The role of autophagy emerging in postinfarction cardiac remodelling

Hiromitsu Kanamori1,2, Genzou Takemura1*, Kazuko Goto1, Rumi Maruyama1, Akiko Tsujimoto1, Atsushi Ogino1, Toshiaki Takeyama1, Tomonori Kawaguchi1, Takatomo Watanabe1, Takako Fujiwara3, Hisayoshi Fujiiwara4, Mitsuru Seishima2, and Shinya Minatoguchi1

1Department of Cardiology, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan; 2Department of Laboratory Medicine, Gifu University Graduate School of Medicine, Gifu, Japan; 3Department of Food Science, Kyoto Women’s University, Kyoto, Japan; and 4Department of Cardiology, Hyogo Prefectural Amagasaki Hospital, Hyogo, Japan

Received 3 November 2010; revised 2 March 2011; accepted 11 March 2011; online publish-ahead-of-print 15 March 2011

Time for primary review: 28 days

1. Introduction

Large myocardial infarctions lead to severe chronic heart failure with adverse remodelling of the left ventricle characterized by cavity dilatation and diminished cardiac performance.1 The most critical determinant of subsequent ventricular remodelling and heart failure is the magnitude of the acute infarction, which can be determined within several hours of an attack.2 The process of cardiac remodelling is complicated; however, and many other factors, including late death or hypertrophy of cardiomyocytes, fibrosis, expression of various cytokines, and non-myocyte dynamics within the infarct tissue, are all associated with disease progression during the chronic stage.3,4

Hibernating myocardium present in the region bordering the infarct is associated with reversible contractile dysfunction, and the cardiomyocytes there are said to be ‘dedifferentiated’ because they show degenerative changes such as myofibrillar loss and mitochondriosis that are somewhat similar to the foetal phenotype.5 In those cells, the transcription factor GATA-4,6 which stimulates expression of various sarcomeric proteins (e.g. myosin heavy chain and tropinin I), is down-regulated. In addition, apoptotic loss of those cardiomyocytes has been reported, which may contribute to progression of postinfarction cardiac remodelling.7 This hypothesis remains highly

Aims

Autophagy is activated in cardiomyocytes in ischaemic heart disease, but its dynamics and functional roles remain unclear after myocardial infarction. We observed the dynamics of cardiomyocyte autophagy and examined its role during postinfarction cardiac remodelling.

Methods and results

Myocardial infarction was induced in mice by ligating the left coronary artery. During both the subacute and chronic stages (1 and 3 weeks postinfarction, respectively), autophagy was found to be activated in surviving cardiomyocytes, as demonstrated by the up-regulated expression of microtubule-associated protein-1 light chain 3-II (LC3-II), p62 and cathepsin D, and by electron microscopic findings. Activation of autophagy, specifically the digestion step, was prominent in cardiomyocytes 1 week postinfarction, especially in those bordering the infarct area, while the formation of autophagosomes was prominent 3 weeks postinfarction. Bafilomycin A1 (an autophagy inhibitor) significantly aggravated postinfarction cardiac dysfunction and remodelling. Cardiac hypertrophy was exacerbated in this group and was accompanied by augmented ventricular expression of atrial natriuretic peptide. In these hearts, autophagic findings (i.e. expression of LC3-II and the presence of autophagosomes) were diminished, and activation of AMP-activated protein kinase was enhanced. Treatment with rapamycin (an autophagy enhancer) brought about opposite outcomes, including mitigation of cardiac dysfunction and adverse remodelling. A combined treatment with bafilomycin A1 and rapamycin offset each effect on cardiomyocyte autophagy and cardiac remodelling in the postinfarction heart.

Conclusion

These findings suggest that cardiomyocyte autophagy is an innate mechanism that protects against progression of postinfarction cardiac remodelling, implying that augmenting autophagy could be a therapeutic strategy.

