The Effect of Oxaliplatin on the Immunogenic Cell Death and Cell Apoptosis of Human Merkel Cell Cancerous Tumor

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Abstract. Oxaliplatin, as previously studied in the paper, is a derivative of Cisplatin that is effective in treating the Lewis Lung Carcinoma (LLC). As it can actively induce immunogenic cell death of the cancer cells, and result in apoptosis, which increases the therapeutic efficacy in the LLC cancer treatment. Merkel cell cancer is a type of skin cancer that is rare but highly aggressive, with high metastasizing and reoccurring rate. In this study, we aim to determine the potential of Oxaliplatin to induce apoptosis and ICD in cancerous Merkel cell line MCC1, in association with the PD-1 inhibitor Nivolumab. The cancer cells will be treated with Oxaliplatin at concentrations 1 mM, 10 mM, or 100 mM. Avelumab and PBS will be used as the positive and negative control, respectively. The treated cells will be measured by checking for tumor size change in confocal microscopy and MTT assay, measuring the ICD using flow cytometry analysis of CRT expression, and conducting Western Blot for Cytokeratin 20 expression. The results of the study will provide insights on the potential of Oxaliplatin as a treatment of Merkel Cell Cancer in the future.

Keywords: Merkel Cell Cancer, Oxaliplatin, T cells, PD-1 and PD-L1

1. Introduction

Merkel cell cancer, or Merkel cell carcinoma (MCC), is a rare but highly aggressive skin cancer [1]. 1 per 130,000 people would have been diagnosed with having Merkel cell cancer in the U.S. Merkel cells are usually found in the top layer of the skin, these cells are very close to the nerve endings that receive the sensation of touch. Therefore, the cancer often comes with neuroendocrine deficiency and related symptoms. The MCC also has a high risk for recurring and metastasizing, often within two to three years after initial diagnosis, making it the second most common cause of skin cancer death after the melanoma [2].

Epithelial-to-mesenchymal transition (EMT) is a process that provides cancer cells with a metastatic phenotype, and thus plays a key role in the progression and chemoresistance of cancers [3]. For cancer cells to metastasize, it is important to lose their cell-cell adhesiveness and detach from the original tumor. One of the factors that
contribute to such a transition is the change in the expression of cadherins, a family of membrane glycoproteins that mediate calcium-dependent cell-cell adhesion. During cancer metastasizing, E-cadherins, which are normally expressed by epithelial cells, are downregulated, while N-cadherins which are expressed by mesenchymal cells, are upregulated, thus resulting in a loss of epithelial phenotypes and a de novo acquisition of mesenchymal phenotypes, allowing the cancer cells to spread to other tissues and organs throughout the human body [3].

With such a significant metastasizing rate, one of the most effective treatments could be the induced immune system response to activate its anti-tumor activities. Immunogenic cell death (ICD) is a form of cell death induced by a series of cytostatic agents, the process involves changes in the composition of the cell surface as well as the release of soluble mediators, occurring in a defined temporal sequence [4]. Such signals operate on a series of receptors expressed by dendritic cells to stimulate the presentation of tumor antigens to T cells. Oxaliplatin is a chemotherapy drug that contains platinum, it is typically used to stop or slow cancer cell growth. Recent studies have discovered that in comparison with its analogue- Cisplatin, Oxaliplatin induces more robust ICD in Lewis lung carcinoma (LLC) cells in the mouse model through activating dendritic cells and enhancing T-cell infiltration, making Oxaliplatin a effective inhibitor to restrict and limit cancer cell metastasizing [4].

PD-1 (Programmed cell death-1) is a protein located in T-cells which helps regulate the immune responses in human body [5]. When PD-1 binds to another protein called PD-L1, the T-cell will lose its function as a killer cell, thus preventing T-cell from killing other cells including cancer cells [5]. PD-L1 is normally expressed in some cells, but are found largely expressed in MCCs, which protects the cancer cells from being eliminated by T-cells. In this case, the introduction of PD-1 inhibitor drug will block the binding of PD-1 to PD-L1 proteins, allowing efficient programmed cell apoptosis [4].

Hypothesis: Since Oxaliplatin can promote immunogenic cell death (ICD) and activate T-cell responses through dendritic cell signaling, it can be hypothesized that increasing concentration and treatment duration with Oxaliplatin combined with Nivolumab (a PD-1 inhibitor) can effectively lead to cancerous Merkel cell apoptosis.

