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Inhibition of c-*myc* in breast and ovarian carcinoma cells by 1,25-dihydroxyvitamin D_3, retinoic acid and dexamethasone

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The role and regulation of the c-*myc* protooncogene in breast and ovarian neoplasms is receiving increased attention. The downregulation of the c-*myc* protooncogene by 1,25-dihydroxyvitamin D_3 (calcitriol), retinoic acid (RA) and dexamethasone (Dex) is closely associated with growth inhibition in leukemic cells. Calcitriol, RA and Dex have anti-proliferative activity in breast and gynecologic carcinoma cells; however, the regulation of c-*myc* by these agents in breast and ovarian cancers is mostly unknown. We have addressed the regulation of c-*myc* in these cancers using an adaptation of a novel method which employs an immunohistochemical procedure to detect c-*myc* protein followed by quantification of c-*myc* staining with computerized image analysis. This system represents an alternative to protein product assay by Western blotting and is straightforward, rapid (1 day), can be carried out on a small scale and provides a sample size that readily facilitates statistical analysis of assay data. In MCF-7 human breast cancer cells, c-*myc* was suppressed 29% by 0.5 nM Dex, 45% by 0.01 nM RA and 54% by 100 nM calcitriol after 24 h of drug treatment. At the same hormone concentrations, growth was inhibited 18% by Dex, 18% by RA and 39% by calcitriol after 3 days of treatment ($p < 0.05$ for all hormones). Similar patterns of growth and c-*myc* inhibition were seen in T47D human breast cancer cells and NIH:OVCAR3 human ovarian cancer cells, with the exception of Dex in T47D cells, which caused no inhibition of c-*myc* or growth. Parallel control experiments in MCF-7, NIH:OVCAR3 and T47D cells showed that none of the three hormones suppressed epithelial membrane antigen, a non-growth-related protein. This suggested that the hormones specifically influenced c-*myc* expression. In conclusion, this study has shown that c-*myc* was repressed by calcitriol, RA and Dex in breast and ovarian carcinoma lines where these same hormonal agents also inhibit growth, and suggests that these hormonal agents have commensurate effects on c-*myc* expression and growth in the breast and ovarian carcinoma lines examined.

Key words: Breast neoplasms, hormones, oncogenes, ovarian neoplasms.

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Introduction

The c-*myc* protooncogene is amplified in breast and ovarian carcinomas,[1,2] and the role and regulation of c-*myc* in breast and ovarian cancers are beginning to receive considerable attention. Particularly in breast cancer, data suggest important roles for c-*myc*. Elevated c-*myc* is associated with breast cancer tumorigenicity in nude mice and with invasiveness in humans.[3,4] Amplification of c-*myc* was reported to have a strong association with reduced disease-free interval and reduced survival in breast cancer patients,[5] but smaller studies did not support these conclusions.[4,6] C-*myc* antisense oligonucleotides, which specifically repress c-*myc* protein levels, caused growth inhibition of breast cancer cells.[7]

The hormone-related anticancer agents, hydroxytamoxifen and medroxyprogesterone acetate downregulate c-*myc* and inhibit the growth of breast cancer cells, while estradiol stimulates c-*myc* expression and DNA synthesis.[8,9] Retinoic acid (RA) has been shown to repress c-*myc* expression in an ovarian carcinoma line.[10] To our knowledge, these are the only hormonal agents to be examined for their effects on c-*myc* levels in breast and ovarian cancers. The downregulation of the c-*myc* by 1,25-dihydroxyvitamin D_3 (calcitriol), (RA) and dexamethasone (Dex) has been shown to be closely associated with growth inhibition and onset of differentiation in leukemic cells.[11–13] Furthermore, calcitriol, RA and Dex have anti-proliferative activity in breast and gynecologic carcinoma cells.[11,14–18] Thus, c-*myc* levels may be repressed by these hormonal agents in breast and ovarian carcinoma cells.

The novel method of Czerniak[19] was adapted to address this question. This technique employs an immunohistochemical procedure to detect c-*myc* protein which was then quantified utilizing

computerized image analysis. Data are presented that show repression of c-*myc* protein levels by calcitriol, RA and Dex in one ovarian and two breast cancer cell lines.

