Rapamycin induces autophagy in the melanoma cell line M14 via regulation of the expression levels of Bcl-2 and Bax

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Abstract. Cancer therapy with rapamycin has been successfully implemented for kidney cancer, glioblastoma and prostate cancer. However, there are few studies concerning the effects of rapamycin on the treatment of human melanoma. In this study, we investigated whether rapamycin may be a promising strategy for the effective treatment of melanoma and explored the possible mechanism for this by culturing M14 cells in vitro and treating with rapamycin at three concentrations (10, 50 or 100 nmol/l). MDC and LC3B staining, western blot analysis, flow cytometry and transmission electron microscopy were employed. We revealed that rapamycin induced autophagy and inhibited the proliferation of M14 cells in a concentration-dependent manner. Furthermore, western blot analysis revealed an upregulated expression of Bcl-2 and downregulated expression of Bax in M14 cells. In conclusion, rapamycin appears to be a promising strategy for the effective treatment of melanoma.

Introduction

Autophagy is an active degradative process that removes or recycles bulk cytoplasmic constituents through the endosomal and lysosomal fusion system, resulting in the formation of autophagosomes in eukaryotic cells. The autophagic process is robustly upregulated in response to cellular stress, such as nutrient or cytokine depletion, hypoxia and oxidative damage, and it is also pivotal to innate intracellular defense mechanisms against certain pathogens. Autophagy has significant roles in tissue development, differentiation and remodeling (1).

It is also implicated in diseases such as in the development of tumors, although its precise role is ambiguous (2).

Melanoma is the most fatal form of skin cancer with increasing incidence throughout the world. There are no efficacious therapies for malignant melanoma at present (3). The alkylating agent dacarbazine, administered as a single agent, remains the current standard treatment. However, few patients are capable of achieving remission from distant metastases and the 5-year survival rate is 10%. Thus, new agents and/or therapeutic strategies with different action targets need to be developed.

Rapamycin, a lipophilic macrolide antibiotic, was originally identified as a fungicide and immunosuppressant (4). However, studies have revealed that rapamycin can potently arrest the growth of cells derived from a broad spectrum of cancers (5). Rapamycin has been shown to specifically inhibit its target, mammalian target of rapamycin (mTOR), which plays a key role in tumor development and progression. Rapamycin binds the immunophilin FK506 binding protein (FKBP12) to form the FKBP12-rapamycin complex, which then interacts with mTOR and inhibits the mTOR-mediated phosphorylation of S6K1 and 4E-BP1. In addition, rapamycin is the best characterized drug that enhances autophagy, a process of ‘self-eating’ that involves both the death and survival of cancer cells. Therefore, rapamycin may interfere with different aspects of the tumor. Certain authors have demonstrated that rapamycin inhibits lung metastasis of B16 melanoma cells through downregulating alpha integrin expression and upregulating apoptosis signaling; autophagy is not involved in the rapamycin-mediated suppression of metastasis (6). However, there are few studies concerning the effects of rapamycin on human melanoma and the interaction with autophagy, thus the impact of rapamycin on M14 cells remains unclear.

Bcl-2 family proteins, which have either pro- or anti-apoptotic activities, have been studied intensively for the past decade owing to their significance in the regulation of apoptosis, tumorigenesis and cellular responses to anti-cancer therapy (7). Aberrant expression of Bcl-2 family members is capable of inappropriately promoting or preventing apoptosis. Bcl-2 is an anti-apoptotic member that prevents the release of cytochrome c from the mitochondrial intermembrane space (IMS) into the cytosol. Oppositely, Bax is a cytosolic protein that translocates to the mitochondria and participates in the release of cytochrome c in response to apoptotic stimuli. There

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is a negative correlation between the expression of Bcl-2 and Bax. In short, Bcl-2 overexpression leads to cell survival and Bax overexpression results in cell death (8).

