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Polylactosamine glycosylation in lung type II epithelial cells following transfection with GlcNAc-TV

Par
Mehrdad Hariri
Département de Pathologie et biologie cellulaire
Faculté de médecine

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Présenté par:

Mehrdad Hariri

A été évalué par un jury composé des personnes suivantes:

Dr. Michel Desjardins.....président-rapporteur

Dr. Ivan Robert Nabi..... .directeur de recherche

Dr. Luis Rokeach.....membre du jury

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SUMMARY

The expression of polylactosamine glycosylation in cancer has been well-studied, and it is known that increased expression of polylactosamine is associated with tumor metastasis. The major lysosomal associated membrane proteins (LAMPs), are heavily glycosylated (15-17 N-linked oligosaccharides per protein) and are a major carrier of polylactosamine glycosylation. However the role of polylactosamine glycosylation and LAMP proteins in the biogenesis of lysosomes is not known. In order to study the role of polylactosamine and LAMPs in lysosomal biogenesis, we used mink lung alveolar type II cells (Mv1Lu) transfected with GlcNAc-TV, the enzyme responsible for adding polylactosamine chaines. This cellular model has previously been shown to exhibit the increased expression of polylactosamine glycosylation of LAMPs. These cells exhibit large lysosomal vacuoles by immunofluorescent microscopy, which were identified by electronic microscopy as multilamellar bodies (MLBs). Using specific inhibitors, MLBs are shown to form via autophagy and lysosomal degradation.

The Mv1Lu cell line is derived from mink lung type II alveolar cells. These cells were transfected with GlcNAc transferase V (GlcNAc-TV), which is responsible for initiation of polylactosamine glycosylation, and labeled with antibody against LAMP-2 (lysosomal membrane glycoprotein). By fluorescent microscopy, large vacuoles were positive for LAMP-2 in Mv1Lu transfected cells. By electron microscopy, these large vacuoles presented the morphology of multilamellar bodies (MLBs). Quantification of the area of cytoplasm covered by MLBs demonstrated that nontransfected Mv1Lu cells contained very few MLBs, whereas in two clones of GlcNAc-TV transfected Mv1Lu cells (clones M1 and M9), which express higher levels of GlcNAc-TV, the cytoplasmic area covered by MLBs was significantly higher. The area covered by MLBs in M1 cells that express the highest level of GlcNAc-TV, is less than that of M9 cells which have a lower level of GlcNAc expression, while M1 cells contain a larger number of MLBs. The lysosomal character of these organelles raised

the question of the role of protein degradation and autophagy in the formation of MLBs.

Following treatment with leupeptin, a protease inhibitor, MLBs showed a progressive transformation to autophagic vacuoles (AVs). Heterologous structures containing lamella and electron-dense regions appeared 15 and 24 hours after leupeptin treatment, indicative of ongoing lysosome-MLB fusion. After 4 days MLBs disappeared and were replaced with AVs. The role of protein degradation in the formation of MLBs in Mv1Lu cells was confirmed when leupeptin was washed out of the medium of the cells and reformation of MLBs was observed. Gradual formation of MLBs 24 and 48 hours after washout of leupeptin resulted in the complete disappearance of AVs and expression of MLBs.

In order to demonstrate that MLBs come from AVs, the cells were treated with 3-methyladenine (3-MA), which is an inhibitor of autophagy at the initial sequestration step. Treatment with 3-MA for 3 days resulted in a significant decrease in the number of MLBs, and accumulation of inclusion bodies resembling nascent autophagic vacuoles (AVi). To confirm that 3-MA indeed blocks autophagy, the cytoplasm of Mv1Lu transfected cells was scrape-loaded with FITC-dextran. After 24-72 hours, FITC-dextran was incorporated into LAMP-2 positive MLBs indicating that active autophagy is involved in MLB formation. In the presence of 3-MA, LAMP-2 positive MLBs were no longer labeled with FITC-dextran, confirming the inhibitory role of 3-MA in autophagy.

To determine whether GlcNAc-TV transfected Mv1Lu cells, which express MLBs, can be used as a model of alveolar type II cells, expression of SP-A was tested. Expression of SP-A in Mv1Lu transfected cells was detected by western blotting and immunofluorescence, using an antibody against SP-A.

Our data demonstrate a role for autophagy and protein degradation in the formation of multilamellar bodies in Mv1Lu transfected cells. Resistance to lysosomal degradation due to the increased expression of polylactosamine glycosylation of MLB specific proteins may be responsible for the stable expression of MLBs in GlcNAc-TV transfected cells.

SOMMAIRE

L'expression de la glycosylation polylactosamine dans le cancer est bien étudiée et il est bien connu qu'une forte expression de polylactosamine est associée avec la métastase des tumeurs. Les protéines majeures associées avec la membrane lysosomiale (LAMPs), sont très glycosylées (15-17 oligosaccharides N-liés par protéine) et portent de la glycosylation polylactosamine. Le rôle de la glycosylation polylactosamine et des protéines LAMPs dans la biogénèse des lysosomes n'est pas encore connu. Afin d'étudier le rôle de la glycosylation polylactosamine et des protéines LAMPs dans la biogénèse des lysosomes, nous avons utilisé des cellules de type II d'alvéoles de poumons de visons (Mv1Lu) transfectées par GlcNAc-TV, l'enzyme responsable de l'ajout des chaines polylactosamines. Il a déjà été montré que ce modèle cellulaire présente une augmentation de l'expression de la glycosylation polylactosamine des LAMPs. Ces cellules possèdent de larges vacuoles lysosomiales observées par microscopie à fluorescence et, par microscopie electronique identifiés comme des corps multilamellaires (MLBs). En utilisant des inhibiteurs spécifiques, nous avons montré que la biogénèse des MLBs se fait par autophagie et dégradation lysosomiale.

La lignée cellulaire Mv1Lu est dérivée de cellules de type II d'alvéoles de poumons de visons. Ces cellules ont été transfectées avec la GlcNAc transferase V (GlcNAc-TV), responsable de l'initiation de la glycosylation de la polylactosamine, et marquées avec les anticorps dirigé contre LAMP-2 (glycoprotéine des membranes lysosomiales). En microscopie à fluorescence, on observe dans les cellules Mv1Lu transfectées, de large vacuoles marquées par LAMP-2. En microscopie électronique, ces larges vacuoles présentent une morphologie de corps multilamellaires (MLBs). La quantification de la surface cytoplasmique couverte par les MLBs démontre que les cellules Mv1Lu nontransfectées ne contiennent pas vraiment de MLBs, alors que dans deux clones de cellules Mv1Lu transfectées avec GlcNAc-TV (les clones M1 et M9) qui expriment un plus haut niveau de la GlcNAc-TV, la surface couverte par les MLBs est plus grande. La surface couverte par les MLBs des cellules M1, qui expriment le plus fort taux de GlcNAc-TV, est moins importante que celle des cellules M9 qui

expriment un taux plus faible, même si les cellules M1 contiennent un grand nombre de MLBs. Le caractère lysosomial de ces organites incite à poser la question sur le rôle de la dégradation des protéines et de l'autophagie dans la formation de MLBs.

Lorsqu'on traite les cellules avec la leupeptine, un inhibiteur de protéases, les MLBs montrent une transformation progressive des vacuoles d'autophagie (AVs). Ces structures hétérologues contiennent des régions lamellaires et denses aux électrons qui apparaissent 15 à 24 heures après le traitement à la leupeptine. Après 4 jours, les MLBs disparaissent et sont remplacés par les AVs. Le rôle de la dégradation des protéines dans la formation des MLBs dans les cellules Mv1Lu est confirmé lorsque la leupeptine est enlevée du milieu des cellules. On observe alors la reformation des MLBs. La reformation graduelle des MLBs en 24 et 48 heures après avoir enlevé la leupeptine résulte d'une disparition complète des AVs et l'expression des MLBs.

Afin de démontrer que les MLBs sont formés à partir des AVs, les cellules ont été traitées avec du 3-MA, un inhibitor de l'autophagie, qui agit à l'étape initiale de la séquestration. Le traitement des cellules avec le 3-MA entraîne une diminition nette du nombre de MLBs, et l'accumulation de corps d'inclusions ressemblant aux vacuoles primaires d'autophagie. Pour confirmer que le 3-MA bloque l'autophagie, les cellules Mv1Lu transfectées ont été grattées puis mises en présence de FITC-dextran. Après 24 à 72 heures, le FITC-dextran est incorporé dans les MLBs marqués par LAMP-2, ce qui indique qu'une autophagie active est impliquée dans la formation des MLBs. En présence de 3-MA, les MLBs marquées par LAMP-2 ne sont pas marquées avec le FITC-dextran, ce qui confirme que le 3-MA inhibe l'autophagie.

Pour détérminer si les cellules Mv1Lu transfectées avec GlcNAc-TV, qui expriment des MLBs, peuvent être utilisées comme des modèles cellulaires de cellules de type II des alveoles, nous avons mesuré l'expression de SP-A. Cette expression dans les cellules Mv1Lu transfectées a été détéctée par western blot et immunofluorescence, en utilisant un anticorps dirigé contre SP-A.

Les résultats démontrent le rôle de l'autophagie et de la dégradation des protéines dans la formation des corps multilamellaire dans les cellules Mv1Lu transfectées. La résistance à la dégradation lysosomiale, provoquée par l'augmentation de l'expression de la glycosylation des polylactosamines des protéines spécifiques des

MLBs, peut être responsable de la stabilité de l'expression des MLBs dans les cellules transfectées avec GlcNAc-TV.

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ABBREVIATIONS

3-MA:	3-Methyladenine
AA:	Arachidonic acid
AVd:	Degradative autophagic vacuole
AVi:	Immature autophagic vacuole
BSA:	Bovine serum albumin
DAG:	Diacylglycerol
DPPC:	Dipalmitoylphosphatidylcholine
EGF:	Epithelial growth factor
EHS:	Engelbreth-Holm-Swarm
EPA:	Eicosapentaenoic acid
ER:	Endoplasmic reticulum
Gal:	Galactose
Glc:	Glucose
GlcNAc:	N-acetylglucosamine
GlcNAc-TV	N-acetylglucosaminyle transferase V
IP3:	Inositoltriphosphate
LAMP:	Lysosomal associated membrane protein
LAP:	Lysosomal acid phosphatase
LIMP:	Lysosomal integrated membrane protein
MLBs:	Multilamellar bodies
Mv1Lu:	Mink lung type II epithelial cells
PBS:	Phosphate buffer saline
PC:	Phosphatydylcholine
PE:	Phosphatidylethanolamine
PG:	Phosphatidylglycerol
PI:	Phosphatidylinositol
PIP:	Phosphatidylinositol diphosphate
PMNs:	Polymorphonuclear cells
PS:	Phosphatidylserine

RER:	Rough endoplasmic reticulum
SDS:	Sodium dodecyl sulfate
SP	Surfactant protein

1. INTRODUCTION

1.1 POLYLACTOSAMINE GLYCOSYLATION

The addition of glucides to proteins is one of the principal functions of the endoplasmic reticulum (ER), which the majority of proteins destined towards the Golgi, lysosomes and plasma membrane are glycosylated with the addition of at least one N-linked oligosaccharide, few cytosolic proteins are glycosylated [1]. Terminal glycosylation occurs in the Golgi. In the Golgi, mannose is removed from the N-linked oligosaccharide and N-acetylglucosamine, galactose and sialic acid are added to the oligosaccharide resulting in the transformation of high-mannose oligosaccharides into complex structures. These modifications result in a high range of diversity in the structure of glycoproteins [1].

Polylactosamine glycosylation consists of repeating Galβ1-4GlcNAcβ1-3 disaccharide units preferentially added to β1-6 GlcNAc linked antennae attached to the trimannosyl core complex-type N-linked oligosaccharides [57]. In different cell lines and under different conditions of differentiation, the expression of specific polylactosamine structures is observed. β1-6 branching and polylactosamine glycosylation are markers for cellular differentiation and transformation [29]. Polylactosamine glycans carry various antigenic structures such as the ABO and I / i blood group antigens [15].

1.1.1 Biosynthesis of polylactosamine branches

The polylactosamine chain is initiated by the addition of N-acetylglucosamine to carbon C-6 of galactose. This reaction is mediated by GlcNAc-TV. In the next step, the formation of polylactosamine chains, galactose is added to the external branch of GlcNAcβ1-6Gal of the polylactosamine chain by β1-4 galactosyltransferase and then N-acetylglucosamine to carbon C-3 of the galactose residue. This is mediated by the extension enzyme β1-3N-acetylglucosaminyl-transferase [1, 15, 29, 101].

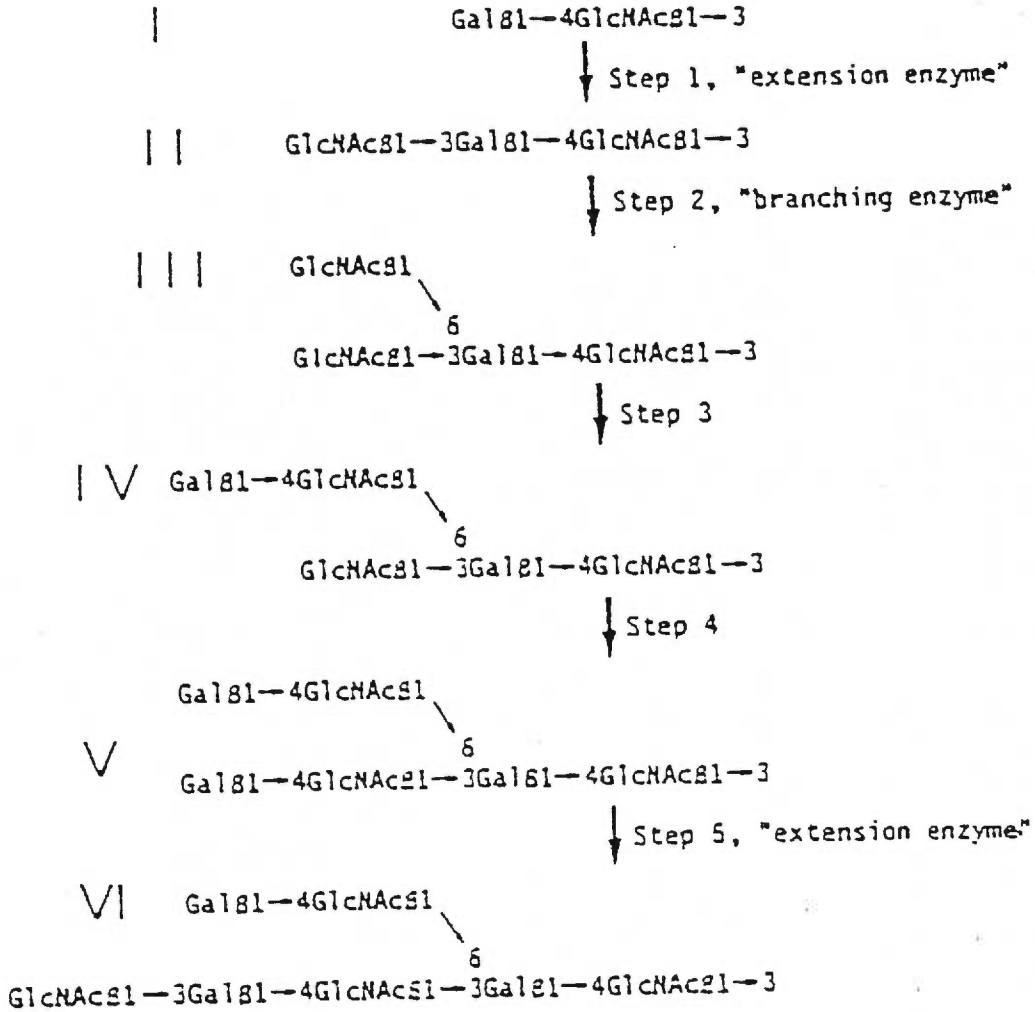

Figure 1: Biosynthesis of branches of polylactosaminoglycanes [29]

1.1.2. Polylactosamine glycosylation and cancer

Neoplastic transformation is usually accompanied by alteration in glycoprotein carbohydrates [29]. Increased expression of β1-6 branching and polylactosamine elongation is associated with tumor cell metastasis [16].