Keywords

Autophagy • Heart failure • Myocardial infarction • Remodelling
Autophagy in postinfarction cardiac remodelling

2. Methods

2.1 Animals and experimental protocols

This study conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996 and was approved by the Institutional Animal Research Committee of Gifu University. Mice were initially anaesthetized with 2% halothane in a mixture of N₂O and O₂ (0.5 L/min each) via a nasal mask and then incubated with a 20G intravenous catheter and a 20G intravenous catheter. Autophagy has survival-oriented functions, occurring under both basal conditions and conditions of stress (e.g. starvation). During autophagic degeneration, degraded membrane lipids and proteins within autophagosomes are recruited to maintain needed levels of ATP production and protein synthesis, thereby promoting cell survival. In the heart, autophagy occurs constitutively in the normal myocardium but is substantially increased in cases of heart failure, cardiac hypertrophy, and ischaemic cardiomyopathy. Inefficient autophagy or its absence causes the myocardium to perform poorly, and inhibition of starvation-induced autophagy results in cardiac dysfunction and dilatation. Moreover, autophagy has been shown to be an adaptive response of the heart that protects the myocardium from haemodynamic overload and acute ischaemic death. On the other hand, autophagy is also a mode of cell death that occurs during tissue and organ development to eliminate unnecessary cells. The functional role of autophagy in heart disease (i.e. whether it mediates cell survival or cell death) and whether it up- or down-regulates cellular function are still poorly understood.

Our aim in the present study was to investigate the functional role of autophagy in the progression of postinfarction cardiac remodelling, taking into consideration the afore-mentioned two aspects of its function: maintenance of energy homeostasis to promote cell survival and induction of cell death. To address that issue, we used a mouse myo-cardiac infarction model, first observing the dynamics of autophagy in the heart during the subacute and chronic stages (1–3 weeks after coronary occlusion) and then examining the effects of agents that alter the autophagic process on the progression of postinfarction left ventricular remodelling and dysfunction.

2.2 Physiological studies

Echocardiography and cardiac catheterization were carried out as previously described. Cardiac catheterization was performed only before sacrifice because of its invasiveness.

2.3 Histology

Once the physiological measurements were complete, mice were sacrificed, and the hearts were removed, weighed and cut into transverse slices at the mid-papillary muscle level. They were fixed in 10% buffered formalin, embedded in paraffin, cut into 4 μm thick sections and stained with haematoxylin–eosin or Masson’s trichrome. Cardiomyocyte size (expressed as the transverse diameter of myocytes cut at the level of the nucleus) was assessed in 20 randomly chosen high-power fields (HPFs; ×600) in each section.

2.4 Immunohistochemistry and immunofluorescence

After deparaffinization, the 4 μm thick sections were incubated with a primary antibody against CD45 (Pharmingen), microtubule-associated protein-1 light chain 3 (LC3; MBL), cathepsin D (Santa Cruz), myoglobin (DAKO), or atrial natriuretic peptide (ANP; Santa Cruz). A Vectastain Elite ABC system (Vector Laboratories) was then used to immunostain the sections; diaminobenzidine served as the chromogen, and the nuclei were counterstained with haematoxylin.

The LC3-immunolabeled sections with Alexa 488 (green; Molecular Probe) were double stained with another antibody against myoglobin (DAKO Japan), endothelial cell (Flk-1; Santa Cruz), macrophage (Mac1; Serotec), or α-smooth muscle actin (DAKO Japan) followed by Alexa 568 (red; Molecular Probe) to observe autophagic activity, respectively, in cardiomyocytes, endothelial cells, macrophages, or myofibroblasts/vascular smooth muscle cells. These sections were then counterstained with Hoechst 33342 and observed under a confocal microscope (LSM510, Zeiss). For in situ terminal dUTP nick end-labeling (TUNEL), tissue sections were first stained with Fluorescein-FragEL™ (Oncogene) and then labelled with anti-myoglobin antibody (DAKO Japan) followed by Alexa 568 (Molecular Probe). Quantitative assessments, including the number of immunopositive cells or immunopositive dots within cells, were carried out in 20 randomly chosen HPFs (×600) using a multipurpose colour image processor (Nireco, Kyoto, Japan). The border area was defined as the area containing both infarction and surviving myocardium within a HPF, while the remote area was the myocardial region remote from any infarction.

