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Autophagy in the stress-induced myocardium

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1. ABSTRACT

Cardiovascular disease is a leading cause of death worldwide, particularly in Western societies. During an ischaemic insult, ventricular pressure from the heart is diminished as a result of cardiac myocyte death by necrosis and apoptosis. Autophagy is a process whereby cells catabolise intracellular proteins in order to generate ATP in times of stress such as nutrient starvation and hypoxia. Emerging evidence suggests that autophagy plays a positive role in cardiac myocyte survival during periods of cellular stress performing an important damage limitation function. By promoting cell survival, cardiac myocyte loss is reduced thereby minimising the potential of heart failure. In contrast, it has been reported that autophagy can also be a form of cell death. By considering the various animal models of autophagy, we examine the role of the Signal Transducers and Activator of Transcription (STAT) proteins in the autophagic response. Additionally we review the role of the tumour suppressor, p53 and its family member p73 and their potential role in the autophagic response.

2. INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death in the UK and contributes to around 17 million deaths worldwide (1). Coronary heart disease (CHD) is the most common disease under the umbrella of CVD particularly in western society (1). CHD results from the occlusion of blood flow through the coronary arteries of the left ventricle and results in local necrotic and apoptotic cell death of terminally differentiated cardiomyocytes (CM). The cessation of coronary blood flow (Ischaemia; I) is often followed by the reestablishment of flow leading to reperfusion (R) injury. Many studies have shown that reperfusion injury further increases cell death thus exacerbating the loss of irreplaceable CM and which appears to be mediated, at least in part, through the JAK/STAT pathway.

Autophagy is a physiological process whereby misfolded proteins and damaged or dysfunctional organelles are degraded and recycled. Basal levels of autophagy are found in unstressed cells, although

autophagic flux is increased under conditions of cellular stress, such as nutrient deprivation (reviewed in (2)). Moreover, mice deficient in a protein essential for autophagosome formation, Atg5, die between birth and the onset of suckling, owing to their inability to provide a source of intracellular nutrients through autophagic failure (3). Thus, autophagy may be a myocyte survival mechanism during I/R, although prolonged autophagy may also lead to cell death (autophagic death) (4).

The Signal Transducer and Activator of Transcription (STAT) proteins have been shown to be involved in modulating myocyte apoptosis whilst negatively regulating autophagy (5,6,7). The use of drugs such as the autophagy activator rapamycin may therefore serve as potential therapeutic agents by activating autophagic signalling during an ischaemic episode thereby promoting cell survival. Other STAT1 interacting proteins such as p53 and p73 appear to play dual roles in autophagic signalling although the exact mechanism of how this occurs is not fully understood (8,9,10,11,12,13).

Here we review the role of free radicals in I/R-induced myocyte death together with the major components of the autophagic signalling pathway. We also highlight the recent advances in understanding how autophagy may contribute to cell death and/or survival in the heart during I/R.

3. FREE RADICAL GENERATION AND CELL DEATH IN THE HEART

Mitochondrial dysfunction is a central mechanism of cell death in I/R injury to the heart. A combination of increased Ca influx and increased generation of Reactive Oxygen Species (ROS) leads to opening of the Mitochondrial Permeability Transition Pore (MPTP) on the inner mitochondrial membrane, resulting in uncoupling of oxidative phosphorylation and reduction in ATP synthesis (reviewed in (14,15)). The intracellular ATP concentration is one determinant of the form of cell death of ischaemically challenged CM. Low levels of ATP result in necrosis while higher levels permit energy-dependent apoptotic death to occur. In relation to apoptosis, increased mitochondrial permeability leads to release of cytochrome c, which forms a complex with Apaf-1, procaspase-9 and XIAP. The subsequent mitochondrial release of Smac/DIABLO neutralises the inhibitory effect of XIAP resulting in activation of caspase-9 and of downstream effector caspases with ordered destruction of proteins essential for cellular integrity (reviewed in (16)).

