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Spliced XBP1 promotes macrophage survival and autophagy by interacting with Beclin-1

Ping-Ge Tian [a, 1], Zhi-Xin Jiang [c, 1], Jian-Hua Li [e], Zhe Zhou [b, **], Qing-Hua Zhang [d, *]

[a] Southern Medical University, Guangzhou, Guangdong 510515, China
[b] Laboratory of Biotechnology, Beijing Institute of Radiation Medicine, Beijing 100850, China
[c] Centre Laboratory, The 305th Hospital of the People's Liberation Army, Beijing 100017, China
[d] Department of Cardiology, The 305th Hospital of the People's Liberation Army, Beijing 100017, China
[e] Department of Geriatric Cardiology, Chinese PLA General Hosptial, Beijing 100853, China

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ABSTRACT

Macrophage autophagy plays an important role in the development of atherosclerosis, but the precise mechanism mediating this process is unclear. The potential role of the X-box binding protein 1 (XBP1), a crucial transduction factor that is involved in endoplasmic reticulum stress and the unfolded protein response, in bone marrow-derived macrophage autophagy is unknown. This study mainly explores the roles of XBP1 mRNA splicing in bone marrow-derived macrophage autophagy. The present study shows that the transient overexpression of spliced XBP1 via adenovirus-mediated gene transfer induces autophagy and promotes proliferation in bone marrow-derived macrophages via the down-regulation of Beclin-1, but that the sustained overexpression of spliced XBP1 leads to apoptosis. When XBP1 is down-regulated in bone marrow-derived macrophages using siRNA, rapamycin-induced autophagosome formation is ablated. Furthermore, we have detected the overexpression of XBP1 in areas of atherosclerotic plaques in the arteries of ApoE−/− mice. These results demonstrate that XBP1 mRNA splicing plays an important role in maintaining the function of bone marrow-derived macrophages and provide new insight into the study and treatment of atherosclerosis.

1. Introduction

It is well known that cardiovascular diseases related to atherosclerosis are the leading cause of mortality worldwide. Autophagy plays a key role in the development of atherosclerosis [1–6]. Oxidized lipids, inflammation, and metabolic stress conditions, which can be detected in advanced atherosclerotic plaques, trigger autophagic responses in atherosclerosis [7]. A growing body of evidence indicates that dysfunction or deficiency of autophagy promotes atherosclerosis via inflammasome hyperactivation [8]. Moreover, the processes of autophagy can be affected by many factors, such as endothelial cell (EC) dysfunction or death, resulting in lossing of vessel wall integrity and promoting the development of atherosclerosis [9], macrophage dysfunction promotes the development of atherosclerosis [10]. In advanced atherosclerosis, apoptosis or death of macrophages promotes the formation of necrotic areas in plaques [10]. Furthermore, Schrijvers DM et al. showed that macrophage autophagy is a potential drug target for plaque stabilization [10]. However, the regulation of macrophage autophagy in atherosclerotic plaques needs to be fully elucidated. Autophagy is a multistep catabolic process that has been shown to be involved in a variety of pathophysiological conditions such as oxidative stress [6,11], hypoxia [12], ischemia-reperfusion [13]. During the process of autophagy, long-lived proteins and organelles are sequestered into a double membrane-bound autophagosome and degraded by a lysosome [14]. Under physiological conditions, autophagy is a reparative and homeostatic process [15]. In cases of excessive induction of autophagy by environmental or intracellular stress, autophagy becomes a cell death pathway [16]. However, the mechanisms underlying the regulation of autophagy still remain unclear.

The X-box binding protein 1 (XBP1) belongs to the basic region/leucine zipper transcription factor family [17]. Under stress

* Corresponding author. 13 Wenjing Streets, The 305th Hospital of the People's Liberation Army, Beijing 100017, China.
** Corresponding author. 27 Taiping Road, Beijing Institute of Radiation Medicine, Beijing 100850, China.
E-mail addresses: zhouzhe76@126.com (Z. Zhou), 1056055170@qq.com (Q.-H. Zhang).
[1] These two authors contributed equally to this work.

