Original Research Article

Oxaliplatin regulates the autophagy of skin squamous cell carcinoma cell line through HMGB1 pathway

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Abstract

Purpose: To determine the influence of oxaliplatin on skin squamous cell carcinoma cell line, and the involvement of autophagy-regulating pathway of high mobility group box 1 (HMGB1) in the process.
Methods: A431 cells cultured in vitro were used. The cells were divided into groups A (treated with different concentrations of oxaliplatin) and B (treated with different concentrations of oxaliplatin combined with autophagy inhibitor 5 mmol/l3-ma). Changes in expression levels of autophagy marker molecules LC3-Ⅰ, LC3-Ⅱ, p62 and HMGB1 in A431 cells treated with different concentrations of oxaliplatin and different concentrations of HMGB1 were evaluated by Western blotting. Viability of A431 cells in both groups was assessed by CCK-8 assay.
Results: With increase in oxaliplatin concentration, LC3-Ⅱ levels in A431 cells were up-regulated, p62 expression decreased, while autophagy level was increased significantly ($p < 0.05$). With increase in HMGB1 protein concentration, LC3-Ⅱ level in A431 cells was raised, while p62 level was reduced, while the level of autophagy was significantly increased ($p < 0.05$). Oxaliplatin treatment led to significantly higher expression level of HMGB1 in the experimental group than in the control group without oxaliplatin treatment. The viability of oxaliplatin-treated group was dose-dependently and significantly lower ($p < 0.05$) than that of the control group. Compared with the control group, the cell viability of the 3-mA + oxaliplatin group also showed a downward trend, and the decrease was greater than that of oxaliplatin-treated group ($p < 0.05$).
Conclusion: Oxaliplatin upregulates autophagy by promoting HMGB1 protein expression, which may be a protective mechanism of tumor cells against oxaliplatin cytotoxicity thereby making HMGB1 protein a potential target in skin cancer therapy.

Keywords: Oxaliplatin, HMGB1, Autophagy, Skin squamous cell carcinoma

INTRODUCTION

Cutaneous squamous cell carcinoma (CSCC) is the second most common skin tumor in humans. It manifests mostly as skin damage at the sites of long-term exposure to UV radiation, such as the head, face, neck and the back of hands, and it is shallow to the epidermis and deep to the dermis [1]. Unlike basal cell carcinoma which has limitations, CSCC is aggressive, with distant metastasis. With progression of the disease, a

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disease spectrum may gradually develop from precancerous lesions (solar keratosis) to carcinoma *in situ*, and finally to metastatic squamous cell carcinoma [2]. The cure rates of most early-detected skin tumors are relatively high. Surgical resection and other local treatment methods produce good results in the treatment of low metastatic CSCC, especially for the primary lesions with clear boundaries and diameter less than 1 cm. For patients with high metastatic risk CSCC, standardized local treatment does not produce satisfactory therapeutic effect [3]. Systemic chemotherapy has become one of the treatment methods for patients with metastatic CSCC, but there are limited number of studies in this area at present.

Oxaliplatin is a third-generation platinum antitumor drug which exerts cytotoxic effects by inhibiting DNA synthesis in tumor cells. Studies have confirmed that it exhibits broad-spectrum antitumor activity *in vitro*, has obvious killing effect on multiple tumor cell lines, and has resulted in good efficacy in the treatment of colorectal tumors, cholangiocarcinoma and other tumors of different systems [4]. Oxaliplatin has been widely recognized for its toxic effect on tumor cells. At the same time, the problem of drug resistance has increasingly become worrying. Many researchers have put forward many hypotheses on the mechanism of resistance to oxaliplatin, and the role of autophagy in oxaliplatin resistance has become an interesting area of research.