Fatty acids are a major metabolite of cardiac tissue which undergo beta-oxidation which contributes to approximately 90 percent of the supplied acetyl-CoA. Acetyl-CoA is then able to enter the Krebs cycle where NADH and $FADH_2$ are generated. During periods of low oxygen saturation the concentration of these two metabolites falls and the available NADH and $FADH_2$ are transported to the mitochondria where they enter oxidative phosphorylation in order to generate additional ATP for cell survival. Since oxygen is the terminal electron acceptor in the oxidative phosphorylation complex, during periods of oxygen starvation, NADH and $FADH_2$ cannot be metabolised and ATP cannot be generated leading to the onset of injury.

The process of oxidative phosphorylation involves the sequential transfer of electrons to oxygen which ultimately leads to the generation of water. However, the process of 'electron leakage' can occur that generates oxygen molecules with odd numbers of electrons in their outer shell. These highly active entities are known as oxygen free radicals (17). The highly active superoxide anion can be generated by the donation of a single electron catalysed by xanthine oxidase (18). The superoxide anion can accept a further electron to form a peroxide anion which can be protonated to form hydrogen peroxide (17).

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are thought to elicit their damaging effects by causing lipid peroxidation, the generation of DNA double strand breaks and the denaturation of cellular enzymes. These anions are devastatingly destructive and so apoptotic and necrotic cell death ensues.

Although ischaemic injury is responsible for the generation of oxygen free radicals, early experiments revealed that reperfusion injury enhanced cellular damage since a burst of free radicals was released upon the reestablishment of normal oxygen tension (19).

3.1. Autophagic signalling

Cells are homeostatic entities, and any challenge such as nutrient deprivation induced by starvation or I/R activates pathways that attempt to maintain cellular integrity. Autophagy is a catabolic process which degrades long-lived proteins, protein aggregates and damaged organelles using the recycled products to fuel metabolism and generate new proteins. Autophagy achieves this by sequestering its targets into double membranous structures known as autophagosomes which then fuse with lysosomes (20). The autophagic process has been shown to occur in the heart and its activity is increased during periods of stress such as starvation and ischaemia/reperfusion injury (21).

The process of autophagy can largely been categorised into 3 main subheadings, namely, initiation, nucleation and elongation/maturation. Initiation primarily occurs through mammalian target of rapamycin (mTOR) (20) which is involved in modulating the other initiator protein Ulk1 (22). The key player in the nucleation step of autophagy occurs through Beclin-1 which results in the sequential activation of various E1 and E2-like ligases or Atg (AuTophaGy) proteins (23) leading to the compartmentalisation of cellular material which is catabolised to generate cellular energy. However, the process of nucleation is not entirely understood although it is believed that the origin of the phagophore membranes may come from mitochondria and the endoplasmic reticulum (24,25,26).

Gene disruption studies on beclin-1 have shown that the homozygous knockout is embryonically lethal (27). No homozygous animals were born from a beclin-$1^{+/-}$ cross even when genotyped at E8.5. Normal Mendelian ratios were only detected at E7.5 and the embryos appear to suffer profound developmental growth retardation. Strong beclin-1 expression was detected in wild type animals at E6.5 suggesting that beclin-1 is required for early embryonic development.

Under basal conditions, mTOR is responsible for suppressing autophagic turnover. However, mTOR acts as a double edged sword since during periods of fasting, it is inhibited resulting in the suppression of protein synthesis and the activation of the autophagic pathway.

Kim and colleagues showed, under glucose starvation, that AMP activated protein kinase (AMPK) promoted autophagy by direct phosphorylation of Ser 317 and Ser 777 of the Atg1 homolog, Ulk1. The authors also demonstrated that over expression of the mTOR activator, RAS-homolog enriched in brain (Rheb), disrupted the interaction of AMPK and Ulk1 by directly phosphorylating Ulk1 at serine 757. The phosphorylation of Ulk1 by mTORC1 (Mammalian Target of Rapamycin Complex 1) could be inhibited by pretreating cells with the autophagy inducer, rapamycin, leading to increased AMPK-Ulk1 co-immunoprecipitation (Figure 1. (22)).

