Autophagy in epithelial homeostasis and defense

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1. ABSTRACT

Autophagy delivers protein aggregates, damaged organelles and intracellular microorganisms to the lysosome for degradation. The epidermis and other epithelia show significant levels of autophagy, however, the functions of autophagy in these tissues have remained elusive until recently. Here we review the experimental approaches for the investigation of autophagy in epithelia and discuss the roles of autophagy in epithelial cells with a focus on epidermal keratinocytes and thymic epithelial cells.

2. INTRODUCTION

2.1. Overview of autophagy

The barrier function of the epidermis and of other epithelia depends on the maintenance of a pool of proliferating cells, cell differentiation, coordinated responses to various kinds of stress and the defense against infections. Among the subcellular processes involved in tissue homeostasis and defense, autophagy has been described in morphological studies for a long time. However, in recent years the molecular regulation of

autophagy has become clearer and new research methods have facilitated the dissection of its functions in epithelia.

Autophagy is an evolutionarily conserved process in which the cell degrades its own components. It is critical for the intracellular quality control of proteins and the maintenance of metabolism during starvation and involved in development and differentiation as well as in anti-bacterial and anti-viral defense (1-3). Macroautophagy, microautophagy, and chaperone-mediated autophagy are different modes of autophagy, of which macroautophagy predominates in mammalian tissues (2, 4). In this review article the term "autophagy" will refer to macroautophagy.

More than thirty autophagy-related genes (*Atg*) control the process of autophagy. The functions of these genes and the mechanism of autophagy have been reviewed extensively (2, 5-7), and only some of the key features will be introduced here. Autophagy is initiated by the formation of a double membrane, most likely originating from the endoplasmic reticulum that encloses a part of the cytoplasm including protein complexes and organelles. This so-called autophagosome fuses with a lysosome so that the interior of the autophagosome is exposed to lysosomal enzymes that eventually degrade the cargo. One of the molecular reactions important for monitoring autophagy is the conjugation of Atg12 and Atg5 in a reaction that is mediated by Atg7 and Atg10. The Atg5-Atg12 conjugation system is essential for attaching LC3, the mammalian homolog of yeast Atg8, to the membrane of the autophagosome. The LC3 protein is produced as pro-LC3, a pro-protein which requires activation by the cysteine protease Atg4. Processing of pro-LC3 generates LC3-I, which is a cytoplasmic protein. During autophagy, LC3 becomes conjugated to phosphatidylethanolamine by the action of the enzymes Atg7 and Atg3 and localizes to the autophagosomal membrane. The lipidated form of LC3, named LC3-II, is present on the inner and the outer membrane of the autophagosome. When the autophagosome fuses with the lysosome, LC3 on the inner membrane is degraded by lysosomal proteases. LC3 on the outer membrane is de-lipidated by Atg4 and moves again to the cytosol (2, 5-7).

Autophagy contributes to cellular survival *in situ*ations of nutrient deprivation but also plays multiple roles in cells that are not starved, as will be described below. It is active in distinct cells of normal and diseased organs as well as in tumors (8). Parkinson's, Alzheimer's, Huntington's disease, Crohn's disease, type II diabetes, and many other diseases show aberrant levels of autophagy (2, 7, 9). Accordingly, the pharmacological modulation autophagy has been recognized as a potential therapeutic target in numerous diseases (10, 11).

2.2. Roles of autophagy in protein recycling and stress response

Autophagy plays a central role in the turnover of cytoplasmic proteins in the basal state of cells and even more so in cells exposed to stressors that lead to protein aggregation (12). Protein degradation via the autophagy –

lysosome route is crucial for supplying amino acids *in situ*ations of nutrient starvation. This supply appears to be relevant for example in the time immediately after birth, as evidenced by perinatal lethality of *Atg5* and *Atg7* knockout mice (13, 14) but also in the unwanted survival of cancer cells within tumors. It is conceivable that some cells within stratified epithelia are confronted with limited nutrient supply and may therefore use autophagy to fuel the synthesis of new proteins. However, this hypothesis has not been tested to the best of our knowledge.