Moreover, Bcl-2 family proteins also target the autophagy pathway. In this study, we set out to observe the autophagy of M14 cells induced by rapamycin; to investigate the effects of rapamycin on regulating the expression of Bcl-2 and Bax and to identify whether rapamycin may be a promising strategy for the effective treatment of melanoma.

Materials and methods

Cell culture. The human melanoma cell line M14 was obtained from Fuxiang Bio-Technology Company (Shanghai, China). Cells were maintained at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco). Cells were inoculated at a density of 1x10⁵ cells/ml and grown for 3 days to reach a phase of exponential growth (log phase), and were then used for the majority of experiments unless specified otherwise.

Autophagy induction and reagent treatment. Cells were plated at a density of approximately 1x10⁵ viable cells/well in 96-well microtiter plates. For autophagy induction, cells were treated with or without rapamycin (10, 50 or 100 nmol/l) for 24h. Following treatment, cells were analyzed as subsequently described.

Monodansylcadaverine (MDC) labeling. Cells were grown on chamber slides, washed with phosphate-buffered saline (PBS) and fixed in 10% formalin solution for 10 min. Autophagic vacuoles were labeled with MDC by incubating cells with 0.05 mmol/l MDC (Sigma-Aldrich, St. Louis, MO, USA) in PBS at 37°C for 10 min. Following incubation, cells were washed three times with PBS and immediately analyzed under a fluorescence microscope (BX50, Olympus).

Immunofluorescent staining of LC3B. M14 cells (1x10⁵/well) were seeded on glass cover slides in 24-well plates and left to attach overnight. Cells were then treated for 24 h with 10, 50 or 100 nmol/l rapamycin. Cells were then washed with PBS and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Subsequently, they were permeabilized by 100 µg/ml digitonin for 15 min, followed by PBS with 3% bovine serum albumin (BSA) for 1 h at room temperature. The anti-LC3B antibody was diluted to 1:200 in PBS, which contained 1% BSA, and then co-incubated with cells overnight at 4°C. After washing twice with PBS, cells were then incubated with Cy3-labeled goat anti-rabbit IgG (Sigma, St. Louis, MO, USA) for 1 h at room temperature in the dark. Then, cells were washed three times with PBS and stained with 4',6-diamidino-2-phenylindole (DAPI; 10 µg/ml) for 5 min. Finally, samples were imaged under a confocal fluorescence microscope (BX50, Olympus).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analyses. Cell viability was measured by MTT assay (Amresco, Solon, Ohio, USA). M14 cells (1x10⁵/100 µl) were seeded in 96-well plates. Following treatment, 20 µl of MTT solution (5 mg/ml) was added to each well and incubated (at 37°C and 5% CO₂) for 4 h. Next, the medium was removed and the wells were allowed to dry. The MTT metabolic product was resuspended in 200 µl of dimethylsulfoxide (DMSO) and placed on a shaking table for 5 min. At this point, the absorbance (optical density) was measured at 530 nm using a microplate reader. The cell proliferation inhibition rate was calculated using the equation: Inhibition rate of proliferation (%) = (Acontrol−Aexperimental)/Acontrol x100 (1).

Western blot analyses for Bcl-2 and Bax. Cellular lysate was prepared using radioimmunoprecipitation assay (RIPA) buffer with protease inhibitors and quantified using the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology Inc., Rockford, IL, USA). Equal amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophotothetically transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA) using a mini trans-blot (Bio-Rad, Hercules, CA, USA). Membranes were then blocked with PBST (PBS with 0.05% Tween-20) containing 5% non-fat dry milk for 1 h and incubated at 4°C overnight with anti-Bcl-2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-Bax antibody (Cell Signaling Technology, Inc., Beverly, MA, USA) in fresh blocking buffer. Membranes were then washed with PBST and incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h. The blots were developed using an enhanced chemiluminescence (ECL) kit (Pierce). Protein levels were normalized against β-actin (Sigma-Aldrich).