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conditions, XBP1 mRNA undergoes unconventional splicing by inositol-requiring enzyme1 to generate the 56 kDa spliced isoform with intact transcriptional activity [18]. The unspliced XBP1 produces a 29 kDa protein, which negatively the spliced isoform [18]. XBP1 mRNA splicing is involved in multiple physiological processes, such as plasma cell differentiation [19]. Because ZengL et al. have reported that XBP1 splicing played diverse roles in endothelial cell inducing EC apoptosis [20], autophagy [21] and proliferation [22], while XBP1 splicing had no effect in smooth muscle cells (SMC) in inducing SMC autophagy. And the more popular cells in atherosclerosis are SMC, EC, and macrophage. And we are interested in macrophage. So in this study we were focused on macrophage. In the present study, we demonstrate that XBP1 mRNA splicing participates in macrophage proliferation, apoptosis, and autophagy via the transcriptional activation of Beclin-1.

2. Methods and materials

2.1. Cell culture

Bone marrow-derived macrophages were harvested from the femurs of wild-type C57BL/6 mice (male, 7 weeks old). Then, the cells were passed through a 0.075 mm cellular filter (Miltenyi, Auburn, CA). Next, these cells were re-suspended in DMEM medium supplemented with 10% fetal bovine serum, 50 ng/ml CSF-1 (PeProTech, Rocky Hill, NJ), and 50 U/ml gentamicin and were cultured for 7 days. F4/80 (FITC-labeled anti-mouse rat IgG2a; eBiosciences) and CD11b (PE-labeled rat anti-mouse IgG2b; eBiosciences) expression were determined by flow cytometry (FACSCanto II; BD Biosciences).

2.2. Adenoviral gene transfer and cell proliferation assays

Ad-XBP1s, Ad-XBP1u, and Ad-Beclin-1 were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China), and were amplified and infected (MOI) of 100 as previously described [20]. Cell proliferation was detected using Cell Counting Kit-8 (CCK8) according to the protocol.

2.3. Transmission electron microscopy assays

Adenovirus was applied to infect macrophages at 100 MOI for 48 h, and si-RNA was applied at 20 pmol to transfect macrophages for 48 h, following by treatment with rapamycin (10 μM) for 6 h. The cells were scraped of, collected by centrifugation (1000 × g, 10 min) and then fixed with 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer for 2 h at 4 °C. After fixation, the samples were observed by transmission electron microscope (Hitachi H-7650 TEM).

2.4. RNA extraction and RT-PCR assays

Total RNA was extracted using the RNA prep pure cell/bacteria kit (Tiangen Biotech, Co., Ltd., Beijing, China) according to the manufacturer's protocol. Then, 1 μg of RNA was reverse-transcribed into cDNA using FastQuant RT Kit (Tiangen Biotech, Co., Ltd., Beijing, China). Next, 50 ng of cDNA was amplified via standard PCR using Taq DNA polymerase (Takara Biotech, Co., Ltd., Beijing, China) and primers. The primers designed using Primer 5 for each gene are as follows: Arg:-5′-CAGAGGCCAATCCACCTGA-3′ and 5′-CTAGTCTGGCTGTTGCCTTGG-3′; Inos:-5′-CGTTCAGCTCACCTTCGAGG-3′ and 5′-AACCTCCAGGCACACAGTTTG-3′; XBP1:-5′-CAGAGCTGGGCATCTCAAACC-3′ and 5′-GGGCCCAGTGTTATGTGGCT-3′; LC3β:-5′-ACTGCAAGTCCAATGCTCCAG-3′ and 5′-GGTGTCCTATAATGGAGGCCG-3′; and for Beclin-1:-5′-AGGTTGAGAAAGGCGAGACA3′ and 5′-TTTTGATGGAATAGGAGCCG-3′.

2.5. Immunoblotting and immunohistochemistry staining assays

Cells were harvested and lysed in RIPA lysis buffer (Applygen Technologies Inc. China) supplemented with protease inhibitors (Roche Applied Science) on ice for 30 min. SDS-PAGE was performed and the protein were transferred to PVDF membrane (Millipore), followed by a standard Western blot procedure. Additionally, the aortas were fixed with 4% paraformaldehyde, followed by standard immunohistochemical staining. Images were captured under a phase-contrast microscope (Olympus, Tokyo, Japan). Moreover, the tissue protein was extracted from mouse aortas using a Tissue Protein Extraction Kit (Beijing Cowin Biotech Co., Ltd., Beijing, China) according to manufacture's protocols.