Several types of stress lead to the aggregation of proteins in the cytoplasm which can have detrimental effects on cell function and survival (10). This is counteracted by ubiquitinylation of protein aggregates, which allows their binding to p62 or NBR1 (next to BRCA1 gene 1). These adaptor proteins bind to lipidated LC3 attached to the forming autophagosome and thereby deliver the protein aggregates to autophagy and lysosomal degradation. Prior to autophagy, many proteins are delivered to so-called aggresomes, i.e. inclusion bodies that are enwrapped by vimentin and typically localize to the vicinity of the nucleus. Both the formation of aggresomes and the fusion of autophagosomes and lysosomes require histone deacetylase 6 (12). Besides macroautophagy, chaperone-mediated autophagy is physiologically very important as it allows highly selective degradation of distinct soluble cytosolic proteins (15).

2.3. Roles of autophagy in cell differentiation

As autophagy, but not the ubiquitin-proteasome system, can degrade organelles, it was hypothesized that it may be crucial for the removal of organelles during differentiation of cell types such as lens cells and erythroblasts (16). The observation of normal organelle degradation in these cells of *Atg5*-deficient mice argued against a crucial role of Atg5-dependent macroautophagy (16). However, later reports showed that Atg5-independent modes of autophagy are important for the removal of mitochondria from terminally differentiating erythroid cells (17). The Atg1 homolog Ulk1, Atg13 and BNIP3L/Nix have essential roles in these alternative autophagy pathways (18, 19). Notably, this mechanism differs from mitochondrial degradation via Parkin and PTEN-induced putative kinase protein 1 (PINK1), two risk factors of Parkinson´s disease (20).

2.4. Roles of autophagy in the defense against microorganisms

Autophagy contributes to the innate immune defense by eliminating intracellular microbes and viruses (3). In this context, sequestosome 1/p62-like receptors (SLRs) serve as a novel category of pattern recognition receptors besides Toll-like receptors, Nod-like receptors and RIG-I-like receptors. While the latter three classes of receptors can induce autophagy but predominantly induce other effects, SLRs function primarily as autophagic adaptors. SLRs bind proteins, organelles and intracellular microbes and target them to autophagy. Besides p62 itself, NBR1, NDP52 and optineurin act as SLRs (21). Once captured in autophagosomes, intracellular pathogens are delivered to lysosomes and degraded. However, several

viruses have evolved mechanisms to inhibit autophagy (22).

3. EXPERIMENTAL MODELS FOR THE INVESTIGATION OF AUTOPHAGY IN EPITHELIA

3.1. Investigation of autophagy in cultures of keratinocytes and other epithelial cells

The methods for monitoring, enhancing or suppressing autophagy have been reviewed extensively (6, 23). Only some exemplary approaches will be discussed here as they have already proven to be useful in studying epithelial autophagy.

In cultured cells autophagy is monitored by several well established methods (6, 23). The most common assays are the detection of LC3 lipidation by Western blot analysis, the immunofluorescence labeling of LC3 on autophagosomes, and the detection of p62 which decreases in the early phase of autophagy whereas it accumulates when autophagy is blocked (23). The recombinant fusion protein, green fluorescent protein (GFP)-microtubule-associated protein light chain 3 (GFP-LC3), is also very useful to detect autophagosomes without the need of immunolabeling. Either keratinocytes from GFP-LC3 transgenic mice or cells transfected with a GFP-LC3 expression vector may be used. A definitive proof of autophagy can be achieved by electron microscopy. Ideally, several methods should be combined to ascertain the occurrence of autophagy.

Autophagy can be activated or suppressed in a relatively specific manner in cultured cells. Rapamycin is most often used to trigger autophagy. The degradation of autophagocytosed proteins can be blocked by the addition of chemical inhibitors of lysosomal proteases. This leads to a characteristic accumulation of LC3-II and p62 (23). Alternatively, autophagy is suppressed by siRNA-mediated knockdown of *Atg* genes. This method of gene knockdown is applicable to epithelial cells in monolayer culture as well as for keratinocytes in 3-dimensional organotypic cultures (24, 25). It is important to note that the culture *in vitro* causes stress to the cells and very likely alters the level of autophagic activity as compared to the *in vivo* situation. Moreover, great care needs to be taken when cell lines are used instead of primary cells because cell immortalization may have selected for cells with altered tendency to undergo autophagy.

