Original Article
Activated autophagy contributes to the development of lymphatic malformations

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Abstract: To investigate the level of autophagy in lymphatic malformations (LMs), and the role of autophagy in the development of LMs, immunohistochemistry was used to examine the expression level of autophagy-related proteins [Beclin-1, Sequestosome 1 p62 (p62), light chain 3 (LC3)], and markers of anti-apoptosis [B cell lymphoma 2 (Bcl-2), phosphorylated extracellular signal-regulated kinase 1/2 (p-Erk1/2)] as well as the maker of proliferation (Ki-67) in samples of LMs and normal skins (SKs). The Pearson’s correlation and cluster analyses were performed to determine to relationships between the proteins we tested. The studies using human dermal lymphatic endothelial cells (HDLECs) were carried out for mechanism investigation. Besides, rat models of LMs were established to evaluate the role of autophagy in LMs. The results showed that the autophagy-related proteins we tested, correlated with each other, were significantly increased in LMs compared with SKs samples. In vitro, autophagy, depended on p-Erk1/2, prevented the apoptosis of HDLECs in FBS starvation conditions. Furthermore, the correlation between the autophagy-related proteins, and p-Erk1/2 as well as Bcl-2 in LMs samples was confirmed in LMs samples. In the LM rat model, the inhibition of autophagy suppressed the development of LMs. Our study indicates that autophagy is elevated in LMs, and plays an important role in LMs.

Keywords: Lymphatic malformations, autophagy, anti-apoptosis, proliferation

Introduction

Lymphatic malformations (LMs) are congenital anomalies related to the dysregulated lymphangiogenesis of the lymphatic system [1]. Compared to the trunk and extremities, the head and neck region is the major site for lymphatic malformations [2]. Due to their location, they cannot only cause aesthetic and development deformities, but also compromise the airway leading to breathing problem, which could threaten the patients’ life [3]. Up to date, management strategies for LMs include surgical resection, sclerotherapy, simple observation, laser, radiofrequency ablation, photodynamic therapy and intralesional endoscopy. Nevertheless, the treatment efficacy for LMs is still unclear as the mechanism behind the pathogenesis of this disease is largely unknown [4].

LMs are composed of abnormal dilated lymphatic channels lined with lymphatic endothelial cells (LECs) [5]. In the embryo stage, lymphatic vessels develop from a subpopulation of Prox1+ endothelial cells which were derived from lymph sacs [6]. Recently, studies have indicated that the higher survival and proliferative potential in the lining LECs of LMs characterized by elevated activation of the PI3K/AKT/mTOR and MEK/ERK signaling pathways, both of which were important regulators of cell growth and survival [1, 5, 7]. However, the accurate mechanism remains to be investigated.

Autophagy, a lysosome-dependent, dynamic degradation process, has been confirmed to be functional in various physiological processes as well as pathogenesis of various diseases [8]. Generally, autophagy is mainly mediated by the formation of double-membrane or multilamellar vesicles which are called autophagosomes that turnover and recycle proteins and organelles. Afterwards, the autophagosomes fuse with the lysosomal vesicles to form autoly-
sosomes where the maturation process could be finally completed [9]. This biological process requires the expression of a series of key genes related to autophagy, including microtubule-associated protein 1 light chain (LC3) [10], Beclin-1 [11], and Sequestosome 1 (p62) [12]. Numerous studies including our previous studies have implicated that the main role of autophagy was to ensure cell growth and survival even under stress conditions [9, 13, 14]. However, to our best knowledge, the status of autophagy and its role in the pathogenesis of LMs, a congenital anomaly of the lymphatic system, are largely unreported.

Therefore, here, the activated status of autophagy in the tissue samples of LMs was, for the first time, evaluated. Meanwhile, we also investigated the relationship of autophagy with cellular anti-apoptosis and proliferation in LMs. Moreover, in vitro studies showed that autophagy, depended on the activation of ERK1/2 pathway, was essential for the survival of human dermal lymphatic endothelial cells (HDL-EDs) in serum starvation condition. More importantly, inhibition of autophagy in a rat model of LMs significantly suppressed the development of LMs.

