Autophagy activator promotes neuronal differentiation of adult adipose-derived stromal cells*

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Abstract

Preliminary research from our group found altered autophagy intensity during adipose-derived stromal cell differentiation into neuronal-like cells, and that this change was associated with morphological changes in differentiated cells. This study aimed to verify the role of rapamycin, an autophagy activator, in the process of adipose-derived stromal cell differentiation into neuronal-like cells. Immunohistochemical staining showed that expression of neuron-specific enolase and neurofilament-200 were gradually upregulated in adipose-derived stromal cells after 5 mM β-mercaptoethanol induction, and the differentiation rate gradually increased with induction time. Using transmission electron microscopy, induced cells were shown to exhibit cytoplasmic autophagosomes, with bilayer membranes, and autolysosomes. After rapamycin (200 µg/L) induction for 1 hour, adipose-derived stromal cells began to extend long processes, similar to the morphology of neuronal-like cells, while untreated cells did not exhibit similar morphologies until 3 hours after induction. Moreover, the differentiation rate was significantly increased after rapamycin treatment. Compared with untreated cells, expression of LC3, an autophagy protein, was also significantly upregulated. Positive LC3 expression tended to concentrate at cell nuclei with increasing induction times. Our experimental findings indicate that autophagy can significantly increase the speed of adipose-derived stromal cell differentiation into neuronal-like cells.

Key Words

neural regeneration; stem cells; adult adipose-derived stromal cells; neuronal-like cells; autophagy; autophagy activator; autophagy protein; rapamycin; differentiation; neuroprotection; neuroregeneration

Research Highlights

(1) The autophagy activator, rapamycin, significantly accelerates adipose-derived stromal cell differentiation into neuronal-like cells and improves the differentiation rate.

(2) After rapamycin treatment, LC3 expression is significantly upregulated during adipose-derived stromal cell differentiation into neuronal-like cells, with expression mainly concentrated around the nucleus.

(3) Autophagic expression (shown by LC3 localization), moves from the cytoplasm to the nucleus, consistent with morphological changes observed during cell differentiation.

INTRODUCTION

A number of studies have shown that mammalian and human adipose-derived stromal cells can differentiate into nerve cells[1-6]. However, adipose-derived stromal cell-derived neurons usually differentiate slowly and do not survive long, and can even become apoptotic within a few hours[7-11]. Autophagy plays a dual role in cell growth. One mechanism induces type II programmed cell death (a different process from apoptosis), while the other protects or delays...
damage caused by environmental changes and promotes cell growth\(^{12\text{-}14}\). Excessive, insufficient or incomplete autophagy may be one of the main initiators of cell apoptosis\(^{15\text{-}16}\). In a previous study, changes in autophagy intensity were related to morphological changes observed during differentiation of adult adipose-derived stromal cells into neuronal-like cells. Moreover, it was suggested that autophagy plays a protective role in the differentiation process.

Rapamycin, a classic autophagy activator, inhibits the activity of mammalian target of rapamycin, to induce and enhance autophagy\(^{17\text{-}19}\). In this study, we used rapamycin to enhance the autophagic response in adipose-derived stromal cell differentiation into neuronal-like cells. Our aim was to enable us to study the biological role of autophagy within this process, thereby providing a basis for improving adipose-derived stromal cell differentiation speed and effectiveness.

**RESULTS**

**Differentiation of adipose-derived stromal cells into neuronal-like cells**

In the control group, neuron specific enolase and neurofilament-200 were not expressed in adult adipose-derived stromal cells. However, with 5 mM β-mercaptoethanol induction, both were positively expressed in the cytoplasm and expression levels increased gradually with time. Expression of neuron specific enolase and neurofilament-200 peaked at 5 hours after induction \( (P < 0.05) \). There was no significant difference between induction groups at 5 and 8 hours \( (P > 0.05; \text{Table 1}) \). Expression of glial fibrillary acidic protein was negative in the non-induction, pre-induction and induction groups at 1, 3, 5 and 8 hours (Figure 1).

| Group                  | Neurofilament-200 | Neuron specific enolase | Glial fibrillary acidic protein |
|------------------------|-------------------|-------------------------|--------------------------------|
| Pre-induction          | 4.80±2.15         | 1.37±1.47               | 0                              |
| Induction for 1 hour   | 33.11±1.32        | 42.23±1.10              | 0                              |
| Induction for 3 hours  | 57.47±2.14        | 62.76±0.43              | 0                              |
| Induction for 5 hours  | 78.77±0.73        | 84.85±1.85              | 0                              |
| Induction for 8 hours  | 77.54±1.19        | 83.93±1.08              | 0                              |

Results are expressed as cell percentages expressing a particular marker. Data are expressed as mean ± SD. Five slices were obtained from each group at each time point, and experiments were performed in triplicate. *\( P < 0.05 \), vs. pre-time point using one-way analysis of variance followed by Student-Newman-Keuls test.

