p27^{Kip1} is upregulated in UMSCV-4 cells corresponding to entering a state of quiescence when treated with clobetasol

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INTRODUCTION

Quiescence, the temporary exit from the cell cycle, presents therapeutic challenges to cancer management since it allows evasion of chemotherapy and radiation treatments. Essential to the study of quiescence in carcinogenesis is an established model system. Our studies have found that clobetasol treatment of the vulvar cancer cell line, UMSCV-4, causes these cells to enter a state of dormancy (we refer to the untreated cells as UMSCV-4NT). Subsequent removal of the cells from clobetasol show a return to normal cell proliferation, even after dormancy for 3 months (referred to as UMSCV-4LT cells) and these cells no longer enter quiescence with subsequent clobetasol treatment. Previous studies in our lab using RT-PCR suggest that a key marker for quiescence, p27^{Kip1} , is upregulated in the UMSCV4 cells upon initial treatment with clobetasol. To further characterize the difference between the UMSCV4NT and LT cells we performed growth and metabolism studies and examined the two different populations for the expression of p27^{Kip1} by Western Blot in both clobetasol and vehicle (ethanol) treated UMSCV-4NT and LT cell populations. Here we present evidence showing that the UMSCV-4 cells enter a state of quiescence within 3 days of clobetasol treatment. Furthermore, the decrease in cell viability is not due to an increase in cell death during this period. This establishes the UMSCV-4 cells as a good model system for the study of clobetasol induced quiescence in vulvar squamous epithelial cells. The importance of this work is underscored by the observation that clobetasol is often used to treat a common inflammatory disease of the vulva known as vulvar lichen sclerosus (VLS) and up to 65% of vulvar carcinomas arise in the background of VLS.

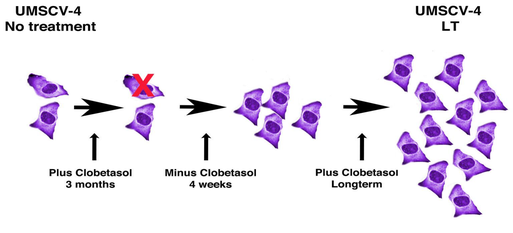


Figure 1. Re-exposure of UMSCV-4 cells to clobetasol results in resistance to the growth inhibition effects of clobetasol. This model represents the process that led to generation of the long-term, clobetasol "resistant" UMSCV-4 cells. Untreated UMSCV-4 cells were exposed to clobetasol for 3 months during 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. Note that 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

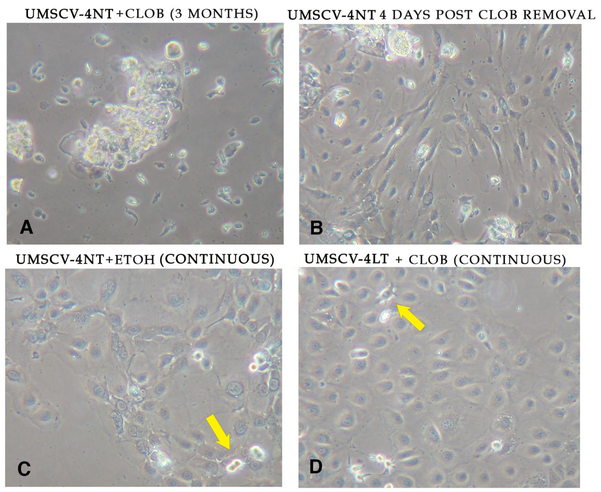


Figure 2. UMSCV-4NT cells can exist in a state of quiescence when treated with clobetasol. Clobetasol (10^{-7}M in ethanol) was added to DMEM/HAMSF12 1:1 +10% FBS medium of actively dividing UMSCV-4 cells. Phase contrast images were taken after cells were incubated for three months (panel A) and 4 days after subsequent removal of the clobetasol containing medium (panel B). UMSCV-4 cells grown in ethanol control (panel C) continued to grow and displayed random mitotic figures (arrows), as did UMSCV-4LT cells that were grown in the clobetasol media (panel D, arrows).

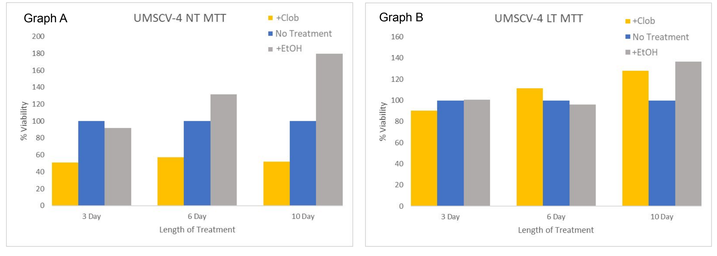


Figure 3. Percent viability as measured by MTT reveals that clobetasol negatively impacts cell metabolism in the UMSCV-4 NT cells within 3 days but does not affect the viability of the UMSCV-4LT cells. Percent viability dropped to 50% at 3 days and remained in that range at 6 and 10 days in UMSCV-4NT cells grown in the presence of clobetasol as compared to cells that were left untreated (Graph A). Percent viability showed a drop to 90% at 3 days of clobetasol treatment in the UMSCV-4LT cells but then showed an increase in viability to 111% and 128% at 6 and 10 days, respectively, when compared to UMSCV-4LT left untreated (Graph B). Growth in ethanol was also compared to growth in the absence of treatment. Interestingly, cells grown in ethanol actually increased cell viability when compared to no treatment in both the UMSCV-4NT and UMSCV-4LT cells at 6 and 10 days (Graphs A and B). This emphasizes the significance of the decreased viability seen in the clobetasol treated UMSCV-4NT cells since the clobetasol is dissolved in ethanol before addition to the medium.