2.5 Electron microscopy

Cardiac tissue was quickly cut into 1 mm cubes, immersion fixed with 2.5% glutaraldehyde in 0.1 molar phosphate buffer (pH 7.4) overnight at 4°C, and postfixed in 1% buffered osmium tetroxide. The specimens were then conventionally processed and were examined under an electron microscope (H-800, Hitachi).
Table 1  Cardiac function during the physiological examination carried out before and after treatments (1 and 3 weeks after surgery, respectively)

<table>
<thead>
<tr>
<th></th>
<th>Sham Saline, n = 3</th>
<th>Myocardial infarction Saline, n = 14</th>
<th>Bafilomycin A1, n = 12</th>
<th>Rapamycin, n = 11</th>
<th>Bafilomycin A1 + Rapamycin, n = 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVDD, mm</td>
<td>3.16 ± 0.03</td>
<td>4.28 ± 0.08*</td>
<td>4.31 ± 0.10*</td>
<td>4.25 ± 0.06*</td>
<td>4.302 ± 0.08*</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>72.6 ± 2.8</td>
<td>31 ± 1.9*</td>
<td>38.2 ± 1.8*</td>
<td>39.4 ± 1.8*</td>
<td>38.4 ± 1.0*</td>
</tr>
<tr>
<td>Heart rate, b.p.m.</td>
<td>474 ± 8</td>
<td>492 ± 1</td>
<td>484 ± 15</td>
<td>495 ± 9</td>
<td>498 ± 14</td>
</tr>
<tr>
<td>After treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVDD, mm</td>
<td>3.17 ± 0.07</td>
<td>4.84 ± 0.05***</td>
<td>5.24 ± 0.06**</td>
<td>4.31 ± 0.09***</td>
<td>4.90 ± 0.06***</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>71.3 ± 1.1</td>
<td>39.5 ± 0.6*</td>
<td>33.2 ± 1.0**</td>
<td>49.2 ± 1.7**</td>
<td>38.9 ± 0.9*</td>
</tr>
<tr>
<td>Heart rate, b.p.m.</td>
<td>489 ± 6</td>
<td>480 ± 7</td>
<td>480 ± 8</td>
<td>495 ± 9</td>
<td>498 ± 14</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>105.3 ± 1.6</td>
<td>81.9 ± 0.9*</td>
<td>83.3 ± 2.3*</td>
<td>82.8 ± 1.3*</td>
<td>82.8 ± 0.9*</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>0.1 ± 0.1</td>
<td>4.1 ± 0.3*</td>
<td>7.6 ± 1.0**</td>
<td>1.8 ± 0.3**</td>
<td>4.5 ± 0.2*</td>
</tr>
<tr>
<td>+dP/dt, mmHg/s</td>
<td>8762 ± 619</td>
<td>4338 ± 150**</td>
<td>2836 ± 260***</td>
<td>5629 ± 237***</td>
<td>3955 ± 196*</td>
</tr>
<tr>
<td>−dP/dt, mmHg/s</td>
<td>−6890 ± 701</td>
<td>−3176 ± 178**</td>
<td>−2100 ± 90***</td>
<td>−4391 ± 222***</td>
<td>−2851 ± 100*</td>
</tr>
</tbody>
</table>

LVDD, end-diastolic left ventricular diameter; LVEF, left ventricular ejection fraction; SBP, systolic blood pressure; LVEDP, left ventricular end-diastolic pressure.

*P < 0.05 vs. the saline-treated sham group.
**P < 0.05 vs. the saline-treated infarction group.
***P < 0.05 vs. before treatment.

