The effect of Oxaliplatin in leading to apoptosis of intestinal cancer cells by inducing PUMA protein expression through reactive oxygen species

Jiayi Gao

Abstract

Previous studies demonstrated Oxaliplatin, a drug used in treating intestinal cancers by inducing cancer cell apoptosis regulated by p53 upregulated modulator of apoptosis (PUMA) protein gene expression. Increasing the reactive oxygen species (ROS) level is possible to speed up the expression of PUMA protein in Oxaliplatin. This study aims to investigate the effect of Oxaliplatin in intestinal cancer cells, leading to its apoptosis by inducing PUMA protein expression through ROS, compared to Cisplatin and PBS. The result of the study will provide important insight into the preclinical effectiveness of Oxaliplatin in intestinal cancer cells, and it also testifies to the underlying mechanism of this drug. Future studies should focus on investigating drug combinations with Oxaliplatin that provides synergism toward the disease and decreases its toxicity.

Keywords: Oxaliplatin, p53, PUMA, intestinal cancer, cell apoptosis, ROS

1. Introduction

Nowadays, an increasing number of people are diagnosed with intestinal cancer and colorectal cancer, especially young people because of a sedentary lifestyle, use of tobacco and alcohol, and unhealthy diets [1]. If cancer spreads to distant parts of the body, the 5-year survival rate is only 14% [2]. As the first-line drug of colorectal carcinoma chemotherapy, oxaliplatin causes damage to tumors by inducing apoptosis. Since it was the first platinum agent with demonstrated effectiveness against colorectal carcinoma, oxaliplatin has been used as the gold standard in colorectal carcinoma care. We already know oxaliplatin fights cancer by inducing the apoptosis of cancer cells, but we have no clear clue about its molecular mechanism. The underlying mechanism may be related to the inhibitory effect of platinum on DNA replication inducing cancer cell death. However, the platinum that kills cancer cells leads to side effects like neurotoxicity since it is harmful to the patient's normal body cells [3]. In this article, I investigate the mechanisms of PUMA in oxaliplatin and its role in the apoptosis of intestinal cancer cells, focusing on its interaction with ROS. The wild type p53 gene can generally induce apoptosis of tumor cells and prevents carcinogenesis. However, the mutant p53 gene promotes cancer development in cells, and there appears to be mutant p53 gene expression in the general type of cancers because the p53 gene inhibits cell cycle progression and promotes cell death in order to limit tumor growth [4]. To prevent the detriment of inappropriate expression and overactivation of the p53 gene while keeping its normal expression still available, PUMA exists to regulate and initiate cell death when there is overwhelming p53 expression PUMA are included in the BH3 protein-only family, which also interacts with Bcl-2 and Bcl-XL at the mitochondrial membrane, causing Bax to change its location on the mitochondrial membrane, multimerize, and release cytochrome C, thus apoptosis occurs [5].

Scientists have already demonstrated that increased amounts of ROS induce cell apoptosis because high levels of reactive oxygen species can cause damage to cells and gene structure. Because protein folding and mitochondrial respiration are two biological processes that naturally produce ROS as byproducts. According to the study, cancerous cells have higher levels of internal oxidative stress than their noncancerous equivalents, which has been linked to unbalanced redox status. Therefore, I will research the relationship between ROS and PUMA and whether ROS contributes to PUMA-inducing cancer cells' apoptosis by its gene expression [6].

2. Materials

Reagents: 3-(4,5-Dimethylthiazol-2-yl) - 2, 5-Diphenyltetrazolium Bromide; 2',7'-dichlorodihydrofluorescein diacetate; RIPA cell lysis; Bull Serum Albumin; Tris Buffered Saline Tween. All the above will be purchased from companies.

Cell lines: Human Caco2 cell line will be used to compare cell signaling by Oxaliplatin, positive control, and negative control. Caco2 cells will be grown in DMEM medium, 0.1 mM of non-essential amino acids, 100 U/mL of penicillin, 0.1 g/mL of streptomycin, 10 mM of sodium bicarbonate, and 10% fetal bovine serum are added to

DMEM, the growth medium [7]. The cultures will be incubated at 37 °C in a humid atmosphere containing 5 % CO2. All required cell lines, medium, and supplemental chemicals will be purchased from companies.