Figure 1  Expression of neuron specific enolase (NSE), neurofilament-200 (NF-200) and glial fibrillary acidic protein (GFAP) in adult adipose-derived stromal cells after 5 mM β-mercaptoethanol induction (immunocytochemistry staining, light microscope, × 100).

Arrows indicate NF-200 and NSE positive cells. Expression of NF-200 and NSE increased gradually in the cytoplasm as the induction time extended and peaked at 5 hours. There was no GFAP expression after induction.
Ultrastructure of adipose-derived stromal cell differentiated neuronal-like cells

After 5 hours of 5 mM β-mercaptoethanol induction, the ultrastructure of normal adipose-derived stromal cell differentiated neuronal-like cells was observed using transmission electron microscopy. Differentiated cells appeared round, or round-like, and had a rough surface with many protrusions, a large round nucleus, a lightly-stained double-layered membrane, and a large amount of euchromatin but little heterochromatin. There were many cytoplasmic organelles including mitochondria, endoplasmic reticulum, Golgi complex structures and a large number of Nissl bodies. In addition, in some neuronal-like cells, the cytoplasm had a large number of outstretched endoplasmic reticulum, swollen mitochondria with increased volumes and ruptured cristae, and double-membrane autophagosomes (some with engulfed mitochondria), autolysosomes (from autophagosomes), and primary lysosomes containing dense phagocytic particles (Figure 2).

Rapamycin promoted adipose-derived stromal cell differentiation of neuronal-like cells

Using an inverted phase contrast microscope, the shape of normal adipose-derived stromal cells was uniform and exhibited a fibroblast-like morphology in swirl-like growth. Both control and rapamycin groups, showed similar changes in cell morphology, namely nuclei became enlarged and rounded, cytoplasm retracted towards the nucleus at the center, and halos were clearly visible around the cell body. In the rapamycin group, 74% of cells had long processes and showed neuronal-like features after 1 hour of induction, similar to the control group after 3 hours of induction. The differentiation rate peaked after 5 hours in both groups, with cells exhibiting a typical neuronal morphology including a small conical cell body, retracted towards the nucleus and thereby enhancing cell body refraction, and elongated axon-like processes, with level ends before branching into two processes. After 8 hours, the morphology and rate of differentiated cells did not change significantly compared with the 5-hour induction group (P > 0.05; Figure 3).

Figure 2. Transmission electron microscope observation of the ultrastructure of normal adipose-derived stromal cell-differentiated neuronal-like cells at 5 hours after 5 mM β-mercaptoethanol induction.

(A) A typical neuron. The arrow indicates a large round nucleus with a large amount of euchromatin but little heterochromatin (× 5000).

(B) Cytoplasmic ultrastructure. The arrow points to a large number of Nissl bodies (× 10 000).

(C) Autophagy. The arrow indicates an autophagic cell with engulfed mitochondria. The arrowhead indicates a swollen mitochondria (× 10 000).

(D) Arrow indicate autolysosomes (× 10 000).

Figure 3. Identification of morphological changes in differentiated adult adipose-derived stromal cells in control and rapamycin groups after 5 mM β-mercaptoethanol induction, using inverted phase contrast microscopy (× 100).

One hour after induction, axon-like processes were visible (arrows). Three hours after induction, cells showed neuronal-like changes (arrows). At 5 and 8 hours after induction, cells exhibited typical neuronal morphologies. Arrows indicate axon- and dendrite-like structures. In the rapamycin group, cell body enhanced refraction was observed.
Overall, we found the cell differentiation rate was significantly higher in the rapamycin group than in controls, at 1 and 3 hours ($P < 0.01$). We also observed this difference at 5 and 8 hours, but it was not statistically significant ($P > 0.05$; Table 2).

### Table 2 Effect of rapamycin on the differentiation rate of adipose-derived stromal cells after 5 mM β-mercaptoethanol induction

| Induction time (hour) | Control group | Rapamycin group |
|-----------------------|---------------|-----------------|
| 1                     | 57.14±6.38    | 73.69±5.33*     |
| 3                     | 72.56±7.13    | 78.75±7.91*     |
| 5                     | 88.67±0.50    | 92.81±6.05*     |
| 8                     | 88.47±0.56    | 92.42±2.08*     |

Data are expressed as mean ± SD. In each group, five slices were obtained at each time point. Results are calculated as the rate of adipose-derived stromal cell differentiation (%), which is the number of cells with long processes and neuronal-like cells/total cells × 100%.