2.6 Western blotting

Proteins (100 µg) extracted from hearts (n = 3–6 from each group) were subjected to 10 or 15% polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes. The membranes were then probed using primary antibodies against LC3 (MBL), cathepsin D (Santa Cruz), p62 (MBL), ANP (Santa Cruz), AMP-activated protein kinase (AMPK), phosphorylated AMPK (p-AMPK), and caspase-3 (all from Cell Signaling), after which the blots were visualized using enhanced chemiluminescence (Amersham). α-Tubulin (analysed using an antibody from Santa Cruz) served as the loading control.

2.7 Statistical analysis

Data are expressed as the means ± SEM. The significance of differences between groups was evaluated using one-way ANOVA with a post hoc Newman–Keul’s multiple comparisons test or a repeated-measures ANOVA (Table 1). Values of P < 0.05 were considered significant.

3. Results

3.1 Cardiomyocyte autophagy during the subacute and chronic stages of myocardial infarction

Over the period between the subacute (1 week) and chronic (3 weeks) stages of myocardial infarction, the mouse heart showed dramatic remodelling of the left ventricle accompanied by dilatation and wall thinning (see Table 1 and Supplementary material online, Figure S1A). A significant increase in the number of LC3 dots within cardiomyocytes was apparent during the subacute stage of infarction, indicating activation of autophagy (Figure 1A). The dots distributed most prominently in the area bordering the infarct, where they were larger in size than those in more remote areas. The number of LC3-positive dots within the border zone was significantly reduced during the chronic stage, though a substantial number remained (Figure 1B). In contrast, the numbers of dots in the remote area were significantly increased during the chronic stage. Electron microscopic examination of cardiomyocytes in the border zone during the subacute stage revealed the presence of cytoplasmic vacuoles that resembled autophagosomes and often contained intracellular organelles, such as degraded mitochondria and membrane-like structures, but these vacuoles were unusually large in size (Figure 2A). In contrast, the cardiomyocytes in the remote area contained more typical (i.e. smaller) autophagosomes. By the chronic stage, the previously huge autophagosomes within cardiomyocytes in the border zone had shrunk in size and now appeared as typical autophagosomes, while lysosomes and autophagosomes became more numerous within the cardiomyocytes in the remote area. Compared with the surviving myocardium, the infarct area showed extremely scarce LC3-positive dots (Figure 1). Since an infarct area contains not only collagen fibres but also cellular components, we examined autophagic activity in those non-cardiomyocytes within the infarct area using cell-specific immunofluorescence. Non-cardiomyocytes, such as endothelial cells, macrophages, and myofibroblasts, were found to contain only very few LC3 dots (see Supplementary material online, Figure S2) and rare autophagosomes were confirmed by electron microscopy (data not shown).

Western blot analysis showed that expression of LC3-II, cathepsin D, and p62 was up-regulated in the infarcted hearts (Figure 2B). The myocardial LC3-II/LC3-I ratio, an established indicator of autophagic turnover, was increased during the subacute and chronic stage to a similar extent. The LC3-binding protein p62 regulates the formation of protein aggregates and is removed by autophagy, while cathepsin D is a lysosomal proteolytic enzyme, and both participate in the digestion steps during autophagy. Both of these proteins were strongly expressed in the myocardium during the subacute stage. The increase in p62 was maintained until the chronic stage, but that in cathepsin D was significantly moderated during the chronic stage (Figure 2B). Collectively, these findings indicate that autophagy is activated within surviving cardiomyocytes in the infarcted heart during both the subacute
and chronic stages of infarction, and suggest that whereas digestion in the border zone is more conspicuous at the subacute stage, autophagic turnover (including production of autophagosomes) in the remote area is more active during the chronic stage.