2. Material and Method

2.1. Drug treatments

Avelumab will be obtained commercially as the positive control drug treatment as it is currently the most effective chemotherapy drug being widely used against the Merkel Cell cancer [6]. PBS will be used as the negative control drug treatment. Oxaliplatin will be obtained commercially and combined with Nivolumab together as the experiment variable drugs.

2.2. Cancer cell line

Merkel Cancer Cell line MCC1 which established from mouse xenografts and exhibits stage 1 tumor formation will be obtained commercially and cultured for experiments [7].
2.3. Cancer cell culture

Merkel Cancer Cell line MCC1 will be transformed and grown in Roswell Park Memorial Institute Medium (RPMI)-1640 supplemented with 2mM L-glutamine, 1.5 g/L sodium bicarbonate, 10mM HEPES, 10% fetal bovine serum (FBS), 4.5 g/L glucose, and 90% 1.0 mM sodium pyruvate. The cell line will be sub-cultured at appropriate times to proliferate [8].

2.4. Confocal Microscopy Tumor size observation

The stage 1 MCC1 cells will be detached from culture medium and treated with 1mM, 10mM, and 100mM of Avelumab or 1mM, 10mM, and 100mM of Oxaliplatin combined with same concentration of Nivolumab. Another group will be treated using same amount of PBS as negative control group. The treated cells will be incubated at 37 degrees Celsius for 48h and then check for tumor size change using confocal microscopy with a time duration of 1 day, 3 days, 7 days, and 30 days after drug treatment and incubation.

2.5. Western Blot

The MCC1 cells will be treated with the same concentrations of Oxaliplatin combined with Nivolumab; Avelumab; and PBS as stated in the previous experiments. The treated cells will be incubated for 48 hours, and then washed twice with 1000 mM of PBS buffer. Using lysis buffer with 50 mM Tris-HCl, 50 mM β-glycerol phosphate, 50 mM NaCl, 1 mM Na3Vo4, 1 mM EDTA, 1 mM EGTA, and 1% NP40. After cell lysis, centrifuge the cell at 16000 rpm for 3 minutes, discard the supernatant and collect the pellet at the bottom into Eppendorf tubes. Load protein samples containing equal amounts of protein (50ul) prepared in sample buffer into SDS-PAGE wells for SDS-PAGE gel electrophoresis. Membrane transfer will be conducted after electrophoresis. The membranes will then be incubated at 4 degrees Celsius overnight with monoclonal antibodies against Cytokeratin 20 which is a sensitive and specific marker for Merkel cell carcinoma [9]. Following primary antibody incubation, the membranes will then be washed with Tris-buffered saline with 0.1% Tween 20 and treated with rabbit anti-Goat IgG Antibody for 1h at room temperatures. After washing in TBST, ECL Western Blotting Substrate will be used to detect and test for Cytokeratin 20 protein expression.

2.6. MTT assay

MTT assay will be performed to examine the effects of Oxaliplatin combined with Nivolumab PD-1 inhibitor on MCC1 cell apoptosis. The MCC1 cells will be incubated overnight at 37 degrees Celsius, and treated with different concentrations of Oxaliplatin combined with Nivolumab; Avelumab; and PBS (1, 10, 100mM) respectively. Add 50 µL of serum-free media and 50 µL of MTT solution into each sample group, and incubate at 37 degrees Celsius for 3 hours. After that, add another 150 µL of MTT solutions, and put the sample plates on an orbital shaker for 15 minutes to fully dissolve the formazan crystals. Read the plates and measure the absorbance at OD=590 nm [10].
2.7. CRT expression analysis

CRT expression analysis will be conducted to examine the apoptosis level of Oxaliplatin & Nivolumab treated MCC1 cells, as the increasing CRT protein level indicates ICD of the cell [4]. After treating the MCC1 cells with Oxaliplatin and incubated for 24h, the cells will be collected and washed using PBS with 10% FCS and 1% of sodiumazide. After centrifugation and discard the supernatant, add formaldehyde into the cells and resuspend using staining buffer. Then, use CRT antibody (Abcam, ab2907) to stain the cells at 4 degrees Celsius for 40 minutes, and incubate using Alexa488 antibody for another 30 minutes. The CRT protein expression at the surface of the MCC1 cell will be analyzed using FACScan, and the data will be analyzed using FlowJo software package [11].