Death associated protein kinase (DAPK) has been shown to phosphorylate the BH3-only protein, Beclin-1 (28) releasing the inhibitory effects of Bcl2 and Bcl_{XL} on Beclin-1 which promotes the nucleation step of autophagy (29,30,31). In addition, it has been found that the proteins tBid, Bad and BNIP3 also disrupt the interaction between Beclin-1 and the anti-apoptotic proteins Bcl2 and Bcl_{XL} (32). VPS34 (vacuolar protein sorting 34) and Beclin-1 activate the association of Atg12 and the E1-like ligase, Atg7 which transfers Atg12 to the E2-like ligase, Atg10. Atg10 conjugates Atg12 to lysine 130 of Atg5 forming a heterodimer (33,34). The Atg16 homodimer conjugates to the Atg12-Atg5 heterodimer forming an Atg12-Atg5-Atg16 complex. The tetrameric Atg12-Atg5-Atg16 complex associates with a forming membrane generating the phagophore (Figure 1;(35,36). Further elongation of the phagophore membrane is achieved by the cleavage of the terminus of the human homolog of Atg8, LC3 by Atg 4 (Figure 1) exposing a terminal glycine residue (37,38). The cleavage of LC3-I by Atg4 enables LC3-I to become lipidated by the addition of phosphotidylethanolamine (PE) to the exposed glycine residue of the cleaved LC3-I and is termed LC3-II (37,38). LC3-II is incorporated into the phagophore contributing to the elongation of the forming membrane (Figure 1;(20)).

Recently, two articles published simultaneously showed two additional Beclin-1-interacting proteins. Atg14L promotes autophagy while Rubicon (RUN domain and cysteine-rich domain containing beclin-1 interacting protein) reduces autophagy by the modulation of Beclin-1 activity (39,40). It was found that Atg14L and Beclin-1 synergistically promoted autophagosome formation (39,40).

Rubicon, however, localises to late endosomes/lysosomes but not to the autophagosome (41) and negatively regulates autophagy as shown by decreased LC3 punctate staining. It is thought that Rubicon negatively regulates autophagy by inhibiting autophagy initiation although the mechanism for this is unclear (41). RNA knockdown of Rubicon using siRNA demonstrated increased autophagosome numbers (39,40) and it is thought that Rubicon impairs the acidification of LC3 associated vacuoles (41).

3.2. Gene disruption of mouse Atg5 leads to post partum lethality

The covalent linkage of Atg5 to Atg12, although not required for targeting to the autophagic membranes, is absolutely required for the elongation of these membranes to enable autophagy to occur (42). Mizushima and colleagues demonstrated this by developing $Atg5^{-/-}$ mouse embryonic stem cells expressing GFP-$Atg5^{K130R}$. It was found that Atg12 did not associate with the Atg^{K130R} mutant but $Atg5^{K130R}$ would still generate characteristic punctate signals which were increased during starvation. Time-lapse microscopy showed the $Atg5^{K130R}$ puncta did not mature into autophagosomes, suggesting that the Atg5-Atg12 interaction is required for membrane elongation to form the cup-shaped isolation structure and the autophagosome (33). Some data has shown that the conjugation of Atg5 and Atg12 can be induced by the RNA component of the ribosome and the authors further suggest that it is indeed required (42).

Autophagy during embryogenesis is low and increases rapidly immediately following birth returning to basal levels after a few days (3). $Atg5^{-/-}$ neonates were shown to be defective in autophagosome formation and furthermore died within 12 hours post-partum during the starvation period between birth and nursing (3). Survival could be elevated to approximately 21 hours if the neonates were force-fed milk. Blood analysis of the animals showed characteristic biochemical markers of energy depletion as well as reduced amino acid concentrations in their plasma and tissues (3). The authors however, were unable to prove whether this observation was due primarily to reduced autophagy since it could not be ruled out that these animals had a defect in suckling (3). However, the data does suggest that the turnover of intracellular constituents is a mechanism by which the cell generates energy during periods of starvation *in vivo*. Furthermore these studies suggest that autophagy is fundamental to survival after birth by providing a source of internal energy in the transition period between placental nutrition and milk consumption. Indeed, many studies have shown that serum/amino acid withdrawal lead to increases in autophagosome formation which can be inhibited using the autophagy inhibitor 3-MA (43,44,45,46,6).