2.6. Statistical analysis

The data were analyzed using a two-tailed Student's test for two group comparisons using SPSS 13.0 (SPSS, Chicago, IL, USA). The differences between more than three groups were determined using one way analysis of variance (ANOVA) with Bonferroni's multiple comparison tests to compare each two groups. A value of $p < 0.05$ was considered to be significant. The data were expressed as the means ± S.D.

3. Results

3.1. The expression of XBP1 is related to atherosclerosis

To evaluate whether XBP1 plays a role in the development of atherosclerosis, we detected the expression of XBP1 via immunohistochemical staining of aherosclerotic sections isolated from 5-month-old (with 3 months high fat diet) $ApoE^{-/-}$ (C57BL/6) and $ApoE^{+/+}$ (C57BL/6J) mice. Immunohistochemical staining indicated that very little XBP1 protein was expressed in the normal aorta (Fig. 1A), but large amount of XBP1 was expressed in the areas of atherosclerotic plaques (Fig. 1B). Because XBP1 has two isoforms (a 29 kDa unspliced isoform and a 56 kDa spliced isoform) and the anti-XBP1 (M186) could not distinguish between them based on Immunohistochemical staining. Thus, we used Western blot to detect the levels of each isoform. The results showed that XBP1s (the 56 kDa spliced isoform) expression was detected at low levels in the $ApoE^{+/+}$ mice but at high levels in the $ApoE^{-/-}$ mice (Fig. 1C and D). XBP1u (the 29 kDa unspliced isoform) was mainly detected in $ApoE^{-/-}$ mice, although at a relatively lower level than the spliced isoform (Fig. 1C and D). According to these results, we concluded that these two isoforms exist in atherosclerotic areas but that XBP1s is the predominant isoform. These data indicate that the expression of XBP1, especially XBP1s, is related to atherosclerosis.

3.2. The expression of spliced XBP1 is related to macrophage proliferation

To test the expression of XBP1 correlates to the biological function of macrophages, down-regulation and overexpression experiments were preformed on macrophages-derived bone marrow. A proliferation assay using the CCK8 method showed that XBP1s overexpression for 24 or 48 h dramatically increased cell survival compared with Ad-XBP1u or Ad-null and XBP1s overexpression for 72 h (Fig. 2A). There was no difference in the survival rate of macrophages between Ad-null and Ad-XBP1u treatment ($p > 0.05$) (Fig. 2A). As only XBP1s showed a significant effect on macrophages, the following experiments mainly focused on this isoform. When XBP1 was silenced using XBP1 siRNA for 24 or 48 h, the cell proliferation rate was decreased compared with the control siRNA (Fig. 2B). Primary macrophages were co-infected with Ad-

