Original Article
In-vitro rescue and recovery studies of human melanoma (BLM) cell growth, adhesion and migration functions after treatment with progesterone

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Abstract: Treatment of human melanoma (BLM) cells for 48 hrs with progesterone resulted in a significant inhibition of cell growth. The mechanism of growth inhibition was due to autophagy and this action of progesterone was not mediated through progesterone receptor. As cells were floating during treatment, adhesion assay was performed, which showed complete loss of adhesion. When cells were allowed to recover after treatment by culturing in growth medium without progesterone, there was recovery in cell growth. Preliminary experiments on adhesion and recovery cell growth prompted us to suppress autophagic lysosomal degradation with 3-methyladenine (3-MA), which resulted in partial rescue of cell growth, adhesion and migration functions. The above experimental design gave rise to two experimental groups viz., progesterone treated and 3-MA rescued. Since, recovery studies also showed improvement in cell growth, progesterone treated and 3-MA rescued groups were allowed to recover on their own for first 48 hrs and then a second 48 hrs. Comparison of in-vitro cell growth, adhesion and migration functions of progesterone treated, 3-MA rescued and recovered human melanoma cells revealed that the recovery of 3-MA rescued cells was better than the recovery of progesterone treated cells in terms of cell growth and adhesion functions. These in-vitro experiments not only provided the scientific basis for epidemiological findings that menstruating females were better protected in melanoma, but also showed the potential of progesterone to act as an anti-cancer agent for melanoma treatment.

Keywords: Progesterone, human melanoma (BLM) cell line, autophagy, 3-methyladenine rescue, in-vitro cell growth, adhesion, migration functions, recovery studies

Introduction

Epidemiological data clearly indicated a female sex advantage in melanoma [1-3]. Doctors knew that menstruating females were better protected in melanoma than post-menopausal women and men of any age [4-9]. People suspected the role of sex steroids in protecting females from melanoma [10-15]. But, there was no direct experimental evidence to link sex steroid hormones with melanoma protection until the publication of our works on effect of sex steroids on mouse (B16F10) [16] and human melanoma (BLM) [17] cell lines. Using mouse melanoma (B16F10) cell line, we showed that among a battery of sex steroids tested, female sex hormone progesterone showed a significant inhibition of melanoma cell growth. Further research work with mouse melanoma cell line showed that the effect of progesterone was not a spurious, toxic or non-specific effect on mouse melanoma cells and that the action of progesterone was not mediated through progesterone receptor [16]. Using human melanoma (BLM) cell line, we showed that the mechanism of inhibition of melanoma cell growth was due to autophagy and this effect of progesterone on human melanoma cell was also not mediated through autophagy receptor [17]. In addition to inhibition of cell growth, we also observed cells floating in the medium during progesterone treatment, suggesting that adhesion might have also been affected. Initial adhesion assay
after 100 uM progesterone treatment showed a complete loss of adhesion. So, progesterone treatment not only affected cell growth but also adhesion of the cells. Preliminary recovery study of cell growth to check whether the inhibition was permanent or reversible after 10 uM progesterone treatment, showed recovery of cell growth close to control (untreated) cells, indicating it was a temporary inhibition of growth at 10 uM concentration of progesterone. This observation prompted us to suppress autophagic lysosomal degradation with 3-methyladenine (3-MA) [18, 19] and investigate the effect on cell growth, adhesion and migration functions. Recovery studies led us to compare recovery of cell growth, adhesion and migration functions of progesterone treated and 3-MA rescued cells. Results showed that the recovery of 3-MA rescued cells was better than progesterone treated cells recovery in terms of cell growth and adhesion functions.

Materials & methods

Chemicals

Progesterone (P), 3-Methyladenine (3-MA), Crystal Violet (CV) and paraformaldehyde were all purchased from Sigma Chemical Company, St. Louis, MO. MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), isopropanol were also obtained from Sigma Chemical Company. Fetal bovine serum (FBS), Trypsin-EDTA (1X), and PBS powder were purchased from Atlanta biologicals, Lawrenceville, GA. RPMI and antibiotic/antimycotic solution 100 × (10,000 IU/ml penicillin, 10 mg/ml streptomycin, 25 mg/ml amphotericin-B) were purchased from Fisher scientific, Houston, TX.