3.3. The interplay between apoptosis and autophagy

The death associated protein kinase (DAPK) is a calcium/calmodulin serine/threonine kinase involved in cell death (47,48). DAPK is involved in signal transduction during apoptosis, autophagy and membrane blebbing (49). Inbal and colleagues showed that DAPK and DRP-1 were

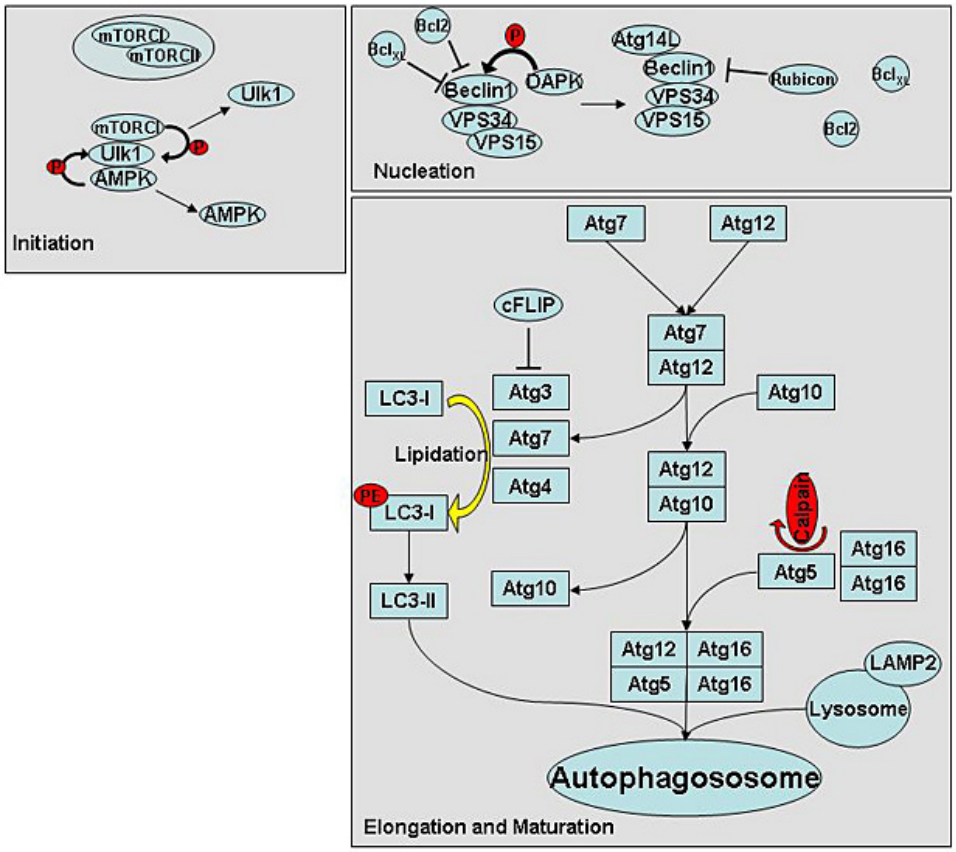

Figure 1. Schematic representation of the autophagic pathway. Autophagy can be divided into three main sub-pathways, initiation, nucleation and maturation/elongation. Proteins are sequestered and surrounded by a double membraned autophagosome where they can be used to generate cellular energy in times of stress such as nutrient starvation and hypoxia/reoxygenation.