### Rapamycin upregulated LC3 expression in adipose-derived stromal cell differentiated neuronal-like cells

Immunohistochemical staining showed increased LC3 expression in the control group with extended induction times, peaking at 5 hours after induction. Significant differences were detected between each time point ($P < 0.01$), except 5 and 8 hours ($P > 0.05$). In the rapamycin group, LC3 expression was increased to 93%, although this was not statistically significant at any time point ($P > 0.05$). However, LC3 expression was significantly increased after 1, 3, 5 and 8 hours of induction in the rapamycin group compared with controls ($P < 0.01$; Table 3).

### Table 3 Effect of rapamycin on LC3 expression in adult adipose-derived stromal cells after 5 mM β-mercaptoethanol induction

| Induction time (hour) | Control group | Rapamycin group |
|-----------------------|---------------|-----------------|
| 0                     | 2.24±2.13     | 2.56±2.62       |
| 1                     | 31.98±1.71    | 93.16±3.70*     |
| 3                     | 51.65±8.81    | 94.32±4.50*     |
| 5                     | 77.29±3.93    | 97.02±3.06*     |
| 8                     | 68.28±1.74    | 96.48±3.17*     |

Results are given as percentage of LC3 positive cells in 100-fold magnification fields. Data are expressed as mean ± SD. In each group, five slices were obtained at each time point and experiments were performed in triplicate. *$P < 0.01$, vs. control group using two-sample t-test.

In the control group, LC3 expression was distributed uniformly and stably within the cytoplasm of whole processes. While in the rapamycin group, LC3 expression was mainly concentrated around the nucleus, with increasing nuclear localization with extended induction times. Weaker or even negative LC3 expression was observed in other parts of the cytoplasm (Figure 4). In addition, the cell differentiation rate and percentage of LC3 cells were significantly different 1, 3, 5 and 8 hours after induction in the control group, peaking at 5 hours after induction ($P < 0.01$). In the rapamycin group, the LC3-positive expression rate was significantly higher than that in controls ($P < 0.01$), accounting for 93% expression at 1 hour after induction (Table 3).

![Figure 4](image)

**Figure 4** LC3 expression in 5 mM β-mercaptoethanol induced adult adipose-derived stromal cells in control and rapamycin groups (immunocytochemistry staining, light microscope, × 100).

In the rapamycin group, LC3-positive expression is mainly concentrated around the nucleus, and more increasingly focused around the nucleus with extension of induction time. Arrows indicate LC3 positive cells with brown cytoplasm and a blue nucleus.
DISCUSSION

In previous studies, autophagy was shown to play an important role in support and protection during cell differentiation\[^{12-13}\], regulating cell growth, differentiation, maturation and death\[^{14-15}\]. Autophagy insufficiency or damage may cause or accelerate apoptosis. However, the role of autophagy in this process is not clear, but can be studied using rapamycin during adipose-derived stromal cell differentiation into neuronal-like cells. Zhao et al\[^{20}\] found that reduced autophagy could inhibit glioma progenitor stem cell differentiation. Many researchers have also demonstrated that apoptosis and autophagy are important to maintain the dynamic balance of physiological processes within cells and tissue\[^{21-22}\]. In this study, we found that in the rapamycin group, 84% of adipose-derived stromal cells differentiated into neuronal-like cells after an induction period of 5 hours, when the differentiation rate peaked. Following this peak, the cell differentiation rate was no longer significantly elevated and the number of viable cells reduced increasingly\[^{9}\]. Typical apoptotic and autophagic ultrastructural changes were found throughout the differentiation process. These results suggest that the cell differentiation rate is not significantly increased 5 hours after induction; consequently, the cell death mechanism and biological role of autophagy will require further analysis.

Expression of LC3, an autophagic marker, peaked 5 hours after induction, showing the same trend as cell differentiation. However, the LC3 positive rate was significantly lower than the cell differentiation rate. These results suggest that autophagy may be one of the most important supportive conditions in cell differentiation. Inadequate autophagy in differentiated cells may result in ineffective metabolite removal leading to cell death, a potential explanation for the lack of a further increase in the cell differentiation rate 5 hours after induction.