Materials and methods

Tissue culture

NIH:OVCAR3 ovarian carcinoma cells and T47D human breast carcinoma cells were purchased from ATCC (Rockville, MD) and grown as a monolayer in Dulbecco's modified eagle medium with HAMS F12 nutrients, 10 μg/ml insulin, 10% donor calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. MCF-7 human breast cancer cells were a gift from Dr Herbert Soule of the Michigan Cancer Foundation and were grown in the same complete medium with 5% donor calf serum. Stock cultures were maintained in T-75 Corning flasks (Baxter Scientific) and transferred to 8-well Lab-Tek slides (Nunc, Naperville, IL) at a density of 10 000 cells/well for experiments. All cells were grown in a humidified 5% CO_2 incubator at 37°C. After 24 h, cells were exposed to fresh medium containing the appropriate concentration of Dex, RA or calcitriol. Dex and RA were obtained from Sigma (St Louis, MO), and calcitriol was obtained from Duphar (Amsterdam, The Netherlands). Hormone treatments and controls were set up in triplicate wells and all were equalized with respect to ethanol vehicle at a concentration below 0.1%.

Immunohistochemistry

After a 24 h incubation with the appropriate hormone, slides were rinsed twice with cold (4°C) phosphate buffered saline (PBS, pH 7.4) and fixed with cold acetone on ice for 10 min. Endogenous peroxidases were blocked with a 3% solution of H_2O_2 in methanol for 10 min at room temperature. After three 2 min rinses in PBS the wells were removed from the slides leaving the attached cells. C-*myc* and Epithelial Membrane Antigen (EMA) were detected immunohistochemically using a Histostain-SP biotin–streptavidin kit (Zymed Laboratories, San Francisco, CA), following the company's protocol. Primary monoclonal antibodies specific for c-*myc*[20] and EMA[21] were obtained from Cambridge Research Biochemicals (Atlanta Beach, NY) and Zymed, respectively. Cells from all treatments were blocked with serum from the species in which the secondary antibody was raised, prior to incubation with primary antibody. Background treatments were incubated with 3% bovine serum albumin (BSA) in PBS in place of primary antibody. Incubation with anti-c-*myc* was carried out at 37°C for 1 h and the incubation with anti-EMA was carried out at room temperature for 1 h. Incubation with a species-specific biotinylated secondary antibody followed. The slides were then incubated with a streptavidin–peroxidase conjugate and visualized with nickel enhanced diaminobenzidine (Zymed). C-*myc* and EMA staining were quantified by placing the stained slides on an Olympus BH12 light microscope and measuring light absorbance using a computerized image analysis system consisting of an MCID™ software package (Imaging Research, St Catherines, Ontario, Canada) operating on a Compaq™ 486 Computer. Nuclear-specific c-*myc* staining was measured by determining the amount of light absorbed by c-*myc* or EMA staining in the cell nuclei. Results are expressed as relative optical density (ROD) units, which are proportional to the concentration of the immunostained antigen, c-*myc* and EMA in this case. EMA staining, though not confined to the nucleus, was measured similarly in order to provide a more closely matched control. Background ROD values were subtracted from control and treatment values to give antigen-specific ROD, which was used for data analysis. The data were evaluated by analysis of variance with Scheffe post-hoc comparisons using an Apple Macintosh Computer™ and Statview 512™ statistical software.

Measurement of growth inhibition

The conditions for drug exposure, concentration and duration were arrived at by prior (unpublished) experiments in this laboratory. For each experiment, cells were harvested at confluence with a trypsin-EDTA solution (Sigma), then passed into a 24-well plate (Corning Glass Works, NY), in quadruplicate. After 24 h incubation, the appropriate hormone was added in 1 ml of fresh complete media. The cells were grown 3 days in the presence of hormone. Cell numbers were then determined by lysing the cells and counting nuclei using the method of Butler.[22] Briefly, the medium was removed and 1 ml hypotonic buffer (0.01 M HEPES, pH 7.4, 1.5 mM $MgCl_2$) was added. The cells were allowed to swell for 5 min. Then, 200 μl lysis solution was added and the plate was shaken for 10 min. The lysis solution consisted of 3 ml

glacial acetic acid and 5 g ethylhexadecyldimethylammonium bromide (Eastman-Kodak Co., Rochester, NY) in 97 ml deionized water. Finally, 1 ml saline:formaldehyde (9 g NaCl/l, 12.5 ml 37% formaldehyde/l) was added. Complete cell lysis was confirmed visually using an inverted microscope. Nuclei were counted using a Coulter Counter Model ZM with a 100 μm aperture (Coulter Electronics, Hialeah, FL). Treatment effects were evaluated by analysis of variance and Sheffe's post-hoc comparisons[23] using Statview™ software on an Apple Macintosh™ computer.