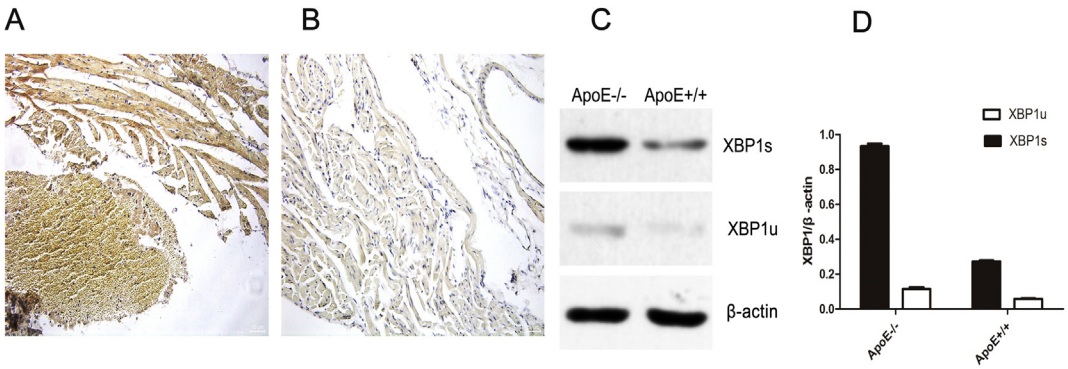

Fig. 1. The expression of the XBP1 mRNA splicing variant (XBP1s) is closely related to atherosclerosis. (A–C) Samples of aortas isolated from ApoE$^{-/-}$ or ApoE$^{+/+}$ (C57/BL6) mice fed a high fat diet for 3 months. (A and B) Immunohistochemistry (IHC) indicating that XBP1 was more highly expressed in the areas of atherosclerosis plaques (B) than in the normal aortas from ApoE$^{+/+}$ mice (A). (C and D) Western blot analysis detected the protein expression of spliced and unspliced XBP1 in ApoE$^{-/-}$ mouse arteries. (C) Representative Western blot bands showing that both isoforms of XBP1 were more highly expressed in the ApoE$^{-/-}$ mice than in the ApoE$^{+/+}$ mice. Note that the protein level of XBP1s in the ApoE$^{-/-}$ mice and ApoE$^{+/+}$ mice was higher than that of XBP1u which was barely detectable in the wild-type ApoE$^{+/+}$ mice. (D) Densitometric analysis of the electrophoretic bands shown in (C). The experiments were repeated 3 times. Error bars, S.D.

XBP1s and Ad-Beclin-1 at 100 MOI each for 24, 48 or 72 h. The results showed that Ad-Beclin-1 significantly increased Ad-XBP1s-infected macrophage survival (Fig. 2C). Additionally, macrophages were co-infected with Ad-XBP1s and Ad-Beclin-1 at 100 MOI each for 72 h, and then, a proliferation assay was performed. The results indicated that Ad-Beclin-1 ameliorated the Ad-XBP1s-induced decrease in cell proliferation (Fig. 2C). The down-regulation of Beclin-1 by adenovirus transfer for 24 or 48 h decreased the rate of macrophage proliferation (Fig. 2D). Besides, we confirmed the phenotype of the bone marrow-derived macrophages cells cultured in complete medium containing CSF-1 (30 ng/ml) by detecting the expression of CD11b and F4-80 via flow cytometry. CD11b and F4-80 are mainly expressed in macrophages. Cells displaying a rate of double-positivity for CD11b and F4-80 of up to 80% were used in these experiments (Fig. 2E and F). The down-regulation or up-regulation of XBP1s, XBP1u and Beclin-1 in macrophages was confirmed via Western Blot (Fig. 2G). Taken together, these data indicated that the transient over-expression of XBP1s in macrophages increased cell proliferation but that the sustained over-expression of XBP1s decreased cell proliferation potentially by inducing apoptosis.

3.3. The transient activation of XBP1 mRNA splicing triggers autophagy in macrophages

Macrophages were infected with the Ad-XBP1s, Ad-XBP1u, or Ad-null for 48 h and then observed via transmission electron microscopy (Fig. 3A–C). The results showed that autophagosomes