3.2. Investigation of autophagy in transgenic and gene knockout mice

Autophagic activity *in vivo* can be detected by the preparation of tissue samples that are then subjected to LC3 and p62 Western blot assays as mentioned above or inspected under the electron microscope. An elegant way to detect autophagy *in vivo* is the use of GFP-LC3 transgenic mice (26, 27). The GFP-LC3 transgene has been crossed into various genetically modified mice and is now widely used to detect autophagy in tissues.

Blockade of autophagy *in vivo* is best achieved by targeted deletion of autophagy-related genes. Constitutive deletions of *Atg3*, *Atg5*, or *Atg7* result in the death of mice on the first day after birth (13, 14, 28). In principle, isolation of epithelial cells from these animals and their investigation are possible but have not been reported yet. The function of autophagy in the thymic epithelium has been investigated by transplanting cells from *Atg5*-deficient embryos to athymic mice (29).

p62 knockout mice are viable (30) and, interestingly for the definition of autophagy in the skin, have an altered fur coat and signs of premature ageing (31). Studies involving the inducible expression of oncogenic Ras in type II alveolar epithelial cells in these mice have shown that p62 is involved in the activation of I-kappaB kinase by the oncogene Ras. Absence of p62 causes high levels of reactive oxygen species that increase apoptosis of tumor cells (32). A detailed investigation of the skin of p62 knockout mice has not been reported so far.

The conditional deletion of *Atg* genes using the Cre-loxP technology facilitates the suppression of autophagy in a cell type-specific manner. An essential region of an *Atg* gene is flanked by loxP sites (floxed) without disturbing the expression of this gene unless gene recombination is induced by Cre, an enzyme that targets lox P sites. The Cre recombinase of the bacteriophage P1 is expressed under the control of a promoter that is active only in distinct cells and at defined stages of development. It recognizes specifically loxP sites and cuts out DNA located between 2 loxP sites. The enzymatic removal of the DNA fragment leads to a recombination event in which the DNA sequences outside of the excised fragment are connected, leaving a single loxP site remains in the final DNA product. The recombination abrogates the expression of the targeted *Atg* gene in the cell expressing Cre and in all progeny cells.

Mice carrying floxed alleles of *Atg5* (33) and *Atg7* (14) have become standard models for the study of autophagy in murine tissues. For gene deletions in epidermal keratinocytes, Cre may be expressed under the control of either the keratin K5 promoter (34) or the keratin K14 promoter (35). Both promoters are active in the basal layer of the interfollicular epidermis as well as in proliferating cells of skin appendages. In addition, they drive the expression of Cre in precursor cells of the thymic epithelium.

4. AUTOPHAGY IN THE EPIDERMIS AND OTHER EPITHELIA

4.1. Autophagy in epidermal keratinocytes
4.1.1. Autophagy during keratinocyte differentiation
4.1.1.1. Autophagy in the interfollicular epidermis

The barrier function of the epidermis is largely maintained by continuous renewal of the stratum corneum. During terminal differentiation and cornification of keratinocytes all organelles, including the nucleus and mitochondria, are degraded, a mechanically resistant cytoskeleton and a stiff cell envelope are formed while

lipids are secreted to establish an intercellular diffusion barrier. The underlying mechanisms are not completely understood at present.

Several reports have addressed potential roles of autophagy in various aspects of keratinocyte differentiation. The stimulation of differentiation upregulates autophagy in freshly isolated human keratinocytes and keratinocyte lines (36). Autophagy is induced by calcipotriol and metabolic stress in keratinocytes (37, 38). Chatterjea and colleagues suggested that autophagy is involved in the control of the differentiation-associated protein filaggrin (39). Morioka *et al.* reported an increase in the abundance of lysosomes from the basal layer to the granular layer of the epidermis (40). Moreover, ultrastructural investigations indicated the presence of mitochondria within lysosomes of stratum granulosum keratinocytes (40). Furthermore, autophagy was suggested to cause cell death in senescent keratinocytes (41, 42).