Laboratory animals: We will acquire 60 male mice that are 4 weeks old and weigh between 15 and 20g on average. The mice will be given a standard rodent meal and given unlimited access to water. Prior to any experiments, the mice will be kept in a sterile setting for seven days to adapt. All animal research and treatment will adhere to institutional authorities' regulations.

3. Methods

3.1 Annexin V/PI assay

To make sure whether a cell is apoptotic or necrotic and find out what a cell is in what stage of apoptosis, I remove the media from the cells to be washed by PBS, then add Annexin V and propidium iodide (PI) working mixture. The cell suspension will then be incubated in the darkness for 15 minutes. The results can be visualized and the relationship between PI and Annexin V can be analyzed by flow cytometry plots.

3.2 Western blot

Wash cell lines by adding phosphate-buffered saline (PBS) and discarding it. Add RIPA cell lysis and place on ice incubate for 30 minutes at a rotation rate of 12000 RPM for 10 minutes at 4 C. Use a cell spatula to scrape down the cells. A BCA Protein Assay Kit is required for consistent protein concentrations in the supernatant. Protein will be electrophoretic by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane at 4 ° C for 90 minutes. Add 5% Bull Serum Albumin in TBST and wash it off for one hour to prevent nonspecific binding. The primary antibody for overnight incubation at 4 ° C on PUMA proteins against (p53 upregulated modulator of apoptosis) was added to a stirrer and room temperature. When finished, the cell membrane will be washed by TBST three times for five minutes each time. Add a secondary antibody to BSA and incubate for one hour. The cell membrane was washed again with TBST 3 times for 5 minutes each time [8]. The results are visualized when the ECL mixture is incubated in the cell membrane for one to two minutes. The expression of PUMA proteins is calculated.

3.3 MTT assay

In order to test the harm of oxaliplatin and the mechanism of PUMA to the mitochondria of cells, I plated the Caco 2 cell lines 24 hours before the assay. During the assay, add 3 - (4,5-Dimethylthiazol-2-yl) - 2, 5-Diphenyltetrazolium Bromide (MTT) reagent to cells in the darkness and incubate for 4 hours. Then add DMSO while incubating for 2 hours. Therefore, we can calculate the viability of cells in the control group (Caco 2 cell lines added PBS) and treated group that Caco 2 cell lines treated by oxaliplatin as concentrations increase (0.2 mg/ml, 0.45 mg/ml, and 0.70 mg/ml which is the highest concentration of oxaliplatin used clinically and know whether the cells are killed by oxaliplatin [9].

3.4 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) staining

ROS could be found by measuring the created products when they interact with specific chemicals [10]. In order to measure ROS, 2',7'-dichlorodihydrofluorescein diacetate is frequently utilized (H2DCFDA). Dichlorofluorescein (DCF), which is produced when ROS oxidizes the sensor, has a strong luminescence [11]. Dissolve 4.85mg of DCFH-DA in 1 ml of normal saline, then mix with DMEM that has been incubated overnight at 37 degrees Celsius, stirring for 10 seconds. Add 500 μL solution to each well and incubate at 37 degrees Celsius for 30 minutes. Discard the solution and add 500 μL of PBS to each well. Fluorescent images were taken for each well using green fluorescent protein on a fluorescent microscope. Afterward, PBS was removed, 200 μL of RIPA was added to each well, and incubated on ice for 5 min, and then the cell lysate was collected and centrifuged at 4 °C for 10 min. 100 μL of supernatant was transferred to a black well plate and the fluorescence intensity was measured using a fluorescent microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm[12]. We will use the same Caco 2 cell lines in two Petri dishes that one added PBS as a negative control group and one treated by Oxaliplatin, then add DCF to both Petri dishes, observe the fluorescence color of the two groups of cells, and record the difference in color as time changes (30 minutes, 60 minutes, and 90 minutes).