Growth medium (GM)

All cell culture works were carried out in RPMI 1640 medium containing 10% FBS +1X Pen/Strep/Ampho. Cell proliferation was quantitated using MTT assay.

MTT proliferation assay [20]

BLM cells were suspended in growth medium (GM) and plated at a density of 1 × 10^4 cells/well in a 96 well plate. Cells were left overnight at 37°C to attach to the plate. Following day growth medium was replaced by GM containing hormone at different concentrations and incubated for 48 hrs. After 48 hrs, medium was replaced by 100 ul of 1 in 10 diluted (in GM) MTT solution and incubated for another 4 hrs at 37°C. After 4 hrs MTT solution was removed. MTT was reduced by metabolically viable cells to a colored (purple) water insoluble formazan salt. The purple color precipitate was solubilized by adding 100 ul of isopropanol and shaken for 20–30 min at room temperature.Intensity of resultant purple color was measured at 570 nm in a SLT spectra plate reader.

Adhesion assay [21, 22]

After 48 hrs of progesterone treatment, cells were harvested by digesting with trypsin. Thirty thousand (30,000) cells were added to each wells in a 96 well plate. Cells were removed at 0, 15, 30, 45 and 60 min intervals and wells were washed with medium to remove any loosely attached cells. Cells which were attached to the bottom of the plate were fixed with 4% paraformaldehyde and crystal violet dye (0.2%) was added to the cells and incubated for 5 min. After 5 min staining, excess CV dye was washed away by PBS. CV was bound to the protein in the cells. Amount of CV bound to protein was proportional to the number of cells in the well. After briefly drying the wells in air, CV was eluted by adding isopropanol and the intensity of purple color was measured at 570 nm in a plate reader.

Migration assay [23]

After 48 hrs of progesterone treatment, cells were harvested from the plate. Four hundred thousand (400,000) cells were added to wells in a 24 well plate. Cells were allowed to settle and attach to the bottom of the plate. Once the cells were completely attached and confluent, a sterile 1 ml pipette tip was used to make a scratch in the middle of the well. Photograph of the area cleared by the tip was taken. This was considered as 0 hr time point. Cells were incubated in GM for 24 hrs. At the end of 24 hrs, cells migrated into the scratch area were photographed. This was considered as migration after 24 hrs and compared to the corresponding 0 hr time picture and the distance covered by the cells were calculated and expressed as percentage of area migrated by cells using a computer software program.

Statistical analysis

All experiments were carried out in triplicate (3 wells). Each experiment was repeated a mini-
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Background

Preliminary adhesion and recovery cell growth assays

As lots of cells were floating during progesterone treatment, adhesion assay was carried out as described in the methods after 48 hrs of progesterone treatment. The assay showed that 100 uM of progesterone treatment completely abolished adhesion (Figure 1A), suggesting that adhesion function was also affected by the progesterone treatment.

Recovery of cell growth was carried out to check whether the inhibition of cell growth by progesterone was permanent or temporary (reversible). Progesterone (10 uM) treated cells were allowed to recover for 72 hrs in GM. Progesterone treated cells recovered close to control (untreated) cells quantitatively as shown by the bar diagram in Figure 1B. Moreover the P-value between recovered progesterone treated cells and untreated cells was not significant compared to the P-value between original progesterone treated cells and untreated control cells, suggesting that cell growth had taken place during recovery period and the inhibition of cell growth by progesterone (at 10 uM concentration) was not permanent. These two preliminary studies on adhesion and recovery cell growth laid the foundation for the present research work.