The role of autophagy in epidermal barrier formation and function was investigated in a recent gene knockout mouse study in which *Atg7* was inactivated by the Cre-loxP system under the control of the keratin K14 promoter (Rossiter, König, Barresi, Buchberger, Ghannadan, Zhang, Mlitz, Gmeiner , Sukseree, Födinger, Eckhart, Tschachler, manuscript submitted). Using a transgenic GFP-LC3 reporter autophagosomes were detected abundantly in the suprabasal layers of the epidermis of normal mice but not in mice lacking *Atg7* in keratinocytes. A constitutive activity of autophagy in the epidermis of normal mice was corroborated by immunoblot analysis of endogenous LC3. In contrast to whole-body knockout of Atg7 (14), the K14-specific deletion of Atg7 did not lead to perinatal lethality and was compatible with a macroscopically normal skin phenotype in adult mice (Rossiter *et al.*, manuscript submitted). The cornification of keratinocytes was essentially normal with the exception of a tendency to thickening of corneocytes in mutant mice. The degradation of organelles as well as the formation of keratinocyte-specific lamellar bodies occurred in the absence of autophagy. Processing of profilaggrin and the expression of keratinocyte differentiation markers was not perturbed by the suppression of autophagy. Transepidermal water loss and the skin resistance to penetration of topically applied dye were normal in mutant mice. Thus, *Atg7*-dependent autophagy is dispensable for the establishment and maintenance of the skin barrier in the mouse. Nevertheless, constitutive autophagy in normal epidermis and subtle differences in the ultrastructure of autophagy-competent and autophagy-deficient keratinocytes suggest as-yet uncharacterized roles of autophagy in the suprabasal epidermis. Further studies involving exposure to various types of stress are necessary to uncover these roles.

Interestingly, autophagy may also be involved in the life cycle of melanosomes that are transferred from melanocytes to epidermal keratinocytes. Ultrastructural investigations by Devillers *et al.* suggested autophagy as the mechanism of degradation of immature melanosomes in keratinocytes in cases of hypomelanosis of Ito (43).

4.1.1.2. Autophagy in skin appendages

Autophagosome-like structures have been detected by electron microscopy of hair and sebaceous glands (44, 45). However, the physiological relevance of autophagy in skin appendages is not well understood at present.

Recently, we have reported on the role of autophagy in the preputial gland which serves as a model for sebaceous glands (46). Preputial glands are located next to the genitals in mice and other mammals but they do not exist in humans. They have a structure similar to those of sebaceous glands in the skin and also produce sebum. Proliferating epithelial cells of preputial glands express keratin K5 and the K5-Cre transgene could be used to inactivate a floxed *Atg5* gene in these glands (46). Autophagy-deficient preputial glands had increased levels of p62 in some but not all differentiated cells, possibly indicating that p62 was partially degraded by non-autophagic mechanisms.

Differentiating preputial gland cells normally lose their nucleus in a gradual way and this process is accompanied by the appearance of DNA fragments that can be labeled by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Strikingly, the frequency of TUNEL-positive nuclei was decreased in autophagy-deficient glands while the speed of DNA degradation appeared to be increased (46). Thus, the relative ratio of areas containing nucleated cells versus areas containing cells without a nucleus was significantly decreased in mutant glands. The mechanism of this accelerated DNA breakdown requires further investigations. Moreover, the differentiation-associated cell death of sebocytes in skin sebaceous glands needs to be investigated in detail in these mice.

Another interesting feature of *Atg5 f/f K5-Cre* preputial glands was an alteration in the hematoxylin-eosin staining pattern. Differentiating autophagy-deficient preputial gland cells showed decreased affinity to hematoxylin and increased affinity to eosin (46). Moreover, the cytoplasmic organisation of these cells was altered. Ultrastructural analyses will be necessary to better define this phenotype.