In transformed cells, there is an increased tail of N-linked oligosaccharides attached to asparagine. This tail is due to an augmentation of sialic acid, increased branching of trimannosyl nuclei, or expression of polylactosamine. Stimulation of GlcNAc-TV and changes in glycosylation at early stage of tumor progression can change the growth of the pre-malignant cell [16]. Increased polylactosamine glycosylation in mink lung type II alveolar cells following transfection with GlcNAc-TV and a phenotype of precancerous cells can be observed in these cells [14]. In highly metastatic carcinoma cells, poly-N-acetyllactosaminyl side chains are expressed more than in the cells with low metastatic potential [69]. Fernandes et al. demonstrated that L-PHA (a plant lectin leukoagglutinin specific for β1-6 branched N-linked oligosaccharides) binding is consistently increased in neoplasias of human breast and colon [27].

1.2. LYSOSOMES

The lysosome is the final repository of macromolecules targeted for degradation derived from the extracellular space or from within the cell [28]. They are membrane limited and contain about 40 types of hydrolytic enzymes that are used for intracellular digestion of macromolecules. These enzymes are hydrolases such as proteases, nucleases, glycosidases, and lipases, that require an acidic environment for optimal activity. The action of proton pumps in the lysosomal membrane generates an intralysosomal pH of about 5. The membrane of lysosomes protects the cytosol from the enzymes inside the lysosome, but the products of degradation like amino acids, nucleotides, and sugars can be transported across the membrane to the cytosol. Lysosomal membrane proteins are highly glycosylated, and are thought to protect the membrane from lysosomal hydrolysis [1].

1.2.1. Glycoproteins of the lysosomal membrane

The proteins of the lysosomal membrane are different from those of the plasma membrane and include: LAMP-1 (lgp-A), LAMP-2 (lgp-B), LIMP I, LIMP II, and LAP [30]. All these glycoproteins are glycosylated with N-linked oligosaccharides. Since they are rich in sialic acid, their isoelectric points are very acidic (between 2 to 4) [30, 48].

1.2.1.1. LAMP-1 and LAMP-2

LAMP-1 and LAMP-2 are the major lysosomal glycoproteins containing poly-N-acetyllactosamines. They are homologous to each other and are similar in structure [28]. These glycoproteins consist of a polypeptide core of about 40 kDa, the major part of which is located in the lumenal part of the lysosome and connected to a short cytoplasmic tail via a transmembrane domain. The intralumenal domain contains 16-20 N-glycans in which some of them are of the polylactosamine type [28, 48]. The high content of carbohydrates and of polylactosamine likely plays a role in the stability of LAMP molecules within the lysosomes [28]. The half-life of LAMP proteins was decreased when they were synthesized in the presence of tunicamycin, which inhibits N-glycosylation [5].

1.2.1.2. LIMP-I and LIMP-II

LIMP I and LIMP II are two other major lysosomal membrane glycoproteins that are smaller than the LAMPs [28]. According to the nomenclature of Wicker and Lodish [92], LIMP-I and LIMP-II are type III glycoproteins. The molecular weight of LIMP-I is 30-35 kDa, and the protein contains four transmembrane domains. Its lumenal domain contains three sites of N-glycosylation, which express polylactosamine chains [30, 63]. Glycoprotein LIMP-II with a molecular mass of 74 kDa, has a small cytoplasmic domain and two transmembrane domains [28, 89]. The intralumenal portion contains 11 potential N-glycosylation sites [28]. There is homology between the cellular adhesion molecule CD36 and the luminal domain of LIMP-II [28, 89].

1.2.1.3. LAP

LAP (lysosomal acid phosphatase) is synthesized as a type I transmembrane precursor. It has a large N-terminal luminal domain, a single transmembrane domain and a short cytoplasmic domain of 19 AA. When attached to the membrane, LAP has a molecular mass of 63 kDa and its luminal domain contains 8 sites of N-glycosylation. The transmembrane precursor is transported from the ER to the lysosomal membrane and proteolytically cleaved within the lysosome to release its luminal domain [63]. The cleavage of LAP includes two proteolytic cleavages. The first one is catalysed by a thiol proteinase, removes the greater part of the cytoplasmic tail and occurs at the outside of the lysosomal membrane. The second cleavage is catalysed by an aspartyl proteinase, which cleaves a peptide bond within the luminal domain. The cleavage within the cytoplasmic tail of LAP is controlled by pH inside the lysosome, and the second cleavage depends on prior processing of the cytoplasmic tail [102]. As a result of these proteolytic changes, LAP is generally considered to be a transitional part of the lysosomal membrane [63].

1.2.2. Lysosomal degradation

The materials digested by the lysosome arrive via three routes. Macromolecules taken up from the outside of the cell are delivered to early endosomes, and after recycling of some of their contents they pass to late endosomes and then to lysosomes. The second pathway of lysosomal degradation is autophagy in which the contents of the cell itself are degraded. The process starts with the enclosure of the cytoplasm by the endoplasmic reticulum membrane, which forms autophagosomes. Autophagosomes subsequently fuse with lysosomes to form autophagolysosomes. The third way is phagocytosis in which large particles are engulfed by the plasma membrane and then fuse with lysosomes [1].

These are not the only ways (or at least not the only classifications) of protein degradation and access to lysosomes. Other ways include microautophagy, crinophagy, and chaperonin mediated and direct uptake of proteins [7].

1.2.2.1. Endocytosis

In endocytosis, material is ingested by invagination of the plasma membrane which forms intracellular vesicles containing ingested material from outside of the cell. Endocytosis occurs primarily via clathrin-coated pits, which consist of about 2% of the plasma membrane. These clathrin-coated vesicles deliver material to endosomes. The endocytic compartments move from the periphery of the cell to the perinuclear region. Two kinds of endocytic vesicles are distinguishable by adding a readily detectable tracer molecule, such as the enzyme peroxidase. Early endosomes in peripheral regions are visible with the tracer molecule after a minute or so, and late endosomes in the perinuclear region after 15 minutes. When the materials reach the late endosome they mix with newly synthesized acid hydrolyses, the pH of the endosomes becomes more acidic and finally they fuse with lysosomes [1].

1.2.2.2. Macroautophagy

Macroautophagy is the most abundant phenomenon of lysosomal degradation in which the proteins are degraded, and is frequently referred to as autophagy. During macroautophagy, portions of cytoplasm are surrounded by a sequestering membrane and form autophagosomes [21]. Later on, autophagosomes fuse with lysosomes to form autophagolysosomes and lysosomal enzymes degrade the proteins inside the autophagosome. For more details refer to sec. 1.3.

1.2.2.3. Microautophagy

In this process, small portions of cytoplasm (ribosomes and cytoplasmic proteins like glycogen) are sequestered by lysosomes. This process results in the formation of membrane-bound intralysosomal vesicles whose contents can be degraded by the lysosomes. Microautophagy is different from macroautophagy since it is insensitive to inhibition by amino acids (see sec. 1.3.4.3.), and is ATP

independent [7, 21]. While it has been suggested that microautophagy declines in long-term starvation [7], others report that nutrient starvation results in an increase in the number of lysosomes with intralysosomal vesicles presumed to arise by microautophagy [21].

1.2.2.4. Crinophagy

In this process, mature secretory vesicles containing proteins that are destined to be secreted from the cell, can fuse with existing lysosomes, autophagosomes, endosomes or amphisomes, to form structures called crinosomes, in which ultimate degradation occurs [7, 21]. Crinophagy is a process that is not well-characterized.

1.2.2.5. Direct uptake of proteins by lysosomes

In some conditions, such as starvation and removal of serum from the medium, proteins can be targeted directly to lysosomes. This is mediated by a peptide Lys-Phe-Glu-Arg-Gln (KFERQ) which is recognized by the cytosolic heat shock protein hsc73 and facilitates the recognition process of degradative proteins by lysosomes. Recently, it has been shown that the lysosomal membrane glycoprotein LGP96 is a receptor for this selective import and degradation of proteins in the lysosomes [101]. This process activates after prolonged starvation [7], and is also called carrier mediated proteolysis [21].

1.2.3. Lysosomal storage diseases

Lysosomal storage diseases comprise more than 30 different diseases in which the common feature is the deficiency of a lysosomal protein that is involved in the degradation of proteins and lipids [32]. Some of the better characterized diseases will be discussed below.

1.2.3.1. Niemann-pick disease

This disease is caused by a deficiency of the enzyme acid sphingomyelinase which hydrolyses sphingomyelin into ceramide and phosphorylcholine [32]. Deficiency of this lysosomal enzyme causes accumulation of sphingomyelin. There

are two types of diseases, the acute form A and chronic form B. There is also a third clinically similar form termed type C. Cells in these patients show impaired intracellular transport of cholesterol from the lysosome [32, 71].

1.2.3.2. Gaucher disease

This disease is caused by deficiency of β-D-glucocerebrosidase. This enzyme hydrolyses glucose from the sphingolipid glucosylceramide. Glucoceramide is an intermediate compound in the synthesis and degradation of complex glycosphingolipids [32].

1.2.3.3. Galactosialidosis

This disease is characterized by the combined deficiency of two enzymes β-D-galactosidase and N-acetyl-α-neuraminidase. This is a result of a deficiency of a protective protein, which associates with these two enzymes, and causes stabilization of β-D-galactosidase and activation of neuraminidase. Neurominidase hydrolyses α-glycosidically linked neuroaminic acid from oligosaccharides and glycolipids. Accumulation of oligosaccharides with terminal neuroaminic acid is seen in the patients with this disease [32].

1.2.3.4. Fabry disease

This is an X linked disorder in which α-D-galactosidase A is deficient. This causes the accumulation of glycosphingolipids with a terminal α-glycosidically linked galactose [32].

1.2.3.5. Fucosidosis

This disease is caused by a deficiency of the enzyme α-L-fucosidase. Fucose is found in N- and O-linked oligosaccharide chains of sphingolipids [32].

1.3. AUTOPHAGY

1.3.1. Formation of the autophagic vacuole

There are different ideas about the origin of the membrane of the autophagic vacuole. Dunn believes that the membrane originates from ribosome-free parts of rough endoplasmic reticulum (RER), so autophagosomes have a double membrane [21, 22]. However, many autophagosomes lack proteins of the

RER which may be due to the recycling or degradation of membrane proteins of RER [21]. It has also been suggested that autophagosomes originate from post-Golgi complex membranes containing complex type N-and/or O-linked oligosaccharide chains [97, 98]. Seglen and Fengsrude proposed that autophagosomes form from a preexisting membrane structure called the phagophore [26, 76].

The sequestered membrane curves and seals and then forms a nascent autophagosome (AVi) via an as yet unknown mechanism. This AVi is a vacuole with a double membrane and its contents are not distinct from the cytosol [72]. It seems that there are some alterations in membrane contents after vacuole formation, which may be due to fusion with other organelles such as preexisting lysosomes and late endosomes [22]. This vacuole has yet to undergo the process of maturation.

The maturation of newly formed AVi occurs in sequential steps by the acquisition of lysosomal membrane proteins and of acid hydrolases from either endosomes or pre-existing lysosomes resulting in the formation of autophagolysosomes, or degradative autophagosomes (AVd) [7, 21, 73]. AVs acquire acid hydrolases from three sources: Golgi/endosomes, RER, and lysosomes [7, 23]. Ongoing maturation induces heterogeneity in AVs. Generally the membrane of AVi is more like RER, while AVd membrane is more similar to lysosomes [23]. However the membrane of AVi/d has characters of both RER and lysosomes [23].

1.3.2. Models of lysosome formation

The formation of late endosomal or lysosomal organelles may occur via three models: maturation, vesicle shuttle, and kiss and run. The first model suggests that by obtaining new elements from fusion with Golgi-derived vesicles, and by losing others via recycling, there will be maturation of early endosomal compartments into later endosomal compartments. The second model or "vesicle shuttle" suggests that membrane elements transfer from early endosomes to late

endosomes and from pre-lysosomes to pre-existing lysosomes via carrier vesicles. The "kiss and run" hypothesis suggests that transient fusion occurs between endosomes, early and late, and lysosomes, and as a result there is a maturation process in which new molecules are taken from endosomes, lysosomes, Golgi derived vesicles and also from cytoplasmic pools [81]. Although not well characterized for autophagic vacuoles, similar mechanisms are likely to be involved in the transformation of AVi to AVd.

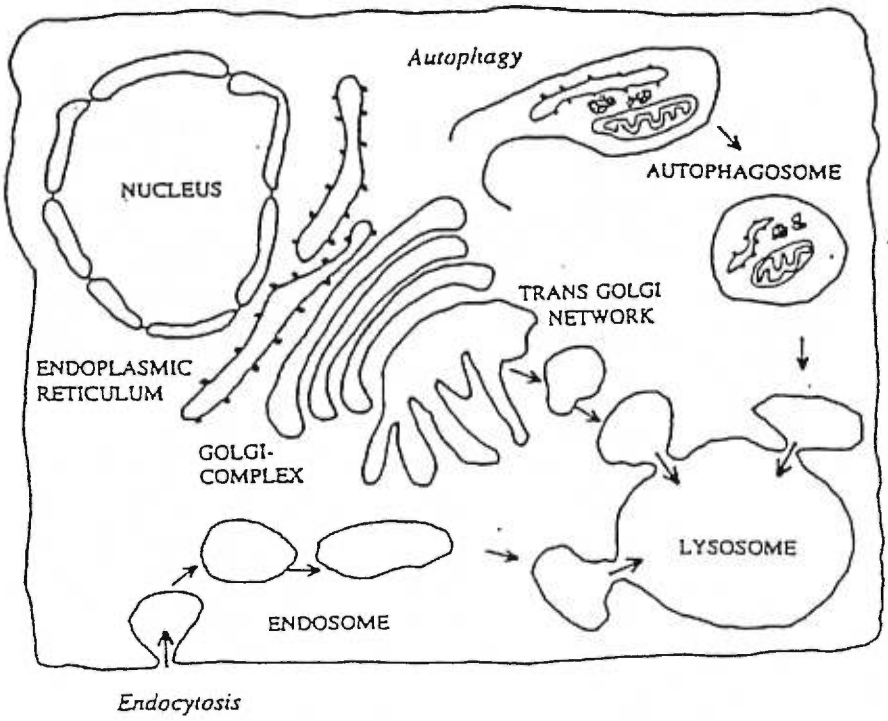

Figure 2: Cell lysosomal system and autophagy [65].

1.3.3. Involvement of cytoskeleton

1.3.3.1. Actin

Aplin et al. [3] evaluated the involvement of the cytoskeleton in autophagy and found that in the presence of drugs that induce the depolymerization of microfilaments (cytochalasin B and D) the formation of autophagosomes was inhibited. Seglen et al. [72] also reported that cytochalasin D and vinblastine blocked autophagic sequestration. Phalloidin, a drug that stabilizes actin filaments, inhibits the formation of AVs [86]. The formation of autophagosomes therefore requires polymerization of actin and depolymerization of actin filaments. It has been suggested before that microfilaments are needed for the invagination of plasma membrane which is necessary for the formation of autophagosome [78]. Dunn et al. believe that probably the same mechanism is involved in invagination of ER to form the autophagosome [3].

1.3.3.2. Microtubules

Microtubules are also involved in the formation of AVs. In general autophagic-lysosomal degradation is strongly suppressed by inhibitors of microtubule formation, probably related to delivery of autophagocytosed material to lysosomes [40]. Vinblastine, an inhibitor of microtubules, inhibits both autophagic and endocytic flux in hepatocytes resulting in the accumulation of autophagosomes as well as endosomes [76]. Maturation from late autophagosomes to autophagolysosomes is facilitated by intact microtubules, since nocodazole treatment results in the accumulation of late autophagosomes [3]. Intact microtubules are required for the fusion of late autophagosomes and lysosomes or late endosomes [3]. Microtubules have also been shown to be required for fusion between secretory vesicles and plasma membrane and between lysosomes and late endosomes [3].

1.3.4. Inhibitors of autophagy

1.3.4.1. Leupeptin

Leupeptin is known as a potent inhibitor of protein degradation, and it has been previously shown that treatment with leupeptin induces the accumulation of autophagolysosomes [31, 41, 49, 85, 100]. Administration of leupeptin results in an increase in the number of autophagic vacuoles, suggesting that leupeptin inhibits the fusion of autophagosomes and pre-lysosomes [41, 50].

