Clobetasol differentially affects the vulvar cancer cell line, UMSCV-4, causing both increases in apoptosis while maintaining a subpopulation in quiescence

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INTRODUCTION

Vulvar cancer is a rare, aggressive form of cancer that remains understudied. It is known that up to 65% of vulvar carcinomas arise in the background of vulvar lichen sclerosus (VLS). Clobetasol propionate, a corticosteroid, is commonly prescribed as a treatment for VLS. Our research has found that exposure of a vulvar cancer cell line, UMSCV-4, to clobetasol leads to apoptosis in a portion of the cells. If our results reflect what is happening *in vivo,* this would indicate that clobetasol may decrease the progression of cancer . However, our studies also indicate that apoptosis is not universal among the clobetasol treated UMSCV-4 cells. A subpopulation appears to enter a state of quiescence upon exposure to clobetasol as evidenced by the return of some cells to normal proliferation shortly after the removal of clobetasol. Cells that were incubated in clobetasol for three months returned to normal cell proliferation upon the removal of clobetasol and are termed UMSCV-4 LT. When compared to cells that have not been previously exposed to clobetasol, called UMSCV-4 NT, differences can be observed in the rates of both apoptosis and quiescence. The differences between the NT and LT populations are highlighted upon their exposure to clobetasol, most notably that the LT population avoids entry into quiescence and even shows increased viability in the presence of clobetasol. Our findings suggest that the NT cell population undergoes increased levels of apoptosis and quiescence when compared to the LT population. Furthermore, differences in the levels of protein expression were also observed between the NT and LT populations suggesting lasting, molecular changes resulting from continued exposure to clobetasol.

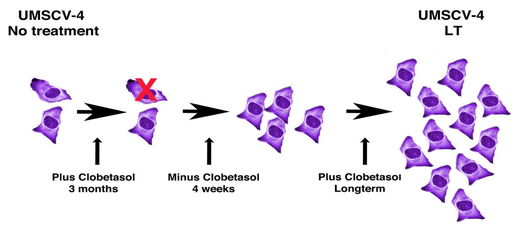

Figure 1. Re-exposure of UMSCV-4 cells to clobetasol results in resistance to the growth inhibition effects of clobetasol. This model represents the process that led to generation of the long-term, clobetasol "resistant" UMSCV-4 cells. Untreated UMSCV-4 cells were exposed to clobetasol for 3 months during which time the clobetasol medium was refreshed periodically to account for evaporation but the cells were not removed or passaged. After 3 months the clobetasol was removed and cells were allowed to "recover" for 4 weeks in medium not containing ethanol or clobetasol. Mitotic cells were visible within 3 days of clobetasol removal. After 4 weeks, proliferating cells were re-exposed to clobetasol. These cells did not experience the growth arrest seen when originally exposed to clobetasol. This population of cells is referred to as UMSCV-4 LT.

RESULTS

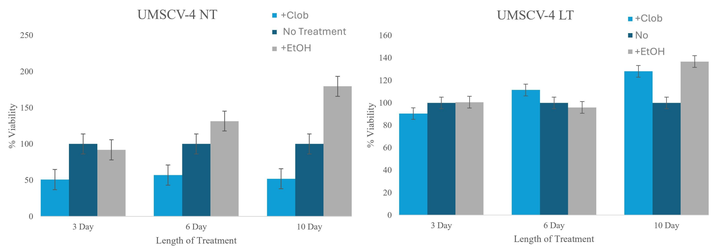

Figure 2. MTT analysis reveals that clobetasol negatively impacts cell metabolism in UMSCV-4 NT cells within 3 days but does not affect the viability of UMSCV-4 LT cells. Percent viability decreased to 50% within 3 days and remained in that range for 6 and 10 days in UMSCV-4 NT cells treated with clobetasol as compared to cells that were left untreated ($p<0.01$). Percent viability showed a drop to 90% in UMSCV-4 LT cells treated with clobetasol for 3 days ($p<0.01$) but then showed an increase in viability to 111% and 128% in 6 and 10 days, respectively when compared to cells left untreated ($p<0.01$). Cells grown in ethanol (as a vehicle control) were also compared to cells grown in the absence of treatment. Interestingly, NT cells showed an increase in viability after 6 days in ethanol ($p<0.001$) while both NT and LT cells showed an increase in viability after 10 days of growth in ethanol ($p<0.001$).This emphasizes the significance of the decreased viability observed in the clobetasol treated NT cells since ethanol is also present in the clobetasol samples as clobetasol is dissolved in ethanol prior to its addition to medium.