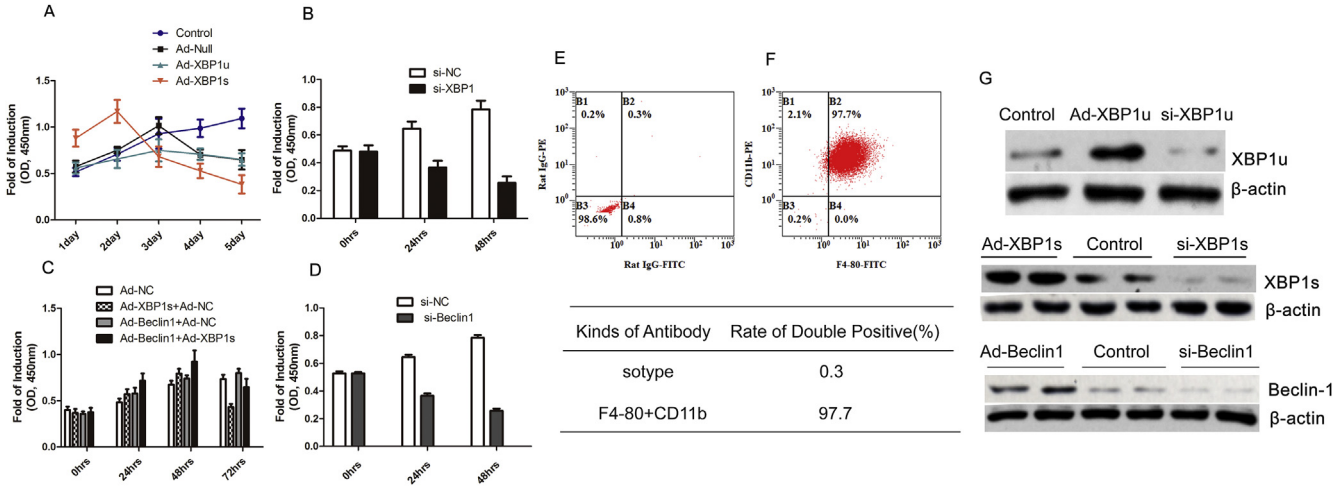

Fig. 2. The expression of the XBP1 mRNA splicing variant (XBP1s) is closely related to macrophage proliferation. (A) The overexpression XBP1s for 24 or 48 h via adenovirus infection increased macrophage proliferation detected using a Cell Counting Kit-8 (CCK8) assay. However, when XBP1s overexpression was sustained for more than 72 h, macrophage proliferation was decreased compared with XBP1u overexpression and empty adenovirus (Ad-null) infection. The proliferation rate of these macrophages infected with adenovirus carrying unspliced XBP1 (Ad-XBP1u) was not significantly different compared with that of the Ad-null and blank controls. (B). XBP1 was silenced in macrophages by siRNA, and proliferation analysis was performed using a CCK8 kit at 24 or 48 h after transfection by CCK8 kit. XBP1 siRNA inhibited cell survival. (C) The over-expression of Beclin-1 via Ad-Beclin-1 infection increased Ad-XBP1s-infected macrophage proliferation. Bone marrow-derived macrophages were co-infected with Ad-XBP1s and Ad-Beclin-1 at 100 MOI each, and the proliferation assay was performed at 24, 48 and 72 h after infection. Empty adenovirus (Ad-null) was used as a control at the identical MOI. The data are presented as the means ± SD of three representative experiments *$p < 0.05$. (D) The down-regulation of Beclin-1 in macrophage using targeted siRNA for 24 or 48 h inhibited the proliferation of macrophages detected by the CCK8 assay. The data are presented as the means ± SD of three repeated experiments. *$p < 0.05$. (E and F) Flow cytometric analysis was used to identify bone marrow-derived macrophages by detecting the expression of CD11b and F4-80 (macrophage surface markers). The double-positive rate was 97.7%. An isotype control antibody was used as a control. (G) Western blot confirmed the over-expression or knockdown of Beclin-1, the unspliced, and the spliced XBP1 isoforms of the XBP1 protein. β-actin was used as a loading control.

mainly appeared in macrophages infected with Ad-XBP1s (Fig. 3C). To further explore whether XBP1 splicing induced macrophage autophagy, we overexpressed XBP1s in macrophages via adenoviral transfer for 24, 48, or 72 h. Then, the mRNA and protein expression levels of the autophagy markers Beclin1 and LC3βII were valuated via routine RT-PCR (Fig. 3D) and Western Blot (Fig. 3I), respectively. The routine RT-PCR results indicated that overexpression XBP1s in macrophages increased the expression of BECLIN-1 and LC3βII (autophagy-related genes) at the mRNA level at 24 and 48 h compared with the control virus-infection (Fig. 3D). However, the expression of the autophagy markers Beclin-1 and LC3βII in macrophages was decreased when XBP1s was overexpressed for 72 h (Fig. 3D). Western blot analysis showed that the expression of Beclin-1 and LC3βII were highly elevated at 24 or 48 h after Ad-XBP1s, which indicated the activation of autophagy (Fig. 3I). The expression of Beclin-1 and LC3βII were decreased at 72 h (Fig. 3I). However, the expression of Chop (apoptosis-related genes) was highly increased at 72 h (Fig. 3I), which indicated that the activation of apoptosis. Flow cytometry detects the percentage of macrophages which were successfully confected by adenovirus. All of the percentage was up to 80% (Fig. 3E–H). Collectively, these results indicate that XBP1s participates in the transcription of Beclin-1 in macrophages. Thus, we conclude that the transient activation of XBP1 mRNA splicing for 24 or 48 h can induce autophagy in macrophages via the transcriptional regulation of Beclin-1.