Flow cytometry with rhodamine 123 (Rh123) staining. Mitochondrial membrane potential (MMP) was assessed by the retention of Rh123, a membrane-permeable fluorescent cationic dye. The uptake of Rh123 by mitochondria is proportional to the MMP. Cells were incubated with Rh123 (0.25 nmol/l) in the dark at room temperature for 20 min. After washing with PBS, the cells were analyzed by FACSScan (Becton-Dickinson, San Jose, CA, USA) with excitation and emission wavelengths of 495 and 535 nm, respectively. Cells treated with rapamycin (10, 50 and 100 nmol/l) for 24 h were incubated with Rh123 (0.25 nmol/l) in the dark at room temperature for 20 min. After washing with PBS, the change in MMP was detected by Rh123 staining using flow cytometry.

Electron microscopy. Cells were harvested by scraping them from the plates. They were then washed twice with PBS and fixed with 2% paraformaldehyde/2% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4). Cell pellets were post-fixed with 1% (v/v) osmic acid in sodium cacodylate and stained with 1% uranyl acetate. Following dehydration, pellets were embedded in Durcopan (Fluka, Sigma). Ultrathin sections were prepared using ULTRACUT S and observed with a JEM 1010 transmission electron microscope. Images were captured and are shown in Results.

Statistical analysis. Data were expressed as mean ± standard deviation. Mean values were compared using a Student's t-test for independent variables. P<0.05 was considered statistically
to indicate a statistically significant difference. All statistical analyses were performed with SPSS 11.0 (SPSS Inc., Chicago, IL, USA).

**Results**

**Vesicular accumulation of MDC following rapamycin treatment.** MDC is an autofluorescent compound and has been proposed as a tracer for autophagic vacuoles. Thus, we studied the incorporation of MDC into M14 cells using fluorescence microscopy. As shown in Fig. 1, M14 cells treated with rapamycin for 24 h demonstrated a punctate pattern of MDC-labeled fluorescence. By contrast, uninfected cells exhibited a diffused distribution of MDC-labeled fluorescence. Rapamycin induced autophagy of M14 in a concentration-dependent manner.

**Rapamycin induced the accumulation of LC3B fluorescence dots in M14 cells.** We investigated the effect of rapamycin treatment on the staining of LC3B protein, which is produced during autophagosome formation. As demonstrated in Fig. 2, the quantity of LC3B fluorescence dots increased with increasing rapamycin concentration.

**Effects of rapamycin treatment on the proliferation of M14 cells.** The proliferation of M14 cells was measured using MTT assay and expressed as a ratio of color intensity from the rapamycin treatment group to that of the DMSO-treated control group. Data were obtained from three independent experimental replicates. Fig. 3 and Table I demonstrated that as the rapamycin concentration increased from 0-100 nmol/l, the strength of the inhibitory effect increased.
Rapamycin inhibited the proliferation of M14 cells. M14 cells were treated with rapamycin (0, 10, 50 and 100 nmol/l) and were incubated for 24 h. Cells were stained with monodansylcadaverine (MDC) as detailed in Materials and methods. The P-value between the control and treatment groups was <0.01. Each treatment group, the 10 nmol/l group and the other two groups were significantly different (P<0.05). However, no significant differences were observed between the 50 and 100 nmol/l groups (P>0.05). * indicates a statistically significant difference.

Table I. Effects of rapamycin treatment on the proliferation of M14 cells (n=4, mean ± SD).

<table>
<thead>
<tr>
<th>Group</th>
<th>Inhibition rate of proliferation (%)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>Rapamycin</td>
<td></td>
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<tr>
<td>10 nmol/l</td>
<td>8.42±2.88c</td>
</tr>
<tr>
<td>50 nmol/l</td>
<td>16.58±4.43b</td>
</tr>
<tr>
<td>100 nmol/l</td>
<td>24.15±3.69c</td>
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Table II. Gray value of Bcl-2 and Bax.