Materials and methods

Samples, immunohistochemistry and double-labelling immunofluorescence

This study was approved by the review board of the Ethics Committee of Hospital of Stomatology, Wuhan University. Twenty-six tissue samples of lymphatic malformations, and 10 clinical samples of normal skins resected during plastic surgery were obtained at Hospital of Stomatology, Wuhan University. Meanwhile, informed consent for this study was got from all the subjects. The basic information for the patients with LMs is shown in Supplementary Table 1. These specimens were fixed in buffered 4% paraformaldehyde before embedded in paraffin. For immunohistochemical and double-labelling immunofluorescence, the procedures were described as our previous studies [15, 16], which were depended on the National Institutes of Health guidelines as to the use of human tissues. Meanwhile, primary antibodies used in our studies were listed in Supplementary Table 2.

Transmission electron microscopy, immunofluorescence for LC3 localization and detection of acidic vesicular organelles (AVO)

Transmission electron microscopy, immunofluorescence for LC3 localization and detection of acidic vesicular organelles (AVO) were performed as our previous description [17, 18] in the Supplementary.

Real-time quantitative PCR, Western blot analysis and flow cytometric analyses

According to our previous protocol [15-17], real-time quantitative PCR (qPCR), western blot (WB) and flow cytometric analyses were carried out, all of which was described in the Supplementary. In addition, the primer nucleotide sequences for PCR are listed in Supplementary Table 3.

Animal model of LMs in rats

All animal experiments were approved by the Institutional Animal Care and Use Committee, Center for Animal Experiment, Wuhan University. Depended on our previous studies [19, 20], fifteen adult Wistar female rats, weighing 250 ± 30 g were purchased from the Hubei Research Center of Laboratory Animals (Wuhan, China). Rats were randomly assigned into three groups: (i) subcutaneous injection of 200 μl PBS every other week; (ii) subcutaneous injection of 100 μl FIA (Santa Cruz Biotechnology, USA) + 100 μl PBS every other week; (iii) subcutaneous injection of 100 μl FIA (Santa Cruz Biotechnology, USA) + 100 μl PBS every other week, and intraperitoneal injection of chloroquine 60 mg/kg 3 times a week. LMs in rats were monitored for every other week. After 2 months, animals were euthanized, and the lesions were collected for further studies [21].

Statistical analysis

All data were represented as mean ± SEM with three independent experiments. Statistical analysis was performed via Student’s t-test, Spearman’s rank correlation test and hierarchical clustering. Statistical significance was defined as P < 0.05.

Results

Activated autophagy in LMs

To investigate the status of autophagy in LMs, the expression of some core autophagy-related
Autophagy in lymphatic malformation

A

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B

**Histoscores**

- Beclin-1
- p62
- LC3

C

**Histograms**

- Histoscores of Beclin-1 vs Histoscores of p62
- Histoscores of p62 vs Histoscores of LC3
- Histoscores of Beclin-1 vs Histoscores of LC3

D

**Regression Lines**

- Y = 0.6250X + 1.125
  - R = 0.5834
  - P = 0.0018

- Y = 0.6025X + 2.061
  - R = 0.5970
  - P = 0.0016

E

**Regression Lines**

- Y = 0.6393X + 1.984
  - R = 0.4308
  - P = 0.0004

F

**Images**

- SK
  - Prox1/Beclin-1/DAPI
  - Prox1/p62/DAPI
  - Prox1/LC3/DAPI

- LM
  - Prox1/Beclin-1/DAPI
  - Prox1/p62/DAPI
  - Prox1/LC3/DAPI
Autophagy in lymphatic malformation