4.1.2. Autophagy in the stress response of epidermal keratinocytes
4.1.2.1. Autophagy in UVA-irradiated epidermal keratinocytes

Human skin is exposed to ultraviolet (UV) radiation in the long (UVA) and short wavelength (UVB) range, both of which cause damage within cells. Recent publications have demonstrated that autophagy is involved in the UV response of epidermal keratinocytes.

Zhao and colleagues reported recently that exposure of keratinocytes to ultraviolet A radiation induces autophagy in keratinocytes (47). In this study cells were isolated from GFP-LC3 transgenic, *Atg7*-floxed K14-Cre mice and control animals. Irradiation with UVA induced lipidation of LC3 and autophagosome formation

in normal but not in *Atg7*-deficient cells. Autophagy-deficient cells showed signs of oxidative stress even in the unirradiated state and accumulated reactive oxidized phospholipids. UVA irradiation and exposure to exogenous oxidized phospholipids led to the accumulation of protein aggregates to which p62 was attached and which could not be cleared in the absence of autophagy. In addition to protein degradation, the removal oxidized lipids was found to depend on autophagy. Finally, the authors demonstrated an important interaction of autophagy and nuclear factor (erythroid-derived-2)-like 2 (Nrf2) which up-regulates the antioxidant response and the transcription of detoxifying enzymes (47).

4.1.2.2. Autophagy in UVB-irradiated epidermal keratinocytes

Also the UVB component of solar radiation induces autophagy (48). At a UVB dose of 10 mJ per square centimeter, LC3-II accumulated in normal human epidermal keratinocytes mostly within the first 6 hours after irradiation but disappeared later. Using mouse embryonic fibroblasts from various gene knockout mice and chemical modulators of autophagy these authors showed that adenosine-monophosphate-activated protein kinase (AMPK) is required for UVB-induced autophagy and that autophagy protects from UVB-induced apoptosis. Autophagy removes p62 which otherwise activates p38, a stress-activated protein kinase. Squamous cell carcinoma samples derived from sun-exposed human skin showed elevated autophagy and high levels of beclin 1 but low levels of p38 as compared to normal human skin (48). Qiang and colleagues concluded that autophagy may reduce the activation of p38 and thereby promote the survival of cancer cells in the presence of genotoxic stress such as exposure to UVB. In a different report, Yang *et al.* proposed that ultraviolet B-induced activation of autophagy involves glycogen synthase kinase GSK3beta signaling in epidermal cells (49).

4.1.2.3. Autophagy in epidermal keratinocytes exposed to other forms of stress

Besides UV irradiation, stress inducers ranging from cadmium to nanoparticles have been reported to affect autophagy of keratinocytes (50, 51). Hypoxia activates autophagy in HaCaT keratinocytes and reduces the levels of p62. Thus it will be important to determine *in vivo* whether autophagy and p62 are involved in the regulation of cancer cell survival in hypoxic tumor areas (52).

Autophagy has been detected in keratinocytes treated with photocytotoxic porphyrin conjugates, that were tested for their usefulness in the photodynamic therapy of basal cell carcinomas and squamous cell carcinomas (53). The immortalized human skin keratinocyte cell line, NCTC 2544, was used in these studies. The combination of broad band red light irradiation with di-O-isopropylidene-alpha-d-galactopyranosyl group but not with delta-aminolevulinic acid, i.e. the precursor of protoporphyrin IX in current photodynamic therapy, induced the formation of GFP-LC3-labeled autophagosomes and a decrease in the

abundance of p62. Both effects were inhibited by the autophagy inhibitor 3-methyladenine (3-MA). Thus, autophagy may be activated to degrade damaged organelles or protein aggregates generated by photodynamic therapy (53).

Many natural and synthetic substances have the potential to induce both autophagy and apoptosis (54-56), yet, the roles of the two cell response modalities have not yet been investigated systematically in epidermal keratinocytes and many other epithelial cells. Apigenin, a natural flavonoid, induces activation of AMPK and subsequently autophagy in human keratinocytes (57). This effect, besides the inducion of apoptosis, may contribute to the protective effects of apigenin against chemical carcinogens and UV exposure on skin.