<table>
<thead>
<tr>
<th>Group</th>
<th>Bcl-2</th>
<th>Bax</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Rapamycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 nmol/l</td>
<td>0.94765±0.042485</td>
<td>1.96733±0.097991</td>
</tr>
<tr>
<td>50 nmol/l</td>
<td>0.70357±0.043447</td>
<td>2.23067±0.372162</td>
</tr>
<tr>
<td>100 nmol/l</td>
<td>0.34717±0.048413</td>
<td>2.89067±0.208375</td>
</tr>
</tbody>
</table>

Figure 5. MMP decreased with increasing rapamycin concentration. M14 cells were treated with rapamycin (0, 10, 50 or 100 nmol/l) and were incubated for 24 h. Cells were stained with rhodamine 123 (Rh123) as detailed in Materials and methods. The fluorescence intensity of Rh123 was significantly lower in the cells exposed to rapamycin relative to the control group (P<0.05). The MMPs of the four groups were 24.26±2.29, 18.24±1.69, 13.11±1.41 and 8.79±1.36, which indicated that fluorescence intensity decreased with increasing rapamycin concentration. RAPA, rapamycin.

Figure 6. Ultrastructural changes induced by rapamycin. M14 cells were treated with or without rapamycin for 24 h. Ultrastructural changes were observed with a transmission electron microscope. (a) M14 cells were treated without rapamycin and the cellular ultrastructure were normal and (b-d) Cells were treated with 100 nmol/l rapamycin for 24 h. Autophagosomes (dysfunctional mitochondria sequestered into a double-membrane-bound vesicle) and lysosomes were observed (x40,000).

Effects of rapamycin treatment on Bcl-2 and Bax expression. M14 cells were treated with various concentrations of rapamycin. The gray values of Bcl-2 in 10, 50 and 100 nmol/l groups were 0.94765±0.042485, 0.70357±0.043447 and 0.34717±0.048413; those of Bax were 1.96733±0.097991, 2.23067±0.372162 and 2.89067±0.208375. *P>0.05; †P<0.01 and ‡P<0.01.

MMP decreased following rapamycin treatment. Changes in mitochondrial function are capable of launching autophagy. In the case of nutritional deficiencies, there may be mitochondrial permeability transition pores (MPTP), which are a marker of impaired mitochondrial function. Following this, mitochondria are engulfed by lysosomes. A decline in MMP is a morphological characteristic of mitochondrial function recession. Rh123 is a cationic lipophilic fluorescent dye. It accumulates in mitochondria and its intake is proportional to the MMP. When the MMP decreases, Rh123 in mitochondria leaks out and the fluorescence intensity in the cells reduces. Thus, Rh123 fluorescence intensity reflects the MMP of mitochondria. As indicated in Fig. 5, the fluorescence intensity of Rh123 was significantly lower in the cells exposed to rapamycin relative to the control group, and the fluorescence intensity decreased with increasing rapamycin concentration. RAPA, rapamycin.

Ultrastructural examination of autophagy following rapamycin treatment. As demonstrated in Fig. 6, the ultrastructure of the control group (6a) was normal compared with the rapamycin group (6b-d). Double-membrane-bound vesicles, swelling of the mitochondria and vacuolization of
certain cells were observed in M14 cells following treatment with 100 nmol/l rapamycin for 24 h. Additionally, abnormal mitochondria, an increased number of lysosomes and autophagosomes were observed.

Discussion

Melanoma is the most fatal form of skin cancer with increasing incidence throughout the world. There are no efficacious therapies for malignant melanoma at present. It is possible to explore new methods of treatment by modulating autophagy. For example, this may be achieved via rapamycin, an autophagy inducer that promotes autophagy by inhibiting mTOR (9). Interest in applying rapamycin in cancer therapy was initiated due to observations of frequent Akt activation in multiple types of cancer and identification of TSC1/TSC2 as tumor suppressors that are inhibited by Akt. Cancer therapy with rapamycin has been successfully implemented for kidney cancer, with the approval of CCI-779 (temsirolimus) for the treatment of renal clear cell carcinoma (RCC) (10). In RCC, rapamycin may be effective due to its inhibition of the production of HIF-1α, a key transcription factor that mediates oncogenic alterations in the cellular metabolism (11,12). Aside from RCC, numerous clinical trials are testing rapamycin in cancers linked to Akt activation, such as glioblastoma and prostate cancer. Therefore, in this study, we set out to investigate rapamycin-induced autophagy and its possible mechanisms.