**Figure 1.** Detection and evaluation of autophagy in lymphatic malformations (LMs). A. In the serial sections of the LM tissue samples, up-regulated expression of autophagy-related proteins (Beclin-1, p62 and LC3) in LMs compared with these in skin (SK) samples. B. Quantification of Beclin-1, p62 and LC3 expression levels in LM and SK samples. C-E. Spearman rank test showed a positive significant between these autophagy-related proteins. F. Double-labelling immunofluorescence staining for autophagy-related proteins (Beclin-1, p62 and LC3) and Prox1 in LM but SK samples. The white arrowheads indicated the colocalization of autophagy-related proteins with Prox1 signals. All data are presented as mean ± SEM. **P < 0.01 ***P < 0.001 versus control groups.

markers was detected and compared in 26 clinical samples of LMs and 10 of normal SKs by immunochemistry. As shown in **Figure 1A**, in the serial sections of tissue samples from LMs, we found that cytoplasmic staining for LC3, p62 and Beclin-1 in the Prox1 lymphatic endothelial cells (LECs), while the immunoreactivities for these proteins in SKs were much weaker. Importantly, the data from the Students t-test for immunochemical staining showed that the expression levels of LC3 (P < 0.0001), p62 (P < 0.0001), and Beclin-1 (P < 0.01) was significantly up-regulated in LMs than these proteins in SKs (**Figure 1B**). Furthermore, Spearman’s rank correlation was performed and the results demonstrated that there was also a significant correlation between autophagy-related proteins (LC3, p62 or Beclin-1) (**Figure 1C-E**). Meanwhile, the double-labelling immunofluorescence analyses for Prox1 and Beclin-1, Prox1 and p62, Prox1 and LC3 were made on the samples both from the LMs and SKs, respectively, and we found that the colocalization of these proteins in LMs samples (**Figure 1F**). Collectively, these results strongly confirm the activation of autophagy in LMs.

**Induction of autophagy with FBS-starvation**

To examine whether autophagy could also be merged in human lymphatic endothelial cells in vitro, we conducted the studies using HDLECs treated with normal FBS (20% FBS) or low FBS (5% FBS). As shown in **Figure 2A, 2B**, the date from TEM analysis showed that exposure of HDLECs to 5% FBS possessed more membranous vacuoles (AVs), resembling the autophagosomes when compared with the control groups. Meanwhile, we also observed autophagic response to low FBS via analysis of recruitment of punctate LC3, as well as appearance of yellow-orange AVOs, which were the hallmarks of autophagy. The date indicated that HDLECs in the control groups came up with weak and diffuse LC3 immunostaining, while the HDLECs in low FBS or rapamycin (positive control) showed punctate pattern of LC3-associated green fluorescence. On the other hand, similar results could also be found in the formation of yellow-orange AVOs. In addition, the treatment of low FBS also resulted in elevated expression of autophagy-associated genes, including LC3, p62, Beclin-1, Atg2, Atg3, Atg5 and Atg7, in HDLECs, as well as autophagic proteins (Beclin-1 and LC3) (**Figure 2C, 2D**). Furthermore, to distinguish the autophagosome accumulation due to a block of downstream steps, we performed LC3 turnover assay via western blot analysis with Bafilomycin A1 (the lysosomal inhibitor) to measure the influence of low FBS on autophagic flux. The results exhibited that the difference in LC3-II levels with or without Bafilomycin A1 was much larger in low FBS when compared with the cells in normal condition, revealed that autophagic flux induced in HDLECs was indeed activated by low FBS (**Figure 2E**).

**Inhibiting autophagy under low FBS enhanced apoptotic cell death**

To examine whether the suppression of autophagy could accelerate the cell death in low serum condition, chloroquine (CQ), a well-characterized inhibitor of autophagic flux, was carried out in combination with low serum starvation. As shown in **Figure 3A**, the results from flow cytometry showed that the population of Annexin V’ apoptotic cells was markedly increased after treatment with CQ before serum starvation. Consistently, the DNA fragmentation assay was performed and the date indicated that incubation of CQ before serum starvation elicited obvious increase of DNA fragments in HDLECs (**Figure 3B**). Furthermore, the results from western blot analyses also demonstrated that the ratio of Bax/Bcl-2 as well as cleaved PARP in HDLECs were significantly up-regulated under the condition of incubation with CQ before serum starvation (**Figure 3C, 3D**). Thus, our results confirm that the apoptosis induced by low FBS could be enhanced by the inhibition of autophagy.
Serum starvation activated autophagy via the Erk1/2 signaling pathway