Western blotting

Cells were lysed at 4°C and the lysate was electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel, followed by transfer to nitrocellulose membranes as described by Towbin *et al.*[24] with minor modifications recommended by RU Simpson (personal communication). After transfer, the sheet was blocked overnight at room temperature with a gelatin solution (20 mM Tris buffer, pH 8.2, 150 mM sodium chloride, 0.02% sodium azide, 0.02 g/ml gelatin, 0.1% Tween 20). The membranes were then cut into strips and incubated overnight at 4°C with 5 μg/ml 610E anti-c-*myc* antibody, in 20 mM Tris buffer, pH 7.2, 150 mM sodium chloride, 0.1% BSA, 0.2% Tween 20 (Buffer B). The strips were then incubated overnight at 4°C with 67 ng/ml goat anti-mouse immunoglobulin conjugated to peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA), in Buffer B. The strips were washed in Buffer B and enzymatically reacted with diaminobenzidine (DAB Substrate Kit) from Pierce (Rockford, IL).

Results

The first two experiments in this study involve the establishment of procedural details and documentation of assay reliability. The remaining experiments probe the effects of calcitriol, RA and Dex on c-*myc* levels. In order to determine the number of cells necessary to provide precise estimates of the population means in each culture well, NIH:OVCAR3 cells were immunostained for c-*myc*. The ROD values for 50 cells were randomly collected. Figure 1 shows a running mean plot for 2–50 cells and indicates that the mean stabilized after analysis of 30 cells. This is consistent with sample size used in other image analysis model systems.[25] Based on these results, 50 cells were analyzed per well to ensure a sufficiently large well sampling to produce accurate estimates of the population means.

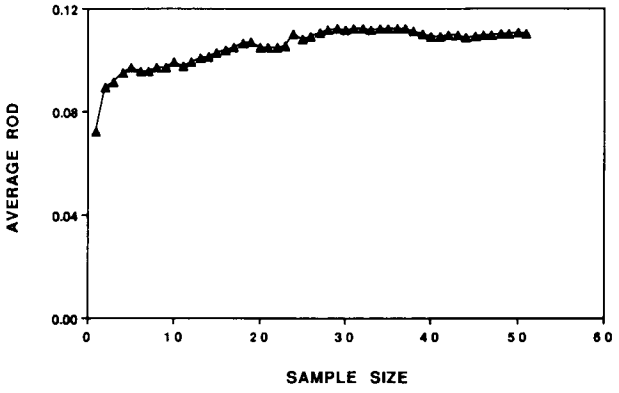

Figure 1. Running mean analysis of cell sampling for c-*myc* quantification. NIH:OVCAR3 cells were plated 10 000/well on 8-well Lab-Tek slides and grown for 48 h as described in Materials and methods. C-*myc* protein was assayed using immunohistochemistry followed by digital image analysis. The running average, in ROD, was calculated for 2–50 cells and plotted against the sample size.

Concern has been raised that immunohistochemical assays exhibit a wide range of inter-assay variability. In the present studies, cell lines were used in order to provide homogeneity in c-*myc* expression, cell size and cell shape, all of which would contribute to consistent readings by the image analyzer. Also, cells were taken out of culture, fixed and assayed immediately, thus eliminating storage parameters as variables. Therefore, variations in image analyzer light readings should be attributable to changes in c-*myc* levels in response to hormone treatments. To deterine inter-assay variability, c-*myc* staining from four independent assays of MCF-7 cells were evaluated (Table 1). The mean c-*myc* level was 0.027 ROD ± 0.003 SD. The calculated inter-assay coefficient of variation (CV) was 10.9%. This compares favorably

Table 1. Inter-assay reproducibility of c-*myc* expression in MCF-7 cells

Mean (ROD)	SD	CV (%)
0.027	0.003	10.9

Cells were plated on 8-well Lab-Tek slides (10 000/well) and grown for 48 h. C-*myc* protein was assayed using immunohistochemistry and digital image analysis. Average c-*myc* levels, in ROD, were calculated for 150 cells in each of four separate experiments. The resulting average values were used to calculate the mean, SD and CV between assays.