Autophagy is a dynamic decomposition process for cells with abnormal metabolism or non-essential components or organelles. The basal autophagy level of normal cells is low so as to maintain the self-stable state of cells. However, under stress state, the autophagy level of cells is increased. When tumor cells are stimulated by drugs or other stimuli, the increased level of autophagy which enables the cells to cope with the adverse stimuli, has a certain protective effect on tumor cells, and it promotes their survival. Endogenous high mobility group protein 1 (HMGB1), an important regulator of tumor cells, may be related to the regulation of autophagy [5,6]. The aim of this research was to determine the effect of oxaliplatin on the viability of skin squamous cell carcinoma cell lines.

EXPERIMENTAL

Cells

Human squamous cell carcinoma A431 cells were used.

Main reagents and instruments

The major reagents and instruments used were DMEM and trypsin (Hyclone, USA); absolute ethanol, BCA protein assay kit, CCK-8 kit, carbon dioxide incubator, inverted microscope, and microplate reader.

Cell culture

A431 cells were seeded in DMEM supplemented with 10 % FBS and cultured in a 5 % CO_2 incubator with saturated humidity at a temperature of 37 ℃, and the culture medium was changed once in 1 - 2 days. The cells were sub-cultured when they grew to a density of 80 – 90 %. Cells in logarithmic growth phase were used for subsequent experiments.

Cell grouping and treatment

Two groups of cells were used: experimental group which received 15 and 35 μM oxaliplatin, and the control group that was not exposed to oxaliplatin.

Western blot assay

The protein expression levels of LC3-Ⅰ, LC3-Ⅱ, p62 and HMGB1 in the different groups of cells were determined with Western blotting method. Total cellular protein was extracted from the cells using 1 x RIPA lysing buffer containing protease inhibitors. Prior to protein extraction, the medium was discarded and the cells were rinsed thrice with chilled PBS buffer. The cell lysate from each group was centrifuged at 12000 rpm for 15 min at 4 ℃, and the total lysate protein content was measured with the BCA procedure. The proteins were separated on SDS-PAGE, transferred to membranes, and incubated overnight at 4 °C with relevant 1° immunoglobulins, followed by incubation with horse radish peroxidase-conjugated 2° immunoglobulin at room temperature for 1 h, and ECL. Grayscale analysis was used to determine relative protein expression levels.

Determination of cell viability

The experimental group was divided into two sub-groups, namely A treated with 2 doses of oxaliplatin (15 and 35 μM) and B treated with combination of oxaliplatin and 5mM 3-mA. The control group (C) was not exposed to oxaliplatin. The A431 cells with different drug treatments were seeded in 96-well plates and incubated for 24h. Then, CCK-8 solution (10 μL) was pipetted into every well, and further incubation was done for 3h. Using a microplate reader, absorbance

was read at 450nm, and the absorbance values were used to calculate cell survival rate.

Statistical analysis

The SPSS 18.0 statistical software package was used for processing of results. Data are presented as mean ± standard deviation (SD), and *t*-test was performed for independent sample comparison. Count data are presented as percentages, and paired comparison was done with χ^2 test. Values of $p < 0.05$ indicate that the differences amongst groups were statistically significant.

RESULTS

Oxaliplatin-induced changes in autophagy level

Western blotting showed that when oxaliplatin concentration was 35 µM, LC3-Ⅱ expression was significantly up-regulated, relative to control, while p62 expression was markedly down-regulated in the experimental group. With increase in oxaliplatin concentration, the expression of LC3-Ⅱ increased, while the expression of p62 decreased. Changes in LC3-Ⅰ protein level were not obvious, and the level of autophagy was significantly up-regulated (Table 1).

Table 1: Oxaliplatin-induced changes in autophagy level in A431 cells

Oxaliplatin concentration	LC3-I	LC3-II	P62
0 (Control)	1.03±0.12	0.16±0.02	0.63±0.11
15 µM	1.08±0.11	0.35±0.04[a]	0.52±0.08[a]
35 µM	1.09±0.13	0.68±0.07[ab]	0.26±0.04[ab]

[a]$P < 0.05$, vs. control; [b]$p < 0.05$, vs. 15 µM oxaliplatin

Changes in HMGB1 level in A431 cells after oxaliplatin treatment

As depicted in Table 2, Western blotting results showed that at oxaliplatin concentration of 35 µM, there was marked increase in expression level of HMGB1 in experimental group, relative to control value.