3.5 Animal studies

Considering some scientific demonstrations of its cancer promotion, I will use mice for this experiment [13]. The mice will be randomly assigned into three groups of 20, and all groups will receive orthotopic microinjection of the same cell line – Caco 2. Ketamine and xylazine were used to sedate naked mice before the appendix were surgically removed. In a clean micropipette, Caco-2 (2 million) was suspended in culture media. Under binoculars, we slowly injected the cell suspension at a 30° angle, inserting the tip for 5 mm into the cecum wall. Then, we drew out the pipette and lightly pressed a cotton swab 2 mm from the injection site before cleaning the region around the injection with 3% iodine. Ultimately,

the peritoneal cavity was closed with surgical grapes after the intestine was reinserted there [14]. Three groups of mice will be injected with cisplatin, oxaliplatin, and PBS, respectively. Moreover, cisplatin and oxaliplatin groups of mice received oral treatment with N-Acetylcysteine, a ROS inhibitor, which is believed to minimize Damage to DNA as an antioxidant [15]. While the negative control group of mice only receive oral treatment with PBS. Watch and record the change of PUMA expression or cell apoptosis within three groups for 20 days, observing the relationship between ROS and PUMA expression by oxaliplatin, and whether treating the groups of cisplatin and oxaliplatin with ROS inhibitors will make their results close to that of PBS group.

3.6 Statistical analysis

For all experiments except animal studies, I will do 10 samples for each treatment with 3 replicates. So, the total number of samples is 30 for every method. The statistical significance of all numerical data acquired from flow cytometry, western blot, MTT assay, DCFH-DA staining, and animal studies will be analyzed using the student's T-test, with the level of significance set to $p < 0.05$(see Table 1).

Table 1. Combination of possible results (PR)

		Observations				
		Oxaliplatin lead to cell apoptosis by flow cytometry?	PUMA expression increased by western blot	Oxaliplatin causes mitochondrial damage by MTT assay?	ROS level increased by DCFH-DA staining?	Support Hypothesis?
Possible Results (PR)	1	+	+	+	+	Yes
	2	-	+	+	+	P
	3	-	-	+	+	P
	4	-	-	-	+	P
	5	+	+	+	-	P
	6	+	+	-	-	P
	7	+	-	-	-	P
	8	+	-	+	-	P
	9	-	+	-	+	P
	10	-	+	+	-	P
	11	+	-	-	+	P
	12	-	+	-	-	P
	13	-	-	+	-	P
	14	+	+	-	+	P
	15	+	-	+	+	P
	16	-	-	-	-	No

Note. A "+" indicates a positive result of the experiment conducted supports the hypothesis. A "-" indicates a negative result that contradicts the hypothesis. A "P" indicates partial support where Oxaliplatin either fails to cause cell apoptosis, increase PUMA proteins expression, lead to mitochondria damage, or increase ROS level.

Oxaliplatin might favor explaining the process of the p53 upregulated modulator of apoptosis protein expression inducing the cell apoptosis death through reactive oxygen species or cannot prove this mechanism (PR1 and PR16, respectively).

In PR5, Oxaliplatin may be favored to demonstrate its mechanisms of PUMA protein expression leading to cell apoptosis by causing mitochondrial damage but fail

to clarify its relationship with ROS. However, in PR4, Oxaliplatin shows its relationship with increased ROS but fails to connect with PUMA protein expression. Oxaliplatin won't be the preferred treatment if Oxaliplatin with ROS inhibitor doesn't change PUMA expression to the same extent as Cisplatin instead performs as well as PBS treatment.

Oxaliplatin might be favored to increase the PUMA expression, which is close to Cisplatin's results in PR2, PR9, and PR14. When Oxaliplatin increases the p53 upregulated modulator of apoptosis (PUMA) protein expression in the cancer cells at previously suggested concentrations to a similar extent as Cisplatin while performing noticeably better compared to the PBS treatment, that will be considered a favorable outcome for the treatment. Moreover, these three possible results showed a high level of ROS in the staining experiment as PUMA expression increased, revealing that increased PUMA expression coincided with a high level of ROS. When Oxaliplatin with ROS inhibitor changes PUMA expression in the cancer cells at previously suggested concentrations to a similar extent as Cisplatin while performing noticeably better compared to the PBS treatment, that will be considered a favorable outcome for the treatment.

The MTT assay didn't reveal the mitochondrial damage for PR6, PR7, PR11, and PR12, which may be favored to the results of PBS instead of Cisplatin. PR6, which may favor PBS results of ROS level, indicates that ROS might not lead to damage to mitochondria. Oxaliplatin won't be the preferred treatment if it doesn't lead to mitochondrial damage to the same extent as Cisplatin instead performs as well as PBS treatment. Within these models, PR7 and PR11 might be against the role of PUMA protein in Oxaliplatin, which researchers have already verified. Therefore, the model of PR7 and PR11 might not favor the results of Cisplatin. Oxaliplatin won't be the preferred treatment if it doesn't cause increased PUMA protein expression to the same extent as Cisplatin instead performs as well as PBS treatment.