1.3.4.2. 3-Methyladenine (3-MA)

Seglen and Gorden have shown that 3-MA can block autophagy at the initial sequestration step [100]. Methyladenine caused lysosome swelling and elevated intra-lysosomal pH, but the mechanism of swelling is not well known [49]. It is thought that sequestration is a process that needs energy, and this might be the reason of the inhibition by 3-MA. Similarly, phosphatase inhibitors such as okadaic acid, and phosphodiesterase inhibitors like theophyline, have also been proposed to inhibit sequestration, though the mechanisms are not yet known [21].

1.3.4.3. Amino acids

Generally, amino acids block autophagy. Based on the concentration and type of the amino acid, the inhibitory effect is different [7]. Amino acids block autophagy at the sequestration step, but in high concentration inhibit the fusion between autophagosomes and lysosomes [39]. Asparagine is the most potent inhibitor among amino acids [75]. The mechanism by which amino acids affect autophagy is not known.

1.3.5. Selectivity of autophagy

In general, autophagy is a bulk process in which cytoplasmic macromolecules are randomly degraded. Phagophores encircle the cytoplasm in a nonselective manner [72, 101]. However there is some evidence that under certain conditions autophagy may be selective. In perfused liver, glucagon accelerates proteolysis but has no effect on RNA degradation. This may be due to the

prevention of excessive degradation of ribosomes under starvation conditions, in order to preserve the ability to synthesize essential proteins [7].

1.4. MULTILAMELLAR BODIES

Multilamellar bodies (MLBs) are subcellular structures that exist in various cell types and in different conditions. They are best investigated in lung type II cells and have, different names in different tissues including: lamellar granules, lamellar lysosomes, membrane coating granules, multilamellar lipid, concentric membranous structures [71]. They are specialized for the storage and secretion of lipids with specific functions such as the secretion of surfactant in lung, and the hydrophobic lining of the gastric mucosa, and of lubricant in the joints. MLBs vary in size from 100 to 2400 nm. They are surrounded by a membrane and contain multilamellar membranes [71]. MLBs have an acidic pH [8] and contain various lysosomal enzymes, therefore present a lysosomal character [17, 38]. MLBs are also present in some pathologic conditions, for example when the lipid metabolism is disrupted including genetic abnormalities, during wound healing, and in degenerative processes in the brain or nervous system [71].

1.4.1. Location and function

MLBs are found in various tissues, though their function in all of them is not completely known. Besides alveolar type II cells, they are located in the mucosa of the nose, the gastrointestinal tract, tongue papillae, the oral epithelium, mucosa cells of stomach, mesodermal layers of sliding surfaces to provide lubrication of the joints, pericardium, peritoneum and plural mesothelium [71]. In type II alveolar cells, they are responsible for secretion of surfactant [36, 43, 71, 94, 96]. In mucosa cells of the stomach, the major phospholipid of MLBs is dipalmitoylphosphatidylcholine (DPPC), which provides a hydrophobic film layer which protects gastric mucosa cells against the gastric juice [71]. It is said that the hydrophobic water-protective barrier of the skin, consisting mainly of neutral lipids, originates from lamellar bodies secreted from epithelial cells. In skin MLBs

were originally named Odland bodies, membrane-coating granules, or keratinosomes. They are synthesized in the cornified layer of the epidermis. Their function is to provide special lipids to the intracellular space of the cornified layer. Membranes prepared from stratum corneum lipids are composed of epidermal ceramids 57%, cholesterol 25%, free fatty acids 15%, and cholesterol sulfate 5%. Multiple lamellae form without the presence of proteins [71]. Electron microscopic studies identified lamellar bodies composed of PC on the articular surface of joints and in synovial fluid, likely produced by synovial type A cells. The lubricating function of phosphatidylcholine (PC) has also been found in sliding surfaces in the pericardium and pleural mesothelium [71].

1.4.2. MLBs in the lung

MLBs are storage forms of lung surfactant in type II alveolar cells of lung (See section 1.6). They have a diameter from 0.2-2 μm, and comprise about 18-24% of the cytoplasm. It has been reported by EM that MLBs are secreted from type II cells via exocytosis. This suggests that the limiting membrane of MLBs fuses with plasma membrane of type II cells [46, 67]. There is some in vitro evidence that purified synexin promotes the fusion of MLBs and the plasma membrane [9]. Several MLBs may release their contents through a single exocytic site [67]. The rate of surfactant secretion is rapid, about 10-30% of the intraalveolar space is replaced per hour under resting conditions [71].

1.5. ALVEOLAR TYPE II CELLS

Respiratory bronchioles are the basic elements of the pulmonary exchange system between blood and air. They are covered by a ciliated cubic epithelium. Located at the ends of the respiratory bronchioles, the most physiologically important parts of the lung are the pulmonary alveoli. They consist of a very thin-walled saccular compartment and are the site of oxygen and carbon dioxide exchange. There are about 150 to 400 million alveoli in a normal lung and they form an extended surface area of up to about 70-80 m^2, for a better exchange of

oxygen with the blood. The alveolar epithelium consists of type I and II pneumocytes. Type I pneumocytes form about 90% of the cellular population of alveolar sacs, and about 10% of the total lung cells. They are attached by occular junctions. These cells have a very tiny cytoplasm (to have a better exchange) and small number of mitochondria and organelles. Type II pneumocytes form about 5-10% of the alveolar cells and are usually located near the angles between the neighboring alveolar septa. They have a rounded apical surface, which is covered by short microvilli [25]. Their cytoplasm is rich in mitochondria and rough and smooth endoplasmic reticulum. They contain multilamellar bodies (MLBs), which contain the base material for lung surfactant [80]. MLBs appear on day 14.2 in mouse, 16 in the rat and in human at week 11 after conception [83].

Type II cells are implicated in the synthesis, storage, and secretion of pulmonary surfactant. They also serve as a source of type I cells after injury of the alveolar epithelium or during the natural replacement of aged cells [64]. Many toxic agents result in both hypertrophy and proliferation of type II cells, and their hypertrophy is accompanied by an increase in the number and size of lamellar bodies, and in the synthesis of surfactant-associated phospholipids. There is a controversial discussion about the relationship between the activation of the surfactant system and proliferation of type II cells after injury. While some believe that there is tight link between the activation of the surfactant system and proliferation of type II cells, others believe that there is no relationship between them [87].

1.5.1. Dedifferentiation of type II cells in culture

Type II cells lose characteristics such as the expression of multilamellar bodies and the secretion of surfactant after a short period of time in culture, and this dedifferentiation limits the use of these cells for in vitro studies [18, 44, 56, 64, 70, 82, 88]. Dedifferentiation of type II cells in culture is accompanied by morphological changes from a cuboidal to a squamous epithelium, and the loss of MLBs, such that they resemble type I cells [18]. Lipid cytochemistry and

differential reactivity of fluorescent stains in type II and type I cells, and immunocytochemical reactivity of protein components of surfactant proteins are among the tools used to measure the transformation from type II to type I cells [70]. Lectins and unique cell surface antigens are also used to distinguish these cells. In addition to these, monoclonal antibodies to plasma membrane molecules of type II cells are helpful [70]. In case of functional characteristics of type II cells, perhaps the most important one is the ability to synthesize phospholipids, specially PC and phosphatydylglycerol (PG). Experiments have shown that this ability of isolated type II cells declines after two or three days of culture [19, 53]. However, adding acellular amniotic membrane reverses this reduction [53]. Shannon et al. [77] tested the secretion of surfactant in isolated type II cells exposed to plastic, EHS tumor extract, or laminin. They found that the cells cultured on plastic and on laminin were negative for SP-A, SP-B, and SP-C, whereas cells on EHS extract were positive for all three, suggesting a strong relationship between extracellular matrix composition and cellular functions [77].

1.5.1.1. The role of the extracellular matrix

The extracellular matrix is considered to be a very important modulator in determination and maintenance of differentiated cellular functions [51, 53, 64, 70, 77]. Insoluble components of extracellular matrices, especially from basement membrane, interact with soluble factors including hormones and growth factors [70]. For instance Ranneles et al. [64] showed that some of the morphological and biochemical changes in cultured type II epithelial cells are due to their own matrix production. In their efforts they grew type II cells on plastic surfaces for six days, and then they removed these cells, but kept their extra cellular matrix, which was rich in fibronectin produced by type II cells, and then reseeded with freshly isolated type II cells. They concluded that a fibronectin-rich matrix might cause increased differentiation and proliferation of cells [64]. Culture of type II cells on a variety of substrates has been done in order to maintain the functional and morphological aspects of type II cells in culture. Kawada et al. showed that serum free, hormonally defined medium (5μg/ml insulin, 5μg/ml transferrin, 10ng/ml epidermal

growth factor, EGF, 1μM hydrocortisone, 0.5mM dibutyryl cAMP and 25nM selenious acid) can maintain type II cells in a more differentiated state than cells grown in serum-supplemented medium [44]. They cultured the isolated type II cells on EHS basement membrane gels. The improvement of differentiated function was demonstrated by an increase in synthesis of phosphatidylglycerol, phosphatidylcholine, and saturated phosphatidylcholine. With respect to ultrastructure, the cells maintained a morphology like that seen in situ, containing lamellar bodies. The effect of hydrocortisone and cAMP on lipid synthesis did not appear if the cells were cultured on tissue culture plastic or floating type I collagen gels. They therefore, concluded that extracellular matrix plays a critical role in maintenance and differentiation of type II cells [44]. Other experiments showed that culturing type II cells on a tumor cell-derived matrix and growth in serum-free medium preserves the differentiated phenotype of type II cells [82].

Type II cells respond in various ways to different basement membranes. Lung basement membrane may regulate its own renewal, which may be one of the mechanisms for the maintenance of the distinct structural domains of the basement membrane. There is also some evidence showing that components of basement membrane are determinants of the locations at which differentiation may occur [56]. Kikkawa et al. suggested that the fate of type II cells in culture is the same as the in the lung after injury [18].

1.5.2. Type II cells as the progenitor of type I cells

Type II cells have the ability to generate type I cells, so it is thought that type II cells might be the progenitor of type I cells [70]. Following exposition of monkey lung to 100% oxygen, the disappearance of type I cells was observed, and shortly after lung injury, type II cells spread out and develop into type I cells. After exposing the cells to nitrogen dioxide, the number of type II cells declines and cells of an intermediate phenotype increase in number and eventually change to type I

to type I cells [88]. With respect to cell division, type II cells act like a stem cell with a long turnover time of 28-35 days in mouse [82].

1.6. SURFACTANT

Surfactant is a complex of protein and lipid secreted by type II pneumocytes, which spreads a film that usually covers the surface of the alveoli. In human it is detectable at the 28^{th} week of gestation [36]. Surfactant has a definitive role in reducing tension at the alveolar surface and in the absence of surfactant secretion the alveoli would collapse. This reduction is accomplished by a monolayer liquid mainly consists of phospholipids at the air/liquid interface [25, 36, 43, 80, 96](see sec. 1.6.3.2). Surfactant also has a role in the defense mechanism of the lung, and stimulates the phagocytic and migrational capabilities of macrophages. Both SP-A and SP-C are essential for the transition between lamellar bodies and tubular myelin [36, 43], which will be discussed in the section 6.3.1.2.

1.6.1. Composition of surfactant

Surfactant is composed of 85-90% lipids, about 10% proteins and 2% carbohydrate. The phospholipids, constituting 80-90% of the total lipid weight, consist of 75% phosphatidylcholine (PC), 10% phosphatydylglycerol (PG), 5% phosphatidylethanolamine (PE) and 5% phosphatidylserine (PS) plus phosphatidylinositol (PI) and less than 5% sphingomyelin. Cholesterol makes up about 6-8% of the lipids. The lipid composition is very similar in all mammals. Almost half of the PC is dipalmitoylphosphatidylcholine (DPPC), which plays an important role in reducing surface tension. The content of DPPC in different animals correlates with the alveolar surface area and ventilatory rate [43].

Surfactant associated proteins include a small amount of albumin and immunoglobulin G, but the major proteins found are four surfactant-specific proteins (SPs) called: SP-A, SP-B, SP-C and SP-D [36].

1.6.1.1 Surfactant protein A

Structure: SP-A is the most abundant surfactant protein at the surface of alveoli (50% of surfactant protein) and the first surfactant associated protein identified. It has a molecular weight of 26-38 kDa. and isoelectric point which varies from 4-5 due to post-translational modifications [43]. SP-A contains two distinct domains, the amino-terminal (one third of the protein) is collagen like and the carboxyl-terminal (two third) has lectin properties [43, 66]. The N-terminal position has a collagen-like amino acid sequence, and has the capacity to bind lipids and carbohydrates and to interact with specific surface receptors [43, 66]. The presence of SP-A in MLBs is controversial; some report that it is concentrated in MLBs, but in vivo experiments suggest that it secreted independently of MLBs [66]. In humans the SP-A gene is located on chromosome 10 and its protein appears in significant quantities in the 30^{th} week of embryogenesis [36].

Functions: SP-A is highly glycosylated and is believed to play a major role in regulating the flow of surfactant [36]. Secreted, SP-A may act as a negative feedback regulator of the synthesis and secretion of surfactant, since some findings show that purified SP-A inhibits secretion of phospholipids from type II cells, though the mechanism of this negative feedback is not understood [20]. It also mediates the endocytosis and reutilization of secreted surfactant components through receptor binding [66]. In vitro studies have shown that SP-A can enhance the uptake of surfactant from the alveoli to type II epithelial cells [66]. Similarities between SP-A and the complement component C1q, suggest a possible antibacterial role for SP-A. SP-A also binds to macrophages and stimulates macrophage migration and phagocytosis of opsonized bacteria [52, 66]. Another property of SP-A is the ability to inhibit the influx of serum proteins into the alveolar space to block the inhibitory effects of different compounds on surfactant functions in that space during pathologic conditions [52]. SP-A may also have a role in rapid formation of the phospholipid surface film in a calcium-dependent way [66].

1.6.1.2. Surfactant proteins B and C

General facts and functions: Two smaller surfactant proteins are SP-B and SP-C with molecular weights of 7-8kD and 4-5kD respectively. They are lipophilic proteins and constitute about 1% of surfactant. They play a major role in spreading the surfactant layer on the surface of alveoli, and in the transformation of MLBs to tubular myelin [36, 66]. In human the SP-B gene is located on chromosome 2 and SP-C on two genes on chromosome 8 [36]. SP-B is a 79-residue polypeptide, and SP-C contains 33-35 amino acid residues. SP-C has an unusual amino acid composition and lacks 8-10 of the 20 common types of amino acid. cDNA studies have shown that SP-B and SP-C are derived from larger precursors, which are not present at the surface of alveoli [43].

1.6.1.3. Surfactant protein D

General facts: Similar to SP-A, SP-D is a highly glycosylated hydrophilic protein of 39 kDa [36, 43]. It is present in Clara cells (Group of cells in bronchiolar epithelium of mammals usually called nonciliated bronchiolar secretory cells [93]), alveolar macrophages, rat bronchoalveolar lavage, and human amniotic fluid but not in MLBs [43]. The native molecule is composed of four subunits, and each of them has three identical disulphide-linked glycosylated peptides of 43 kDa [43].

Functions: The functional role of SP-D is not known yet, but it increases the production of free radicals in alveolar macrophages, so it may have a role in the host defense system [43]. It interacts with *Escherichia coli* in a calcium-dependent manner. SP-D binds to macrophages, and at a high concentrations causes agglutination of bacteria [66].

1.6.2. Surfactant synthesis

It has been shown that DPPC, which is the major lipid component of surfactant, is synthesized in the ER. The lipids are transferred from the ER, via the Golgi apparatus to MLBs. Surfactant proteins are synthesized in the ER and

transferred from the Golgi via multivesicular bodies to MLBs. The synthesis of surfactant is regulated during gestation, as well as hormonally after birth [43].

1.6.3. Secretion of surfactant

Surfactant is secreted from type II cells via exocytosis of MLBs [36, 43, 95, 96].

1.6.3.1. Stimulation of type II cells

Secretion of surfactant from type II alveolar cells occurs by transmembrane signaling including: activation of adenylyl cyclase, activation of protein kinase C, and breakdown of phosphatidylinositol diphosphate (PIP) to inositoltriphosphate (IP3) and diacylglycerol (DAG) with subsequent increase of cytosolic free calcium [95]. The stimulation of surfactant secretion by calcium ionophores suggests that surfactant secretion may be regulated by calcium-dependent protein kinases [91].

