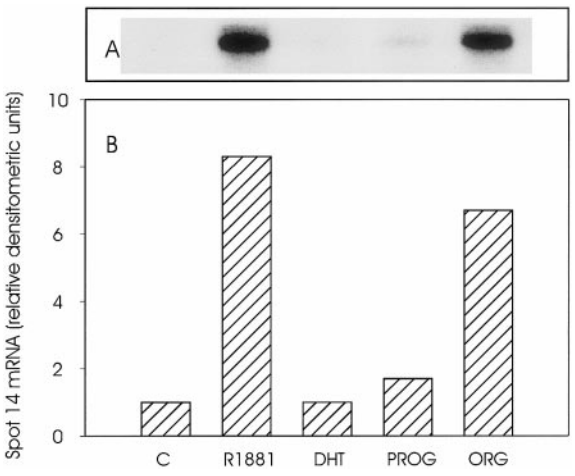

FIG. 1. Effects of androgens and progestins on S14 mRNA expression in T47-D cells. T47-D cells were cultured in the presence of R1881, DHT, progesterone (prog), Org 2058 (ORG) or ethanol vehicle (C) for 2 days. RNA was isolated and a ribonuclease protection assay was performed as described under Materials and Methods. Protected fragments were visualized by autoradiography (A). Levels of S14 mRNA were quantitated using PhosphorImager screens. Values are expressed as relative densitometric units, taking the values obtained in the absence of added steroids (C) as 1 (B).

Zeneca Pharmaceuticals (Manchester, United Kingdom) and is a trademark of Zeneca Ltd. RU486 (mifepristone) was obtained from Biomol (Plymouth Meeting, PA).

RNA isolation. T47-D cells were seeded in 150-mm dishes at a density of 3×10^6 cells/dish in medium containing 5% CT-FCS. Two days later, medium was replaced and cells were treated with steroids and/or steroid receptor antagonists or ethanol vehicle. After the indicated period of time, cell cultures were washed with phosphate-buffered saline (PBS; Life Technologies), quick-frozen in liquid nitrogen, and stored at −80°C. Total RNA was prepared by using a modified guanidinium/CsCl ultracentrifugation method as described (13).

Ribonuclease protection assay. A 345-bp S14 fragment was generated by PCR using the primer pair listed as S14 345 by Moncur *et al.* (10) and cDNA from T47-D cells as template. The resulting PCR fragment was cloned into a pGEM-T vector (Promega, Madison, WI) and verified by nucleotide sequencing using an ALF (automated laser fluorescence) sequencer (Pharmacia, Uppsala, Sweden). After linearization with *ApaI*, the plasmid was used to generate radiolabeled antisense S14 transcripts using a Riboprobe *in vitro* transcription system (Promega) with [α-^{32}P] CTP (Amersham, 400 Ci/mmole). Gel-purified transcripts (20,000 cpm) were hybridized with 20 μg of total RNA of T47-D cells (treated with steroids, steroid receptor antagonists or ethanol vehicle for the indicated period of time) and digested with RNase A/T1 by using the Hyb Speed Ribonuclease Protection kit (Ambion, Uden, The Netherlands). Protected fragments were separated by electrophoresis on a 4% acrylamide/8 M urea gel and visualized by autoradiography. Hybridization signals were quantitated with PhosphorImager screens (Molecular Dynamics, Sunnyvale, CA).

RESULTS AND DISCUSSION

In a series of preliminary experiments we confirmed earlier observations (11, 14–16) showing that treatment of T47D breast tumor cells with progestins (in our experiments: Org 2058) or androgens (in our experiments: R1881) results in: increased synthesis of triglycerides (as measured by incorporation of 2-^{14}C-acetate); induction of FAS mRNA (as measured by Northern blotting); and increased intracellular accumulation of neutral lipids (as shown by staining with oil red O). Increased lipid accumulation could also be demonstrated with the natural steroids progesterone and 5α-dihydrotestosterone (DHT) but because of the rapid metabolism of these compounds the latter effects were less pronounced (data not shown).

In view of recent evidence indicating that increased production of FAS and other lipogenic enzymes in breast tumor samples may be related to increased expression of Spot 14, a putative metabolic integrator that increases lipogenesis both in normal and in cancerous tissues under a number of conditions (2), we explored whether androgens and progestins affect Spot 14 expression in T47D cells. Since antibodies for immunological detection are not routinely available we developed an Rnase protection assay to measure Spot 14 expression.