3.2 Functional role for autophagy in postinfarction cardiac remodelling

We assessed the functional role of autophagy in the infarcted heart by examining the effects of inhibiting autophagy using bafilomycin A1 or stimulating the process using rapamycin. However, bafilomycin A1 and rapamycin were reported to affect macrophage function, fibroblast phenotype, inflammatory, and fibrotic responses in vitro. These actions of the compounds are crucial when considering that many factors other than the magnitude of acute infarct, including late death or hypertrophy of cardiomyocytes, fibrosis, expression of various cytokines, and non-myocyte dynamics within the infarct tissue, are all associated with disease progression during the chronic stage. In the hearts 2 weeks postinfarction, inflammation was significantly attenuated compared with those 1 week postinfarction (see Supplementary material online, Figure S1), while autophagic activity was markedly sustained (Figure 1). Therefore, we started treatment 2 weeks postinfarction to minimize the effect of the reagents on the inflammatory process.

In the remote and border areas, the LC3 dots in cardiomyocytes were reduced by bafilomycin A1, while they were increased by rapamycin (Figure 3A). The LC3 dots were rare in the infarct area, and the dot number was not affected by either treatment. Ultrastructurally, the surviving cardiomyocytes in the bafilomycin A1-treated hearts lacked indicators of autophagic activation, while cardiomyocytes in rapamycin-treated hearts contained abundant lysosomes and autophagosomes (Figure 3B). We found that the LC3-II/LC3-I ratio was significantly reduced in the bafilomycin A1-treated hearts, where compared with the LC3 dots dynamics, not only the expression of LC3-II, but also that of LC3-I was reduced (Figure 3C). Likewise, myocardial levels of both p62 and cathepsin D were reduced in the bafilomycin A1-treated hearts compared with the untreated hearts. Following treatment with rapamycin, the myocardial LC3-II/LC3-I ratio was significantly increased, though levels of p62 and cathepsin D were decreased, suggesting accelerated autophagic turnover. Overall, these findings suggest that whereas rapamycin increases autophagic turnover, bafilomycin A1 impairs it. Moreover, not only

---

**Figure 1** Activation of autophagy in cardiomyocytes during postinfarction cardiac remodelling. (A) Immunofluorescent labelling of LC3 (green) and myoglobin (red) in ventricular tissue in an area remote from the infarct (upper panels), in tissue bordering the infarct (middle panels), and in the infarct area (lower panels). Bars, 10 μm. (B) Numbers of LC3 dots per HPF (×600) in the remote, border, and infarct areas. N = 3 per group. *p < 0.05 vs. with the sham-operated group; #p < 0.05 vs. group with 1-week-old infarctions.
did bafilomycin A1 exert an effect at the digestion step, as previously reported,\textsuperscript{20} it also appeared via an unknown mechanism to negatively affect the initiation of autophagy (phagophore formation) as deduced by the down-regulated LC3-I in this group. In the group treated with a combination of bafilomycin A1 and rapamycin, LC3 dots in cardiomyocytes and myocardial level of LC3 were found similar to those in the control group and myocardial levels of p62 and cathepsin D were reduced compared with the control group (Figure 3).