1.6.3.2. Formation of tubular myelin structure

Extracellular stimuli modulate intracellular chemical events, which result in movement of MLBs towards the apical surface of the type II cells. Then the limiting membrane of MLBs fuses with the plasma membrane and the contents of MLBs are extruded into the alveolus [96]. There is some evidence that surfactant lipids and proteins separate after entrance of lipids into the monolayer surface, probably due to the fact that pressures at low lung volumes causes proteins to exit the lipid monolayer [66]. After secretion from type II cells, MLBs unwind to form a lattice-like structure of double-layered tubular lipid called tubular myelin, which is believed to be the major precursor of the monolayer film [96]. Tubular myelin is the main intra-alveolar reservoir of surfactant and the monolayer at the air/liquid interface is eventually formed from this tubular myelin layer [34, 45]. The hydrophobic acyl lipid chains are oriented towards the air, and the polar head groups interface with the liquid surface. During the compression of alveoli, this layer forms a rigid structure, consisting mainly of DPPC, which reduces the surface tension to near zero [43, 66]. Spreading of phospholipids from tubular myelin into

a monolayer at the air/liquid interface occurs in less than 1 second [43]. In vitro and in the presence of Ca++, it has been shown that multilamellar bodies can form tubular myelin [62]. The formation of surface films from other intra-alveolar membranes is possible and nonlipid surfactant components, especially surfactant proteins, are important factors in this process [45].

1.6.3.3. Mechanisms of regulation

The regulation of secretion can be categorized via three ways: systemic factors, local factors, and local autoregulation.

1. Systemic factors: β-adrenergic stimulation by adrenaline is the most important one and also increases the uptake of surfactant phospholipids by type II cells [95]. An association between membrane methyltransferase activity and surfactant secretion has been observed, and phospholipid methylation may modulate β-adrenergic receptor function, and consequently the secretion of surfactant [37]. The secretion of surfactant in cultured type II cells is stimulated either by receptor-mediated agonists like β-adrenergic agonists, P1- and P2-purinergic agonists or non-receptor-mediated agonists like phorbol esters, calcium ionophores and cytochalasins [84]. Acetylcholine is also a stimulatory agent, in situations of hyperventilation [61], as are terbutaline and vasopressin [95]. Glucocorticoids increase the synthesis of SP-B and SP-C, but for SP-A, both inhibition [59] and stimulation [4] have been reported. Known systemic inhibitors of surfactant secretion include acidosis and alkalosis [95]. Toxic agents like paraquat and 3-methylindole inhibit the secretion of surfactant [95]. Transforming growth factor β decreases the synthesis of SP-A [96].

2. Local factors: Mediators produced locally by inflammatory cells including PMNs or macrophage and lymphocytes in certain conditions can stimulate the secretion of surfactant. Examples of these mediators are leukotrienes, and prostaglandin E2 [95]. Both histaminic [10] and antihistaminic [33] agents have been reported to stimulate the secretion of surfactant in type II cells in culture. EGF-R has a role in lung development, since EGF-/- knocked–out mice

have an impaired lung development, which often results in visible respiratory failure. The expression of SP-C mRNA and protein is increased in the presence of EGF [55]. The same effect has been reported for SP-A m-RNA [96]. Amniotic fluid contains high levels of EGF, and the activation of EGF-R signal transduction by amniotic fluid may play an important role in embryonic lung development [55]. Furthermore; some inhibitory factors like hypoxia and oxygen radicals may damage the type II cells, and thereby affect surfactant secretion [95].

3. Autoregulation: SP-A is a major inhibitor of surfactant secretion. This effect is probably because of its lectin domain, since the same effect has been shown using other lectins like concanavalin A and wheat germ agglutinin, although the mechanism remains unknown [96]. Other factors that are involved in autoregulation of secretion are mechanical distension like sighing or yawning [95]. Arachidonic acid (AA), and eicosapentaenoic acid (EPA) stimulate the secretion of surfactant in type II cells of rat. The physiological mechanism for this secretion is unknown, but the level of stimulation for highly unsaturated fatty acids was highest, compared to saturated and monosaturated fatty acids. It was also shown that highly unsaturated fatty acids like AA facilitate the fusion of secretory vesicles with the plasma membrane, suggesting a mechanism related to changes in membrane fluidity, and facilitated fusion of MLBs with cell membrane [6].

1.6.4. Surfactant recycling

Since there is active secretion of surfactant and the volume of alveoli is limited, the surfactant must also be removed [66]. Clearance of surfactant occurs via three ways:

1.6.4.1. Recycling

Surfactant is taken up by type II cells and reused again, though the mechanism of uptake is not well studied [96]. How surfactant leaves the alveolus is not known, but may be in the form of small vesicles. SP-A, SP-B and SP-C reenter type II cells, but there is no evidence for SP-D recycling [66]. Receptor-mediated

endocytosis of biotinylated SP-A in cultured type II cells has been reported [67], but still there is no evidence of receptor mediated endocytosis for SP-B and SP-C [43, 66, 96]. The existence of a high affinity receptor for SP-A has been suggested, which is consistent with the receptor-mediated endocytosis of SP-A [51].

1.6.4.2. Degradation and reutilization

Surfactant recycling is associated both with degradation and reutilization of material in order to form new surfactant. Some surfactant phospholipids are degraded during the clearance process and the constituents are incorporated into newly synthesized lipids. Alveolar type II cells degrade phosphatidylcholine probably by phospholipase A and lysophospholipase. Biochemical evidence shows that the majority of phospholipids leaving the alveolus reenter type II cells [96]. Some specific receptors on type II cells may be involved in the clearance of neutral lipids (cholesterol) and phospholipids. Also alveolar macrophages internalize surfactant components like tubular myelin and SP-A [66].

1.6.4.3. Removal from surfactant system

Surfactant is also cleared from the whole lung. This removal occurs either as intact molecules or as degradation products. Clearance probably occurs via the bronchiolar muco-ciliary escalator to the esophagus to be swallowed, or via transfer across the epithelial/endothelial barrier into the blood or lymph, or by degradation within the lung and transport of the degraded material into other organs [96].

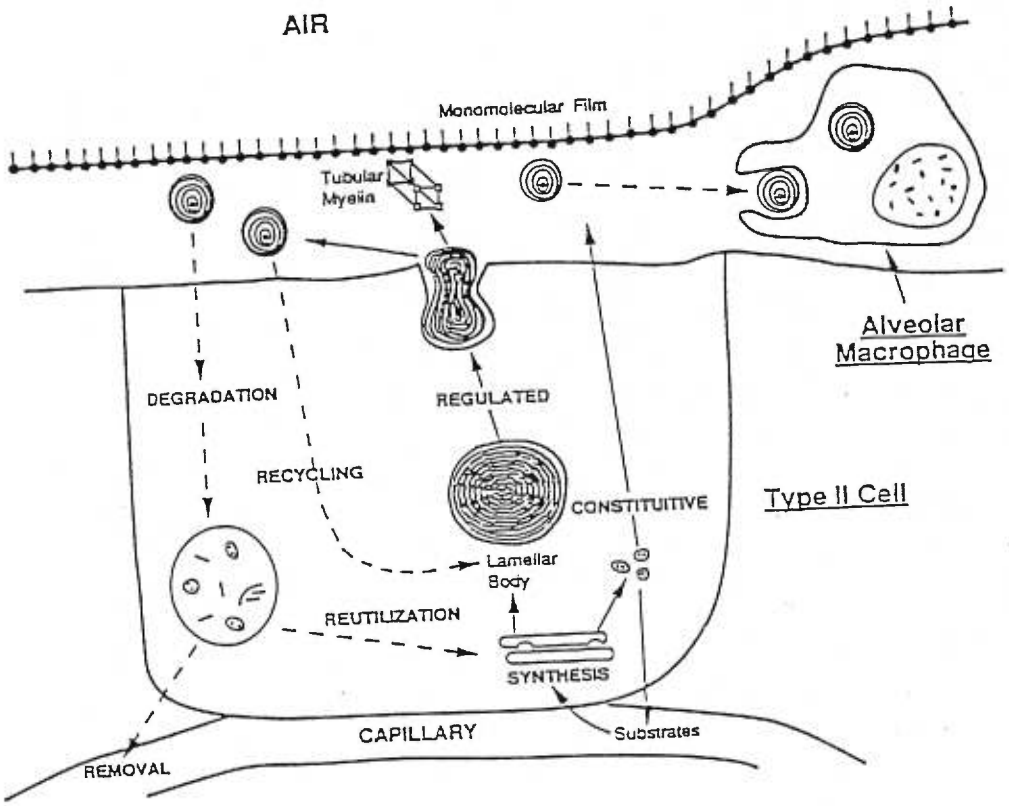

Figure 3: Three pathways of surfactant recycling [96].

1.7. OBJECTIVES

Mv1Lu is an immortalized cell line derived from mink lung type II alveolar cells. Following transfection with GlcNAc-TV, a glycosyltransferase responsible for initiation of polylactosamine glycosylation, these cells exhibit increased glycosylation of LAMP proteins (lysosomal membrane glycoproteins), and expression of MLBs. These cells are the only available GlcNAc-TV transfected cellular model and due to their availability from our collaborator, J. W. Dennis (University of Toronto) were chosen for this study. The MLBs were positive for LAMP by fluorescence microscopy, which indicates the lysosomal character of these organelles. Previous experiments in the laboratory had shown that treatment of cells with leupeptin, a protease inhibitor, resulted in disappearance of MLBs and formation of autophagic vacuoles. Washout of leupeptin from the medium of the cells resulted in the appearance of MLBs and disappearance of AVs in a progressive manner.

My objectives were to complete the previous work, quantify the differential expression of MLBs in GlcNAc-TV transfected cells, establish the role of protein degradation and autophagy in the formation of MLBs, and assess whether these lung type II epithelial derived cells represent a model for surfactant secretion in culture. We determined to address whether or not treatment with leupeptin can cause a gradual transformation of MLBs to autophagic vacuoles and to determine whether inhibitors of autophagy, such as 3-methyladenine, prevent MLB formation. We also determined to test whether Mv1Lu cells express the surfactant associated protein A (SP-A) using an antibody against SP-A for immunofluorescent microscopy and western blotting.

2.ARTICLE

Biogenesis of Multilamellar Bodies via Autophagy

Mehrdad Hariri*, Ghania Millane*, Marie-Pierre Guimond, Ginette Guay, James W. Dennis† and Ivan R. Nabi§

Department of Pathology and Cell Biology, University of Montreal, Montreal, Quebec, Canada H3C 3J7 and †Samuel Lunenfeld Research Institute, Mount Sinai Hospital, University of Toronto, Toronto, Ontario, Canada M5G 1X5

* These authors contributed equally to this work

§ Corresponding author. E-mail address: ivan.robert.nabi@umontreal.ca

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Corresponding author:

Dr. Ivan R. Nabi

Tel: (514) 343-6291

Département de pathologie et biologie cellulaire Fax: (514) 343-5755

Université de Montréal E-mail: ivan.robert.nabi@umontreal.ca

C. P. 6128, succursale A

Montréal, Québec, Canada H3C 3J7

Courier address:

Université de Montréal, Pavillion principal N-812

2900 Blvd. Edouard Montpetit

Montréal, Québec, Canada H3T 1J4

This article has been accepted by Molecular Biology of the Cell. My contribution to the work is as follows:

1. The labeling of large vacuoles with LAMP-2 antibody (Fig. 4), colocalization of LAMP-2 with LPH-A (Fig. 5), and the demonstration of the presence MLBs in Mv1Lu transfected cells by electron microscopy (Fig. 6) that was done previously and for the first time by Ghania Millane. However I repeated the experiments and figures were prepared using my data.

2. The quantification of the cytoplasmic area covered by MLBs in different clones of cells (Table 1).

3. The gradual transformation of MLBs to AVs following treatment with leupeptin (Fig. 7)

4. The appearance of inclusion bodies following treatment with 3-MA demonstrating by fluorescence and electronic microscopy (Fig. 9 and 10).

5. Scrape-loading with FITC-dextran of cells in the presence and absence of 3-MA (Fig. 11).

2.1. ABSTRACT

Transfection of Mv1Lu mink lung type II alveolar cells with GlcNAc-transferase V (GlcNAc-TV) is associated with the expression of large lysosomal vacuoles which are immunofluorescently labeled for the lysosomal glycoprotein LAMP-2 and the ß1-6 branched N-glycan specific lectin L-PHA. By electron microscopy, the vacuoles present the morphology of multilamellar bodies (MLBs). Treatment of the cells with the lysosomal protease inhibitor, leupeptin, results in the progressive transformation of the MLBs into electron-dense autophagic vacuoles (AV_d) and eventual disappearance of MLBs after 4 days treatment. Heterologous structures containing both membrane lamellae and peripheral electron-dense regions appear 15 hours after leupeptin addition and are indicative of ongoing lysosome-MLB fusion. Leupeptin washout is associated with the formation after 24 and 48 hours of single or multiple foci of lamellae within the autophagic vacuoles which give rise to MLBs after 72 hours. Treatment with 3-methyladenine (3-MA), an inhibitor of autophagic sequestration, results in the significantly reduced expression of multilamellar bodies and the accumulation of inclusion bodies resembling nascent or immature autophagic vacuoles (AV_i). Scrape-loaded cytoplasmic FITC-dextran is incorporated into LAMP-2 positive MLBs and this process is inhibited by 3-MA demonstrating that active autophagy is involved in MLB formation. Our results indicate that selective resistance to lysosomal degradation within the autophagic vacuole results in the formation of a microenvironment propicious for the formation of membrane lamella.

2.2. INTRODUCTION

Multilamellar bodies (MLBs) are membrane-bound cellular organelles which vary in size from 100-2400 nm, are composed of concentric membrane layers and frequently exhibit an electron-dense core. MLBs are found in numerous cell types where they function in lipid storage and secretion (Schmitz and Müller, 1991). In lung type II alveolar cells, MLBs function as secretory granules whose exocytosis results in the deposition of the tubular myelin forms of surfactant on the surface of the alveolae (Hatasa and Nakamura, 1965; Ryan *et al.*, 1975; Williams, 1977). The surfactant film over the alveolar epithelium regulates the surface tension at the air cell interface and protects the alveola from collapse during respiration (Haagman and van Golde, 1991).

While the secretory function of MLBs in type II alveolar cells is well established, the precise mechanism of MLB biogenesis remains unclear. Autoradiographic studies of murine type II alveolar cells of mouse lungs showed that while phospholipids labeled with ^{3}H-choline are delivered directly from the Golgi to the MLB, proteins metabolically labeled with ^{3}H-leucine are visualized within multivesicular bodies prior to delivery to MLBs (Chevalier and Collet, 1972). Surfactant proteins A, B and C are delivered via multivesicular bodies to MLBs and multivesicular bodies are proposed as the site of processing of surfactant precursor to mature forms; both multivesicular bodies and MLBs express the lysosomal marker CD63 and are therefore part of the lysosomal pathway (Voorhut *et al.*, 1992; Voorhut *et al.*, 1993). The lysosomal nature of the MLB has been demonstrated by the localization of various lysosomal enzymes to this organelle (Balis and Conen, 1964; Hatasa and Nakamura, 1965; Goldfischer *et al.*, 1968; Hoffman, 1972; DiAugustine, 1974; Heath *et al.*, 1976; Hook and Gilmore, 1982; de Vries *et al.*, 1985).

It has been previously suggested based on morphological criteria that MLBs form via cellular autophagy (Balis and Conen, 1964; Sorokin, 1967; Flaks

and Flaks, 1972; Stratton, 1978). Autophagy is a normal degradative process that exists in all eukaryotic cells and is stimulated in response to a variety of environmental stresses which necessitate the use of autophagic mechanisms to enable cellular survival (Seglen and Bohley, 1992; Dunn, 1994). Degradative autophagic vacuoles (AV_d) form following acquisition of lysosomal properties by nascent autophagic vacuoles or AV_i, which present multiple limiting membranes and are considered to form by the sequestration of cytoplasm by smooth ER membranes (Dunn, 1990; Furuno *et al.*, 1990; Ueno *et al.*, 1991). The lysosomal nature of both the autophagic vacuole and the MLB supports a relationship between the two organelles however definitive evidence of a role for autophagy in MLB biogenesis has yet to be demonstrated.