Autophagy contributes to the control of inflammatory responses of keratinocytes at least *in vitro* (58). Application of a diacylated lipopeptide that is recognized by TLR2/6 or lipopolysaccharide, i.e. the ligand of TLR4, triggered autophagy and reduced p62 levels in human keratinocytes. Suppression of autophagy led to elevated abundance of p62 and upregulation of pro-inflammatory cytokines via activation of NF-kappa B. P62 was reported to be increased in psoriasis but not in atopic dermatitis, indicating a role of p62 in the induction or maintenance of a pro-inflammatory milieu in psoriatic lesions and the potential anti-inflammatory activity of autophagy in normal epidermis.

One of the standard inducers of autophagy in studies of cultured cells is rapamycin, which is also used, in topical formulations, to treat skin diseases (59). Although the therapeutic effect of rapamycin is mediated primarily by an arrest of T-cell proliferation, it also affects epidermal keratinocytes. Mills and colleagues reported that rapamycin promotes autophagy of gamma delta T cells and inhibits their proliferation whereas it did not suppress the proliferation of keratinocytes at wound sites in murine skin (60).

Notably, keratinocytes show a significant level of baseline autophagy when they are cultured *in vitro* (47). A systematic investigation of autophagic flux during the different phases of keratinocyte culture, in comparison to cells *in vivo*, should be performed to define variations in baseline autophagy and to allow for better comparability of *in vitro* studies.

4.1.3. Autophagy in antimicrobial and antiviral defense of the epidermis

Two clinically important skin pathogens, *Streptococcus sp.* (61) and *Staphylococcus aureus* (62), interfere with the autophagy. Invasive skin infection with group A *Streptococcus* are characterized by the prevention of cellular uptake of bacteria due to encapsulation (63). If bacteria are taken up by keratinocytes, the majority of streptococci are killed within a few hours (63). Nakagawa *et al.* showed that autophagy is responsible for the killing activity (61). Although, some bacteria survive, the reduction of the number of extracellular streptococci is

likely to have a partially protective effect. As the mechanistic studies of the action of autophagy against group A *Streptococcus* have not been performed in keratinocytes (61) additional studies will be necessary to understand the relevance and efficiency of this putative antibacterial strategy in the skin.

S. aureus induces autophagy via its alpha-toxin (62, 64). Pore-forming toxins cause a drop in nutrient and energy levels that trigger autophagy as a rescue mechanism to re-establish cellular homoeostasis (65). Whether autophagy suppresses or enhances *S. aureus* infection in the skin *in vivo* remains to be determined.

Recent reports suggest an important role of autophagy in the defense of keratinocytes against human papilloma virus (HPV) 16 (66, 67). Surviladze and colleagues knocked down mTOR with siRNAs in HaCaT keratinocytes and found significantly reduced infection by HPV16 (66). Pharmacological induction of autophagy blocked HPV16 infection of HaCaT cells and inhibition of autophagy increased infection levels. Similarly, the knockdown of beclin 1 and Atg7 enhanced HPV16 infection. Interestingly, this study also indicated that HPV suppresses host cell autophagy to promote infection (66). Griffin and colleagues showed that primary keratinocytes have higher level of autophagy than HaCaT cells which is associated with a much lower infectivity of HPV (67). The autophagy inhibitor 3-MA and the knockdown of either class III phosphatidylinositol-3 kinase, which is the target of 3-MA, or Atg7 enhanced HPV16 infectivity. This study provided also evidence that autophagy degrades HPV16 L1 capsid proteins during the entry of the virus into primary keratinocytes. Taken together, these studies suggest that autophagy protects against HPV16 infection. However, it is noteworthy that, later in the life cycle of HPV, autophagy may be beneficial to the virus as it helps the host cell to cope with metabolic stress induced by HPV16 E7 expression (68). Roles of autophagy in the response of viruses were also suggested by the detection of autophagosomes in infected keratinocytes from zoster and varicella vesicles (69).