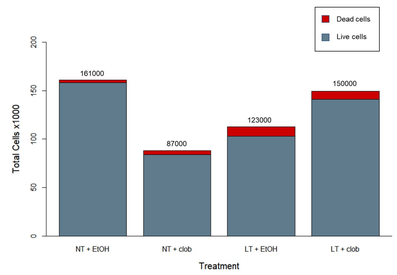
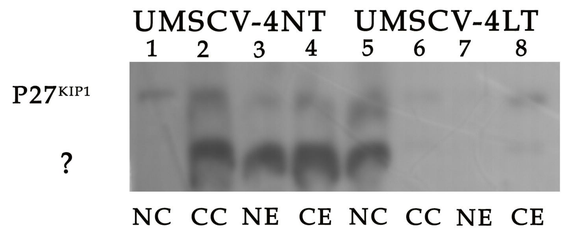


Figure 4. The initial decrease in cell viability is not due to an increase in cell death in UMSCV-4NT cells treated with clobetasol. UMSCV-4NT and UMSCV-4LT cells were treated with either clobetasol or ethanol (in triplicate). After 3 days, cells were counted and cell death was calculated on a subfraction of the cells using a Trypan Blue assay. The number of dead cells (red block). The percent of live cells to total cells was 95% and 98% for UMSCV-4NT treated with clobetasol or ethanol, respectively. The percent of live cells to total cells was 94% and 91% for UMSCV-4LT treated with clobetasol or ethanol, respectively. This may reflect the increased cell growth in the UMSCV-4LT +clobetasol but also reflects that the decreased viability of the UMSCV-4NT cells grown in clobetasol is not due to an increase in cell death. Total number of cells is displayed over each bar.



NC= nuclear fraction + clobetasol; CC = cytoplasmic fraction + clobetasol; NE = nuclear fraction + ethanol; CE = cytoplasmic fraction + ethanol.

Figure 5. Clobetasol treatment of UMSCV-4NT cells appears to increase the amount of p27^{Kip1} protein when compared to the ethanol treated UMSCV-4NT cells and the clobetasol and ethanol treated UMSCV-4LT cells. UMSCV-4NT and UMSCV-4LT cells were treated with either clobetasol or ethanol for 3 days, followed by extraction of proteins from the nuclear and cytoplasmic fractions. Western blots showed a slight increase in p27^{Kip1} in the UMSCV-4NT cells treated with clobetasol when compared to those treated with ethanol. It shows a significant increase in the UMSCV-4LT cells treated with either clobetasol or ethanol. The increased levels of p27^{Kip1} appeared to reside mostly in the cytoplasmic fraction in the UMSCV-4NT cells (compare Lanes 1 and 2). Interestingly, the nuclear fraction from the UMSCV-4LT cells treated with clobetasol (lane 5) did show a level of p27^{Kip1} comparable to that seen in the ethanol treated UMSCV-4NT cells (lanes 3 and 4). NC= nuclear fraction + clobetasol; CC = cytoplasmic fraction + clobetasol; NE = nuclear fraction + ethanol; CE = cytoplasmic fraction + ethanol. The question mark (?) denotes a band that may represent p27^{Kip1} breakdown but has yet to be confirmed.

CONCLUSIONS

- UMSCV-4NT cells show a decreased metabolism and cell replication within 3 days of treatment with clobetasol.
- The decrease in viability does not correspond to an increase in cell death.
- An increase in p27^{Kip1} is seen in the UMSCV-4NT cells within 3 days of treatment with clobetasol. There is, however, a general increase in p27^{Kip1} in the UMSCV-4NT that have not previously been treated with clobetasol as compared to the UMSCV-4LT cells suggesting that a loss of p27^{Kip1} expression occurs after long-term treatment with clobetasol (that resulted in the generation of the UMSCV-4LT cells).
- This may confer an ability to remain viable even in the presence of clobetasol.
- These results suggest UMSCV-4 cells are a model system to study quiescence.

FURTHER STUDIES

- The expression of p27^{Kip-1} will be more extensively examined by RT-PCR to determine when transcription increases post addition of clobetasol.
- Cell viability will be examined for times past 10 days to 1 month after addition of clobetasol to see if the trend for a decrease in viability with time continues.
- Cell death will also be measured for times past 10 days to 1 month after addition of clobetasol to see if there is an increase in cell death over that seen at earlier time points.
- 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 1 X 10^4 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.

Trypan Blue assay

UMSCV4-NT and UMSCV-4LT cells were plated at 1x10^4 cells per well in 6 well plates. Cells were grown in medium containing either clobetasol or ethanol (as described above). After 3 days cells were trypsinized and resuspended in 500 µL of their respective mediums. Cells were incubated for 1 minute in Trypan Blue solution (0.4%) at a 100:100 µL ratio in Eppendorf tubes. A Neubauer chamber was used to count dead and total cells from each sample.

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 blot was probed with anti-p27^{Kip1} (Cell Signaling Technology, SC53G8.5) followed by anti-mouse HRP conjugated secondary antibody. Supersignal West Pico Plus Chemiluminescent Substrate (ThermoFisher) was used to visualize the bands.

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