3. Statistical Analysis

Each experiment is conducted and repeated for three times, and all the data are represented as mean ± standard deviation (SD). T-test is used for statistical analysis, and the different treatment groups are compared using one-way ANOVA followed by Tukey’s post hoc tests. P-value is calculated using GraphPad Prism 8 with significant threshold set to 0.05 (see Table 1).

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Table 1. Combination of Possible results

Note that “+” indicated a positive result which Oxaliplatin combined with Nivolumab (PD-1 inhibitor) successfully lead to MCC1 cell apoptosis and ICD. While a “-” indicated a negative result, which Oxaliplatin combined with Nivolumab fails to cause results stated above.
4. Possible results

In result 1, the tumor size of treated cells have decreased, and also decreased cell proliferation level can be observed from the MTT assay. While increased CRT and decreased Cytokeratin 20 protein expression in CRT expression analysis and Western blot are observed respectively.

In result 2, the treated cells successfully lead to decreased tumor size, decreased cell proliferation level, and increased CRT expression. However, decreased Cytokeratin 20 protein expression is not observed in the treated MCC1 cells.

In result 3, the treated MCC1 cells successfully lead to decreased tumor size, decreased cell proliferation level, and decreased Cytokeratin 20 expression, but fail to have increased CRT protein expression at the surface of the cell using CRT expression analysis.

In result 4, the MCC1 cells successfully lead to decreased tumor size and proliferation activity, while fail to have both increased CRT expression as well as decreased Cytokeratin 20 expression.

In result 5, the increasing concentration of Oxaliplatin combined with PD-1 inhibitor lead to decreased tumor size, increased CRT & decreased Cytokeratin 20 protein expression, but the cell proliferation level is not decreased by MTT assay.

In result 6, the drug treatment resulted in the decreased tumor size, and increased CRT expression, while the cell proliferation level is not decreased, and the Cytokeratin 20 expression is not decreased either.

In result 7, tumor size is decreased by confocal microscopy, and decreased Cytokeratin 20 expression is increased by Western Blot, while the cell proliferation level is not decreased, and the CRT expression level is not increased.

In result 8, only decreased tumor size is successfully observed, while the cell proliferation level fails to decreased, CRT expression is not increased, and Cytokeratin 20 expression level is not decreased in the treated MCC1 cell.

In result 9, tumor size is not decreased by confocal microscopy, cell proliferation level is decreased by MTT assay, increased CRT and decreased Cytokeratin 20 expression level are observed.

In result 10, tumor size is not decreased by confocal microscopy, cell proliferation level is decreased by MTT assay, CRT expression level is increased, and Cytokeratin 20 expression level is not decreased by Western Blot.

In result 11, tumor size is not decreased by confocal microscopy, cell proliferation level is decreased by MTT assay, CRT expression level is not increased, and Cytokeratin 20 expression level is decreased by Western Blot.

In result 12, tumor size is not decreased by confocal microscopy, cell proliferation level is successfully decreased by MTT assay, CRT expression level is not increased, and Cytokeratin 20 expression level is not decreased.

In result 13, tumor size is not decreased by confocal microscopy, cell proliferation level is not decreased by MTT assay, the CRT expression level is successfully increased, while the Cytokeratin 20 expression level is not decreased.

In result 14, tumor size is not decreased by confocal microscopy, cell proliferation level is not decreased by MTT assay, the CRT expression level is not increased, while the Cytokeratin 20 expression level is successfully decreased by Western Blot.

In result 15, tumor size is not decreased by confocal microscopy, cell proliferation level is not decreased by MTT assay. However, CRT level is increased and Cytokeratin 20 expression level is found to be successfully decreased.
In result 16, tumor size is not decreased by confocal microscopy, cell proliferation level is not decreased by MTT assay, and CRT level is not increased, Cytokeratin 20 expression level is not decreased, either.

5. Discussion

Result 1 fully supports the hypothesis, in which 100mM concentration of Oxaliplatin combined with Nivolumab successfully leads to shrink in tumor size, decreased cell proliferation level, increased CRT and decreased Cytokeratin 20 expression, indicating cell apoptosis and ICD is successfully induced by Oxaliplatin, which is consistent with the effects of the positive control Avelumab. For future experiments, we can investigate whether Oxaliplatin can induce apoptosis and ICD of the cancer cells without combining with the PD-1 inhibitor Nivolumab by compare the results of only Oxaliplatin to the results generated using Oxaliplatin combined with Nivolumab and PBS as the negative control.