To confirm this hypothesis, we used rapamycin as a potential autophagy activator. Rapamycin increased the autophagy intensity when added to induction medium, with the number of autophagic cells and autophagy intensity significantly enhanced. Immunohistochemistry showed LC3 expression reached 93% 1 hour after induction and did not change significantly during the remaining induction process. In the control group, although autophagy increased with increasing induction time, the LC3 positive rate reached a peak value of only 77% at 5 hours. Moreover, compared with the control group, the number of neuronal-like cells in the rapamycin group increased from 57% to 74% 1 hour after induction, from 77% to 97% 5 hours after induction, and stabilized 8 hours after induction. Throughout the differentiation process, the autophagic rate was significantly higher than the cell differentiation rate in the rapamycin group, confirming a role for autophagy in protection and support. Adequate autophagy ensured cell differentiation, a necessary condition to obtain differentiated cells. Studies have shown rapamycin inhibits differentiation, proliferation and migration of smooth muscle progenitor cells\[^{23}\]. Similarly, rapamycin was less effective in bone marrow hematopoietic stem cell differentiation into dendritic cells, resulting in dendritic cells that were in a stable yet immature state\[^{24}\]. Autophagy mechanisms in the differentiation of different cell types, needs further study.

In both the control and rapamycin groups, cell morphology changes were similar; however, the speed and extent of cell differentiation were significantly different. In the rapamycin group, 74% of cells had long processes and showed neuronal-like cells 1 hour after induction, similar to controls 3 hours after induction. Three and 5 hours after induction, cells exhibited elongated axon-like processes, with level ends and two branches. After 8 hours, cell morphology did not change significantly, indicating that autophagy activators activate autophagy immediately and enhance autophagic intensity, but also accelerate differentiation of adipose-derived stromal cells. However, autophagy regulation is complex and involves many signaling pathways, including target of rapamycin or mammalian target of rapamycin, phosphatidylinositol 3-kinase-l/protein kinase B, GTPases, calcium and protein synthesis\[^{25-26}\]. The molecular mechanisms and autophagic regulation involved in this study are not yet known.

Transmission electron microscopy showed that during adipose-derived stromal cell differentiation into neuronal-like cells, the cytoplasm of some neuronal-like cells contained a large number of outstretched endoplasmic reticulum, swollen mitochondria with increased volumes and ruptured cristae, double-membrane autophagosomes, autolysosomes with engulfed mitochondria, and autolysosomes (from autophagosomes) and primary lysosomes containing dense phagocytic particles. The detection of these subcellular structures is consistent with the occurrence of autophagy. Our results also show that in the presence and absence of rapamycin, cell morphological...
changes are similar, namely nuclei become enlarged and rounded, the cytoplasm retracts towards the nucleus at the center, and halos are clearly visible around the cell body. With regard to LC3 expression, both groups were different. In the control group, LC3 was distributed uniformly and stably within the cytoplasm, throughout the differentiation process. However, in the rapamycin group, LC3 expression was mainly concentrated around the nucleus, and increasingly focused around the nucleus with extension of induction times, consistent with the morphological changes observed during differentiation. Autophagy was distributed uniformly and weakly in the cytoplasm in the control group, incompatible with retraction of the cytoplasm toward the nucleus. Therefore, inadequate autophagy could not effectively remove metabolites causing stress to the endoplasmic reticulum, leading to cell death. Enhanced autophagy in nerve cells was neuroprotective. Therefore, autophagy activators may be useful in prevention and treatment of neural degenerative diseases, especially early onset Parkinson’s disease, Alzheimer’s disease and peripheral myelin protein 22-related demyelinating polyneuropathy[27-30].

In summary, autophagy plays an important role in protection and support during adipose-derived stromal cell differentiation into neuronal-like cells. When adipose-derived stromal cells were not treated with rapamycin, autophagy was unable to fulfill the requirements of the cytoplasm retracting towards the nucleus. Inadequate autophagy was unable to effectively remove metabolites, outstretching the endoplasmic reticulum and leading to cell death. In the rapamycin group, LC3 expression mainly concentrated around the nucleus, and increasingly focused around the nucleus with extended induction times, consistent with the morphological changes observed during differentiation. Rapamycin significantly enhanced autophagy intensity and accelerated the speed of cell differentiation, improving the differentiation effect.

MATERIALS AND METHODS

Design
A cytological, in vitro controlled observation experiment.

Time and setting
Experiments were performed at the Central Laboratory of Hebei Union University from March 2010 to November 2011.

Materials
Abdominal, subcutaneous, fatty tissues were obtained with the needle method, from volunteers of healthy adults (Physical Check-up Center, Kailuan Hospital, China), aged 20–35 years old, from Kailuan General Hospital, Tangshan, China. The study was in accordance with the Administrative Regulations on Medical Institution, formulated by the State Council of China. Written informed consent was obtained.