4.2. Autophagy in the thymic epithelium

Autophagy has been proposed to have an essential function in the epithelium of the thymus (29). Thymic epithelial cells show a high level of constitutive, starvation-independent autophagy (26). To investigate the biological significance of this process, Nedjic and colleagues transplanted the thymus of *Atg5*-deficient embryos under the kidney capsule of autophagy-competent adult mice. In comparison to thymus grafts from normal embryos, thymi of autophagy-deficient embryos remained smaller but developed a normal structural organization into a cortex and a medulla. When thymi were grafted into athymic (nude) mice, the recipients of *Atg5*-negative thymi developed a higher frequency of activated CD4 T cells than recipients of *Atg5*-positive thymi. These mice had enlarged lymph nodes, flaky skin, atrophy of the uterus, absence of fat pads, and an enlarged colon (29). Moreover, the colon, liver, lung, uterus and Harderian glands of mice receiving an autophagy-deficient thymus showed massive

inflammatory infiltrates on thin sections stained by hematoxylin and eosin (H&E). Mice bearing an *Atg5*-negative thymus started to lose weight approximately 1 month after grafting and later had to be killed because of severe autoimmune disease. The primary and critical role of autophagy in thymic epithelial cells as opposed to hematopoietic cells was suggested by various control experiments (29, 70). Together with another study demonstrating that autophagic compartments gain access to the MHC class II compartments in thymic epithelium (71), these data have established the concept of autophagy-dependent antigen processing in thymic epithelial cells (70, 72, 73). Nedjic *et al.* suggested that this process is essential for negative selection of T cells and for the development of self tolerance (29).

This hypothesis was later tested by suppressing autophagy in the thymic epithelium but not in other cells of the thymus and determining the effects on the abundance of tissue inflammation (46, 74). First, *Atg7* was deleted in epithelial cells that express keratin K14 using the Cre-loxP system. The Cre recombinase was expressed under the control of the K14 promoter which is active in the precursor of all epithelial cells of the thymus (75) and therefore caused the deletion of floxed *Atg7* in these cells (as well as in epidermal keratinocytes). The efficiency of autophagy suppression was confirmed by LC3 Western blot analysis and by introducion of the reporter gene GFP-LC3 (74). As expected, the absence of autophagy was associated with the accumulation of p62 in epithelial cells of the thymus which showed a normal organization and size. However, in contrast to the predicted failure to negatively select autoreactive T-cells (29), the mutant mice did not show more tissue inflammation than control mice and also appeared to have normal T-cell differentiation (74).

In a second mouse model, autophagy was suppressed by the expression of the Cre recombinase under the control of the keratin K5 promoter and a floxed *Atg5* gene was used as a target for recombination (46). Again, the efficiency of autophagy abrogation in the thymic epithelium was appropriately controlled and no signs of increased autoimmunity were detected. Unexpectedly, the blockade in K5-positive epithelial cells led to a defect in the preputial glands as described in section 4.1.1.2 of this review. Together, the two studies of autophagy suppression in the thymic epithelium provided evidence against the putative essential role of thymic epithelial autophagy in the control of autoimmunity. Nevertheless, autophagy was confirmed to be constitutively active in the epithelium of the thymus which indicates a yet unknown physiological function.

4.3. Autophagy in epithelia of the intestine, lung and other organs

Autophagy has been reported to be active in various epithelia such those of the intestine and the lung (76-78). Autophagy plays important roles in the barrier function of these epithelia (79, 80). Importantly, alterations of epithelial autophagy are involved in diseases affecting large parts of the population (81, 82). Moreover, cancers

arising from epithelia show alterations in autophagy. Therefore, autophagy is emerging as an important target of pharmaceutical treatments.

It is now generally accepted that autophagy contributes to maintaining the homeostasis of the intestinal epithelium and that defects of autophagy may cause inflammatory bowel disease (79, 80). In particular, *Atg16L1* is involved in the pathogenesis of Crohn's disease and possibly ulcerative colitis (82).