3.4. XBP1 overexpression in macrophages as detected by an indirect immunofluorescence assay results in typical "punctate staining" for the autophagy marker LC3β

To further confirm the hypothesis that XBP1 mRNA splicing can trigger macrophage autophagy, immunofluorescence double-staining for LC3β (a typical autophagy marker) and XBP1s was employed to detect their expression in macrophages in 40 × magnifications (Fig. 4A–R). The results showed a large amount of "punctate staining" for the LC3β marker in macrophages infected with Ad-XBP1s (Fig. 4A–C) but not Ad-null (Fig. 4G–I). The percentage of XBP1 and LC3β double-positive cells per field were counted in 5 random fields in 40 × magnifications, and the average percents of positive cell were about 94.62% (Fig. 4T). Moreover, when macrophages infected with Ad-XBP1s were treated with chloroquine, which inhibits the fusion of autophagosomes with lysosomes, the amount of "punctate staining" was further increased (Fig. 4D–F). Additionally, when the macrophages infected with XBP1 siRNA were treated with rapamycin (an inhibitor of mTOR), the amount of "punctate staining" was decreased (Fig. 4P–R). Real-time quantitative RT-PCR was used to confirm the down-regulation of XBP1s in bone marrow-derived macrophages (Fig. 4N). Taken together, these data confirmed that XBP1s mRNA induced autophagy in macrophages.

4. Discussion

In this study, we have demonstrated that XBP1s promotes CSF-1-differentiated bone marrow-derived macrophage (CSF-1 macrophage) survival and autophagy by interacting with Beclin-1. CSF-1 macrophages in which XBP1 was down-regulated using siRNA, failed to induce autophagy, and the basal level of LC3β expression was reduced. Additionally, we have found that the transient overexpression of XBP1s promotes macrophage proliferation and survival but that the sustained overexpression of XBP1s induces CSF-1 macrophage apoptosis. The elevated expression of XBP1 was detected in areas of atherosclerotic plaques. Thus, it appears that XBP1s may play an important role in the process of atherosclerosis by protecting CSF-1 macrophages from survival and autophagy, at least in part. Atherosclerosis is a multistep process that involves many factors, such as platelet aggregation [23], hypertension [24]. In recent years, researchers have become interested in autophagy, which can be triggered by oxidative stress [11] or hypoxia [12]. However, the factors that can induce autophagy are still unclear.