Physiological examination revealed that neither bafilomycin A1 nor rapamycin affected left ventricular geometry or systolic function in sham-operated mice (data not shown). In mice bearing myocardial infarcts, however, bafilomycin A1 significantly aggravated and rapamycin significantly mitigated postinfarction left ventricular dilatation and both systolic and diastolic dysfunction as shown by markedly decreased +dP/dt (Table 1). Histologically, treatment with bafilomycin A1 led to elongation of the infarcted wall segment and increases in the heart-to-body weight ratios and cardiomyocyte size, while treatment with rapamycin brought about the reverse (Figure 4A). In addition, western blot and immunohistochemical analyses showed that expression of ANP in cardiomyocytes in the border zone was augmented in bafilomycin A1-treated hearts, but was only weakly expressed in rapamycin-treated hearts (Figure 4B). Finally, the
Figure 3  Effects of inhibiting or enhancing myocardial autophagy during postinfarction remodelling on expression of LC3-immunopositive dots, the ultrastructure of cardiomyocytes, and expression of autophagy-related proteins. (A) Number of the LC3 dots per HPF (×600) in the remote, border, and infarct area. S, sham; V, vehicle; B, bafilomycin A1; R, rapamycin. N = 3 per group. *P < 0.05 vs. with the sham-operated group; **P < 0.05 vs. the vehicle-treated control group. (B) Electron micrographs of cardiomyocytes in bafilomycin A1- and rapamycin-treated hearts 3 weeks postinfarction. Arrows indicate lysosomes. N, nucleus. Bars, 1 μm. (C) Expression of autophagy-related proteins, LC3, cathepsin D, and p62, during progression of postinfarction cardiac remodelling. S, sham; Veh/V, vehicle; Baf/B, bafilomycin A1; Rap/R, rapamycin. *P < 0.05 vs. with the sham-operated group; **P < 0.05 vs. the vehicle-treated control group.
combined treatment with bafilomycin A1 and rapamycin was found to offset one another in the effect on cardiac function and remodelling and myocardial ANP expression (Figure 4B). Thus, it is suggested that bafilomycin A1 exacerbates, while rapamycin mitigates, postinfarction cardiac remodelling, and dysfunction, by respectively inhibiting and enhancing cardiomyocyte autophagy.

AMPK belongs to a conserved family of protein kinases activated by ATP depletion and the resultant AMP accumulation. We observed significant increases in the myocardial levels of p-AMPK 3 weeks postinfarction, which confirmed the activation of AMPK; moreover, treatment with bafilomycin A1 increased the levels of p-AMPK further, while rapamycin diminished them (Figure 5). The combination offset the effect of each on the other. This suggests bafilomycin A1-mediated inhibition of autophagy interferes with the supply of ATP to the ischaemic myocardium, leading to activation of AMPK, while rapamycin-mediated activation of autophagy does the reverse.

Because earlier studies reported that autophagic cell death could switch to apoptotic cell death when autophagy was inhibited, we next assessed the incidence of apoptosis in the present model.
Double labelling cells using TUNEL and an anti-myoglobin antibody showed the incidence of TUNEL positivity among both cardiomyocytes and non-myocytes to be significantly increased in hearts 3 weeks postinfarction. That said, the rate was very low among cardiomyocytes, and was unaffected by any treatment (see Supplementary material online, Figure S3A). The rate of TUNEL positivity among non-myocytes was also unaffected by either treatment. Myocardial caspase-3 activation was assessed by western blotting for cleaved caspase-3 and confirmed the increase in apoptosis (see Supplementary material online, Figure S3B), the majority of which was probably of non-myocyte origin, and was not affected by any treatment.

4. Discussion

4.1 Cardiomyocyte autophagy during progression of postinfarction cardiac remodelling

Autophagy is known to be activated in ischaemic heart disease and has been well documented following ischaemia–reperfusion injury and in hibernating myocardium. In the present study, we observed augmented autophagy in surviving cardiomyocytes during subacute and chronic stages of myocardial infarction. Autophagosomes in surviving cardiomyocytes showed a unique morphology and localization that was dependent on the stage of infarction. At the subacute stage, autophagy was more prominent in cardiomyocytes in the border zone than in those in more remote areas, and the size of the autophagosomes was exceedingly large in the former. At the chronic stage, in contrast, autophagic activity was stronger in the remote cardiomyocytes than in those in the border zone. Ultrastructurally, the remote cardiomyocytes showed many lysosomes as well as autophagosomes, suggesting activated initiation step of autophagy. At present, we do not know the aetiology or significance of the huge autophagosomes observed in cardiomyocytes in the border zone during the subacute stage, but these cells are exposed to both increased local mechanical stress and active inflammation in the granulation tissue. This excessive stimulation may have resulted in formation of exceedingly large autophagosomes. Another interesting finding is the strong activity of autophagy in remote myocardium at the chronic stage. Along with the progression of cardiac remodelling, wall stress increases in parallel with the ventricular dilatation (Laplace’s law), causing tissue hypoxia even in the remote myocardium. These factors might have induced such activity. On the other hand, we have no idea why at the chronic stage autophagic activity becomes attenuated in the border area.