Autophagy is a process by which cells degrade macromolecular intracellular material via sequestration into a double-membranous structure, known as an autophagosome, which then delivers the enclosed material to a lysosome for degradation. Initially believed to be a system dedicated to the ‘recycling’ of macromolecular material within the cell, autophagy is now known to be involved in a multitude of cellular processes including immunity, tumorigenesis, programmed cell death, the selective degradation of organelles, aging and numerous neurodegenerative conditions (13). There are currently 33 identified autophagy-related genes shown to play a role in autophagy, and many techniques are available to detect autophagy, including transmission electron microscopy, half-life assessments of long-lived proteins, detection of LC3 maturation/aggregation, fluorescence microscopy and co-localization of mitochondrial or endoplasmic reticulum-specific markers with lysosomal proteins (14).

In this study, we observed autophagy in M14 cells treated with various concentrations of rapamycin for 24 h by MDC labeling, LC3B protein staining and transmission electron microscopy. The results showed that rapamycin induced autophagy in M14 cells in a concentration-dependent manner. By using electron microscopy (the most reliable method of observing cell ultrastructure) we observed autophagosomes (dysfunctional mitochondria sequestered into a double-membrane-bound vesicle) and mitochondria that had lost their cristae in cells treated with rapamycin compared with those in the control group. Mitochondria that were abnormal in appearance and an increased number of lysosomes were also observed.

Mitochondria are vital organelles for cellular metabolism and bioenergetics, but they are also the key regulators of cell death (15). Notably, in many (if not all) paradigms of apoptosis, MMP represents the point of no return in the cascade of events that ultimately leads to cell death (16). MMP results in the leakage of potentially toxic proteins from the mitochondrial IMS. In addition, mitochondria play a role in stress responses and can produce reactive oxygen species (ROS) when damaged. Selective degradation of mitochondria by autophagy is also known as ‘mitophagy’ and is considered to be promoted by their functional impairment and/or by MMP. Mitophagy may ensure the removal of damaged and potentially dangerous mitochondria, thus acting as a quality control mechanism. We used Rh123 to detect the changes in MMP by flow cytometry and discovered that the fluorescence intensity of Rh123 was significantly lower in the cells exposed to rapamycin relative to the control group (P<0.05). Thus, combined with the electron microscopy results, we conclude that rapamycin may affect the regulation mechanism of mitochondria and cause swelling and vacuolization of mitochondria.

The proliferation of M14 cells was measured by the inhibition rate of proliferation using MTT assay (Table II). The results showed that the proliferation of melanoma cells was significantly inhibited in the rapamycin group (P<0.01). Compared with the other two treatment groups, the 10 nmol/l group demonstrated notably higher gray values (P<0.05); however, no significant differences were observed between the 50 nmol/l and 100 nmol/l groups (P>0.05). Thus, it is suggested that rapamycin possesses a biphasic effect. On one hand it is capable of inducing autophagy, on the other hand it may inhibit proliferation.

The correlation between autophagy and apoptosis is complex and controversial (17). It varies with cell type and stress stages. Depending on the cellular context and stimulus, autophagy may be indispensable for apoptosis by initiating the process (18). In other cellular settings, autophagy may rather antagonize or delay apoptosis and inhibition of autophagy may increase the sensitivity of the cells to apoptotic signals (19). In certain cell systems, two processes can occur independently. Numerous signaling pathway overlaps are found between autophagy and apoptosis, including various kinases such as PI3K, PKB/Akt, Bcl-2 family members, PTEN, c-Myc and Ras.