Recently, the Erk1/2 signaling pathway was proved to be pivotal in the activation of autophagy [10]. Thus, we tried to investigate whether Erk1/2 could be the upstream mediators for low serum-induced autophagy. As shown in Figure 4, low serum led to a rise in the expression level of Erk1/2 phosphorylation (p-Erk1/2) with a significant difference at 3 h post treatment. Meanwhile, low FBS-induced p-Erk1/2 was greatly inhibited when HDLECs were pretreated with PD98059, a specific small-molecule inhibitor for p-Erk1/2 (Figure 4A, 4B). To our interests, PD98059 also significantly down-regulated the expression levels of Beclin-1 and the LC3II/LC3I ratio (Figure 4C-F). These results reveal that the activation of autophagy in HDLECs is depended on the activation of Erk1/2 signaling pathway under FBS starvation.
Autophagy in lymphatic malformation

Figure 3. Low FBS-induced apoptosis is enhanced by inhibiting autophagy. A. HDLECs pre-treated with or without chloroquine (CQ) were cultured in low FBS condition for 24 h, and apoptosis rate was detected by Annexin V-FITC/PI dual labelling using flow cytometry. B. An augmentation of DNA fragmentation of low FBS-treated HDLECs was found after CQ pre-treatment. C. The expression levels of apoptosis-related proteins were determined by using western blot analysis. D. Relative quantitative data for apoptosis-related proteins were calculated by Image J. *P < 0.05, **P < 0.01 versus control groups.
Autophagy in lymphatic malformation

Significant correlation between autophagy-related proteins and p-Erk1/2, Bcl-2 and Ki-67 in LMs clinical samples

To further explore the role of autophagy in the cellular anti-apoptosis and proliferation of LMs. We detected the expression of p-Erk1/2, Bcl-2, and Ki-67 in LMs samples via immunohistochemistry. The representative images are shown in Figure 5A. We found that the expression levels of p-Erk1/2, Bcl-2 and Ki-67 in LECs were significantly increased in lymphatic vessels of LMs when compared with these proteins in SKs (Figure 5A, 5B). Furthermore, Spearman’s rank correlation revealed that there was closely positive correlation between LC3 and Bcl-2 or Ki-67, p62 and Ki-67, p-Erk1/2 and LC3, Bcl-2 or Ki-67 (Figure 5C-H). Moreover, clustering analysis for these tested markers was conducted, which was visualized with a heat map (Figure 5I). As shown in the heat map, almost all the LMs samples clustered together, which was obviously distinct from the cluster of SKs samples. Besides, this clustering indicated that the expression of autophagy-related markers, was closely associated with the expression of Bcl-2, p-Erk1/2 and Ki-67, implicating internal relations between these proteins we tested.

Suppression of autophagy inhibited the development of IFA-induced LMs

Previous researches including our studies showed that Freund’s incomplete adjuvant (FIA) could be used to induce LMs in rats [19, 22] but the mechanism behind is unclear. To ascertain whether or not targeting autophagy in LMs can block or delay the development of LMs, we performed a chemopreventive treatment on IFA-induced LMs with chloroquine (CQ). As shown in Figure 6A, 6B, CQ significantly prevented the growth of FIA-induced LMs. On the other hand, the systemic toxicity was also preliminarily assessed. None of weight loss (Figure 6C), ulceration in the skin, toxic response and death was found. Moreover, the H&E stained sections of heart, liver, spleen, lung and kidney showed no organ hemorrhage or other significant histopathological lesions could be found in the organs with the treatment of FIA and CQ (Supplementary Figure 1). Furthermore, by performing immunohistochemistry, we found that the expression of Ki-67, Bcl-2 as well as VEGF-C in LMs treated with CQ was largely reduced when compared with the control groups in Figure 6D and Supplementary Figure 2. All of the above results indicate that inhibiting autophagy with CQ could effectively suppress the development of IFA-induced LMs with high systematic biocompatibility.