Transfection of the immortalized Mv1Lu cell line, derived from mink lung type II alveolar cells, with ß1-6N-acetylglucosaminyl transferase V (GlcNAc-TV), the enzyme responsible for the ß1-6 branching of N-glycans which favors the addition of elongated polylactosamine chains, results in the loss of contact inhibition, decreased substrate adhesion, increased susceptibility to apoptosis and increased tumorigenicity in nude mice (Demetriou *et al.*, 1995). GlcNAc-TV transfected Mv1Lu cells also exhibit increased L-PHA reactivity of LAMP-2 demonstrating that increased GlcNAc-TV activity alters the ß1-6 branching of this heavily glycosylated lysosomal glycoprotein (Demetriou *et al.*, 1995). We show here that in contrast to untransfected Mv1Lu cells, which exhibit none or at best few MLBs, GlcNAc-TV transfected Mv1Lu cells stably express numerous cytoplasmic MLBs. MLB formation in the GlcNAc-TV transfectants is reversibly regulated by leupeptin, an inhibitor of lysosomal proteases, demonstrating that lysosomal degradation is necessary for the formation of the membrane lamella of the MLB. It is also inhibited by 3-methyladenine (3-MA), a specific inhibitor of early stages in autophagic vacuole formation (Seglen and Gordon, 1982), and we

demonstrate the necessary role for autophagy and autophagic vacuole biogenesis in MLB formation.

2.3. MATERIALS AND METHODS

2.3.1. Cell culture

Mv1Lu mink lung epithelial cells, mock transfected Mv1Lu cells (C1) and the GlcNAc-TV transfected Mv1Lu cell lines (R2, M9, M1) (Demetriou *et al.*, 1995) were grown in DME supplemented with glutamine, non-essential amino acids (Gibco Laboratories, Oakville, Ontario) and 10% FBS (Immunocorp, Laval, Quebec) in an air-5% CO_2 atmosphere at constant humidity at 37°C. The medium of the transfected cell lines (C1, R2, M9, M1) was supplemented with 600 µg/ml G418 in order to maintain the transfected phenotype. For all experiments, cells were plated at a density of 40,000 cells/cm^2 and the medium was replaced every two days. Leupeptin (Roche Diagnostics, Laval, Quebec) was added to cell cultures at a concentration of 2 µg/ml and 3-MA (Sigma, St. Louis, MI) at a concentration of 10 mM.

2.3.2. Immunofluorescence

Cells cultured on glass coverslips were fixed by the addition of precooled (-80°C) methanol/acetone (80%/20% v/v) directly to the coverslips and then placed at -20°C for 15 min. After fixation, the cells were rinsed extensively with PBS (pH 7.4) supplemented with 0.1 mM Ca^{++} and 1 mM Mg^{++} (PBS/CM), and then incubated for 15 min with PBS/CM containing 0.5% BSA (PBS/CM/BSA) at room temperature to reduce nonspecific binding. LAMP-2 distribution was determined using the AC17 anti-LAMP-2 antibody (Nabi *et al.*, 1991; Nabi and Rodriguez-Boulan, 1993) followed by FITC or Texas Red conjugated secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA). Detection of the distribution of L-PHA reactivity was performed using rhodamine-conjugated

L-PHA (E-Y Laboratories, San Mateo, CA). After labeling, the coverslips were mounted in Airvol (Air Products and Chemicals Inc, Allentown, PA). Labeled cells were viewed in a Zeiss Axioskop fluorescent microscope equipped with a 63X Planapochromat objective and fluorochrome selective filters. Images were photographed using Kodak T-Max 400 film. Confocal microscopy was performed with the 60X Nikon Plan Apochromat objective of a dual channel BioRad MRC 600 laser scanning confocal microscope equipped with a krypton/argon laser and printed using a Polaroid TX1500 video printer.

2.3.3. Electron microscopy

Cells grown on petri dishes were rinsed with 0.1 M sodium cacodylate pH 7.3 and fixed with 2% glutaraldehyde for 60 minutes at 4°C. The fixed cells were rinsed in cacodylate buffer, scraped from the petri dish and collected by centrifugation. The cell pellet was post-fixed for 60 minutes with 2% osmium tetroxide at 4°C, dehydrated and embedded in LR-White resin. Ultra-thin sections (80 nm) were contrasted with uranyl acetate and lead citrate and visualized with a Phillips 300 or Zeiss CEM902 electron microscope. Quantification of the expression of MLBs and of inclusion bodies in the 3-MA experiments was determined by circumscribing the cytoplasm (excluding the nucleus), the MLBs and the inclusion bodies from 10 images at 4,400 fold magnification and determining the area of the circumscribed regions. MLBs were defined as membrane bound cytoplasmic organelles which present at least 3 distinct circumferential concentric membrane lamellae. MLBs were composed either completely of concentric lamella or of concentric lamella surrounding a single dense core. Inclusion bodies, or immature autophagic vacuoles (AV_i), were defined by the presence of multiple internal structures surrounded by a limiting membrane composed of single or multiple membranes and could be morphologically distinguished from MLBs.

2.3.4. Scrape-loading of FITC-dextran

FITC-dextran was scrape-loaded into M9 cells essentially as previously described (McNeil *et al.*, 1984). Cells were plated overnight to semiconfluence and then rinsed 3 times in cold PBS/CM and incubated on ice for 15 minutes to chill the cultures. 0.5 ml cold PBS/CM containing 2.5 mg/ml lysine-fixable 10,000 MW FITC-dextran (Molecular Probes, Eugene, OR) was added to the culture dish and the cells were immediately scraped from the dish in the concentrated FITC-dextran solution. The cell suspension was rapidly diluted in 40 ml cold PBS/CM and centrifuged in the cold to pellet the cells. The scrape-loading was performed at 4°C and the cells rapidly diluted in cold PBS/CM in order to reduce the possibility of FITC-dextran uptake by fluid phase endocytosis. The cell pellet was resuspended in culture medium and the cells plated for 2, 24, 48 or 72 hours in regular medium or in medium containing 10 mM 3-MA prior to fixation with 3% paraformaldehyde and immunofluorescence labeling with anti-LAMP-2 and Texas Red conjugated secondary antibodies. Quantification of autophagic activity was performed by counting the number of FITC-dextran loaded cells exhibiting FITC labeling of LAMP-2 positive lysosomal structures.

2.4. RESULTS

2.4.1. Increased expression of MLBs in GlcNAc-TV transfected Mv1Lu cells

GlcNAc-TV transfection of Mv1Lu cells resulted in the obtention of clones M9 and M1 which exhibited significantly higher expression levels of GlcNAc-TV activity and increased L-PHA reactivity of LAMP-2 (Demetriou *et al.*, 1995). To assess the distribution of LAMP-2 and of lysosomes in these cells, 6 day confluent cultures of untransfected Mv1Lu cells and M9 and M1 GlcNAc-TV transfectants were immunofluorescently labeled with antibodies to LAMP-2 (Figure 4). Clusters of LAMP-2 labeled lysosomes, as indicated by the arrows, correspond to the perinuclear concetnration of lysosomal organelles in an individual cell. In contrast to the punctate distribution of the lysosomal marker in Mv1Lu cells, anti-LAMP-2 antibodies label large vacuolar structures in both the

M9 and M1 cell lines (Figure 4 B, C). To determine whether the increased ß1-6 branched N-glycans of the GlcNAc-TV transfectants are indeed localized to the large LAMP-2 positive lysosomal vacuoles present in the M9 and M1 transfectants, Mv1Lu cells and M9 cells were double immunofluorescently labeled for LAMP-2 and L-PHA, a lectin specific for the ß1-6 branching of polylactosamine chains.

By confocal microscopy, LAMP-2 and L-PHA colocalize in both the punctate LAMP-2 lysosomal labeling of Mv1Lu cells as well as in the swollen LAMP-2 labeled vacuoles of M9 cells (Figure 5).

Electron microscopy of the different cell types revealed the presence of large MLBs in the M9 and M1 cell lines but not in the untransfected Mv1Lu cells (Figure 6). MLBs of M1 cells frequently exhibit a dense core surrounded by lamellae while those of M9 cells exhibited a more uniform lamellar morphology and are larger. Expression of MLBs in untransfected Mv1Lu cells, mock transfected C1 cells and the GlcNAc-TV transfectants R2, M9 and M1 exhibiting increasing levels of GlcNAc-TV activity ((Demetriou *et al.*, 1995); Table 1) was quantified by determining the extent of cytoplasmic area that was filled by MLBs in the different cells. Both the number of MLBs and the proportion of cytoplasmic area which they cover is significantly greater in high GlcNAc-TV expressing M9 and M1 cells compared to either untransfected Mv1Lu, mock transfected C1 or low-expressing R2 cells (Table 1). Curiously, the area covered by MLBs is greater in M9 cells than in M1 cells which express higher GlcNAc-TV levels; the number of MLBs in M1 cells is larger than in M9 cells indicating that the difference between the two cell types is due to an increased size of MLBs in the M9 cells. MLBs larger than 2 μ^2 in area are not observed in untransfected Mv1Lu, mock transfected C1 or low GlcNAc-TV expressing R2 cells. Untransfected Mv1Lu cells can express MLBs, albeit very few, suggesting that these cells may have partially retained a differentiated type II phenotype. Mock transfected C1 and low GlcNAc-TV expressing R2 cells (Demetriou *et al.*, 1995) exhibit more MLBs than untransfected Mv1Lu cells. However, the difference between the MLB/cytoplasm

ratio of C1 and R2 cells and of Mv1Lu cells is not statistically significant ($0.05<p<0.1$) and suggests that MLB expression in C1 and R2 cells may be due to subtle changes in the phenotype of the cells following transfection.

Figure 4: Expression of large lysosomal vacuoles in GlcNAc-TV transfected Mv1Lu cells. Untransfected Mv1Lu cells (A) or GlcNAc-TV transfected clones M9 (B) and M1 (C) plated for 6 days were immunofluorescently labeled with LAMP-2 to reveal the distribution of lysosomes in these cells. Clusters of lysosomes representing the lysosomes of individual cells are indicated by the arrows. Distinct swollen LAMP-2 positive lysosomal vacuoles are present in M9 and in M1 cells but not in untransfected Mv1Lu cells.

Bar = 10 μm

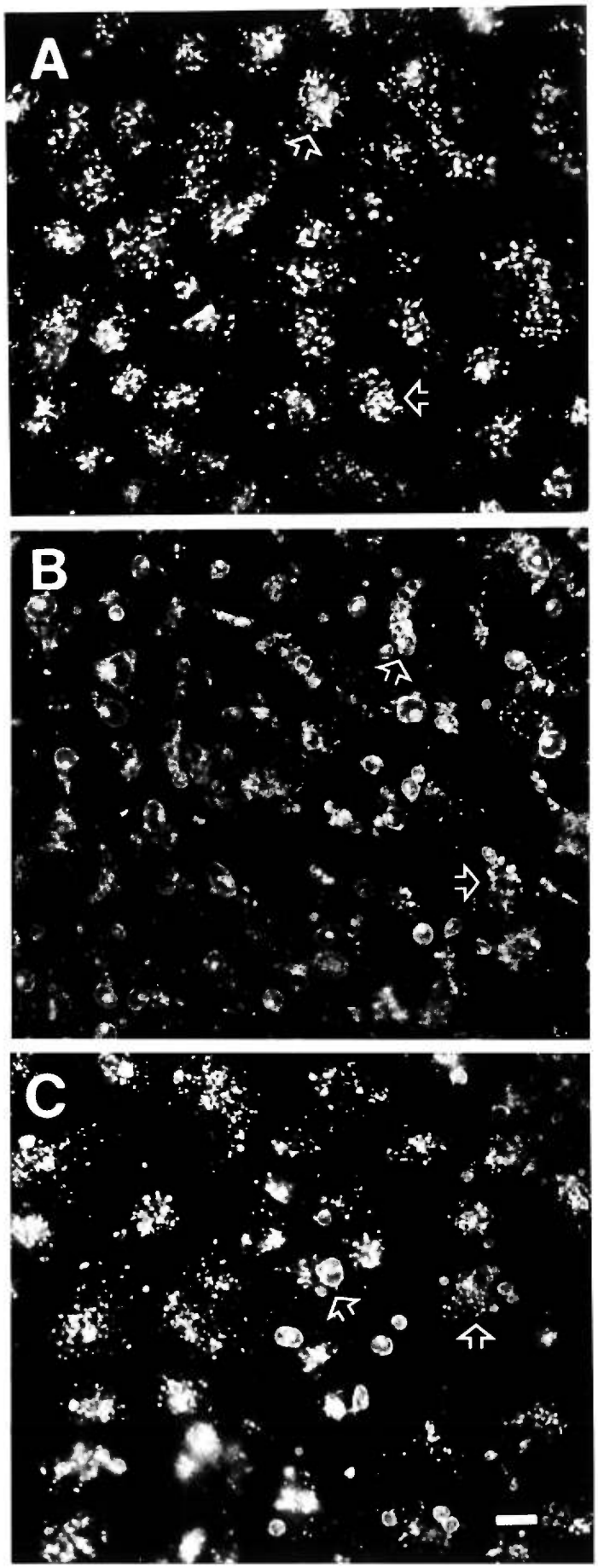
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Figure 5: The swollen lysosomal vacuoles of GlcNAc-TV transfectants are labeled with L-PHA. Untransfected Mv1Lu (A, B, C) or GlcNAc-TV transfected M9 (D,E,F) cells plated for 6 days were double immunofluorescently labeled with anti-LAMP-2 followed by FITC-conjugated anti-mouse secondary antibody (A, D) or with rhodamine-conjugated L-PHA (B, E). Merged images are presented in C and F (LAMP-2 in green; L-PHA in red). LAMP-2 and L-PHA reactive ß1-6 branched oligosaccharides are localized to lysosomes of Mv1Lu cells as well as to the large lysosomal vacuoles of GlcNAc-TV M9 transfectants. Bar = 10 μm.

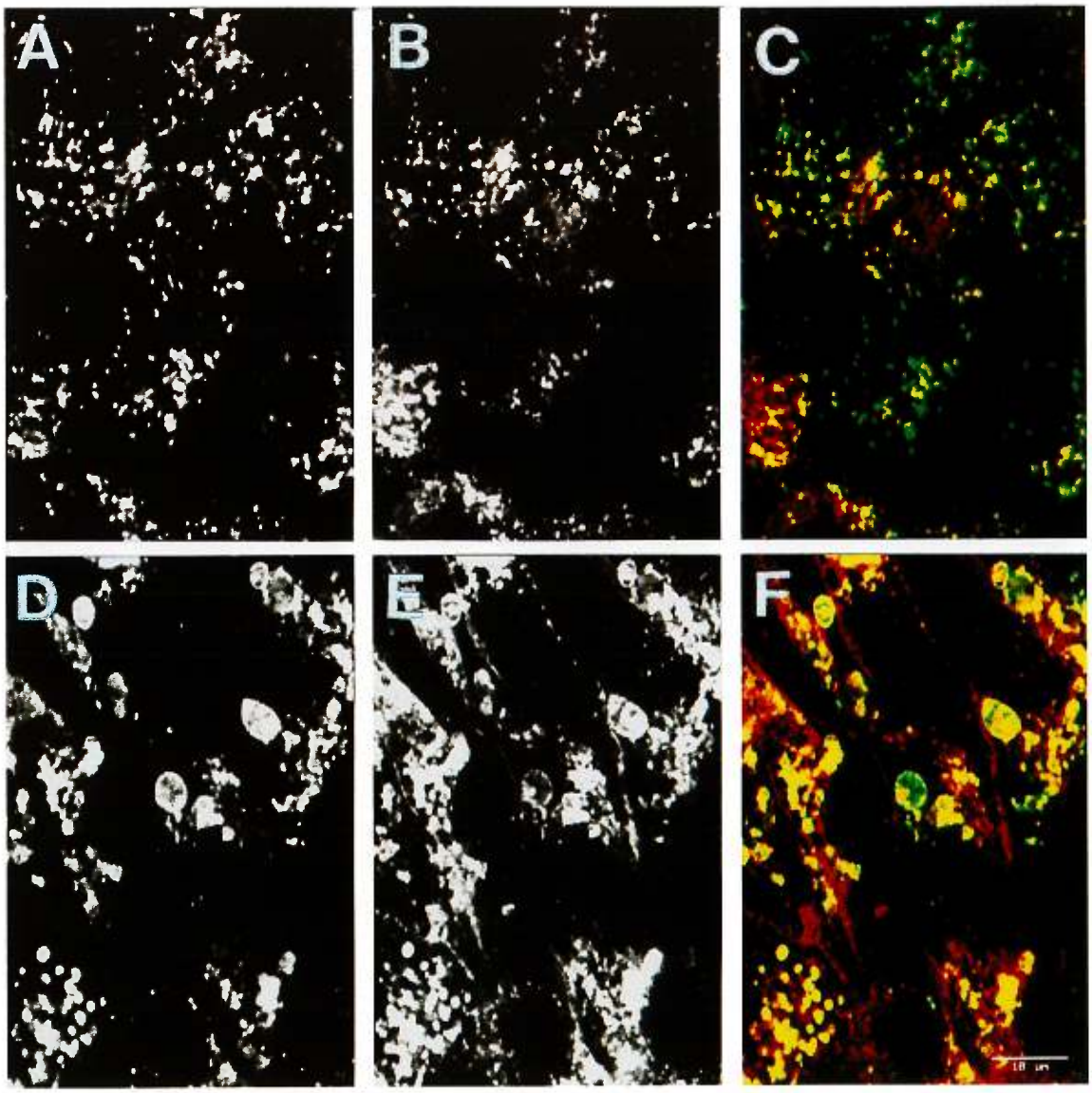
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Figure 6: GlcNAc-TV transfectants express MLBs. Untransfected Mv1Lu cells (A) or GlcNAc-TV transfected clones M9 (B, D) and M1 (C) cells plated for 6 days were processed for electron microscopy. Distinct MLBs are present in the cytoplasm of both M9 (B) and M1 (C) cells but are absent from untransfected Mv1Lu cells (A). The lamellar morphology of large MLBs is particularly evident in M9 cells (D). Bar = 1 μM (A,B,C) or 0.5 μM (D).