Table 2: Effect of oxaliplatin on HMGB1 protein in tumor cells

Oxaliplatin concentration	HMGB1
0 (Control)	0.36±0.06
15 µM	0.53±0.08[a]
35 µM	0.76±0.07[ab]

[a]$P < 0.05$, vs. control; [b]$p < 0.05$, vs. 15 µM oxaliplatin

Changes in autophagy level due to HMGB1 exposure

Western blotting results showed that A431 cells treated with different concentrations of HMGB1 showed dose-dependent changes in levels of autophagy-related proteins. With increase in HMGB1 protein concentration, the expression of LC3-Ⅱ showed an upward trend, while the expression of p62 showed a downward trend. The changes in LC3 - Ⅰ protein level were not obvious, while the level of autophagy was significantly up-regulated. These results are presented in Table 3.

Effect of oxaliplatin on the activity of A431 cells

Cell viability of group A was significantly lower than that of group C. With increase in oxaliplatin concentration, cell viability was decreased in a dose-reliant fashion. Relative to group C, the cell viability of group B also showed a downward trend, and the decreases were greater than those of group A which also showed a dose-dependent trend. These results indicate that oxaliplatin exerted a killing effect on A431 cells, while autophagy inhibitors had significantly enhanced effects on its cell killing potential (Table 4).

DISCUSSION

One of the most common tumors of human skin is CSCC which is of immense importance to clinicians because of its significantly higher invasiveness than basal cell carcinoma. It occurs mostly in the head, face, neck, back of hands and other parts of the body that are exposed to UV radiation for a long time, and it affects the skin, from superficial layer to the deep dermis.

Table 3: Changes in autophagy level of A431 cells after treatment with different concentrations of HMGB1

HMGB1 concentration	LC3- I	LC3-II	P62
0 (Control)	1.05±0.08	0.18±0.03	0.66±0.09
500 ng/mL	1.07±0.09	0.37±0.05A	0.48±0.07a
1000 ng/mL	1.10±0.11	0.62±0.08ab	0.31±0.05ab

[a]$P < 0.05$, compared with control; [b]$p < 0.05$, compared with 500 ng/mL dose of HMGB1

Table 4: Effect of different concentrations of oxaliplatin on survival rate of A431 cells (%)

Group	0	15 µM	35µM
A	94.65±5.53	58.49±6.23[a]	10.84 ±1.34[a]
B	95.63±5.68	24.81±3.17[ab]	5.96±1.03[ab]
C	96.86±4.84	66.78 ±7.92	18.65 ± 1.88

[a]$P < 0.05$, compared with group C; [b]$p < 0.05$, compared with group A.

Due to limitations in educational and economic levels, skin neoplastic diseases are often ignored in developing countries, and patients go to see doctors only when the diseases seriously affect normal life, an attitude which results in low rate of diagnosis and treatment [7]. Although less than 5% of CSCC patients degenerate to metastatic CSCC, its level has shown an upward trend in recent years, and it has increasingly become an important public health problem. Some studies have found that some clinical manifestations of CSCC are not highly malignant, but the disease may already have acquired aggressive behavior [8]. Other studies suggest that CSCC may be associated with genetic susceptibility, and once this kind of population develops, CSCC progression is extremely rapid. Therefore, in order to actively diagnose and treat CSCC, basic research on it is very necessary [9].