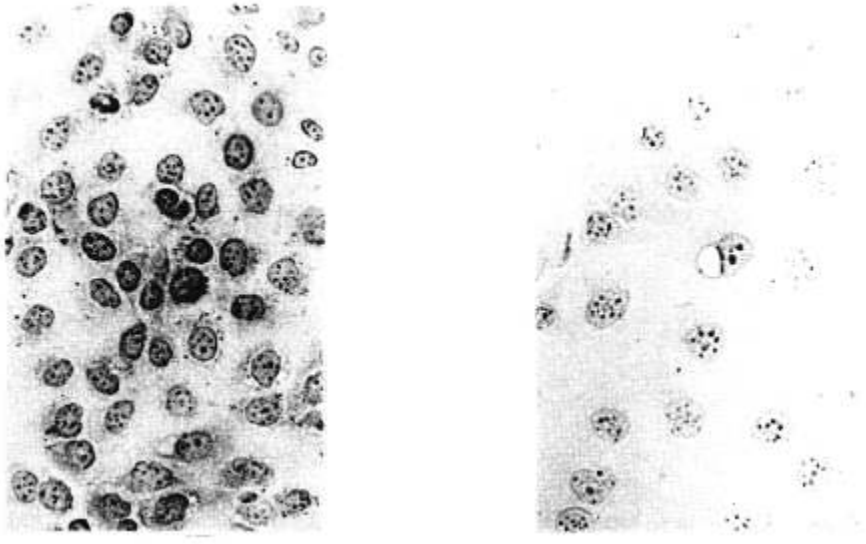

Figure 2. Photomicrographs of control and dexamethasone treated NIH:OVCAR3 cells stained for c-*myc*. Cells were plated 10 000/well on 8-well Lab-Tek slides on Day 0. On Day 1, cells were dosed with 0.05 nM Dex in fresh complete medium. After a 24 h incubation, determination and quantitation of c-*myc* was carried out using immunohistochemistry as described in Materials and methods. Photos were taken at the time of image analysis using identical light settings.

with 15% CV, a value commonly applied for quality control analysis in clinical laboratories and indicates that the model system utilized for these studies yields reproducible data.

The goal of the following experiments was to determine if Dex, RA and calcitriol inhibited c-*myc* protein levels in epithelial breast and ovarian carcinoma cells and to determine if these hormones also inhibited proliferation of these cell lines. Optimal hormone concentrations for regulation of c-*myc* were determined empirically in prior experiments (data not shown). The photomicrographs in Figure 2 are typical representations of control and hormone treated cells after immunohistochemical detection of c-*myc* protein. In this particular case, the effect of Dex on c-*myc* expression in NIH:OVCAR3 ovarian cancer cells is shown. Cells incubated with pre-immune serum in place of primary antibody exhibited virtually no staining (data not shown). The data from pre-immune serum-exposed cells were used for subtraction of background ROD levels from control and treated cells (see Figure 3). The cytoplasm and plasma membranes were not visible because no counterstain was used (Figure 2); however, all slides in these studies were examined by phase contrast microscopy and the cells in all treatment modalities were determined to be intact. Photomicrographs of cells treated with calcitriol and RA showed similar reductions in c-*myc* (data not shown). These results were quantified for all treatments and cell lines by computerized image analysis and presented below.

MCF-7 cells

Immunohistochemical assays showed that c-*myc* was suppressed 29% by 0.5 nM Dex, 45% by 0.01 nM RA and 54% by 100 nM calcitriol after 24 h of drug treatment ($p < 0.05$ for all hormones) (Figure 3A). Since c-*myc* levels are frequently correlated with growth rate, experiments were undertaken to determine if c-*myc* and growth were effected similarly by calcitriol, RA and Dex. MCF-7 cells were inhibited 18, 18 and 39% by Dex, RA and calcitriol. These results indicate that in MCF-7 cells, Dex, RA and calcitriol inhibited both c-*myc* and growth.