involved in the formation of autophagic vesicles (50). Recently, however, Zalckvar *et al* demonstrated that DAPK was involved in regulating autophagy early in the signalling pathway by mediating the phosphorylation of beclin-1 (29,30). This study showed that the phosphorylation of Thr119 of the BH3 domain of beclin-1 promoted disassociation of beclin-1 from its inhibitor, Bcl-$X_{L,}$ thereby promoting the onset of autophagy (29,30). Immunoprecipitation and pull-down assays using phosphomimetics and phospho-silencing mutants of the BH3 domain of beclin-1, showed that the interaction between beclin-1 and bcl-2 could be modulated. The phosphomimicking mutant T119E strongly diminished the interaction of beclin-1 with bcl-2 family members while the phospho-silencing mutant increased the association of these two proteins. *In silico* analysis showed that the binding pockets of the BH3 domains of bcl-2 and Bcl-X_L were conserved apart from the solvent exposed A104 residue. The authors suggest that since the BH3 domains of bcl-2 and Bcl-X_L are highly conserved, they would function in a similar manner. Importantly, these studies show that autophagy and apoptosis are not mutually exclusive processes, but that significant crosstalk exists between them in order to coordinate cell survival.

3.4. Lysosome-associated membrane protein-2 (LAMP-2) deficiency causes vacuolar cardiomyopathy and myopathy

A clinical triad of cardiomyopathy, myopathy and mental retardation was reported by Danon and colleagues in 1981 and thus is known as Danon's disease (51,52). Inheritance patterns showed no male to male transmission thus the disease is X-linked with mothers presenting with milder and later onset of symptoms (53).

Lamp-2 is ubiquitously expressed and the protein product highly glycosylated (54,55). It has been implicated as an important component of the lysosomal membrane although its precise function remains unknown. Gene sequencing data from a cohort of 11 probands with Danon's disease revealed, in all but one proband, mutations in the lamp-2 gene (53). The authors identified eight different mutations consisting of three microdeletions/insertions, two nonsense mutations, two intronic point mutations and one 10bp-deletion which were found at an intron-exon boundary (53). Immunostaining failed to detect LAMP-2 protein which the authors suggest shows that the mutant LAMP-2 protein is not retained in the endoplasmic reticulum or other membrane structures.

Investigators have performed gene disruption of lamp-2 in mice (56). Lamp-2 mutant animals were found to accumulate autophagic vacuoles in the kidney, pancreas, skeletal and cardiac muscle. This study also showed that accumulation of autophagosomes leads to cardiomyopathy and myopathy in LAMP-2 knockout mice resulting in increased postnatal mortality (56) Wild-type littermate controls were approximately fifty percent larger than LAMP-2 deficient animals between day 20 and 30 of surviving mice and post-partum survival of LAMP-2 deficient animals was only about fifty percent. Ultrastructural observation of both skeletal and cardiac muscle showed accumulation of vacuoles, with mice that died early having more pronounced vacuolar accumulation (56).

Since autophagy mainly degrades long lived proteins, *in vitro* studies were performed to examine the effect of LAMP-2 deficiency and serum/amino acid withdrawal on the cells ability to degrade long lived proteins. Wild type cells degraded proteins at a rate of 3.4% per hour which could be inhibited by adding the autophagosome formation inhibitor 3-methyadenine. The protein degradation rate remained unchanged in LAMP-2 deficient cells when serum and amino acid availability was withdrawn further suggesting that LAMP-2 is involved in protein degradation by the autophagosomal pathway.

3.5. Autophagy: protector or destroyer of the cell?

The role of autophagy in cell death is not fully understood. Nonetheless, autophagic programmed cell death appears to occur and early studies showed evidence of autophagy in the regressing fruit fly salivary gland (57,58). Additionally, it is believed that self-digestion is a mechanism by which tissues perform the extensive cell elimination to facilitate remodelling (reviewed in (57)).