In PR8 and PR15, we might detect the amounts of propidium iodine and the binding of Annexin-five to phosphatidylserine compared to that in PBS changed in the Annexin-PI assay. Moreover, the color of PR8 and PR15 will change to purple after conducting an MTT assay, in which the sample color is close to Cisplatin samples and PBS samples might stay yellow in contrast. Therefore, these two combinations of possible results favored the positive control group of Cisplatin on the results of mitochondrial damage and cell apoptosis of intestinal cancer cells. When Oxaliplatin leads to mitochondrial damage at previously suggested concentrations to a similar extent as Cisplatin while performing noticeably better compared to the PBS treatment, that will be considered a favorable outcome for the treatment.

However, Oxaliplatin might be close to the PBS' results in PR3, PR4, and PR13 because there is no even cell apoptosis happening as well as not increased PUMA expression, which is totally opposite to the basic mechanism of Oxaliplatin that scientists affirmed previously. When Oxaliplatin causes cell death in the cancer cells at previously suggested concentrations to a similar extent as Cisplatin while performing noticeably better compared to the PBS treatment, that will be considered a favorable outcome for the treatment. Oxaliplatin won't be the preferred treatment if it doesn't cause cancer cells apoptosis to the same extent as Cisplatin does instead performing as well as PBS treatment.

As the concentration of Oxaliplatin increases during MTT assay, the color of cell lines samples will change its color more obviously as well as the Cisplatin, but PBS might stay yellow color because there is no Oxaliplatin in it which would not cause mitochondrial damage. As time changes from 30 minutes to 90 minutes in the DCFH-DA staining experiment, the ROS level within the Caco 2 cell lines samples might be increasing as logarithmic growth, which appeared the similar speed as Cisplatin. When Oxaliplatin raises the total ROS level in the cancer cells at previously suggested concentrations to a similar extent as Cisplatin while performing noticeably better compared to the PBS treatment, that will be considered a favorable outcome for the treatment. Nevertheless, the PBS samples might have a constant rate of increasing ROS level in the cell lines or stay no change. Oxaliplatin won't be the preferred treatment if it doesn't raise total ROS level to the same extent as Cisplatin does instead performing as well as PBS treatment.

4. Discussion

Previous studies frequently demonstrated that Oxaliplatin exhibits anti-cancer properties in various types of colorectal cancers because PUMA protein is involved in the cell apoptosis [16]. However, little is known about the effect of ROS level in Oxaliplatin in the body along with Oxaliplatin-induced cell apoptosis PUMA protein expression. Therefore, to test the effects of ROS level to Oxaliplatin and PUMA expression in colorectal cancer cells, this study applies Oxaliplatin/Cisplatin treatment and PBS treatment (which only protect cells from shrinking due to osmosis) to Caco 2 cell lines to establish the cell apoptosis, increased PUMA expression, and cell killing under Oxaliplatin treatment instead of PBS treatment as well as using orthotopically transplanted mice to indicate

the decreased PUMA expression under a treatment of ROS inhibitor in the Oxaliplatin treatment[17].

Possible result (RR) 1 is consistent with the findings of previous studies investigating the PUMA expression of Oxaliplatin [18]. We found apoptotic cancer cells and increased PUMA expression present after the Annexin V/PI assay and Western blot respectively for the Oxaliplatin treated groups than for the negative control groups (PBS), indicating the potential of Oxaliplatin-induced cell apoptosis due to PUMA expression. Furthermore, after the MTT assay, we discovered the existence of cell killing, which should be consistent with the positive results of first two assay. Because MTT assay demonstrated the cell viability by adding mitochondrial reductase, as we already mentioned in the introduction that PUMA expression causes translocation of BAX protein in mitochondria [19]. Therefore, possible results (PR) that we found apoptotic cancer cells and increased PUMA expression but no cell killing by MTT assay, PR 6, PR 14, PR 13 should be rejected since they are contradicting to previous studies.