T47D cells

Incubation of T47D breast cancer cells with 100 nM Dex, 100 nM RA and 100 nM calcitriol

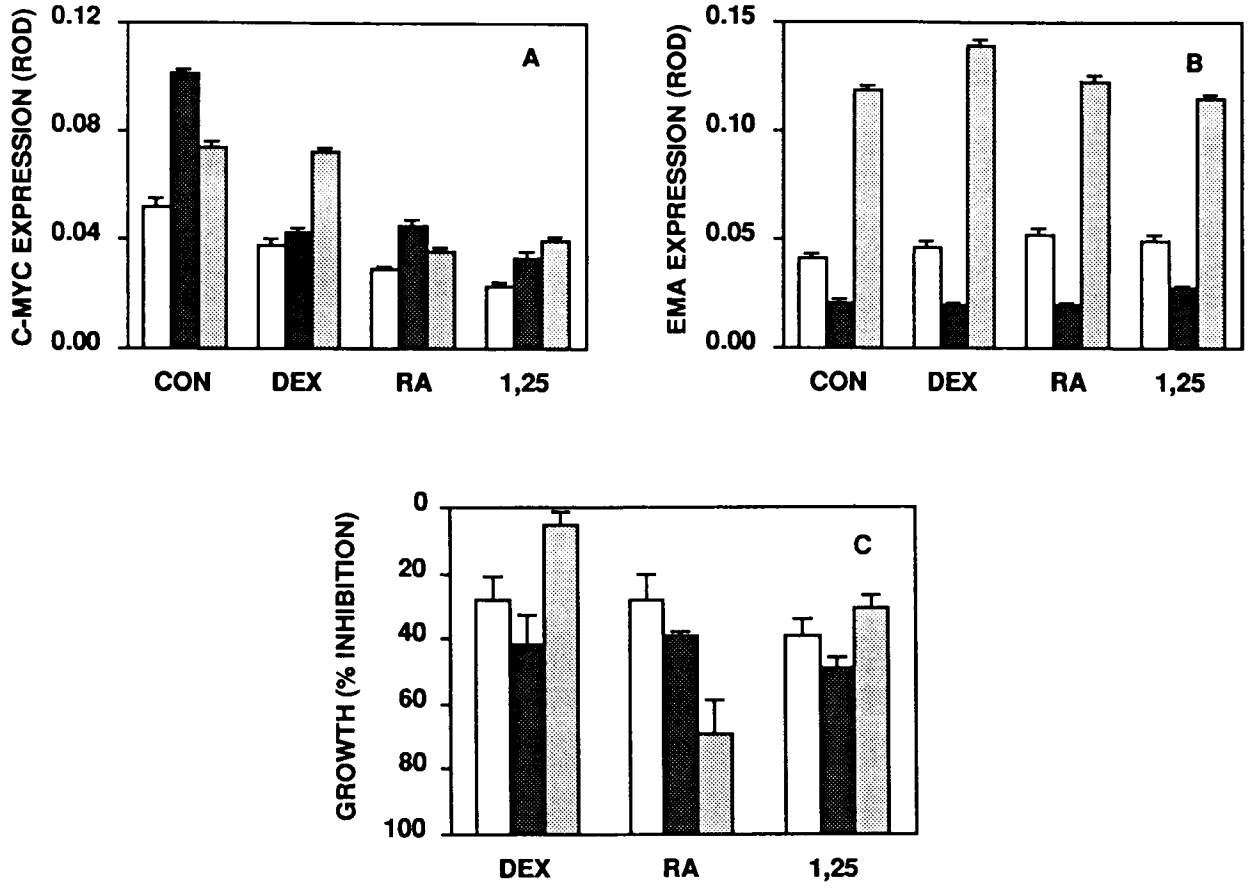

Figure 3. Hormonal regulation of c-*myc* and growth in MCF-7 (□), NIH:OVCAR3 (▩), and T47D (▨) cells. (A) Bar chart depicting c-*myc* expression. Cells were plated 10 000/well on 8-well Lab-Tek slides on Day 0. On Day 1, previously determined optimal concentrations of Dex (0.5 nM for MCF-7, 0.05 nM for OVCAR3, 100 nM for T47D), RA (0.01 nM for MCF-7, 100 nM for OVCAR3 and T47D) and calcitriol (100 nM for all cell lines) were added in fresh complete medium. Control cells received an equivalent volume of ethanol vehicle. After a 24 h incubation, determination of c-*myc* levels was carried out using immunohistochemistry as described in Materials and methods. C-*myc* levels were quantified using computerized image analysis and, after subtraction of background, expressed as ROD. Results are the average of 150 cells (50 cells taken from each of three treatment wells). With the exception of Dex on T47D cells, all hormone treatments on all cell lines yielded statistically significant differences ($p < 0.05$) compared with control using analysis of variance and Scheffe post-hoc comparisons. (B) Bar chart depicting EMA expression in a parallel experiment. Cells were passaged and dosed as in (A). EMA was detected by immunohistochemistry and quantified as in (A). Using analysis of variance, none of the treatments resulted in a statistically significant reduction in EMA when compared with control. (C) Bar chart showing hormonal effects on growth. Cells were plated 15 000/well on 24 well plates on Day 0. Cells were dosed on Day 1 using optimal concentrations of the hormones as described in (A). After 3 days of growth cells were lysed and nuclei counted as detailed in Materials and methods. Bars represent means and standard errors of four wells. Growth of all cell lines were inhibited ($p < 0.05$ versus control) by all hormones with the exception of Dex on T47D cells.