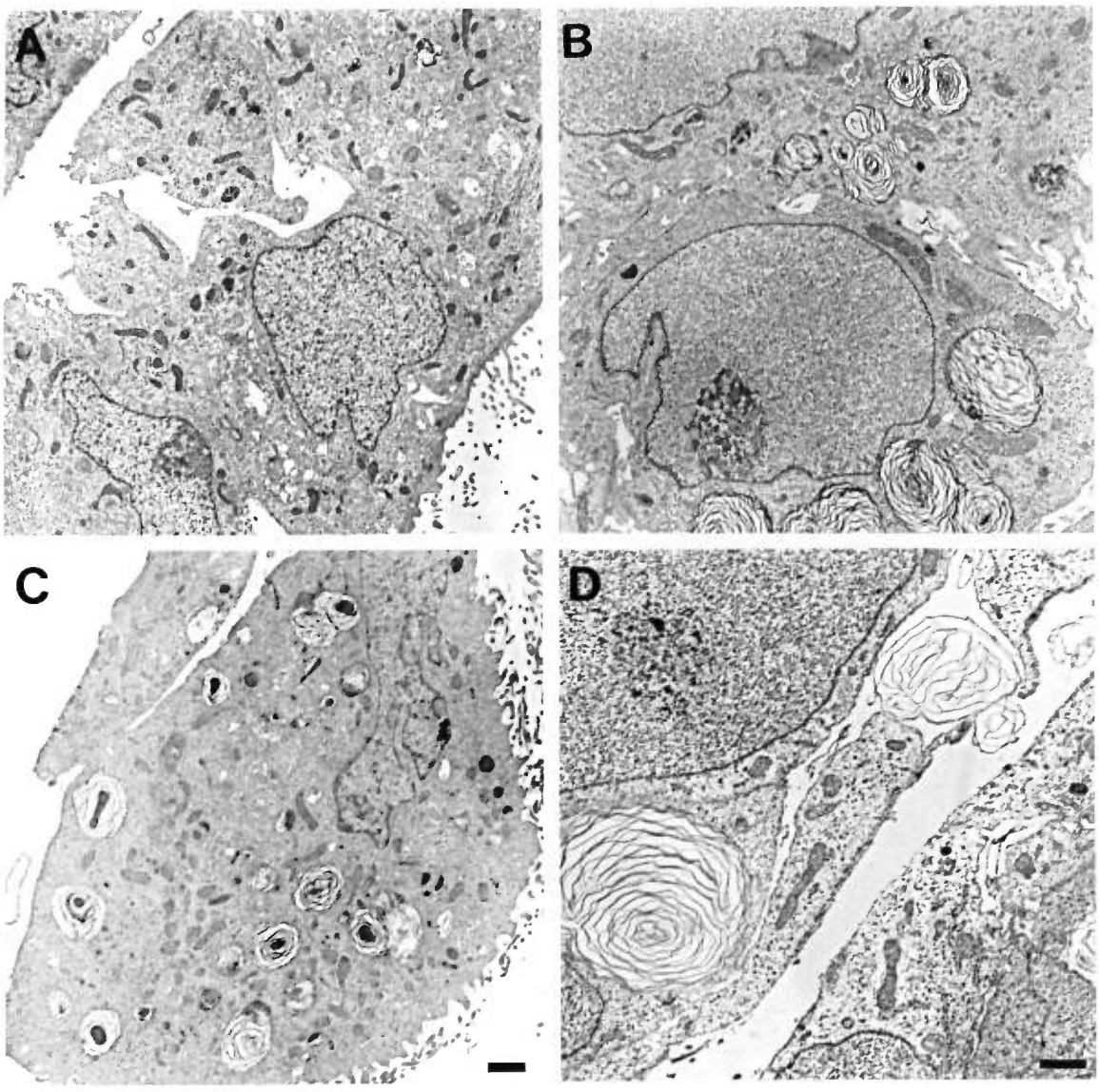
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Table 1: Quantification of MLB expression in GlcNAc-TV transfected Mv1Lu cells

Cell type	GlcNAc-TV *(pmol/mg/ h)**	Total # of MLBs	Range of MLB size *(μ^2)*	Total cytoplasmic area *(μ^2)*	Total MLB area *(μ^2)*	% area MLB/ cytoplasm
Mv1Lu	63 ± 6	1	0.53-0.53	2022	0.53	0.03 ± 0.03
C1	59 ± 5	29	0.27-2.00	1875	22.5	1.1 ± 0.6
R2	151 ± 15	15	0.33-1.60	1882	12.3	0.6 ± 0.3
M9	567 ± 265	78	0.23-16.3	1690	223	13.0 ± 1.1
M1	1082 ± 308	98	0.22-14.0	1821	109	6.1 ± 1.6

From 10 electron microscopy images (4,400X) of each cell line, the area covered by MLBs and by cytoplasm (excluding the nucleus) in the complete image was determined. Using a two-tailed student t-test, the ratio of MLB to cytoplasmic area in M9 cells is significantly different from both Mv1Lu and C1 cells ($p<10^{-5}$). For M1 cells, the ratio of MLB to cytoplasmic area is also significantly different from both Mv1Lu ($p<0.005$) and C1 ($p<0.01$) cells. The significance of the difference in MLB/cytoplasm area between either C1 or R2 cells and Mv1Lu cells is of the order of $0.05<p<0.1$. *The values for GlcNAc-TV expression were taken from Dimetriou et al (1995).

2.4.2. Lysosomal degradation is necessary for MLB biogenesis

The only cytoplasmic structures visualized in M9 or M1 cells by electron microscopy large enough to correspond to the large LAMP-2/L-PHA positive vacuoles identified in these cells by immunofluorescence labeling are MLBs. Furthermore, the expression of LAMP-2 in the MLBs of GlcNAc-TV transfected Mv1Lu cells is consistent with the previously described lysosomal nature of this organelle (Balis and Conen, 1964; Hatasa and Nakamura, 1965; Goldfischer *et al.*, 1968; Hoffman, 1972; DiAugustine, 1974; Heath *et al.*, 1976; Hook and Gilmore, 1982; de Vries *et al.*, 1985; Voorhut *et al.*, 1992). To assess the role of lysosomal degradation on MLB expression, M1 cells were treated with the lysosomal protease inhibitor leupeptin (Figure 7). After 15 hours of leupeptin treatment, MLBs exhibit electron-dense material around the periphery of the vacuole due to the apparent fusion of MLBs with other lysosomal organelles and transfer of non-lamellar electron dense material. In addition to these heterogeneous structures, smaller dense vacuoles accumulate. With increasing time in leupeptin containing media, MLBs are no longer evident and after 4 days incubation with leupeptin, only large dense vacuoles are present in M1 cells (Figure 7E, 8A). Leupeptin treatment has been previously shown to induce the accumulation of AV_d (Furuno *et al.*, 1982; Ueno *et al.*, 1991; Yokota *et al.*, 1995) and the leupeptin induced vacuoles in M1 cells are morphologically equivalent to AV_d (Figure 7E, 8A).

To determine whether removal of leupeptin and activation of lysosomal degradation could reverse this process and lead to the formation of MLBs, M1 cells treated with leupeptin for 4 days were then incubated in the absence of leupeptin and analyzed by electron microscopy. 15 hours after leupeptin washout, dense AV_d are still present in M1 cells however appear to have lost some internal structure (Figure 8 B). After 24 hours the morphological transformation of the vacuoles commenced and numerous dense core bodies are present with some exhibiting internal lamellae (Figure 8 C).

Figure 7: Gradual transformation of MLBs into AV_d by leupeptin treatment. GlcNAc-TV transfected M1 cells were plated for 2 days in regular medium and then incubated for 15 (A, B), 48 (C), 72 (D) or 96 (E) hours in the presence of 2 μg/ml leupeptin and processed for electron microscopy. At early times following addition of leupeptin dense material can be observed at the periphery of MLBs (A,B) and at later times dense vacuoles proliferate such that after 96 hours autophagic vacuoles predominate and MLBs are no longer present. Bar = 0.5 μm.

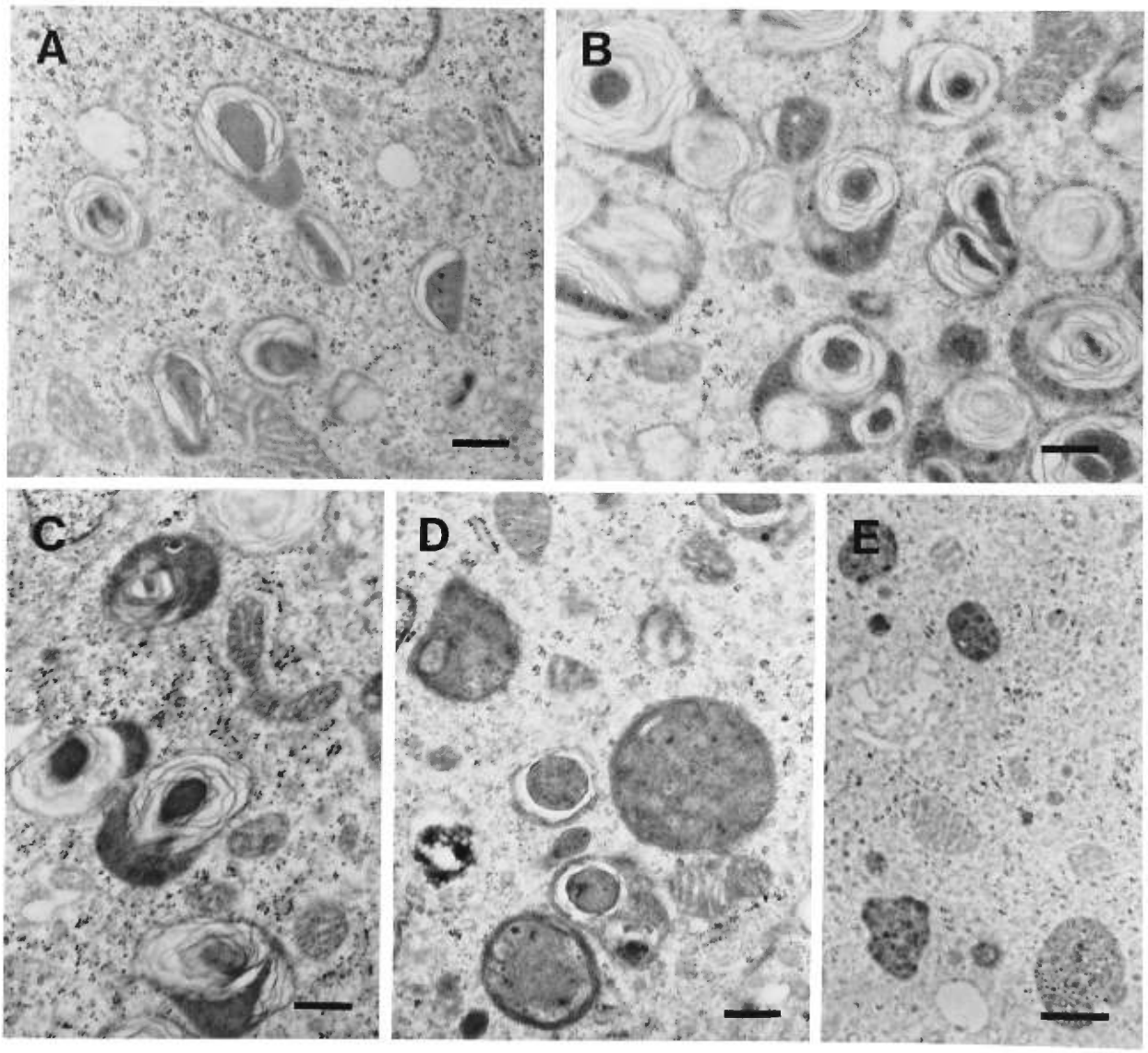
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Figure 8: Formation of MLBs via autophagic vacuole degradation following leupeptin washout. GlcNAc-TV transfected M1 cells were plated for 2 days and then incubated for 4 days in the presence of 2 μg/ml leupeptin (A) and then washed and incubated in leupeptin-free medium for 15 (B), 24 (C), 48 (D, E) or 72 (F) hours before being processed for electron microscopy. Following leupeptin treatment, MLBs disappear and large autophagic vacuoles are present (A). After 24 and 48 hours, single or multiple foci of lamella form within the autophagic vacuole (C, D, E) and after 72 hours the autophagic vacuoles transform into lamellar structures resembling those of untreated cells (F). Bar = 1 μm (A, B and F) or 0.2 μm (C, D, E).