Autophagy is a general term for decomposing abnormal or non-essential components or organelles in eukaryotic cells through lysosomal pathways. Normal cells have low levels of basal autophagy which is highly conserved, and it is an important protective mechanism against apoptosis [10]. Under stress, the level of autophagy increases, to support cellular survival before initiation of apoptotic signals. Autophagy is related to the occurrence of many diseases. Neurological research has shown that when the level of autophagy in neurons is blocked, intracellular error proteins are not degraded and cleared in time, resulting in degenerative lesions in neurons [11]. Therefore, for normal cells, autophagy supports cell survival by degrading proteins harmful to their own cells, and impaired autophagy is pathogenic for cells with physiological functions. The existence of autophagy blocks the accumulation of proteins or other components harmful to tumor cells, thereby enhancing tumor cell survival. This has become an important mechanism underlying anti-tumor drug resistance [12]. Oxaliplatin is a well-defined anti-tumor drug, but the resistance of tumor cells to oxaliplatin has seriously affected its therapeutic effect. The relationship between autophagy and oxaliplatin resistance has become an interesting area of research. This study has demonstrated that with increase in oxaliplatin concentration, the expression of LC3-

Ⅱ showed an upward trend, while changes in LC3-Ⅰ protein level were not obvious, and the level of autophagy was significantly up-regulated. When stimulated, LC3-Ⅰ binds to the autophagosome membrane lipids. During autophagy, LC3-Ⅰ protein is transformed into LC3-Ⅱ form which also returns to the autophagosome membrane to form autophagolysosomal degradation complex. The level of LC3-Ⅱ also decreases. Therefore, the ratio of LC3-Ⅱ/LC3-1 may be used as an important indicator of the level of autophagy. The autophagy substrate P62 is of great significance for detecting autophagic flux, that is, the autophagosome breakdown mechanism downstream of autophagy [13]. In the present work, an increase in LC3-Ⅱ level was accompanied by a decrease in p62 level, which suggests that oxaliplatin improved the autophagic activity of tumor cells, and that the autophagy flow was smooth. However, the mechanism by which it upregulates autophagy is still unclear. The balance between autophagy (programmed cell survival) and apoptosis (programmed cell death) is very important in cells, and HMGB1 is an important molecule that regulates the balance between apoptosis and autophagy. When HMGB1 is transferred from the nucleus to the cytosol, on the one hand, its semi-oxidative state further activates apoptosis-related proteins, thereby promoting apoptosis. On the other hand, its binding to autophagy-related proteins promotes the formation of autophagosomes and maintains the level of autophagy [14]. Therefore, endogenous HMGB1 is a pro-autophagic substance that supports cell survival and inhibits apoptosis [15]. In this research, the expression level of HMGB1 was markedly superior in oxaliplatin-exposed cells than in cells untreated with oxaliplatin, indicating that oxaliplatin up-regulated tumor cell HMGB1 protein. With increase in HMGB1 protein concentration, the expression of LC3-Ⅱ in cells showed up-regulated status, while the expression of p62 was down-regulated. The changes in LC3-Ⅰ protein were not obvious, and the level of autophagy was significantly up-regulated. This shows that HMGB1 upregulated the level of autophagy, which is consistent with the result of oxaliplatin treatment. Thus,

oxaliplatin upregulated the autophagy level of A431 cells by promoting the expression of HMGB1. Further analysis of the relationship between autophagy induced by oxaliplatin and its drug resistance showed marked reduction in viability of oxaliplatin-treated cells, relative to control group, indicating that oxaliplatin exerted a killing effect on tumor cells. With increase in oxaliplatin concentration, the viability of the cells decreased in a dose-dependent manner. Studies have shown that 3-mA, a common autophagy inhibitor, exerts a selective inhibitory effect on PI3K [16]. In this study, the viability of cells treated with oxaliplatin in combination with 3-mA also showed a downward trend, relative to control group, with larger decreases, indicating that the killing effect of oxaliplatin increased when autophagy was inhibited, that is, tumor cells were more sensitive to chemotherapy drugs after autophagy inhibition.

CONCLUSION

Oxaliplatin promotes HMGB1 protein A expression, boosts the cytotoxic effect of chemotherapy drugs by up-regulating the level of autophagy, and enhances the sensitivity of tumor cells to oxaliplatin, thereby making HMGB1 protein a potential target in skin cancer therapy.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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