produced c-*myc* inhibition of 3, 53 and 47%, respectively. Growth inhibition of 5, 69 and 31% was observed in response to Dex, RA and calcitriol administration to T47D cells at the same concentrations as above. Those hormones that inhibited c-*myc* expression (RA and calcitriol) also inhibited growth, while Dex had no effect on c-*myc* or growth.

NIH:OVCAR3 Cells

The effects of these hormones on c-*myc* expression were examined in another hormone-responsive epithelial carcinoma line, NIH:OVCAR3. The results in OVCAR3 cells were qualitatively similar to those obtained in MCF-7 cells. Figure 2(A) shows that c-*myc* was reduced 53% by 0.05 nM Dex,

45% by 100 nM RA and 54% by 100 nM calcitriol ($p < 0.05$ for each). Growth of OVCAR3 cells was inhibited 42% by Dex, 39% by RA and 49% by calcitriol. As in MCF-7 cells, the pattern of growth inhibition in OVCAR3 cells was quite similar to the pattern of c-*myc* inhibition.

Investigation of the BG-1 ovarian carcinoma cell line indicated that it too was growth-inhibited by Dex, RA and calcitriol but c-*myc* levels were below the limits of detection for both immunohistochemical and western blot assays (data not shown), thus conclusions regarding the role of c-*myc* in these cells await more sensitive assay methods.

EMA

To ascertain the specificity of hormonal effects on c-*myc* expression, parallel control assays were carried out to determine the effects of calcitriol, RA and Dex on the expression of a non-growth-related product, EMA.[21] EMA levels were not significantly reduced by any of the hormones in any of the cell lines ($p < 0.05$), results which suggest that the hormones used in this specifically repressed c-*myc* levels and did not induce a global repression of all cellular products (Figure 3B).

Western blots

Suppression of c-*myc* by hormones was observed not only by immunohistochemical assays, but by Western blotting as well (Figure 4). Two selected previous experiments were replicated using Western blots to measure the effects of calcitriol on c-*myc* in MCF-7 and NIH:OVCAR3 cells. A 62 kDa species of c-*myc* was shown to be repressed by 100 nM calcitriol in both cell lines, consistent with the immunohistochemical results depicted in Figure 3(A). A *myc*-cross-reacting peptide appeared at 45 kDa which was coordinately downregulated by calcitriol in both cell lines. These results provide further support for the c-*myc* regulation observed using immunohistochemical methods above.