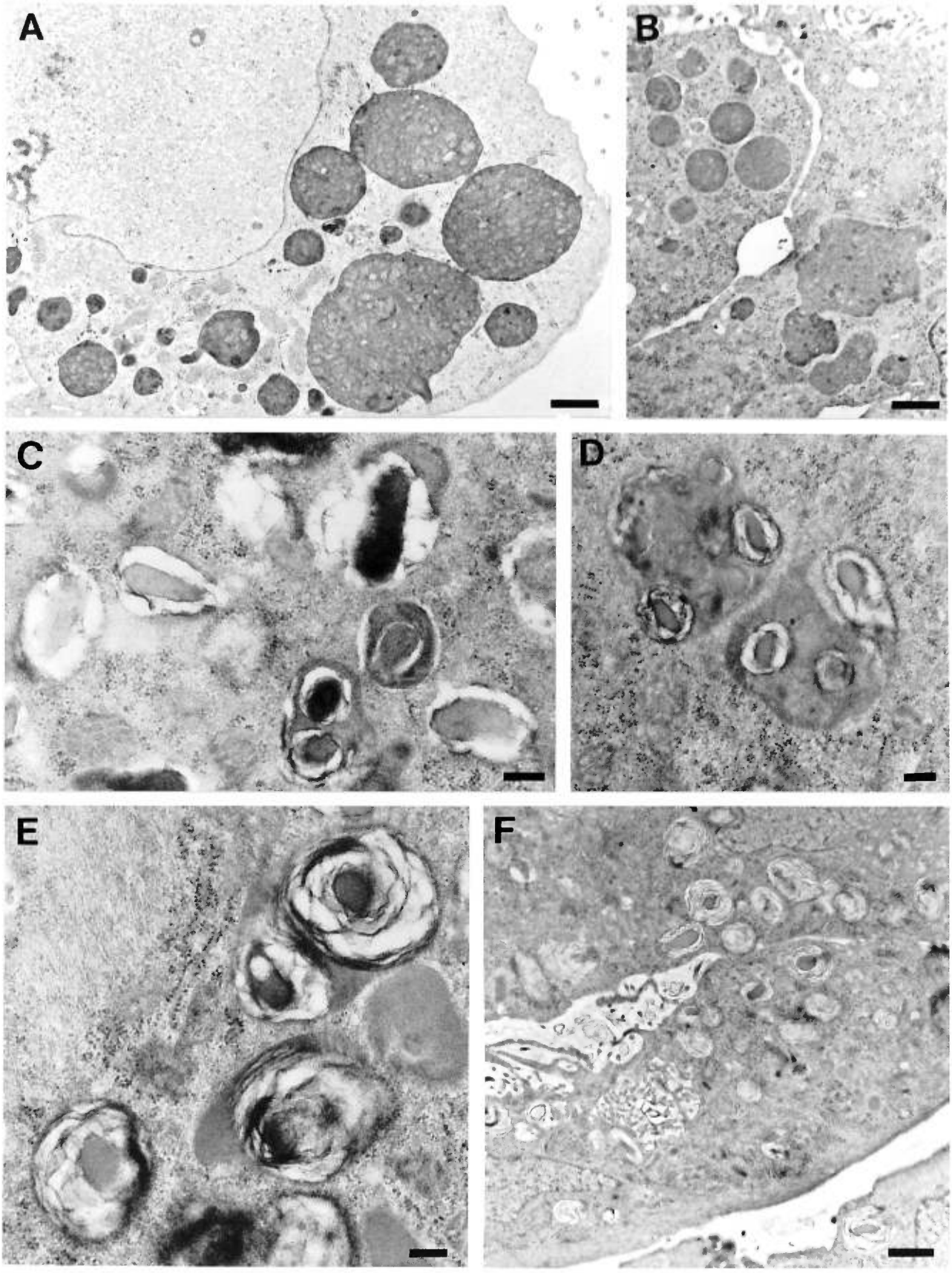
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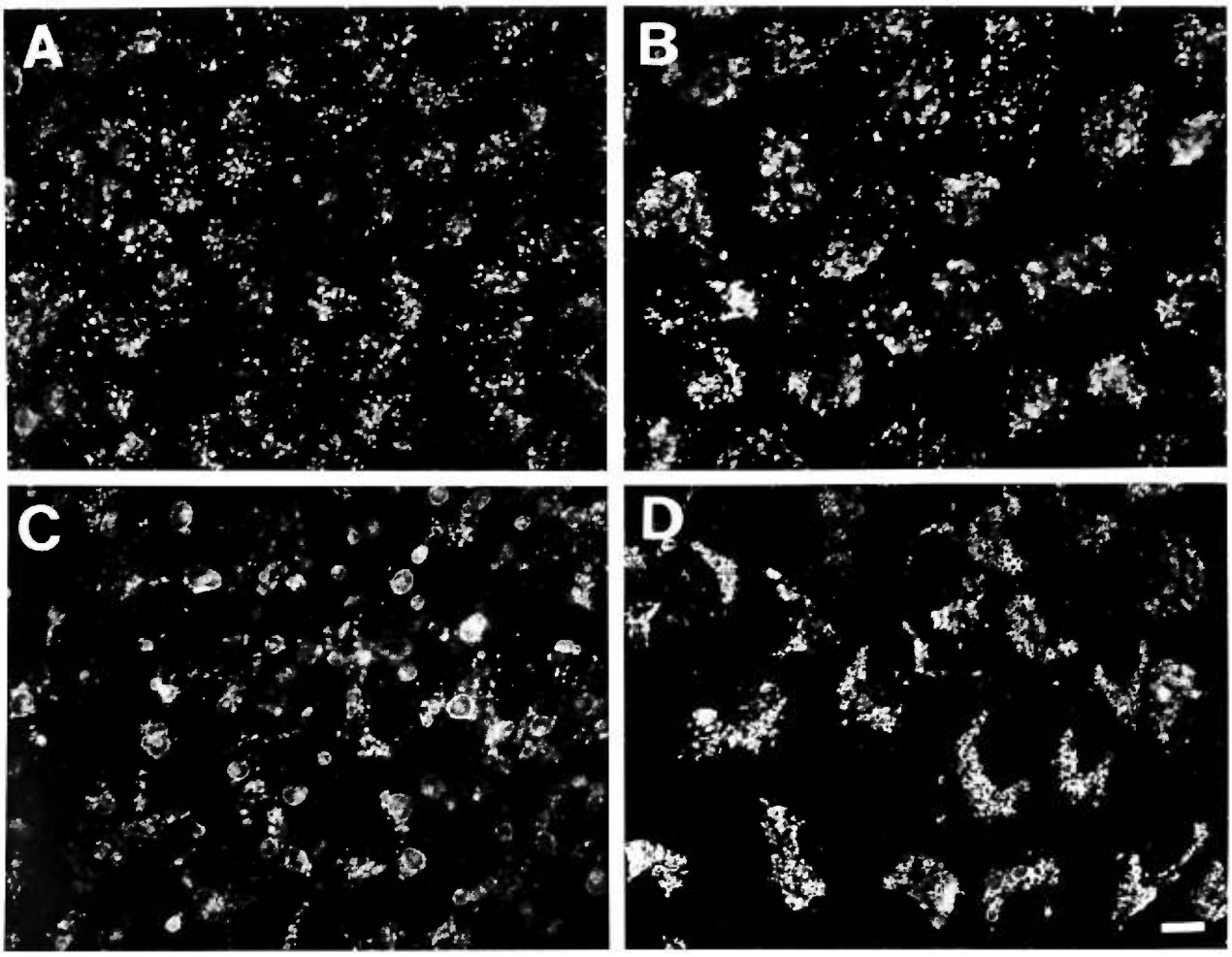
Figure 9: Inhibition of autophagy by 3-MA results in the disappearance of swollen LAMP-2 positive MLBs. Untransfected Mv1Lu (A, B) and GlcNAc-TV transfected M9 (C, D) cells were incubated in regular medium (A, C) or medium supplemented with 10 mM 3-MA for 3 days (B, D) and then immunofluorescently labeled for LAMP-2. While 3-MA treatment induces the formation of slightly swollen lysosomal structures in both Mv1Lu and M9 cells (B, D), it also results in the disappearance of the large LAMP-2 positive vacuoles which correspond to MLBs in M9 cells (D). Bar = 10 μm.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48 hours after leupeptin removal, intermediates in the transformation of AV_d into MLBs can be visualized (Figure 8 D,E). The formation of single or multiple dense core lamellar structures within individual autophagic vacuoles can be clearly visualized. After 72 hours in the absence of leupeptin the cells exhibit MLBs similar to untreated cells (Figure 8 F). The reversible regulation of MLB expression by leupeptin in GlcNAc-TV transfected M1 cells demonstrates that lysosome fusion with MLBs and subsequent degradation of MLB contents by lysosomal hydrolases regulates lamella formation.

2.4.3. Role of autophagy in MLB biogenesis

Leupeptin is a general inhibitor of lysosomal protease activity, and while it blocks degradation of AV_d (Furuno *et al.*, 1982; Kovacs *et al.*, 1982; Ueno *et al.*, 1991; Yokota *et al.*, 1995), it is not a specific inhibitor of autophagy. In order to determine whether autophagy is specifically involved in MLB formation, we used 3-MA which has previously been demonstrated to block autophagy at the initial sequestration step (Seglen and Gordon, 1982). By immunofluorescence, 3-MA treatment results in the disappearance of large LAMP-2 positive vacuoles in M9 cells (Figure 9). The LAMP-2 positive lysosomes in both Mv1Lu and M9 cells treated with 3-MA (Figure 9 B,D) are slightly swollen compared to those of untreated Mv1Lu cells (Figure 9 A) however the large LAMP-2 positive vacuoles corresponding to MLBs in M9 cells (Figure 9 C) are no longer visible in 3-MA treated M9 cells (Figure 9 D).

The ability of 3-MA to induce the disappearance of MLBs was confirmed by electron microscopy (Figure 10). In cells treated with 3-MA, distinctive inclusion bodies accumulate which do not present the circumferential membrane layers of MLBs (Figure 10, arrows). The limiting membrane of the inclusion bodies is formed of double or multiple membranes and surround multiple internal structures including multilamellar structures. These inclusion bodies can be morphologically distinguished from MLBs whose concentric membrane layers surround only a single dense core. These inclusion bodies can be morphologically

distinguished from MLBs whose concentric membrane layers surround only a single dense core. These inclusion bodies are morphologically equivalent to AVi and their expression following 3-MA treatment is consistent with the role of 3-MA as an inhibitor of autophagy. Quantitative analysis of the effect of 3-MA treatment on MLB expression in M9 and M1 cells demonstrates that cells cultured in 10 mM 3-MA for three days exhibit significantly decreased expression of MLBs (Table 2). In some experiments, we observed the complete disappearance of MLBs. Relative to MLB expression in control cells, expression of inclusion bodies in 3-MA treated cells is significantly reduced in all experiments.The ability of 3-MA to inhibit MLB formation identifies a role for autophagic vacuole maturation in MLB formation.

In order to demonstrate that 3-MA indeed blocks autophagy in GlcNAc-TV transfected Mv1Lu cells and to confirm the role of autophagy in MLB biogenesis, M9 cells were scrape-loaded with FITC-dextran. The procedure was performed at 4°C to minimize endocytic capture of FITC-dextran such that the fluorescent marker was incorporated only into the cytoplasm of the cell. The scrape-loaded cells were washed in the cold to eliminate any free FITC-dextran and then fixed at various times after plating in regular or 3-MA supplemented medium. At early times after plating in regular medium (2 hours) the majority of cells exhibit a cytoplasmic distribution of FITC-dextran which is excluded from vacuolar structures (Figure 11 A,B). After 48 hours, the majority of cells plated in regular medium exhibit an accumulation of FITC-dextran in LAMP-2 positive vacuoles including the swollen structures equivalent to MLBs (Figure 11 C,D). However, if the cells are replated in 3-MA containing medium after scraping, the FITC-dextran remains cytosolic and no LAMP-2 positive vacuoles are labeled (Figure 11 E,F). The number of cells presenting FITC-dextran labeling in LAMP-2 positive vacuoles was counted from 6 experiments (Figure 12). With time an increasing number of cells exhibit autophagic incorporation of FITC-dextran into LAMP-2 positive vacuoles. In the presence of 3-MA, essentially no cells exhibit a vacuolar labeling irrespective of the time of incubation in culture medium. The limited incorporation of scrape-loaded FITC-dextran into lysosomal vacuoles in

the presence of 3-MA demonstrates that endocytic uptake of FITC-dextran by the cells was minimal and that 3-MA does indeed block autophagy in M9 cells. FITC-dextran transfer from the cytosol to large vacuoles of M9 cells which correspond to MLBs therefore demonstrates that active autophagy is involved in the formation of these organelles.

Figure 10: Expression of inclusion bodies in 3-MA treated GlcNAc-TV transfectants. Treatment of GlcNAc-TV transfected M1 cells with 10 mM 3-MA for three days resulted in the disappearance of MLBs and the appearance of morphologically distinct inclusion bodies (see arrows) exhibiting multiple external membranes and resembling AV_i (A-D). Bar = 0.5 μm (A) or 0.2 μ (B, C, D).

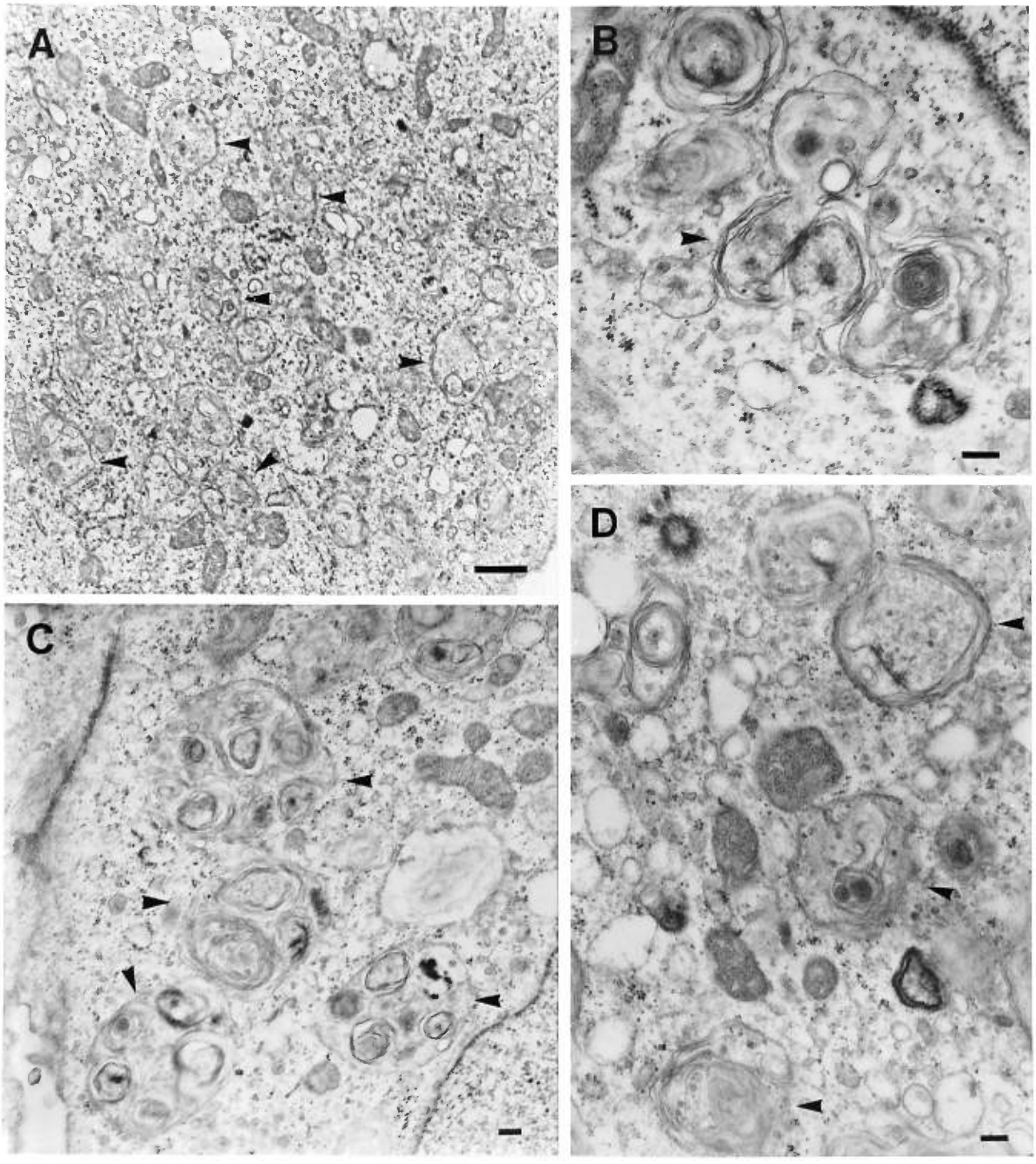
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Figure 11: Incorporation of cytosolic FITC-dextran into MLBs is inhibited by 3-MA. M9 cells were scraped from plastic dishes in the presence of 2.5 mg/ml FITC-dextran, washed extensively, replated on cover slips in regular medium (A, B, C, D) or medium containing 10 mM 3-MA (E, F) and then fixed after 2 (A, B) or 48 (C, D, E, F) hours and labeled for LAMP-2 using Texas Red conjugated secondary antibodies. The distribution of FITC-dextran (A, C, E) and of LAMP-2 (B, D, F) in the same cells is presented. After only two hours of plating, FITC-dextran is cytosolic and excluded form large LAMP-2 positive vacuoles (A, B) however with time FITC-dextran is incorporated via autophagy into LAMP-2 positive perinuclear vacuoles equivalent to MLBs (C, D). Autophagic incorporation of FITC-dextran into MLBs is inhibited by 3-MA (E, F). Bar = 10 μm.