Methods
Extraction and culture of adult adipose-derived stromal cells
Adult adipose-derived stromal cells were isolated and cultured from adipose tissues of healthy volunteers according to the method by Liu et al[5]. Isolated adipose-derived stromal cells were cultured in a humidified incubator at 37°C in 5% CO₂ with Dulbecco’s modified Eagle’s medium (DMEM)-high glucose (Hyclone, Logan, UT, USA), and the medium was changed every 2–3 days. Cells were confluent after 10–14 days and subsequently passaged at a ratio of 1:2. Passaged cells were maintained.

In vitro induction and differentiation of adult adipose-derived stromal cells into neuronal-like cells, and rapamycin treatment
Adult adipose-derived stromal cells, at passages 3–6, were digested and seeded onto culture plates. Initially, cells were not induced by culturing in pre-induction medium containing 1 mM β-mercaptoethanol (Sigma, St. Louis, MO, USA), 20% fetal calf serum and Dulbecco’s modified Eagle’s medium-high glucose, for 24 hours until reaching 70–80% confluency. The cells were then cultured in formal induction medium (containing 5 mM β-mercaptoethanol and DMEM), in both control and rapamycin groups. In the rapamycin group, the medium also contained 200 µg/L rapamycin (Sigma). Control and rapamycin groups included a pre-induction subgroup and induction subgroups at 1, 3, 5 and 8 hours. Cell morphology was observed using an inverted phase contrast microscope (Nikon, Tokyo, Japan). Within each group, and at each time point, five slices were obtained in triplicate. The rate of adipose-derived stromal cell differentiation (%) = the number of cells with long processes and neuronal-like cells/total cells × 100%.

Neurofilament-200, neuron specific enolase and glial fibrillary acidic protein expression detected by immunocytochemistry
Cover slips were removed from culture plates and cells
fixed with 4% paraformaldehyde for 30 minutes, treated with 0.1% Triton X-100 for 8 minutes, followed by 3% 
H₂O₂ incubation for 10 minutes to eliminate endogenous peroxidases. Next, cells were incubated with primary 
monoclonal antibodies: rabbit anti-human neurofilament-200, neuron specific enolase and glial 
fibrillary acidic protein (all 1:100; Beijing Biosynthesis Biotechnology, Beijing, China) at 4°C overnight. Goat 
anti-rabbit IgG-horseradish peroxidase polymer (ready to use; Beijing Zhongshan Gold Bridge, Beijing, China) was 
added and incubated for 15 minutes at room temperature. 

A diaminobenzidine kit (Beijing Zhongshan Gold Bridge) was used to detect antibody signal, with a 10-minute 
incubation followed by termination of the reaction with tap water. Cells were counterstained with hematoxylin, and 
dehydrated with gradient alcohol, followed by xylene. Stained cells were imaged using a light microscope 
(Nikon). The percentage of positive cells in 100-fold magnification fields was calculated. Each slice was 
quantified three times, and an average value was obtained from five slices.

Transmission electron microscopy of induced adipose-derived stromal cell ultrastructure

Cells induced for 5 hours were collected, digested and then centrifuged. Cells were fixed with 3% 
glutaraldehyde and 1% osmic acid, followed by propionaldehyde dehydration and epoxy resin 
embedding. Cells were sliced using a thin slicing machine at 40 μm thickness and stained using 2% uranyl 
acetate and lead citrate. Images were obtained using a transmission electron microscope (Hitachi, Tokyo, 
Japan).

LC3 expression determined by immunocytochemistry

Immunocytochemical staining was performed as previously described. In brief, cells in control and 
rapamycin groups were incubated with rabbit anti-LC3 polyclonal antibody (1:100; Beijing Biosynthesis 
Biotechnology) at 4°C overnight. Next, goat anti-rabbit IgG-horseradish peroxidase polymer (ready to use; 
Beijing Zhongshan Gold Bridge) was added for 15 minutes at room temperature. The remaining steps 
were the same as described above. The percentage of positive cells in 100-fold fields was calculated. Each slice 
was quantified three times, and an average value was obtained from five slices.

Statistical analysis

All experimental data were collected using Microsoft Excel 2003 (Microsoft, Redmond, WA, USA) and 
analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA). Data were expressed as mean ± SD. Multiple 
group differences were compared using one-way analysis of variance, and intergroup differences were 
compared using the Student-Newman-Keuls test. Differences between the control and rapamycin groups 
were compared using two-sample t-tests. A value of P < 0.05 was considered statistically significant.

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arrangement. Xiaodong Yuan was responsible for study design, organization, data analysis and manuscript authorization. Ya Ou 
and Qiaoyu Sun assisted in study design and performed experiments. All authors approved the final version of the paper.

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