Discussion

Here, we demonstrated the autophagy process in LMs for the first time, and implicated that it might contribute to the anti-apoptosis and proliferation of lymphatic endothelial cells in LMs. Moreover, we also showed autophagia activa-

Figure 4. Low FBS-induced autophagy involves the ERK pathways in HDLECs. A. HDLECs exposed to low FBS for 3, 6, 12 and 24 h and showed an increase in Erk1/2 phosphorylation compared with those in normal FBS condition. B. Densitometric analysis of p-Erk1/2 level to total Erk1/2 level by using Image J. C. Expression levels of Beclin-1 and the ratio of p-Erk to total p-Erk, LC3-I to LC3-II, were down-regulated at 24 h using the p-Erk inhibitor (PD98059). D-F. Quantification of protein levels with the PD98059 treatment showed that the expression level of Beclin-1, and the ratio of p-Erk1/2 to total p-Erk1/2, LC3-I to LC3-II, were significantly down-regulated in 24 h. *P < 0.05, **P < 0.01 ***P < 0.001 versus control groups.
Figure 5. Elevated levels of p-Erk1/2, Bcl-2 and Ki-67 in human LM tissues and correlation analyses for the tested proteins in LM samples. A. Higher levels of p-Erk1/2, Bcl-2 and Ki-67 in the LM samples when compared with these in SK samples. B. Quantification of p-Erk1/2, Bcl-2 and Ki-67 expression levels in both of LM and SK samples. C. The
expression levels of LC3 showed significant positive correlation with Bcl-2. D. The expression levels of LC3 showed significant positive correlation with Ki-67. E. The expression levels of p62 showed significant positive correlation with Ki-67. F. The expression levels of p-Erk1/2 showed significant positive correlation with LC3. G. The expression levels of p-Erk1/2 showed significant positive correlation with Bcl-2. H. The expression levels of p-Erk1/2 showed significant positive correlation with Ki-67. I. Hierarchical clustering analyses for immunohistochemical staining of p62, LC3, Beclin-1, Bcl-2, p-Erk1/2, Ki-67 and VEGF-C in human LM samples. ***P < 0.001 versus control groups.

Figure 6. Suppression of autophagy prevented the anti-apoptosis and growth of IFA-induced LMs in rats. A. LMs regression was observed in IFA-induced LMs in rats treated with CQ. B. The size of IFA-induced LMs of rats in both CQ- and vehicle-treated groups was assessed every other week. C. Body weight of rats was measured every other week and showed that no side effect of CQ treatment. D. Immunohistochemical staining of the indicated biomarkers in IFA-induced LMs of rats treated with or without CQ treatment, which showed that decreased expression of Bcl-2, Ki-67 and VEGF-C was observed in FIA-induced LMs of rats treated with CQ when compared with the control groups.
Autophagy in lymphatic malformation

tion in lymphatic endothelial cells (HDLECs), with the activation of ERK1/2 signal pathway in low FBS condition. In addition, our in vivo study also revealed that autophagy could be a promising target for the treatment of FIA induced-LMs in rats.

To date, more than 30 autophagic proteins have been recognized during autophagy-related processes [9, 13]. Among them, LC3, generally used to monitor the status of autophagy, is documented as the first mammalian protein bounded with autophagic membranes [8, 9]. p62 is selectively merged into autophagosomes by directly bonding with LC3 [9]. Besides, Beclin-1 is essential for vacuolar transport during autophagic processes [11]. Our study showed that these autophagic proteins, correlated with each other, were significantly increased, indicating an activated statue of autophagy in LMs.