The precise role of autophagy in neurodegenerative disease currently remains unclear. Studies using animal models, and human brain dissection of Alzheimer's, Parkinson's and Huntington's disease patients, have shown accumulation of autophagosomal bodies suggesting that autophagic cell death contributes to neurodegeneration (57). However, since autophagy is required to eliminate protein aggregates, it has been suggested that autophagy could potentially have a protective role (57). Poly-glutamine tract accumulation in Huntington's disease appears to be responsible for clinical neurodegeneration, and defective autophagy leading to the inability to remove these mutant protein aggregates may contribute to disease progression (59,60). Ravikumar and colleagues used GFP-tagged alanine repeats as well as expanded poly-glutamine repeats of exon 1 of the Huntington disease gene and found that these protein aggregates accumulated in autophagosomes when treated with autophagy inhibitors but were degraded when treated with rapamycin (61). Recent experiments have exploited this phenomenon to measure autophagic flux by comparing degradation of luciferase-tagging polyQ80 with polyQ19 tracts for its qualitative measurement (62). However, Li and colleagues compared the inhibition of ubiquitin-mediated proteasomal degradation system with autophagic degradation in the clearance of mutant huntingtin aggregates. The authors suggested that inhibition of the ubiquitin-mediated proteasomal system had a more profound negative effect on the clearance of protein aggregates than inhibiting the autophagic pathway (63).

The STAT1 transcription factor has been heavily implicated in apoptotic cell death and is known to be involved in promoting death in response to ischaemia/reperfusion injury (7,64,65,66,67), a role which requires the C-terminal domain (68,5). Experiments in our laboratory using cultured neonatal rat cardiomyocytes, and *in vivo* and *ex vivo* studies have shown that STAT1 promotes cell death by apoptosis, an effect which can be partially rescued by free radical scavenging and treatment with polyphenols such as epigallocatechin-3-gallate (69,6,70). The intravenous injection of the free radical scavenger, tempol, significantly reduced infarct size in rats subjected to left anterior coronary artery ligation. Furthermore, intravenous injection of interferon-γ reversed the protection elicited by tempol by inducing STAT1 phosphorylation (69).

Our laboratory has also shown that cultured primary cardiomyocytes which were exposed to a single round of simulated ischaemia/reperfusion underwent autophagic cell death which could be rescued by the addition of the cardioprotective peptide, urocortin (71). The reduction of autophagy under these conditions appeared to be by the inhibition of beclin-1 expression thereby inhibiting the onset of autophagy (71). However, in a study using the chronically ischaemic porcine myocardium, it was demonstrated that apoptotic cell death declined and autophagosome numbers appeared to increase between 3 and 6 cycles of ischaemia/reperfusion injury (72). The authors suggested that the increase in autophagosomes was a protective mechanism since it occurred following the apoptotic cell death phase of ischaemia/reperfusion injury. It is possible that cells are able to be rescued from apoptosis by degrading the free-radical damaged proteins of the reperfusion cycle, thereby limiting the extent of cell loss in the risk area of the myocardium.

Work conducted using the mouse atrial cardiac muscle cell line, HL-1, found that cells subjected to hypoxia/reperfusion injury showed impaired degradation of autophagosomes (73). Furthermore, the overexpression of beclin-1 enhanced autophagy and simultaneously reduced GFP-Bax translocation in response to hypoxia/reperfusion injury (73). Similarly, inhibition of beclin-1 expression using RNAi resulted in reduced autophagic flux thus confirming the above observation. The expression of a dominant negative form of Atg5 ($Atg5^{K130R}$), which is defective in Atg12 conjugation, also reduced autophagic flux during hypoxia/reperfusion injury and resulted in GFP-Bax clustering. Together, these data suggest that beclin-1 is involved in protecting the myocardium from the damage elicited by hypoxia/reperfusion injury.

3.6. Positive and negative regulators of autophagy

3.6.1. Signal transducer and activator of transcription 1

Recently, we demonstrated that STAT1 was involved in the autophagy pathway and appeared to act as a negative regulator of autophagy during *ex vivo* ischaemia/reperfusion injury (6). Hearts from $STAT1^{-/-}$

mice exposed to *ex vivo* ischaemia/reperfusion injury demonstrated significantly smaller infarct sizes with increased levels of autophagy (6). It appears that STAT1 is able to promote apoptosis and inhibit autophagy thereby pushing the balance towards cell death. These data, together with our previous studies, suggests that inhibition of STAT1 may be cardio protective at two levels. Thus, STAT1 inhibition will reduce the pro-apoptotic signalling cascade initiated by STAT1, and also promote pro-survival autophagy signals enabling the cell to generate the required ATP during a period of ischaemia.