Figure 1 illustrates that both R1881 and Org 2058 markedly stimulate Spot 14 mRNA steady state levels. The average level of induction in a series of independent experiments was 7.0 ± 2.0 (mean ± SD; n = 5) for R1881 (10^{-8} M) and 4.4 ± 2.0 (n = 3) for Org 2058. Under the same experimental conditions no effects were observed with DHT and only marginal effects with progesterone (Fig. 1) or testosterone (not shown). A dose-response curve with R1881 revealed that stimulation was already evident at 10^{-10} M and that maximal stimulation was seen at about 10^{-7} M (Fig. 2). Time course studies demonstrated a detectable increase after 16 h of treatment with R1881 and a max-

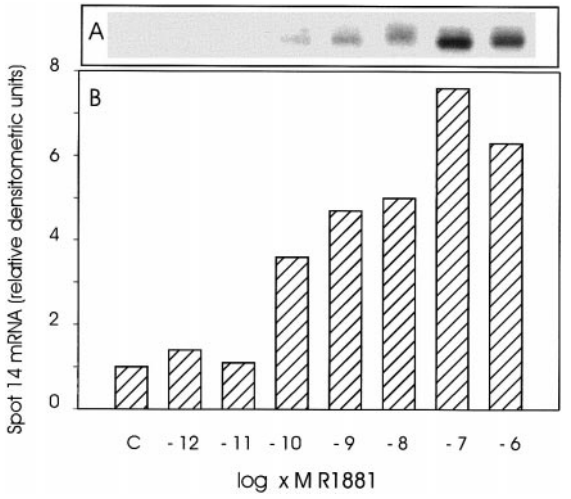

FIG. 2. Dose dependence of the effects of R1881 on S14 mRNA expression in T47-D cells. T47-D cells were cultured for 48 h in the absence (C) or in the presence of increasing concentrations of R1881. RNA was isolated and a ribonuclease protection assay was performed as described under Materials and Methods (A). Levels of S14 mRNA were quantitated using PhosphorImager screens. Values are expressed relative to the values of the control condition (C) (B).

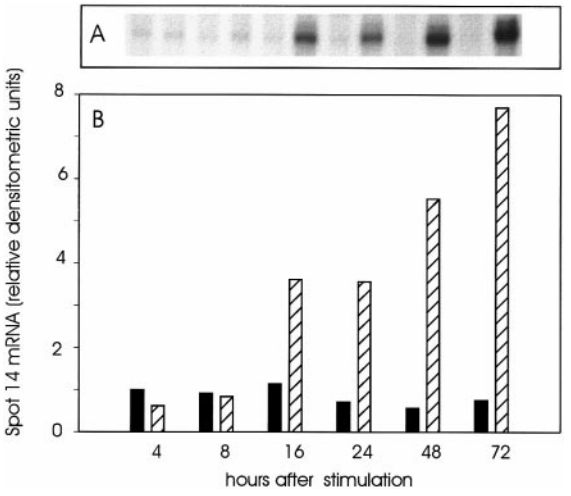

FIG. 3. Time course of the response of S14 mRNA to R1881 in T47-D cells. T47-D cells were cultured in the absence (black bars) or presence of 10^{-8} M R1881 (hatched bars). At the indicated periods of time, RNA was extracted and subjected to a ribonuclease protection assay as described under Materials and Methods (A). Levels of S14 mRNA were quantitated using PhosphorImager screens, and mRNA values were expressed as relative densitometric units taking the value of untreated cells at the 4-h time point as 1 (B).

imal response after about 2 days (Fig. 3). These responses are relatively slow as compared to those observed in hypothyroid rats given T3, where a 20-fold induction is already evident after 4 h of treatment (17). Accordingly the possibility should be considered that the induction by steroid hormones may be indirect.

Since R1881 is known to stimulate not only androgen but also progesterone receptors we tried to use antagonists to distinguish between these two signaling pathways. At the lower concentration of R1881 (10^{-10} M) the effect on Spot 14 expression is apparently mediated by the androgen receptor. Indeed, as shown in Fig. 4 (left panel) induction by R1881 is almost completely inhibited by Casodex (10^{-5} M), a selective androgen receptor antagonist, and by RU486 which possesses both androgen receptor and progesterone receptor antagonistic properties. At higher concentrations of R1881 (10^{-8} M) however, induction appears to be mediated primarily by the progesterone receptor (Fig. 4, right panel). Under these conditions 80% inhibition is observed with RU486 whereas Casodex enhances rather than inhibits the effect of R1881. Similar effects of both antagonists are observed when the progestin Org 2058 rather than R1881 is used as an inducer. At the moment we can only speculate about the mechanism underlying the unexpected but consistent potentiating effects of Casodex in the presence of high concentrations of R1881 and Org 2058. The effectiveness of the androgen receptor antagonist at low concentration (and not at high concentration) of R1881 can probably be explained by the fact that the concentration of the progesterone receptor in T47D cells is up to 40-fold higher than that of the androgen receptor (18). Accordingly, at lower concentrations of the agonist a higher fraction of R1881 may be bound to the androgen receptor and displacement by an excess of Casodex may be easier to demonstrate.