Autophagy is a conserved catabolic process where damaged organelles, bacteria and misfolded proteins are transported into lysosomes for degradation [9, 10]. Numerous studies have confirmed that the primary role of autophagy was to ensure cellular survival under stress condition [8, 9, 14]. However, it is still vague that autophagy could be activated and its accurate role in lymphatic endothelial cells. Thus, in vitro studies using HDLECs were performed. As expected, autophagic activation could be observed in HDLECs, which was confirmed by our date from TEM, immunofluorescent, AVO staining, qPCR and western blot. Moreover, the date from flow cytometry, DNA fragments detection and western blot showed an increased apoptosis after inhibiting autophagy with CQ, a widely used autophagic pathway inhibitor [23], which indicated a pro-survival role of autophagy in HDLECs in low FBS condition. Therefore, based on the above results, we can draw a conclusion that autophagy could be activated in HDLECs and may contribute to the survival of these endothelial cells.

According to previous studies [14], there are two pathways well known to regulate autophagic response to nutrition starvation in mammalian cells: the class I phosphatidylinositol 3-phosphate kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR)/p70 ribosomal protein S6 kinase (p70S6K) signaling pathway, and the Ras/Raf-1/mitogen-activated protein kinase 1/2 (MEK1/2)/extracellular signal-regulated kinase 1/2 (Erk1/2) pathway. Among these, the Erk1/2 signal pathway positively regulates autophagy [24]. Correspondingly, our studies show that inhibition of the Erk1/2 signal pathway by a MAPK kinase (MAPKK) MEK1/2 inhibitor (PD98059) could inhibit FBS starvation-induced autophagy. Our date clearly showed that FBS-starvation up-regulated the Erk1/2 signaling pathway, leading to the activation of autophagy in HDLECs. More importantly, we found that in LMs samples, there were close correlations between autophagy core marker (LC3) and p-Erk1/2, Bcl-2 or Ki-67, which were confirmed by Spearman rank correlation test and the clustering analysis. Collectively, our results strongly suggested that autophagy may promote the anti-apoptosis and proliferation of lymphatic endothelial cells through the activation of Erk1/2 signaling pathway in LMs.

For clinical significance, we also used CQ to suppress the growth of IFA-induced LMs. Interestingly, the sizes of the IFA-induced LMs in rats were largely decreased after the treatment with CQ, followed by the weaken capacity of anti-apoptosis, proliferation and pro-lymphangiogenesis in lymphatic endothelial cells. Although further studies are needed to elucidate the mechanism, these findings suggest that defect of autophagy could restraint the development of IFA-induced LMs.

Here, our present study reported the activated status of autophagy in LMs and, simultaneously examined the underlying mechanism and pro-survival role of autophagy in the pathological processes of LMs. Our results also implicated that inhibition of autophagy could significantly suppress the development of LMs, revealing a new therapeutic strategy for LMs. Nevertheless, deeper investigation is still need to uncover the precise molecular mechanism in order to understand the pathogenic effect of autophagy for LMs.

Acknowledgements

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Disclosure of conflict of interest

None.

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References


Autophagy in lymphatic malformation


# Supplementary Table 1. Summary of clinical features of patients with lymphatic malformations

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# Supplementary Table 2. Summary of antibodies used in immunochemistry (IHC), immunofluorescence (IF-P) and western blot (WB)

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<td>VEGF-C</td>
<td>Goat pAb</td>
<td>SantaCruz (27128)</td>
<td>IHC (1:400)</td>
</tr>
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<td>Rabbit mAb</td>
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<td>WB (1:1000)</td>
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<td>Total Erk1/2</td>
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<td>WB (1:1000)</td>
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Supplementary information

Supplementary materials and methods

Cell culture and cell viability assay

As our previous description [1], human dermal lymphatic endothelial cells (HDLECs) were obtained from Sciencell (Carlsbad, USA). HDLECs were cultured in endothelial cell medium (ECM; Sciencell, USA) with 20% fetal bovine serum (FBS), penicillin-streptomycin mixture and SingleQuot (Cambrex Bio Science). In our present study, cells in passages 2-7 were used. When the cells were grown to 70% confluence, they were cultured with low FBS (5% FBS) or normal FBS (20% FBS) for 24 h, respectively.