In contrast with the inhibitory effects of STAT1 on autophagy as exemplified by the STAT1 knockout mice, other data suggests that STAT1 can also act as a positive regulator of autophagy. Using EGFP-LC3 (Atg8) it was shown that addition of IFN-gamma induced autophagy, characterised by the lipidation of LC3I to LC3II, in wild type MEFs but not in $Atg5^{-/-}$ cells. While immunostaining revealed normal expression of both IFN receptor subunits, IFNGR1 and IFNGR2, the addition of IFN-gamma in $Atg5^{-/-}$ cells did not inhibit the formation of the IFNGR2-JAK2 complex required for STAT1 phosphorylation. However, Atg5 deficiency prevented IFN-gamma tyrosine phosphorylation of the JAK2 intracellular domain at residues 1007 and 1008 (74). The inability of JAK2 to undergo phosphorylation results in the sequential inability for STAT1 to dock at the JAK2 intracellular domain, hence STAT1 cannot undergo phosphorylation at Tyr701. Time lapse microscopy was additionally used to visualise autophagic flux induced by IFN-gamma without inducing cell death while cells deficient in Atg5 or Atg7 were insensitive to IFN-gamma induced autophagy (74).

3.6.2. Signal transducer and activator of transcription 3

The STAT proteins have been implicated in the DNA damage response, immunity, ischaemia/reperfusion injury, apoptosis, autophagy, hypertrophy and heart failure (75,76,77,5,6,68,78,71,79,80). Indeed, work from our laboratory has shown that the release of free-radicals in the heart activates STAT3 and so initiates a survival pathway (69). These data indicate that STAT1 and STAT3 may act in a yin-yang fashion insofar as STAT3 confers protection whilst STAT1 appears to be pro-death. STAT3 has been shown to be upregulated in many cancers suggesting a role in oncogenesis, while STAT1 has been implicated as a tumour suppressor (81,82). *In vivo* studies in STAT3 transgenic mice have demonstrated that constitutively activated STAT3 protects the heart during ischemia/reperfusion injury and is thought to function through metallothionein induction (83). Ischaemic preconditioning experiments have also shown that STAT3 is required to confer protection in the brain, liver and heart (84,85,86,87) whilst Huang and colleagues suggest induction of autophagy by ischaemic preconditioning is required for efficient cardioprotection (88).

MEF cells deficient in STAT3 are more sensitive to oxidative stress when exposed to hydrogen peroxide in a dose and time dependent manner (75). To investigate whether STAT3 is also important in cardiomyocyte death, we used a phospho-silencing mutant (Y705F) which has been shown to act as a dominant negative (89). Adenoviral delivery was exploited to achieve high transduction efficiency and it was shown that $STAT3^{Y705F}$ in neonatal rat ventricular myocytes enhanced apoptotic cell death, further illustrating the crucial role of STAT3 in cell survival under oxygen deprivation, as well as the importance of the phosphorylation site at position 705 (75).

Although the role of STAT3 in cell survival and apoptotic cell death during ischaemia/reperfusion injury is well established, its role in autophagy is not fully understood. Some studies have suggested that STAT3 may be involved in negatively regulating autophagy. The use of T-oligos has been shown to inhibit mTOR and STAT3 thereby inducing autophagy in human glioma cells (90). Genome-wide siRNA screens have further illustrated that a number of growth factors and cytokines, and also STAT3, inhibited type III PI3 kinases through multiple pathways thereby regulating autophagy (91). Conversely, however, G-CSF was found to activate Akt and STAT3 along with reduction in myocardial TNF-alpha and an increase in metalloproteinases (92). The authors showed improved cell survival, cardiac function and remodelling in an animal model of heart failure (92) with concomitant activation of Akt and STAT3. Thus, the authors suggest that activation of STAT3 by G-CSF may confer protection from autophagic cell death (92).