Trial rescue of cell growth with 3-methyladenine (3-MA)

As shown in other studies [18, 19], autophagic lysosomal degradation was suppressed by adding 3-MA. As usual cells were treated with pro-
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Figure 2. A. Trial rescue assay with the addition of 2 mM 3-MA: Comparison of cell growth between control and P-10 uM treated cells showed a difference in growth between them. However, when 3-MA was added along with P-10 uM, cell growth between control and 3-MA rescued cell group was not statistically significant as shown by the P-value of 0.46. B. Adhesion dose and time curves assays: Adhesion was lost completely following 100 uM progesterone treatment. It was decided to find out the dose effect of progesterone on adhesion because we observed a dose-dependent effect of progesterone on cell growth earlier [17]. We found a dose-dependent effect on adhesion, with 10 uM treatment closely paralleling the untreated control cells. After the determination of dose effect of progesterone on adhesion, it was decided to find out the time of incubation of progesterone on adhesion, using a single concentration (100 uM) of progesterone. Cells were harvested after incubation with progesterone for 12, 24 and 48 hrs. Adhesion assays were carried out, which showed a significant decrease in adhesion after 48 hrs of incubation with progesterone.

Preliminary adhesion experiment with 100 uM of progesterone treatment showed complete loss of adhesion. Since, progesterone showed a dose-dependent inhibition on cell growth, we expected a dose-dependent loss of adhesion. Adhesion assays were carried out at 10 and 50 uM progesterone concentrations along with untreated control, which showed a dose-dependent loss of adhesion (Figure 2B). Since adhesion assay was carried out after 48 hrs of treatment, we checked adhesion at earlier time point of progesterone treatment such as 12 and 24 hrs. Adhesion assay showed a time dependent decrease in adhesion with a maximum loss of adhesion after 48 hrs treatment of progesterone (Figure 2B).

Rescue and recovery studies on in-vitro cell growth function

Having standardized rescue and recovery studies, we focused initially on in-vitro cell growth
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Figure 3. A. Rescue and recovery studies on cell growth-cell pictures: Photomicrograph of cells showed a decrease in cell number and differences in the structure of cells in the treated row under P-50 uM column. But in the same treated row, P-50 uM + 3-MA column showed an increase in cell number and differences in the structure of cells. Similarly in the 3rd row, second 48 hrs recovery under P-50 uM + 3-MA column, there was an increase in cell number and change in the structure of cells compared to cells in the same row under P-50 + 3-MA column, there was an increase in cell. B. Rescue and recovery studies on cell growth-graph: The decrease in cell growth between untreated
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cell growth in P-50 uM treated cells was statistically significant as shown by a P-value of 0.004 (in Treated graph). First 48 hrs recovery did not show any significant changes. However, after second 48 hrs recovery, the difference in cell growth between P-50 uM treated and 3-MA rescued cells was significant, as shown by a P-value of 0.001. Again, the difference in cell growth between control and 3-MA rescued cells was not significant as shown by a P-value 0.18. The difference in cell growth in 3-MA rescued cells and second 48 hrs recovered 3-MA rescued cell was significant as shown by the P-value of less than 0.000 (shown in all combined in bar diagram).

**Figure 4.** Rescue and recovery studies on adhesion function: The difference in adhesion between untreated control and P-50 uM treated cells was significant as shown by a P-value of 0.000. But in the second 48 hrs recovery graph the difference in adhesion between P-treated and 3-MA rescued cells was not significant as shown by the P-value 0.11. In the same graph, the difference in adhesion between control and 3-MA rescued was also not significant as shown by a P-value of 0.08 (because there was a recovery in adhesion). Comparison of the difference in adhesion between first time 3-MA rescued and second 48 hrs recovered 3-MA rescued cells was significant P-0.001 (shown in all combined in bar diagram) indicating a significant recovery. Similarly, comparison of the difference in adhesion between original P-treated and second 48 hrs (P-50 uM) recovered cells was significant as shown by a P-value of 0.000 indicating a significant recovery in adhesion function after 96 hrs of recovery. The experiment also suggested that the suppression of adhesion was temporary.