Transmission electron microscopy

As our previous description [2], for the transmission electron microscopy (TEM), HDLECs (2*10^5) were seeded into 6-cm dishes for overnight attachment, and then incubated with 10% FBS or 5% FBS for indicated time. Then, these HLECs were fixed by using 2.5% electron microscopy grade glutaraldehyde, and further postfixed in 1% osmium tetroxide with 0.1% potassium ferricyanide, followed by dehydrated through an orderly series of ethanol (30-90%). After embedded in Epon, ultrathin sections (65 nm) of cells were cut, routinely stained with 2% uranyl acetate as well as Reynold’s lead citrate. Finally, these sections were observed using a transmission electron microscope (Hitachi H-600).

Immunofluorescence for LC3 localization

As we described previously [3, 4], HDLECs (2*10^5) were seeded to coverslips in 6-cm dishes and allowed to attach after overnight culture. After incubated with 5% FBS or 10% FBS for indicated time, these cells were fixed with 4% paraformaldehyde, and then permeabilized in 0.1% Triton X-100 for 20 min. Later, the cells were blocked with bovine serum albumin buffer for 1 h at 37°C before incubated with the anti-LC3 antibody (1:50) overnight at 4°C. Next, the cells were treated with fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit second antibody (West Grove) for 1 h. Then, the cellular nuclei were stained with DAPI, observed and photographed under a fluorescence microscope (Leica).

Detection of acidic vesicular organelles (AVO)

According our previous procedures [2, 4], HDLECs (2*10^5) were plated on coverslips in 6-cm dishes. After attached on the coverslips, the cells were treated with 5% FBS or 10% FBS for definite time and then stained with 1 mg/ml acridine orange for 15 min. Immediately, these cells was photographed under a fluorescence microscope (Leica).

Real-time quantitative PCR

According to our previous protocol [5], real-time quantitative PCR (qPCR) was performed. Briefly, total RNA from HDLECs was isolated with TRIzol Reagent (Invitrogen). Afterwards, 2 µg RNA was reverse transcribed to cDNA with Oligo(dT) and AMV reverse transcriptase (Fermentas). Then, the cDNA was used for polymerase chain reaction (PCR) by using FastStart Universal SYBR Green Master (Roche) with a 7900HT Real-time PCR System (Applied Biosystems). In this assay, GAPDH was chosen to be the endogenous control, and the primer nucleotide sequences for PCR are presented in Supplementary Table 3.

Western blot analysis

According to our previous protocol [2], the western blot was carried out. Briefly, HDLECs were lysed to the total protein which was then transferred onto polyvinylidene fluoride membranes (Millipore). Next, the blots were blocked in 5% non-fat dry milk for 1 h, followed by incubation with corresponding primary antibodies. After incubated with horseradish peroxidase-conjugated secondary antibody (Pierce Chemical), the blots were immersed in a SuperEnhanced chemiluminescence solution (Pierce, Rockford).
Flow cytometric analyses

Depended on our previous studies [5], after culture in 5% FBS and 20% FBS with or without CQ, 1*10^6 HDLECs were collected, stained with FITC-Annexin V and PE-PI for flow cytometry with BD fluorescent-activated cell sorter system (Calibur, CO) according to the manufacturer’s instructions. Finally, the date was analyzed with FlowJo 7.6.1 software.

References


Autophagy in lymphatic malformation

Supplementary Table 3. Primer sequences used for real-time quantitative PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward (5'-3')</th>
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Supplementary Figure 1. Photographs of hematoxylin-eosin staining for cardiac muscle, liver, spleen, lung and kidney organs of rats after the FIA treatment with or without CQ.

Supplementary Figure 2. Semi-quantification of immunohistochemical staining of Bcl-2, Ki-67 and VEGF-C in IFA-induced LMs of rats treated with or without chloroquine (CQ). **P < 0.01 ***P < 0.001 versus control groups.