AMPK is activated by ATP depletion and the resultant AMP accumulation, and is an important regulator of autophagy stimulated by cellular starvation. In failing hearts with adverse remodelling, increased wall stress represents a significant barrier against which cardiomyocytes must contract. These cells would be expected to require a greater energy supply than those in a healthy heart, which could lead to relative hyponutrition, trigger AMPK activation, and induce autophagy. On the other hand, it was recently reported that there is latent hypoxia in the failing heart, which could interfere with ATP synthesis and reduce both the contraction and relaxation of cardiomyocytes. Thus, a relative scarcity of energy may be another trigger of cardiomyocyte autophagy in the failing postinfarction heart.

4.2 Role of autophagy in postinfarction cardiac remodelling

We next investigated the functional role of augmented autophagic activity during progression of postinfarction cardiac remodelling. Treatment with bafilomycin A1 for 1 week inhibited autophagy and significantly exacerbated cardiac dysfunction and remodelling. Conversely, treatment with rapamycin augmented autophagic activity and significantly mitigated cardiac dysfunction and remodelling. The latter confirms the findings of the recent study by Buss et al., demonstrating that mTOR inhibition using everolimus increased autophagy and attenuated adverse remodelling following myocardial infarction. These suggest that autophagy in surviving cardiomyocytes is one of the compensatory mechanisms that mitigates postinfarction cardiac dysfunction and remodelling. In the rapamycin-treated group, however, blots of LC3-I and II are relatively small (Figure 3C), appearing inconsistent with increase in immunofluorescent LC3 dots (Figure 3A). Western blot was performed using the whole ventricles including the basal portion of the heart far from the infarct. As a haemodynamic stress can stimulate innate autophagic activity, the innate autophagic activity could be attenuated in the rapamycin-treated heart with less remodelling and this tendency might be greater in the portion far from the infarct. Since western blots using the whole ventricles do not always reflect the local event near the infarct, apparent inconsistency mentioned above might have been caused.

Autophagy also occurs during tissue and organ development to eliminate unnecessary cells while autophagic cell death is a morphological term derived from electron microscopic observations and indicates a form of cell death in which abundant autophagic vacuoles are present in the cytoplasm. Electron microscopic examination revealed numerous autophagic vacuoles in surviving cardiomyocytes in postinfarction hearts. However, none of these cardiomyocytes were judged to be dead, as their nuclei, plasma membranes, organelles, and cytoplasm appeared intact. In other words, we noted
autophagic degeneration but not autophagic cell death in surviving cardiomyocytes during the subacute and chronic stages of myocardial infarction. It appears that autophagy is activated in cardiomyocytes under stress, and that autophagic activity is inversely related to postinfarction cardiac remodelling and dysfunction. This suggests that activation of autophagic machinery is a compensatory response aimed at increasing the energy supply to meet the cellular demand in the face of excessive wall stress and hypoxia, which can cause a low-energy state in cardiomyocytes.

It has been reported that apoptotic loss of surviving cardiomyocytes may contribute to the progression of postinfarction cardiac remodelling and dysfunction. In addition, inactivation of apoptosis reportedly triggers apoptosis in cell lines. In the present study, bafilomycin A1 inhibited autophagy in cardiomyocytes but did not affect the rate of TUNEL positivity, and cardiac non-myocytes showed relatively little autophagic activity after infarction, as indicated by the absence of LC3-II immunopositivity. The low rate of apoptosis specifically among cardiomyocytes may be due to the relatively low sensitivity of cardiomyocytes to apoptotic stimuli such as Fas stimulation.