Members of the Bcl-2 family proteins, including Bcl-2, Bax and Bak, are thought to play regulatory roles in the apoptotic execution of the cells (20). However, more and more studies have revealed that Bcl-2 family proteins also target the autophagy pathway. Further biochemical and genetic data has led to a resurgence of interest in the role of autophagy in tumor suppression (21). In addition to the discovery that an autophagy execution protein, Beclin 1, is a tumor suppressor protein, oncogenic signaling molecules are capable of suppressing autophagy and tumor suppressors are able to stimulate autophagy.

Bcl-2 and Beclin 1 interact in mammalian cells. Firstly, Bcl-2 inhibits Beclin 1-dependent autophagy. To explore the mechanism, an autophagy-competent cell line (HT-29 colon carcinoma cells) that expresses endogenous Beclin 1 has been used. In HT-29 cells, stable transfection of Bcl-2 inhibits starvation-induced autophagy decreases the association of Bcl-2 with Beclin 1 (22). In vitro, Bcl-2 overexpression blocks the forma-
tion of the autophagy-promoting Beclin 1-Vps34 complex. Additionally, Bcl-2 inhibits Beclin 1-dependent autophagic cell death. Bcl-2, apoptosis-inhibiting gene and Bax, apoptosis inducing-gene, are two important members of the Bcl-2 family; the ratio between the two determines the survival of cells. There is a negative correlation between the expression of Bcl-2 and Bax. Bcl-2 overexpression leads to cell proliferation, while alternatively Bax leads to cell death. In this study, we investigated the effects of rapamycin treatment on Bcl-2 and Bax expression. As described previously, there was a significant dose-dependent reduction in the level of Bcl-2 expression after rapamycin treatment for 24 h of 10, 50 or 100 nmol/l. By contrast, the level of Bax expression increased. This is speculated to be caused by MPTP. Certain studies suggest that MPTP is formed by the interaction with Bcl-2 family proteins and other proteins outside the mitochondria; Bax may promote the opening of MPTP. Besides, certain authors consider Bcl-2 protein to inhibit the release of Ca\(^{2+}\) from the endoplasmic reticulum, while Bax may promote this release. Rapamycin reduces the expression of Bcl-2 and increases the expression of Bax, which results in an overload of Ca\(^{2+}\) in the mitochondria and promotes the opening of MPTP. Then, mitochondria swell, their outer membranes collapse and exit into the cytoplasm, thus initiating apoptosis.

The ability of Bcl-2 to inhibit autophagy through a direct interaction with Beclin 1 is of particular interest with regards to cancer. The role of autophagy in tumorigenesis and cancer treatment is complex. Autophagy has roles in both tumor prevention and survival, as well as in treatment resistance. As autophagy protects cells from metabolic stress, it is speculated that the upregulation of autophagy preserves cellular fitness and genomic integrity, and thus prevents tumorigenesis (22). On the contrary, established tumor cells utilize autophagy to survive stresses such as nutrient limitation and hypoxia. Furthermore, tumor cells activate autophagy as a stress response to survive cancer treatment. A recent study has demonstrated that under certain situations, such as in radiation-resistant melanoma, inhibition of autophagy may be exploited to prevent resistance to treatment (23). Notably, modulation of autophagy has significant potential in cancer diagnosis and treatment. At present, there are few studies concerning the effects of rapamycin on melanoma cells; we speculate that rapamycin may be a promising strategy for the effective treatment of melanoma by modulating autophagy and regulating the expression of Bcl-2 family proteins. However, in view of the biphasic effect of rapamycin (in inducing autophagy and apoptosis) there is a requirement to establish the appropriate concentration of rapamycin that is capable of inducing mitophagy, promoting tumor cell apoptosis and activating autophagy, so as to provide a new approach to treating malignant melanoma.

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References