Moreover, studying the previous studies, we can reject all possible results that we observed increased PUMA expression without cell apoptosis, like PR 2, PR9, PR10, PR12. We may not detect any or a little cell apoptosis in these four possible results which might not exhibit the effectiveness to PBS treatment group of colorectal cancer cells. Instead, we could discover necrotic cells by Annexin V/PI assay because of unhealthy eating habits. However, there might be colossal apoptotic cancer cells in the experiment group (Oxaliplatin treatment) and Cisplatin group because of using drugs which activates PUMA proteins to start expression.

In PR9 and PR4, no matter High level ROS are related to increased PUMA protein expression, there are neither cell killing nor cancer cell apoptosis. On the contrary, in PR5 and PR8, there are both increased cancer cell apoptosis and cell killing without high ROS level. The insight from these four possible results is opposite with previous study that demonstrating a reasonable quantity of ROS causes cell damage, DNA mutation, and inflammation, encouraging the beginning and growth of cancer; Overly high levels of ROS cause the apoptosis of cancer cells, showing an anti-cancer effect [20].

In PR 4, we didn't observe any positive results from the first three conducted experiments, so it doesn't support the hypothesis in this article. After DCFH-DA staining, we find that high ROS level is similar to PR 1, even if the results for other experiments are all negative. PR4 is still valuable and possible, since reactive oxygen species can cause damage on DNA, which might lead to cancer cell death. In the future, we can study on how utilize ROS to better treat colorectal cancer cells with less toxicity because Oxaliplatin still has some serious side effects even it is already good enough compared to Cisplatin. Same as PR 3 and PR 11, we still have room for improvement and research on the potential mechanisms of ROS-induced cancer cell death.

PR 7 and PR 8 partially support the hypothesis although it has nothing to do with PUMA expression and high ROS level. It might propose a new hypothesis for future research to turn the direction from PUMA protein to others. Although it doesn't show any increased PUMA expression and ROS level, we still discovered cell killing and cell apoptosis. Thus, it reveals that there might be other potential mechanisms to kill colorectal cancer cells except PUMA expression and ROS.

In PR5 and PR6, we didn't find that high ROS level compared to PR 1 after DCFH-DA staining. These two possible results can still be possible and partially support the hypothesis. However, they are in favor of previous studies instead of investigating the relation of reactive oxygen species

[21,22]. Therefore, if these two results really happened after the experiments, it would be contradicted to my hypothesis that Oxaliplatin-induced cell apoptosis is related to ROS level. In other words, a mass of ROS won't increase PUMA protein expression, instead, increased PUMA protein expression could directly lead to cell apoptosis without the existence of quantity of ROS.

In PR11 and PR15, we discovered high ROS level inducing cell apoptosis and cell killing without the expression of PUMA protein, which provide a beneficial insight for our future studies. Recalling the introduction part, PUMA protein induced cell apoptosis by translocating the BAX protein at mitochondrial membrane and releasing cytochrome, which I doubt the toxicity to normal cells. Due to the results of model 11 and 15, future research can make an effort on only utilizing ROS as a treatment to colorectal cancers, seeking the slighter toxicity of the drug by less inducing the PUMA protein expression.

CR16 is the least expected outcome in all the possible results, it demonstrates that the results of every experiment conducted contradict the hypothesis. It would suggest that Oxaliplatin doesn't induce cell apoptosis by PUMA expression nor exhibits high ROS level and cell killing, which suggested that Oxaliplatin can't treat colorectal cancer cells successfully as studies concluded or it indicated that there might be other mechanisms of Oxaliplatin to kill colorectal cancer cells.

5. Conclusion

This study explores the effect of Oxaliplatin on intestinal cancer. The result of the experiment would indicate whether the cancer cell apoptosis (including its mechanism of PUMA protein expression) will be affected by the ROS level. The results of this study would also provide insights into the direction of future studies in the same field. The mechanism of interaction between Oxaliplatin and ROS can be further investigated, thus providing insights into the cell signaling pathways of intestinal cancer. A similar drug, Cisplatin, can be revealed to more therapeutic targets potentially. The combination of Oxaliplatin with other drugs can also be investigated for synergistic effects between drugs. For future research, we can focus on the better effectiveness and less toxicity based on this article that lays the foundation for the association of ROS and Oxaliplatin.

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