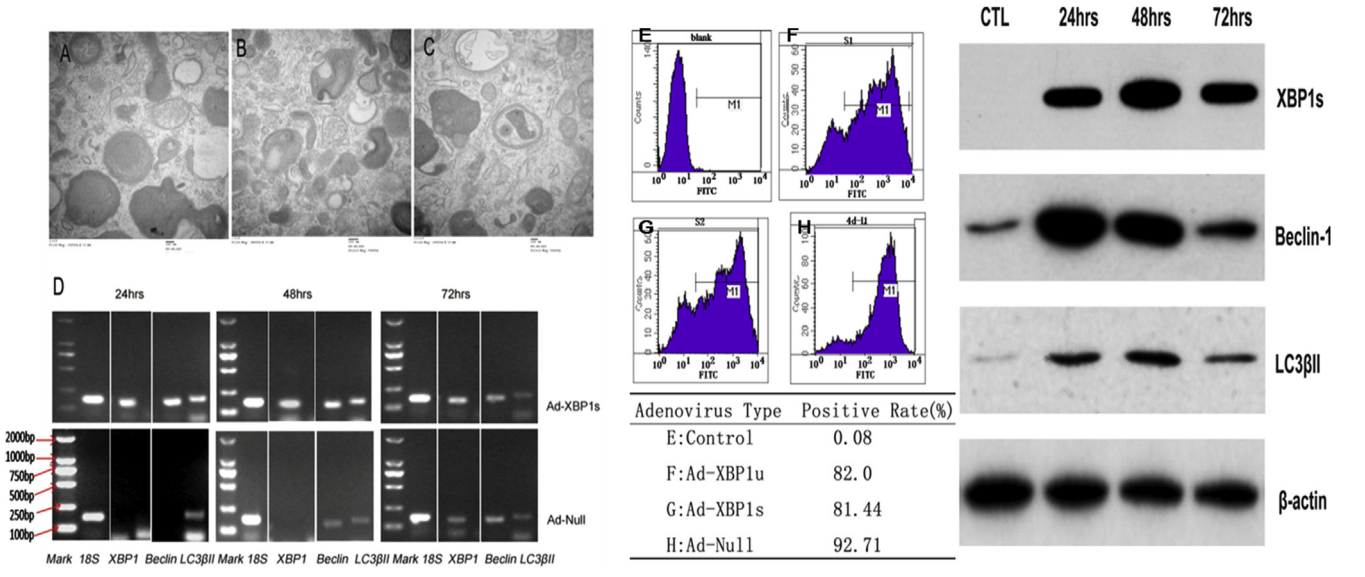

Fig. 3. The transient over-expression XBP1s triggers macrophage autophagy via Beclin-1. (A–C) Macrophages were infected with Ad-null, Ad-XBP1s, or Ad-XBP1u at 100 MOI for 6 h and then cultured for 48 h, followed by transmission electron microscopy (scale bars, 100 nm). (A and B) Few autophagosomes were observed in macrophages infected with Ad-XBP1u or Ad-null. (C) Many autophagosomes were observed in macrophages infected by Ad-XBP1s for 48 h. (D) Routine RT-PCR was performed to detect the gene expression of Beclin-1 and LC3β (an autophagy marker) in macrophages after infection with Ad-XBP1s for 24, 48 and 72 h. The results show that the expression of Beclin-1 and LC3βII increased or decreased as XBP1s expression in macrophages was up-regulated or down-regulated. (E–H) Flow cytometry detects the percentage of macrophages which were successfully confected by adenovirus. All of the percentage was up to 80%. (I) Overexpression XBP1s by Ad-XBP1s in macrophages for 24 h, 48 h, and 72 h, then western blot profermed to detected the protein expression of Beclin-1, LC3βII (autophagy related protein) and Chop (apoptosis-related protein). In the intial terms of 48 h, Beclin-1 and LC3βII were increased as XBP1s increasing. But the expression of Beclin-1 and LC3βII were decreased and Chop was increased at 72 h.