To the best of our knowledge this is the first demonstration that sex steroids modulate the expression of S14 in cancer cells. The implication of this finding is that, apart from the amplification of the S14 gene observed in breast tumors and breast cancer cell lines including T47D (10), a second (steroid-controlled) pathway may contribute to increased S14 expression and the accompanying stimulatory effects on the expression of lipogenic enzymes. The exact relationship between steroid hormone receptor activation, S14 expression and enhanced lipogenesis certainly warrants further investigation. Nonetheless, the above described data seem to corroborate a number of recent reports demonstrating an association between FAS expression and progesterone receptor status (19, 20) or estrogen and progesterone receptor status in breast tumors (21) and in endometrial carcinoma (22). It should be stressed, however, that S14 may not represent the only link between steroids and increased expression of lipogenic enzymes in hormone responsive tumors. In the prostate cancer cell line LNCaP e.g., androgen exposure results in a marked accumulation of lipids provoked by activation of FAS and several

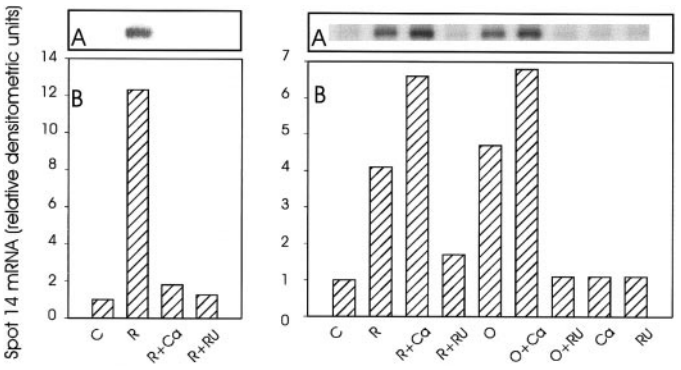

FIG. 4. Effects of androgen and progestin agonists and antagonists on S14 mRNA expression in T47-D cells. Left panel: T47-D cells were cultured in the presence of 10^{-10} M R1881 (R), the combination of 10^{-10} M R1881 and either 10^{-5} M Casodex (Ca) or 10^{-7} M RU486 (RU), or ethanol vehicle (C) for 2 days. RNA was isolated and a ribonuclease protection assay was performed as described under Materials and Methods (A). Levels of S14 mRNA were quantitated using PhosphorImager screens. Values are expressed as relative densitometric units, taking the values obtained in the absence of added steroids (C) as 1 (B). Right panel: T47-D cells were treated with ethanol vehicle (C), 10^{-8} M R1881 (R), the combination of 10^{-8} M R1881 and either 10^{-5} M Casodex (Ca) or 10^{-5} M RU486 (RU), 10^{-8} M Org 2058 (O), the combination of 10^{-8} M Org 2058 and either 10^{-5} M Casodex or 10^{-5} M RU486, 10^{-5} M Casodex or 10^{-5} M RU486 for 2 days. RNA was isolated and a ribonuclease protection assay was performed as described under Materials and Methods (A). Levels of S14 mRNA were quantitated using PhosporImager screens. Values are expressed relative to the values of the control condition (B).

other lipogenic enzymes, but in this case the effect of androgens appears to be mediated by sterol regulatory element-binding protein (SREBP), a transcription factor with a pivotal role in intracellular lipid homeostasis (23–25). Preliminary experiments revealed no stimulatory effects of androgens on S14 expression in LNCaP cells. Conversely we were unable to show steroid-induced nuclear accumulation of active SREBP in T47-D cells (data not shown).

In conclusion, we show that apart from gene amplification there is another, steroid-regulated pathway that may enhance Spot 14 expression and lipogenesis in tumor cells. The question should be raised whether the clinical and prognostic significance of increased lipogenesis induced by these two pathways is identical.

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