Result 2 partially supports the hypothesis, in which tumor size shrink and decreased cell proliferation level is successfully generated using MTT assay, indicating cancer cell induced apoptosis with Oxaliplatin & Nivolumab, this can be further justified by the increased CRT expression level at the surface of the cell, which serves as a sensitive marker for ICD. However, the Cytokeratin 20 protein expression level is not decreased by Western Blot, indicating that the number of MCC1 cancer cells is not being actively reduced, which is contrast to the hypothesis. The reason of this finding could be that the treatment duration is not long enough after treated with 100mM Oxaliplatin & Nivolumab, resulting in remained Cytokeratin 20 expression. Another possible explanation could be that since Cytokeratin 20 is not only a marker for the presence of Merkel Cell Carcinoma, but also a sign for metastasizing activity. Thus, the not decreased Cytokeratin 20 level indicates that the cancer cell is still spreading to the surroundings before ICD is induced, which means Nivolumab is ineffective in preventing cell metastasizing. Result 4 also generates the similar results as compared to result 2, which CRT level and Cytokeratin 20 level are not as expected. For future experiments, other PD-1 inhibitors could be used to test for its effectiveness in preventing cancer cell spreading and ICD.

Result 3 partially supports the hypothesis, as the decreased tumor size, decreased Cytokeratin 20 and decreased cell proliferation activity is observed and consistent with the expected results. However, the CRT expression level is not increased, which serves as a marker for ICD of the cancer cell. Possible explanation could be that the CRT protein expression is time sensitive, as the treatment duration time is not long enough, resulting in not obvious increase in the CRT level. Same explanation could be used for the result 5 and 9, which the cell proliferation level and tumor size is not decreased respectively. For future experiment, the CRT expression analysis could be done at a longer time after the drug treatment, and tumor size.

Result 6, 7, 10, 11, and 15 partially supports the hypothesis, as the results only exhibit two expected outcomes but never the four expected outcomes. This could be due to the low concentration of the Oxaliplatin & Nivolumab added into the MCC1 cells. Since only 10mM of Oxaliplatin & Nivolumab is added, it can be concluded that low concentration of Oxaliplatin and PD-1 inhibitor can only partially and ineffectively leads to cell apoptosis and ICD, and cannot prevent cancer cells from metastasizing to the surroundings. For future experiments, we can divide the MCC1 cells into two treatment
groups, one group is treated with high concentration of Oxaliplatin and low concentration of Nivolumab, the other group is treated with low concentration of Oxaliplatin and high concentration of Nivolumab. Therefore, we can further study the specific function of Oxaliplatin and Nivolumab, respectively, in treating with Merkel cell cancer.

Result 8, 12, 13, and 14 partially support the hypothesis, as all the groups only generated one expected result, while the rest of the measurements are inconsistent with the hypothesis. Which indicates that increasing concentration of Oxaliplatin and PD-1 inhibitor would likely repress each other’s functions, resulting in only one expected result at a time no matter how long the treatment duration is. The results also indicates that the ICD and apoptosis might not be necessarily coupled, and the mechanism of inducing ICD or cancer cell apoptosis remains unknown with the techniques currently performed and used in the experiment. For future experiments, Oxaliplatin can be used individually to examine its effects on Merkel cell cancer, and whether it can induce cell apoptosis and ICD.

Result 16 does not support the hypothesis that Oxaliplatin combined with Nivolumab can effectively lead to MCC1 cell apoptosis and ICD as the treated MCC1 cells did not demonstrate significant effectiveness in decreasing the tumor size and cancer cell proliferation activity, and failed to cause the decrease in Cytokeratin 20 expression, and increase in CRT expression level. This result suggests that Oxaliplatin combined with Nivolumab would have no effects at all to the treatment of Merkel cell cancer. However, it could be possible that the lack of ineffectiveness might be due to the dose concentration added and the treatment duration being too long or too short. For future experiments, it is suggested that other kind of drugs can be used to test for the effectiveness of treating the Merkel cell cancer.

6. Conclusion

In conclusion, this paper examines the potential of Oxaliplatin combined with Nivolumab to cause cancerous Merkel cell apoptosis and induce Immunogenic cell death (ICD). The results have shown all the possible outcomes of the experiments performed, and indicates the possibilities of whether Oxaliplatin is an effective drug that can be used for the treatment of Merkel cell cancer. The experiments provide insights for future studies of Merkel cell cancer treatment, as well as the research of the functions and effectiveness of Oxaliplatin on other types of cancer.

References