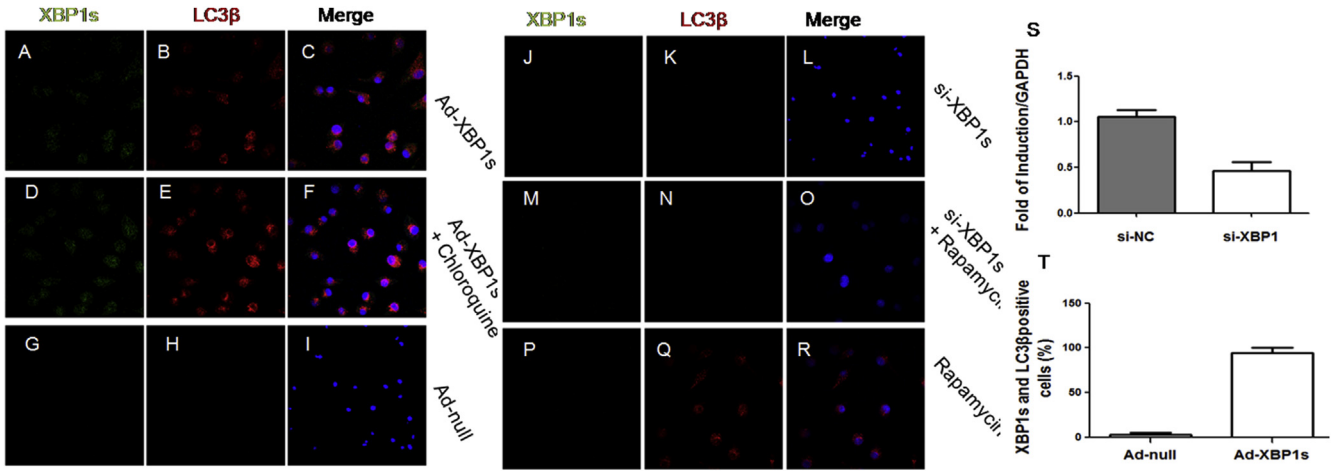

Fig. 4. XBP1s overexpression increases "Punctate Staining" of LC3β in macrophage. Macrophages were infected with Ad-null or Ad-XBP1s at 100 MOI for 48 h and then stained with anti-LC3β and anti-XBP1 antibodies to detect exogenous XBP1s expression. in 40 × magnifications. (A–R) Macrophages were analyzed via immunofluorescence double-staining for LC3β (a typical autophagy marker) and XBP1s (A–C) XBP1s overexpression increased the expression of LC3β, which shows increased "Punctate Staining" compared with that of control. (T) The percentage of XBP1- and LC3β-positive macrophages of (A–C) was calculated to be approximately 94.6%. (D–F) Macrophages were infected with Ad-null or Ad-XBP1s for 48 h and treated with chloroquine for the final 6 h. Then, Western blot for LC3β were performed. The expression of LC3β, which shows increased "Punctate Staining", was increased. (G–I) Ad-null-infected cells were used as controls. (J–L) XBP1 was silenced in macrophages using siRNA. Then, immunofluorescence staining was used to detect the expression of LC3β, which showed no typical punctate staining in the siXBP1s-transfected cells. (M–O) Macrophages were transfected with siXBP1, treated with rapamycin for 12 h, and then observed via immunofluorescence staining. The results show that little "punctate staining" in these cells. (P–R) Rapamycin induces macrophage autophagy, as demonstrated by increased "Punctate Staining". (S) Macrophages were infected with Ad-XBP1s or si-XBP1 for 48 h and real time quantitative RT-PCR was performed for XBP1. The results showed that XBP1 was successfully overexpressed or silenced in macrophages. The presented data are representative of three independent experiments. *Error bars*, S.D.

XBP1 plays an important role in the transcriptional control of the ER stress response [25], which can trigger autophagy [11,26]. The results from the report by Andriana Margarit et al. [26] showed that XBP1 overexpression triggers autophagy in endothelial cells. Moreover, they indicated that autophagy induced by XBP1 is cell-specific. Thus, whether XBP1 can trigger CSF-1 macrophage autophagy is unknown. To answer this question, in this study, we explored the role of XBP1 in CSF-1 macrophages autophagy and demonstrated that XBP1s can induce CSF-1 macrophage autophagy.

Macrophage dysfunction plays an important role in the development of atherosclerosis [10]. Autophagy, which can be activated by rapamycin (an mTOR inhibitor) [27], plays a critical role in maintaining the functions of macrophages [27]. In this study, we showed that the expression of autophagy gene when XBP1 was silenced in CSF-1 macrophages using siRNA, was down-regulated. These results further confirm that XBP1s induces CSF-1 macrophage autophagy. Beclin-1 plays a key role in both autophagosome formation and autolysosome fusion during the early stages of autophagy [28]. It has been reported that Beclin1 can interact with Bcl-2 family protein, resulting in the induction or inhibition of autophagy. Moreover, XBP1s may be a target of acetylation and deacetylation by p300 and SIRT1, respectively, and these events may regulate the transcriptional activity of XBP1s [25,28]. Additionally, Biochem J. et al. have demonstrated that IRE1/XBP1 regulates the acetylation status of the ER by regulating the influx of acetyl-coA thereby modulating autophagy [25]. Based on this evidence, we conclude that the over-expression of XBP1s may activate the ER stress response, which increases the phosphorylation of Beclin1 and promotes the dissociation of Beclin1 from Bcl-2, resulting in the induction of autophagy. Recently, increasing evidence has shown a macrophage autophagy response in advanced atherosclerotic plaques [29]. In the present study, our results show that the transient over-expression of XBP1s induces an autophagy response in CSF-1 macrophages but that the sustained activation of XBP1s leads to CSF-1 macrophages apoptosis. Thus, we conclude that the duration of XBP1s overexpression is tightly regulated to modulate the functions of macrophages. Moreover, it seems that some key molecule may determine the threshold for autophagic responses. Our data show that XBP1s overexpression for 24 or 48 h induces CSF-1 macrophage autophagy, but that XBP1s overexpression for 72 h leads to apoptosis. In 2012, Sirois I et al. showed that autophagy and apoptosis are two closely related processes that regulate the extracellular export of autophagic vacuoles via activated caspases [30]. In conclusion, our data demonstrate for the first time that the transient over-expression of XBP1s induces an autophagic response in CSF-1 macrophage and promotes their proliferation and survival via the transcriptional regulation of Beclin-1, but that the sustained overexpression of XBP1s induces apoptosis. In the future, it will be helpful to further learn about the threshold for the macrophage autophagy. Targeting for XBP1 mRNA splicing may serve as a novel therapeutic strategy for atherosclerosis.

Conflict of interest

None.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.061.

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