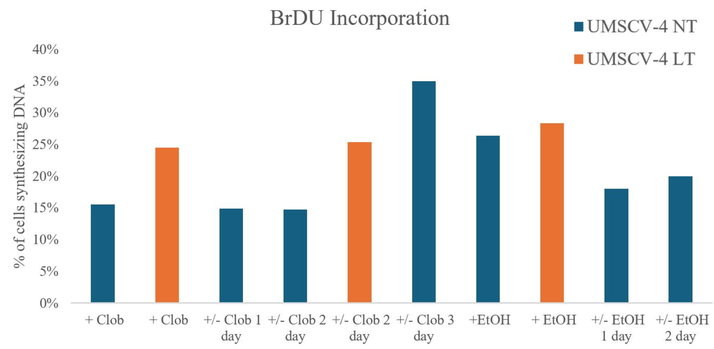

Figure 3. BrDU incorporation assays reveal that the UMSCV-4 NT cells increase their rates of DNA synthesis within 3 days of removal from clobetasol. UMSCV-4 NT cells were treated with clobetasol for 4 days followed by removal of clobetasol for 1, 2, or 3 days. The final amount of time for the cells left in clobetasol was 7 days. The cells left in clobetasol showed only a 15% incorporation of BrDU while those that had been removed at day 4 from clobetasol showed a 35% incorporation of BrDU. This reflected how quickly the cells recovered from clobetasol treatment. The UMSCV-4 LT cells showed a 25% incorporation of BrDU in both the presence and absence of clobetasol reflecting their indifference to clobetasol. In contrast, the UMSCV-4 NT cells grown in ethanol (as a vehicle control) showed an increase in the rate of DNA synthesis as reflected by the 26% incorporation of BrDU. UMSCV-4 LT cells also showed increased levels of DNA synthesis in the presence of ethanol.

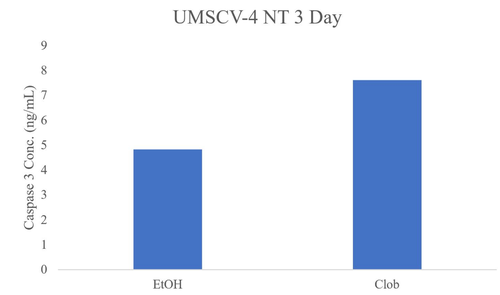

Figure 4. Caspase 3 quantification reveals that exposure of UMSCV-4 NT cells to clobetasol for 3 days leads to an increase in apoptosis. UMSCV-4 NT cells were treated with either clobetasol or ethanol for three days. After 3 days cells were counted and total cell lysate was isolated. Cell lysate was dispensed into microwells coated with a caspase 3 antibody. Elevated levels of caspase 3 in the clobetasol treated cells indicate an increase in apoptosis.

Figure 5. Phosphorylated $p27^{Kip1}$ is localized in the nucleus while total $p27^{Kip1}$ and glucocorticoid receptor levels show decreased levels in UMSCV-4 LT cells. UMSCV-4 cells were treated with either clobetasol or ethanol (vehicle) for 6 days followed by extraction of proteins from the nuclear and cytoplasmic fractions. Western blots revealed that glucocorticoid receptor (GR) levels were diminished in UMSCV-4 LT cells and were highest in the cytoplasmic UMSCV-4 NT fractions (lanes 2, 4). Probing for the phosphorylated form of $p27^{Kip1}$ revealed its localization in the nucleus for both the NT and LT cells (lanes 1, 3, 5, 7). $p27^{Kip1}$ total protein levels showed an elevated presence in the NT cells (lanes 1-4) when compared to the LT cells (lanes 5-8). Among the NT cells, $p27^{Kip1}$ levels are higher in the cytoplasmic fractions (lanes 2, 4) than the nuclear fractions (lanes 1, 3). Additionally, the higher molecular weight bands present in the nuclear fractions corresponding to the phosphorylated form of $p27^{Kip1}$ were observed. Of these bands, levels were highest in the NT nuclear fraction that had been treated with clobetasol.