function using a single concentration of progesterone (50 uM), as it fell in between 10 and 100 uM concentration range in results. Representative pictures in **Figure 3A**, indicated a decrease in cell number in P-50 uM treated column compared to control column. However, after second 48 hrs recovery, there was an increase in cell numbers in 3-MA rescued cells shown by the red arrow at the bottom of the picture. For comparison sake control cells were also indicated by red arrow at the top of the picture. Fifty uM Progesterone treatment resulted in 42% cell growth compared to 100% growth in untreated control (**Figure 3B**, Treated). But, 3-MA addition only marginally rescued cell growth to 46.7% (**Figure 3B**, Treated). When both treated and rescued cells were allowed to recover initially for 48 hrs, P-50 uM treated cells showed improvement in cell growth to 50%, whereas 3-MA rescued cells showed
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57.5% growth (Figure 3B, First 48 hrs recovery). Again, when both cells were allowed to recover for a second 48 hrs, 3-MA rescued cells showed a cell growth of 87%, whereas P-treated cells...
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maintained the same level of cell growth at 52.9%, as the previous level of growth (Figure 3B, Second 48 hrs recovery). So recovery of cell growth in 3-MA rescued cells was better than the recovery of cell growth in progesterone treated cells.

Rescue and recovery studies on adhesion function

Partial recovery of cell growth in 3-MA rescued cells prompted us to check recovery of adhesion function. Progesterone 50 μM treatment resulted in 71.8% adhesion compared to 100% in untreated control (Figure 4, Treated). But, 3-MA marginally rescued adhesion to 76.9% (Figure 4, Treated). When both progesterone treated and 3-MA rescued cells were allowed to recover for first 48 hrs, rescued cells showed improved adhesion (86.5%) than treated cells (71%) (Figure 4, First 48 hrs recovery). Again, when both cells were allowed to recover for second 48 hrs, 3-MA rescued and progesterone treated cells showed almost equal recovery of adhesion 91.2% and 87.9% respectively (Figure 4, Second 48 hrs recovery), indicating suppression of adhesion was a temporary effect. The fact that adhesion recovered after first 48 hrs of recovery, suggested that the suppression of adhesion could be at the protein level.

Rescue and recovery studies on migration function

Adhesion is essential for migration of cancer cells. As adhesion was affected by progesterone treatment, problem with migration was anticipated. So, scratch migration assay was...
carried out as described in the method. The difference in migration between control and progesterone treated cells was shown qualitatively in the picture (Figure 5A). Fifty μM progesterone treatment decreased migration to 23% compared to 100% migration in untreated control (Figure 5B, treated). 3-MA addition partially rescued migration to 45%. But, an initial 48 hrs recovery did not improve migration function in both progesterone treated and 3-MA rescued cells (Figure 5B, First 48 hrs recovery).

Comparison of rescue and recovery studies on in-vitro cell growth and adhesion functions

Comparison of cell growth between control and progesterone treated cells showed there was no recovery of cell growth even after second 48 hrs recovery period (Figure 6A). It implied that the inhibition by progesterone was permanent and could be at the gene level. Several genes involved in cell growth, such as cell cycle genes and other protein factors might have been affected. So, cells could not recover even after 96 hrs of recovery. However, when 3-MA was added there was a significant recovery in 3-MA rescued cells after second 48 hrs recovery. Similarly when adhesion assay was compared between control and progesterone treated cells, progesterone treated cells showed only marginal recovery in the first 48 hrs recovery. By the end of second 48 hrs recovery period, progesterone treated cells adhesion and 3-MA rescued cells adhesion were very close, indicating that the inhibition of adhesion function was not permanent. Based on the recovery study, it appeared that cell growth and adhesion functions did not go hand in hand.

Discussion

Our earlier study with human melanoma (BLM) cells showed that progesterone significantly inhibited cell growth by inducing autophagy [17] and also caused cells to float in the medium during treatment. Initial adhesion assay showed that adhesion was completely lost after 100 μM progesterone treatment. Preliminary recovery cell growth study showed that progesterone (10 μM) treated cells recovered cell growth close to untreated control level after 72 hrs of recovery. These two results gave the idea to rescue progesterone treated cells by suppressing autophagic lysosomal degradation with 3-MA [18, 19] and monitor cell growth, adhesion and migration functions. Trial experiment with the addition of 3-MA, showed its ability to suppress autophagy and to partially rescue cell growth. Similarly trial experiments for time and dose-curve studies of adhesion showed an optimal 48 hrs of incubation of progesterone at 50 μM concentration, was sufficient to study in-vitro adhesion and migration functions. The two experimental group of cells viz., progesterone treated and 3-MA rescued were allowed to recover for first 48 hrs. There were marginal recoveries in cell growth and adhesion functions. This partial recovery prompted us to extend the recovery period to a second 48 hrs in the hope of a complete recovery comparable to the control level. When cells were allowed to recover for a second 48 hrs., 3-MA rescued cells recovered close to untreated control cell level in terms of cell growth and adhesion functions than progesterone treated cells.