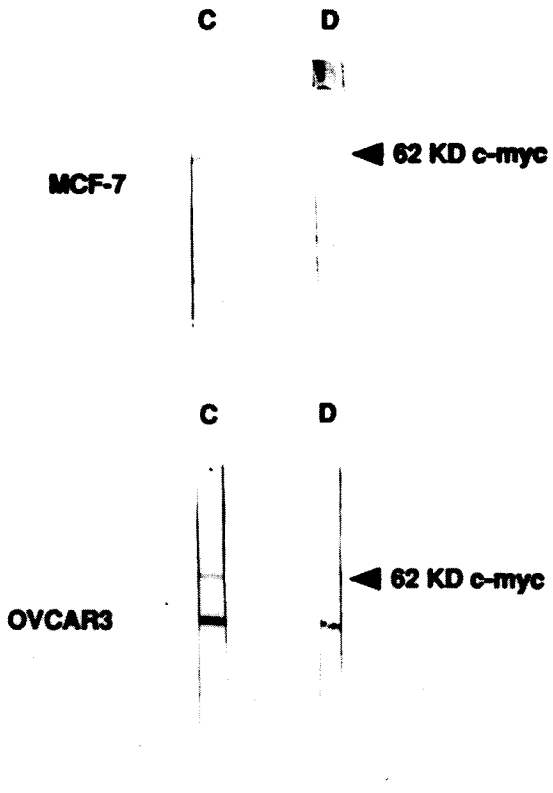

Figure 4. Western blots showing effects of calcitriol on c-*myc* levels in MCF-7 and NIH:OVCAR3 cells. Cells were plated 2.5 × 10^6/plate in 6 cm tissue culture plates. After 24 h, cells were exposed to 100 nM calcitriol for 4 h (previously determined to be as effective as the 24 h exposure utilized in Figure 2, data not shown). Prior to lysis, cells were counted, and cell concentrations were adjusted to 25 × 10^6 cells/ml and 22 μl was loaded per lane. Western blotting was carried out according to the protocol in Materials and methods. (C) denotes control cells and (D) denotes calcitriol exposure.

Discussion

The data show that calcitriol, RA, and Dex, three hormonal agents that inhibit growth of MCF-7 breast, T47D and NIH:OVCAR3 ovarian carcinomas, all specifically repressed c-*myc* protein levels in these cells. This is the first demonstration of repression of c-*myc* by these agents in breast or ovarian carcinomas, with the exception of a previous report showing repression of c-*myc* by RA in HOC-7 ovarian carcinoma cells.[10] These data are consistent with prior reports showing repression of c-*myc* and growth inhibition by tamoxifen and medroxyprogesterone acetate in breast cancer cells. Also, previous experiments using estradiol showed an *increase* in both c-*myc* expression and a growth marker.[26] Including the present study, there are now six different hormonal agents that cause commensurate up- or downregulation of both c-*myc* levels and growth in breast cancer cells.

There is increasing evidence for c-*myc* having an important role in breast cancer. This evidence includes its amplification as well as its association

with tumorigenicity, invasiveness, survival and growth rate.[3–5,7] Less is known about c-*myc* in ovarian cancers, but the many similarities between breast and ovarian cancers suggest c-*myc* may also be of importance in ovarian neoplasms. Demonstration of a direct role for one or more oncogenes in the control of epithelial cancer cell growth would be of tremendous importance in understanding the growth regulation of these cells and in designing strategies to block their growth.

While immunohistochemistry has been used to detect c-*myc* for several years, primarily in tumor sections, rigorous quantification of the c-*myc* signal by computerized image analysis is a novel concept put forth by Czerniak *et al.*[19] This technique has been adapted by our laboratory to provide the first report of precise quantification of hormonal effects on c-*myc* expression when assayed by immunohistochemistry. This system represents an alternative to protein product assay by Western blotting and is straightforward, rapid (1 day), can be carried out on a small scale and easily provides a sample size that readily facilitates statistical analysis of assay data.

Several lines of evidence support the specificity of the immunohistochemical assay for the c-*myc* protein product. The 610E antibody utilized in this study is a monoclonal specific for the c-*myc* protein.[20] Background reactivity (pre-immune serum control) was evaluated for every assay and found to be negligible in all cases. The c-*myc* signal was present almost exclusively in the nucleus, an observation which is consistent with the demonstrated nuclear localization of the c-*myc* protein. In a separate time course study by this laboratory, c-*myc* was undetectable just after passage while cells were in lag phase but then increased in parallel to the increase in proliferation as the cells entered the exponential phase of growth (data not shown). This result is consistent with other studies where c-*myc* expression was shown to be highly correlated with growth in solid tumors.[2] Lastly, selected immunohistochemical results were confirmed biochemically by Western blotting, which showed specific bands at 62 kDa, consistent with the reported size for human c-*myc* protein.[20] Incubation of OVCAR3 and MCF-7 cells with calcitriol caused c-*myc* to be downregulated in the western blot experiments, results similar to those obtained by immunohistochemical methods (Figures 3A and 4), and which further validate immunohistochemical results. The 45 kDa bands also observed in the western blots are not uncommon when using antibodies directed against members of the *myc* gene family. The 45 kDa species is non-nuclear[28] and thus not a factor in the immunohistochemical assays described in this manuscript, since only the nuclei were evaluated for c-*myc* immunostaining.

Conclusion

This study has shown that c-*myc* was repressed by calcitriol, RA and Dex in breast and ovarian carcinoma lines where these same hormones also inhibited growth. The results suggest that these hormonal agents have commensurate effects on c-*myc* expression and growth in the breast and ovarian carcinoma lines examined.

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