3.6.3. The tumour suppressors, p53 and p73, positively and negatively modulate autophagy

p53 is a critical regulator of apoptosis, and it has been shown that inactivation of p53 induces autophagy (12). Cancer cells deficient in p53 exhibit enhanced survival under hypoxic conditions by allowing them to maintain high ATP concentrations during a period of oxidative phosphorylation uncoupling (11). Studies using enucleated cells resulted in the activation of autophagy, while inhibition of autophagy using the p53 pharmacological inhibitor, cyclic pifithrin-α, and siRNA knockdown of p53 resulted in the activation of autophagy in multiple cell lines characterised by the depletion of p62/SQSTM1, LC3 lipidation and accumulation of autophagosomes (12). Transfection of $p53^{-/-}$ cells with wild type p53 and a p53 mutant where the nuclear localisation sequence has been deleted, resulted in inhibition of autophagy (11). Treatment of cells with autophagy inducers such as rapamycin, serum starvation and tunicamycin, have all been shown to induce rapid degradation of p53 and activate autophagy (13). It was further shown that inhibition of the p53-specific E3 ubiquitin ligase, HDM2, prevents p53 turnover and concomitantly inhibits the activation of autophagy (11). The deletion of the HDM2 ubiquitinylation site of p53 results in the stabilisation of p53 and also inhibits the onset of autophagy (11). The authors concluded that p53 plays a dual role in autophagy by transcriptionally activating autophagic genes while cytoplasmic p53 is involved in the inhibition of autophagy (11).

The lysosomal protein, damage-regulated autophagy modulator (DRAM), induces autophagy and is a p53 target (8). Crighton and colleagues (2006) showed

that, while p53 induced autophagy in a DRAM dependent manner, DRAM was essential for p53-mediated apoptosis (8). The same authors also investigated the role of p73 in regulating autophagy and found that its autophagic effects are independent of DRAM, unlike its family member p53 (9). However, it has also been shown that ΔN-p73 negatively regulates p53 and p73-induced autophagy but was not responsible for regulating nutrient-depletion induced autophagy (9). These data indicate that the regulation of autophagy occurs at many different levels and that there may be different pathways for autophagy induction in response to various stimuli.

Work in our laboratory has indicated that STAT1 interacts with p53 in response to DNA damage to modulate apoptosis and was also shown to be a negative regulator of mdm2 (78). Additionally, STAT1 has also been shown to modulate p73 induced Bax gene expression thereby regulating apoptotic cell death (93). As indicated above, STAT1 is further involved in the autophagic response which we suggest may involve STAT1 modulating p53 and p73 in autophagy. The dual role of p53 in autophagy regulation suggests that there are many complex levels of modulation in the control of the autophagic response to stressful stimuli.

4. CONCLUDING REMARKS

The role of autophagy as a cardioprotective versus destructive mechanism is not entirely clear. However, emerging data from our lab and others is beginning to paint a picture that autophagy may be a guardian of cellular integrity by removing damaged proteins from the cellular environment. In the course of this review we have seen that autophagy appears to be involved in the turnover of proteins in neurodegenerative disorders, but again, whether this is detrimental or helpful to the cell is yet to be fully elucidated. As a consequence, the process of autophagy is a fascinating conundrum which requires more investigation so we can exploit its therapeutic potential. Indeed, it is possible that the protective versus the death-promoting effects of autophagy are dependent on the duration of the insult, shorter periods allowing recycling of intracellular proteins and organelles to provide an endogenous nutrient source, while, once this resource is exhausted over a longer time period, cell death is the inevitable result.

Whilst the core machinery of autophagic signalling is understood, the role of 'ancillary' proteins in the intricate regulation in response to external stimuli such as hypoxia and nutrient deprivation are only just beginning to be investigated. We have seen that proteins such as p53 play dual roles in the modulation of autophagy depending on their cellular location.

Whilst autophagy is seen as an adaptive response to stress in order to generate ATP required for cell survival, it has also been established to play a role in type II programmed cell death and thus seen as a death inducing mechanism. Indeed, the idea that autophagy can generate the necessary energetic requirements of the cell during ischaemia/reperfusion injury is a very attractive one for the development of therapeutics to counter the damaging effects of free radicals.

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