With respect to rescue and recovery studies on cell growth, progesterone 50 uM treated cells decreased cell growth to 42%, compared to untreated control cell growth at 100%. However, 3-MA was able to marginally rescue cell growth to 46.7%. Both progesterone treated and 3-MA rescued cells were allowed to recover for 48 hrs. Initially cell growth rose to 50% and 57.5% respectively with progesterone treated and 3-MA rescued cells. When both groups were allowed to recover for a second 48 hrs, 3-MA rescued cells showed a cell growth of 87%, whereas progesterone treated cells maintained the previous level of 52.9% growth. The result indicated that it was a permanent inhibition of cell growth by progesterone and suggested the inhibition could be at the gene level.

With respect to rescue and recovery studies on adhesion, progesterone 50 uM treated cells showed an adhesion of 71.8%, whereas 3-MA rescued cells showed an adhesion of 76.9%. When progesterone treated and 3-MA rescued cells were allowed to recover for first 48 hrs, 3-MA rescued cells showed an improved adhesion (86.5%) than progesterone treated cells (71%). But, when both cells were allowed to recover for a second 48 hrs, both treated and rescued cells showed almost an equal level of recovery in adhesion 87.9% and 91.2% respectively. This showed that suppression of adhesion was not a permanent effect of progesterone and suggested the suppression could be at the protein level. Moreover, the experiments...
indicated that the effect of progesterone on cell growth and adhesion functions did not go hand in hand.

Adhesion is essential for migration function. As a proof of concept, if adhesion was affected, migration would also have been affected as proved by scratch migration assay. As expected progesterone decreased migration to 23%, but 3-MA partially rescued migration to 45%. However, initial 48 hrs recovery did not improve migration function in both progesterone treated and 3-MA rescued cells.

Comparison of progesterone treated, 3-MA rescued and recovered studies on cell growth showed that 3-MA rescued cells recovered better than progesterone treated cells recovery. Progesterone treated cells showed minimal recovery of cell growth over a period of 96 hrs, indicating that the inhibition was permanent. Even 96 hrs after treatment, cells were not able to recover cell growth indicating permanent inhibition of genes involved in cell growth. Similarly comparison of recovery of progesterone treated and 3-MA rescued cells with respect to adhesion function showed better recovery of adhesion in both groups. However, progesterone treated cells did not show any recovery in the first 48 hrs recovery period. It took another 48 hrs for progesterone treated cells to recover adhesion function close to control level of adhesion, indicating the involvement of protein(s) which was/were suppressed by progesterone treatment. But when allowed to recover after the first 48 hrs recovery period, protein synthesis might have occurred and cells recovered adhesion function close to control level. So suppression of adhesion was temporary.

As a proof of concept, when adhesion was affected, migration would also have been affected as proved by progesterone treatment. The experiment results showed that when adhesion was affected, migration was also affected. Similarly, when 3-MA partially rescued adhesion, migration was also partially rescued.

Progesterone not only affected cell growth by inducing autophagy, but also affected other functions such as adhesion and migration. Recovery experiments indicated progesterone treatment affected cell growth permanently, whereas adhesion function was suppressed temporarily, but recovered during second 48 hrs recovery period. Moreover 3-MA recovery further confirmed that the mechanism of inhibition of cell growth by progesterone was due to autophagy as reported earlier[17].

In conclusion progesterone not only affected cell growth but also adhesion and migration functions essential for metastasis. Hence, progesterone has the potential to be an anti-cancer agent for melanoma treatment. Moreover, these in-vitro experiments provided the scientific basis for epidemiological findings that menstruating females were better protected in melanoma.

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Disclosure of conflict of interest
None.

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