Surprisingly, autophagy deficiency due to mutation of *Atg16L1* confers protection from infection *Escherichia coli* in epithelia of the urinary tract (83). The mechanism may involve structural abnormalities of superficial epithelia that were found in *Atg16L1* knockout mice. However, in this system the absence of autophagy in the hematopoietic compartment, especially in macrophages and granulocytes, appears to have a major role in protection (83) so that the role of autophagy in uroepithelial antibacterial defense remains uncertain.

Recently, *Atg7* was deleted specifically in the intestinal epithelium of the mouse (76, 84). This was achieved by crossing mice carrying floxed *Atg7* alleles with mice carrying Cre under the control of the villin promoter. The mutant mice had smaller than normal granules and lower levels of lysozyme in Paneth cells. Nevertheless, the homeostasis of the gut appeared normal and mutant mice were not more susceptible to dextran sodium sulphate-induced colitis than control mice (76). In another study, *Atg7*-dependent autophagy in the intestinal epithelium was found to decrease inflammatory responses to endotoxin via inhibiting the activation of NF-kappa B (84). These data were reminiscent of the role of *Atg16L1* in suppressing endotoxin-induced interleukin (IL)-1beta production in macrophages (85). Moreover, *Atg7* is required for the resistance of the murine intestinal epithelium to infection by *Citrobacter rodentium* (86). These studies demonstrated that the autophagy plays distinct roles in response to different inflammatory stimuli and supported a crucial role of autophagy in the protection against bacterial infection of gut epithelium.

Autophagy was also reported to contribute to the homeostasis of airway epithelial cells. The floxed *Atg7* gene was ablated in a doxycycline-inducible system in the epithelial cells of the conducting airway of adult mice (78). Autophagy deficiency was associated with the swelling of bronchiolar epithelial cells, which had elevated levels of p62 and Nrf2. Perhaps due to mechanical airway constriction, the lungs of mutant doxycycline-treated mice were hyper-responsive to cholinergic stimuli. Interestingly, cystic fibrosis is associated with a defect in autophagy that leads to the accumulation of protein aggregates in airway epithelia (87). Thus, autophagy appears to play significant roles in the physiology of the epithelia of the normal and diseased lung and of the upper airways.

The reports on autophagy in the epithelia of the gut, the lung and other organs suggest that the determination of functions of autophagy requires careful analysis of multiple stimuli in complementary experimental systems. It is likely that the roles of autophagy in the skin barrier will also become clearer when different study approaches yield their results. A particular focus on the roles of autophagy in the control of skin inflammation and in the defense against pathogens seems to be warranted in the view of autophagy functions in other epithelia.

5. CONCLUSIONS

Several important functions of autophagy in epithelia have been reported recently and autophagy is emerging as a main contributor to the barrier functions of diverse epithelia. While rapidly renewing epithelia are less prone to protein aggregation defects than tissues that consist mostly of non-proliferating cells (2), autophagy contributes to cell differentiation and antimicrobial defense of epithelia. Epidermal keratinocytes have a basal level of autophagy that can be increased by various types of stress, most notably UV irradiation. New tools such as cell type-specific *Atg* gene knockout mice provide ideal tools to close gaps in our understanding of physiological autophagy in the skin. Moreover, autophagy may emerge as a target of existing and new pharmaceutical substances for treating epithelial disorders.

6. ACKNOWLEDGEMENT

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Abbreviations: AMPK: adenosine-monophosphate-activated protein kinase, Atg: autophagy-related gene, GFP: green fluorescent protein, H&E: hematoxylin and eosin, HPV: human papilloma virus, IL: interleukin, LC3: Microtubule-associated protein 1A/1B-light chain 3, MA: methyladenine, MHC: major histocompatibility complex, NBR1: next to BRCA1 gene 1, NF-kappa B: nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, Nrf2: nuclear factor (erythroid-derived-2)-like 2, PINK1: PTEN-induced putative kinase protein 1, PTEN: phosphatase and tensin homolog, siRNA: short interfering RNA, SLR: sequestosome 1/p62-like receptor, TLR: toll-like receptor, TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling, UV: ultraviolet

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