4.3 Study limitations

Treatment with bafilomycin A1 reportedly interferes with fusion between autophagosomes and lysosomes to inhibit formation of autophagolysosomes, resulting in suppression of the final digestion step in autophagy. Consequently, LC3-II protein and undisgested autophagosomes would be expected to accumulate in hearts treated with bafilomycin A1. Curiously, however, we did not observe this accumulation. We previously observed the same phenomenon (decreased LC3-I) when we administered bafilomycin A1 to mice that had been starved for a period. Bafilomycin A1 was found to activate AMPK and thus was assumed to disturb energy supply or recycle. Instead, we found that the LC3-immunopositive dots and levels of both LC3-II and LC3-I were significantly reduced, suggesting that the initial step in the formation of autophagosomes (phagophore formation) is also inhibited by bafilomycin A1. Impaired formation of autophagosomes in the bafilomycin A1-treated heart was actually confirmed by electron microscopy. Thus, bafilomycin A1 does effectively inhibit autophagy but the yet unknown mechanisms must be involved (e.g. a negative feedback to LC3-I synthesis), which should be clarified in future.

The agents we used in the present study do not specifically target autophagy or the heart; both have several actions other than modulation of autophagy, and both affect systemic metabolism. Thus, we cannot say that the profound alterations in postinfarction cardiac remodelling and function we observed resulted solely from manipulation of cardiomyocyte autophagy because of possible actions of the reagents independent of modulating autophagy such as anti-inflammatory one. However, we confirmed that neither bafilomycin A1 nor rapamycin affected the structure or function of normal (sham-operated) hearts, and importantly, we started the treatments 2 weeks postinfarction when inflammatory response in the infarct tissue is substantially diminishing in mice. Non-cardiomyocytes within infarct area showed rare autophagic activity which was not affected by either bafilomycin A1 or rapamycin. A majority of autophagic signals in the postinfarction heart are thus considered to originate from the cardiomyocytes, supporting the notion that a cardiomyocyte is one of the cell type in which autophagy is markedly activated. Furthermore, the combined treatment with bafilomycin A1 and rapamycin was found to offset one another in the effect on the autophagic findings and cardiac remodelling. Since a completely inverse correlation was noted between autophagic findings in cardiomyocytes and postinfarction cardiac remodelling, it would be allowed to conclude that the altered postinfarction cardiac remodelling is most likely through modulating cardiomyocyte autophagy, but less possibly through the other bafilomycin A1- or rapamycin-specific actions. A dose escalating study with each reagent may bring about more convincing results. Another effective means of resolving the issue may be through the use of conditional knockout or transgenic mice expressing an autophagy-related gene. However, such approaches may also lack specificity, as it was recently reported that there is an alternative autophagic pathway that is independent of Atg5 and Atg7, which had been believed to be essential for mammalian autophagy. On the other hand, our pharmacological approach has a merit in this problem—e.g. bafilomycin A1 inhibits autophagosome-lysosome fusion to prevent the final step of both classic and alternative pathways of autophagy.

4.4 Conclusions

The present study demonstrates the dynamics of cardiomyocyte autophagy and suggests its protective role during postinfarction cardiac remodelling. The latter finding implies that augmenting autophagy can be a therapeutic strategy for preventing the progression of postinfarction cardiac remodelling.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Acknowledgements

We thank the staff of Kyoto Women’s University (Chikako Koda, Aya Sakagami, Makiko Takeuchi, Megumi Tanigaki, Yuko Nakajima, Ayako Nozu) for technical assistance.

Conflict of interest: none declared.

Funding

This study was supported in part by Grants-in-Aid for scientific research from The Ministry of Education, Science, and Culture of Japan, Grant-in-Aid of The Japanese Medical Association, and Research Grant from Gifu University.

References


H. Kanamori et al.
Autophagy in postinfarction cardiac remodelling