CONCLUSIONS

- UMSCV-4 NT cells show decreased metabolism and cell replication within 3 days of treatment with clobetasol
- UMSCV-4 LT cells show increased viability in the presence of clobetasol
- UMSCV-4 NT cells begin DNA replication after 3 days when removed from clobetasol
- A portion of UMSCV-4 NT cells treated with clobetasol undergo apoptosis within 3 days of treatment
- There are increased levels of glucocorticoid receptor protein present in the UMSCV-4 NT cells when compared to UMSCV-4 LT cells
- Phosphorylated $p27^{Kip1}$ is localized in the nuclei of UMSCV-4 cells and is present in the highest concentration in the UMSCV-4 NT cells treated with clobetasol
- $p27^{Kip1}$ is expressed at a higher concentration in UMSCV-4 NT cells than the UMSCV-4 LT cells
- These results suggest UMSCV-4 cells are a model system to study quiescence.

FURTHER STUDIES

- Cell viability and apoptosis will be examined for times past 10 days to 1 month after addition of clobetasol to see if the observed trends continue over a longer period of treatment
- Caspase 3 tests will observe trends in UMSCV-4 LT cells to provide insight into the difference in apoptosis between the NT and LT populations
- RNA seq will be performed to compare the overall expression levels of important cell cycle regulators in the UMSCV-4 NT vs. UMSCV-4LT cells, particularly those that are involved in exit from quiescence and progression into G1/S phase.

MATERIALS & METHODS

Cell growth and treatment
UMSCV-4NT cells (obtained from the University of Michigan Cell Culture Bank) were grown in DMEM/HAMSF12 1:1 + 10% FBS. Cells were grown in the presence of clobetasol (10^{-7} M diluted in a solution of 95% ethanol) or absence of clobetasol (equal amount of 95% ethanol) for times indicated in each experiment.

MTT assay
Cells were plated in 96 well plates $1x10^4$ cells per well. On day 2 medium was changed to either +ethanol or +clobetasol or left in medium without any additional treatment. On day 3, 6 or 10, after addition of treatment, respectively, MTT (5 mg/ml) was added to the cells and incubated for an additional 5 hours. Cells were processed using NaOH + 1M HCl according to standard protocols and absorbance was measured at 570 nm. Samples for each treatment were averaged.

BrDU assay
UMSCV-4NT and LT cells were plated at $1X10^5$ cells/well in 12 well plates on poly-L-lysine coated coverslips. Cells were treated for 4 days with either clobetasol (10^{-7}M) or an equal amount of ethanol (vehicle control), followed by removal of treatment for indicated days. BrDU was added for 3 hours followed by fixation in Histochoice (Amersham) and denaturation of DNA using 2M HCl followed by blocking in 10% goat serum. BrDU was localized using mouse anti-BrDU (Developmental Hybridoma Studies Bank) and Alexafluor 488 secondary. Coverslips were mounted with Vectashield mounting medium containing DAPI and 3 random areas for each treatment were captured using a Zeiss Axiophot Immunofluorescence microscope. Image J was used to analyze the number or BrDU stained cells divided by the number of DAPI stained cells and the percentage of BrDU staining was calculated from these results.

Caspase 3 assay
UMSCV-4NT cells were plated at $5x10^5$ cells per well in 6 well plates. On day 2 medium was changed to either +ethanol or +clobetasol. 3 days later cells were harvested by scraping the wells in 1x PBS. A cell pellet was isolated and then resuspended in 1x lysis buffer (triton X-100). The supernatant was then added into the microwell strip provided in the Human Caspase 3 Instant ELISA Kit (ThermoFisher Scientific) and processed according to the manufacturer's protocol. Absorbances were read on a Synergy HTX microplate reader immediately after processing.

Western blot (isolation of nuclear vs. cytoplasmic)
UMSCV-4NT and UMSCV-4LT cells were grown for 3 days in medium containing either clobetasol or ethanol as described above. Cells were trypsinized and nuclear and cytoplasmic fractions were isolated by standard procedures. Briefly, trypsinized cells were washed in 1X PBS followed by suspension in a Hepes Buffer containing $MgCl_2$, KCl, DTT and NP40. Nuclei were pelleted by centrifugation and resuspended in a high salt buffer containing Hepes, $MgCl_2$, NaCl, glycerol, DTT and EDTA. Protease inhibitors were added to each of the buffers. Protein was measured using a Bio-Rad protein reagent and equal amounts of protein were separated on a 7% PAGE Gel followed by transfer to nitrocellulose.
The following antibodies were used: anti-$p27^{Kip1}$ (Cell Signaling Technology), anti-phospho-p27KIP- PTHR198 (Millipore Sigma), anti-glucocorticoid antibody (BS13385R, BIOSS). Secondary antibodies were either anti-mouse or anti rabbit AP conjugates and proteins were visualized using NBT and BCIP.

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