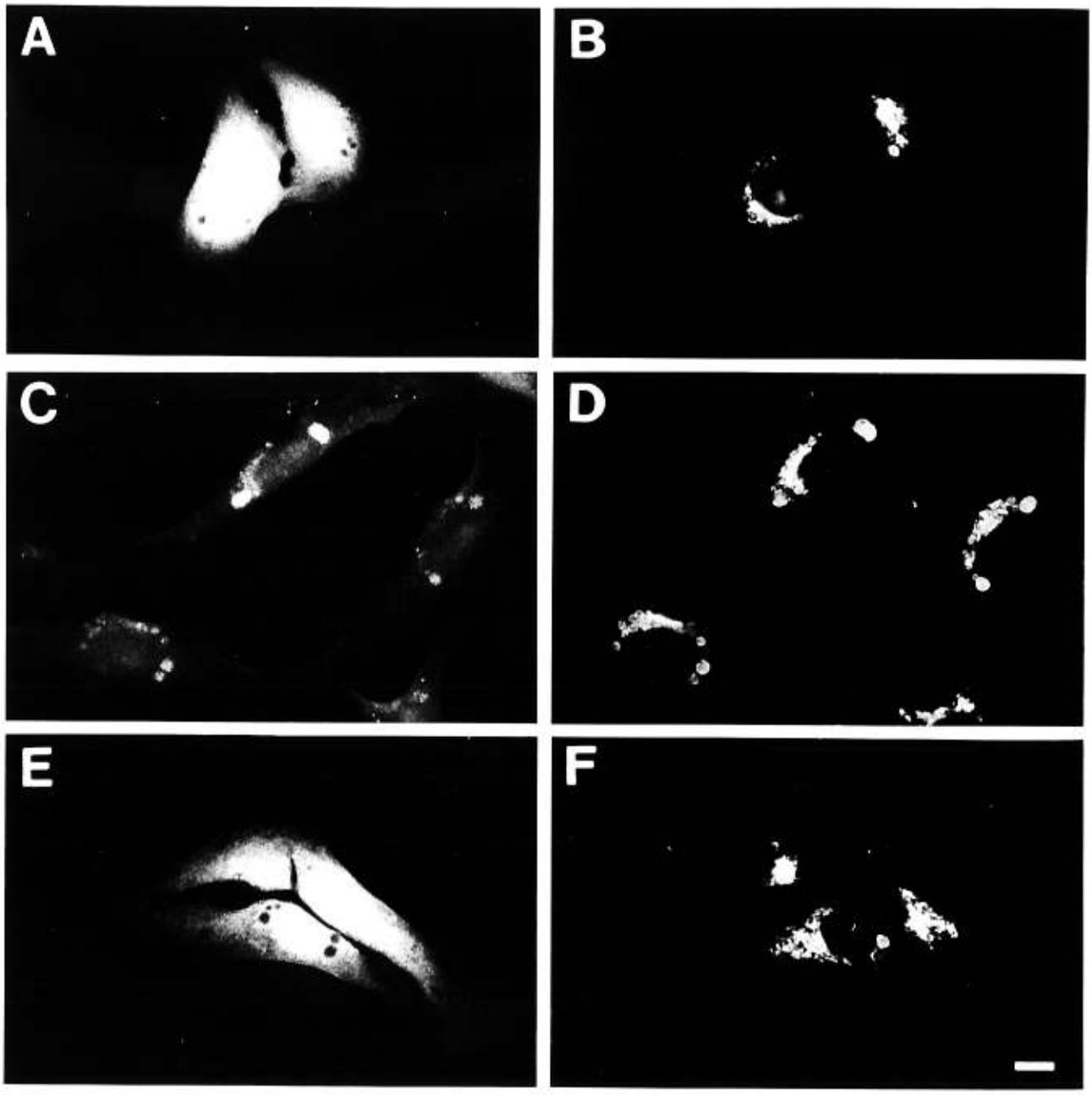
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Figure 12: Autophagy of scrape loaded FITC-dextran in GlcNAc-TV transfected cells. FITC-dextran was scrape-loaded into M9 cells as in Figure 11 and incubated in regular medium (filled bars) or medium supplemented with 10 mM 3-MA (empty bars) for 2, 24, 48 or 72 hours (as indicated) prior to fixation and labeling for LAMP-2 as in Figure 8. 50 FITC-dextran loaded cells per slide were assessed for the presence of FITC labeling in LAMP-2 positive vacuoles. The percent of cells which exhibit FITC labeling of lysosomal vacuoles is presented and represents the average (± S.D.) of 6 experiments.

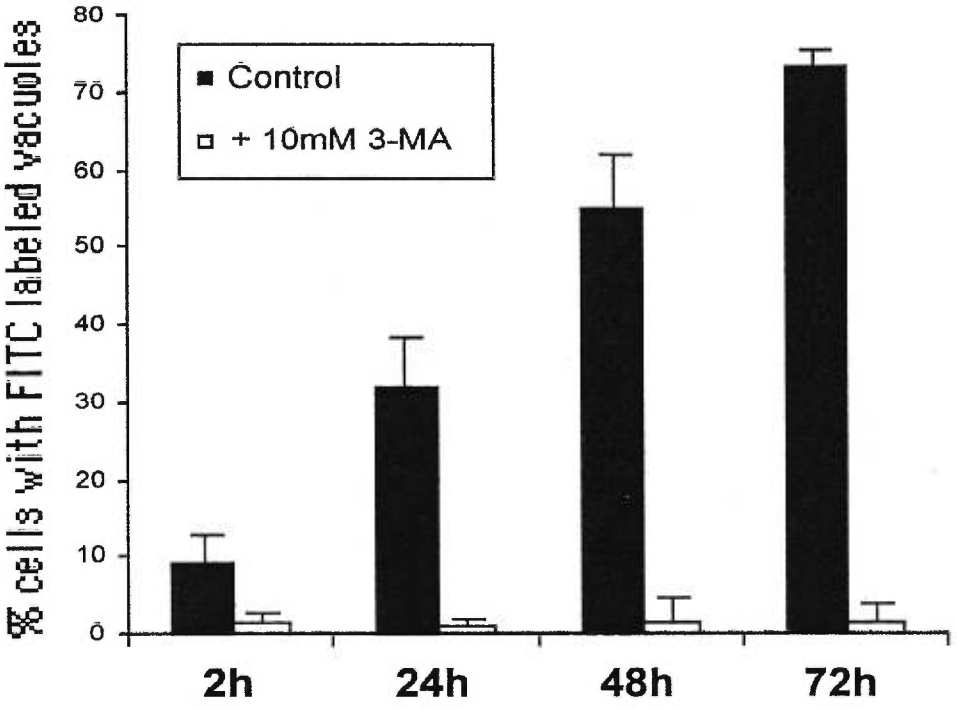
% cells with FITC labeled vacuoles
80
70
60
50
40
30
20
10
0
Control
+ 10mM 3-MA
2h
24h
48h
72h

Table 2: 3-Methyl adenine decreases multilamellar body expression

	Experiment 1	Experiment 2	Experiment 3	Experiment 4
M1 control				
MLBs	5.6 ± 1.1%	8.6 ± 1.7 %	9.1 ± 1.6 %	ND
M1 +3-MA				
MLBs	0.05 ± 0.04 %***	2.1 ± 0.1 %**	0.08 ± 0.06 %***	ND
Inclusion bodies	3.1 ± 1.1 %	2.8 ± 0.6 %	5.5 ± 1.3%	ND
M9 control				
MLBs	13.7 ± 2.1 %	24.4 ± 4.3 %	12.3 ± 2.8 %	11.8 ± 3.5 %
M9 +3-MA				
MLBs	5.2 ± 1.6 %**	10.5 ± 1.5 %*	0.3 ± 0.3%***	2.3 ± 1.4 %*
Inclusion bodies	1.2 ± 0.1%	1.8 ± 0.6 %	1.8 ± 0.5%	0.5 ± 0.1%

GlcNAc-TV transfected M1 and M9 Mv1Lu clones were plated for 6 days in regular medium (control). For the 3-methyl adenine treated cells (+3-MA), the culture medium was supplemented with 10 mM 3-methyladenine for the final three days. The area of MLBs and of cytoplasm was quantified as for Table 1. The area covered by inclusion bodies, morphologically distinguished from MLBs (see Materials and Methods), in the 3-MA treated cells was also measured. Inclusion bodies were not detected in control cells. The significance of the difference in MLB/cytoplasm area between control and 3-MA treated cells is as follows: * $p<0.05$; ** $p<0.01$; *** $p<0.005$. ND: Not determined.

2.5. DISCUSSION

2.5.1. Expression of MLBs in GlcNAc-TV transfected Mv1Lu cells

ß1-6 branching of complex N-linked oligosaccharides is initiated by ß1-6N-acetylglucosaminyltransferase V (GlcNAc-TV) and produces the preferred substrate for ß1-3GlcNAc-T(i), the rate-limiting enzyme implicated in polylactosamine elongation (Holmes *et al.*, 1987; Yousefi *et al.*, 1991). Increased expression of polylactosamine and the associated Lewis and blood-group antigens are carcinoma markers (Fukuda, 1985; Hakamori, 1989). Modified expression of polylactosamine is also associated with cellular differentiation of various cell types (Spillmann and Finne, 1987; Youakim *et al.*, 1989; Amos and Lotan, 1990; Lee *et al.*, 1990; Tuo *et al.*, 1992; Nabi and Rodriguez-Boulan, 1993). The decreasing polylactosamine glycosylation of the lysosomal LAMP glycoproteins in cultured epithelial cells with time in culture is modulated independently of glycosyltransferase activities (Brockhausen *et al.*, 1991; Nabi and Dennis, 1998) and polylactosamine glycosylation has been shown to be regulated by the Golgi residence time of the protein (Wang *et al.*, 1991; Nabi and Rodriguez-Boulan, 1993; Nabi and Dennis, 1998). Increased GlcNAc-TV expression is associated with increased polylactosamine glycosylation in oncogenically transformed and undifferentiated cell lines (Yamashita *et al.*, 1985; Heffernan *et al.*, 1989; Yousefi *et al.*, 1991) indicating that GlcNAc-TV expression levels can regulate the expression of polylactosamine oligosaccharides.

Transfection of the contact-inhibited lung epithelial cell line with GlcNAc-TV resulted in increased expression of L-PHA reactive ß1-6 branched N-glycans and the expression of a partially transformed phenotype including loss of the contact-inhibited phenotype, tumorigenicity in nude mice and an increased propensity to apoptosis (Demetriou *et al.*, 1995). GlcNAc-TV expression in Mv1Lu cells is therefore associated with the expression of early events in cellular transformation. Mv1Lu cells are derived from lung type II alveolar cells, responsible for the elaboration of alveolar surfactant in the lung. Surfactant secretion by type II lung alveolar cells is mediated by MLBs whose presence in

type II cells is a phenotypic characteristic of these cells (Haagman and van Golde, 1991). However, the differentiated type II alveolar phenotype is highly unstable in culture and the expression of MLBs by primary type II cell cultures is maintained for only days after establishment of the cultures (Diglio and Kikkawa, 1977; Dobbs *et al.*, 1985). The ability of GlcNAc-TV transfection to induce the formation of MLBs in a cultured type II alveolar derived cell line identifies a role for protein glycosylation in organelle biogenesis and in the expression of a differentiated phenotype by this lung type II derived alveolar cell line in culture.

The immunofluorescent L-PHA labeling of the large LAMP-2 positive vacuoles localizes ß1-6 branched L-PHA substrates to MLBs. Increased L-PHA reactivity of LAMP-2 was demonstrated in M1 and M9 cells compared to untransfected Mv1Lu cells (Demetriou *et al.*, 1995). In both M9 and M1 cells, LAMP-2 migrates more slowly in SDS-PAGE than in Mv1Lu cells even though M1 cells exhibit increased L-PHA reactivity of LAMP-2 relative to M9 cells, corresponding to their two-fold increased expression of GlcNAc-TV (Demetriou *et al.*, 1995). The basis for the increased MLB expression of M9 cells compared to M1 cells is not clear and the specific aspect of polylactosamine glycosylation that induces MLB formation is not known. Direct demonstration of a role for polylactosamine glycosylation in MLB biogenesis proved difficult as inhibitors of the glycosylation biosynthetic pathway also inhibit the corresponding lysosomal glycosidases thereby preventing autophagic vacuole degradation (Tulsiani and Touster, 1992). Putative ß1-6 branching and polylactosamine glycosylation of MLB glycoproteins might enhance their resistance to degradation by lysosomal proteases or modify interactions between MLB components thereby favoring lamella formation.

2.5.2. Role of lysosomal degradation in MLB biogenesis

The large vacuoles immunofluorescently labeled with antibodies to LAMP-2 are present predominantly in the M9 and M1 GlcNAc-TV transfectants as are morphologically identifiable MLBs by electron microscopy. The fact that no other structure comparable in size to the MLBs is present in the transfected cells

identifies the large fluorescently labeled LAMP-2-positive/L-PHA positive vacuoles as MLBs. Deficiency in lysosomal galactosidases and sialidases is associated with the accumulation of lamellar bodies demonstrating that impaired lysosomal degradation of glycoproteins or glycolipids can be associated with the formation of lamellar bodies (Amano *et al.*, 1983; Alroy *et al.*, 1985; Allegranza *et al.*, 1989; Ohshima *et al.*, 1997).

A definitive role for lysosomal degradation in MLB formation was demonstrated by leupeptin treatment of GlcNAc-TV transfectants (Figures 7 and 8). Over a period of 3-4 days, MLBs are gradually replaced by AV_d implicating leupeptin inhibition of lysosomal proteases in the prevention of de novo formation of MLBs from AV_d. In the absence of new synthesis of MLBs, the disappearance of MLBs could occur via normal turnover mechanisms which may include dilution due to cell division or secretion. Lamellar membrane structures can be visualized in the extracellular space of GlcNAc-TV transfected Mv1Lu cells (Figure 6 D). However, the presence of peripheral dense regions in MLBs 15 hours after addition of leupeptin is also indicative of the fusion of MLBs with endosomes and/or lysosomes whose contents are not transformed into membrane lamellae in the absence of lysosomal degradation. The appearance of heterologous transforming vacuoles following leupeptin treatment suggests that MLBs are continually fusing with lysosomes and that transformation of newly incorporated material into membrane lamellae requires lysosomal degradation. The endocytic pathway has been shown to deliver material to nascent autophagic vacuoles and the autophagic pathway is therefore accessible at early stages (Gordon and Seglen, 1988; Tooze *et al.*, 1990; Liou *et al.*, 1997). Fusion of lysosomes with degradative autophagic vacuoles has also been documented (Ericsson, 1969; Lawrence and Brown, 1992; Yokota *et al.*, 1995). Our data support the idea that heterologous fusion events between lysosomes and MLBs are continually occuring; whether these fusion events represent complete incorporation of the lysosome into the MLB or rather a kiss and run mechanism is not clear (Storrie and Desjardins, 1996).

The transformation of leupeptin-induced AV_d into MLBs following leupeptin washout demonstrates that lysosomal degradation is a critical element in the formation of membrane lamellae. The formation of lamellae within distinct subregions of the autophagic vacuole (Figure 8 D, E) further indicates that localized degradation is responsible for the formation of a microenvironment propicious for lamellae formation. In GlcNAc-TV transfected Mv1Lu cells, ß1-6 branching of N-glycans of LAMPs and possibly other as yet unidentified MLB glycoproteins therefore generates a lipid-protein mix which is conducive to the formation of membrane lamellae within the degradative lysosomal environment of the AV_d. Continuing lysosome fusion could generate large lysosomal organelles whose contents cannot be degraded by lysosomal hydrolases resulting in the formation of a residual body of lysosomal degradation or a MLB. However, the ability to inhibit MLB formation with 3-MA, a specific inhibitor of autophagy, demonstrates that in the cell system studied here, lysosome fusion with autophagic vacuoles is necessarily involved in MLB biogenesis.

2.5.3. Biogenesis of MLBs via autophagy

A specific role for autophagic sequestration in MLB biogenesis was demonstrated by the ability of 3-MA to prevent the formation of MLBs in GlcNAc-TV transfected cells (Figure 9; Table 2). 3-MA treatment is associated with increased lysosomal pH and decreased lysosomal density and with inhibition of late endosome to lysosome transport (Caro *et al.*, 1988; Punnonen *et al.*, 1994) which may explain the slight enlargement of LAMP-2 positive structures in both GlcNAc-TV transfected and untransfected Mv1Lu cells (Figure 9 B, D). Nevertheless, 3-MA treatment of GlcNAc-TV transfectants results in the disappearance of large LAMP-2 labeled vacuoles corresponding to MLBs as well as the significant reduction in morphologically identifiable MLBs by electron microscopy. Inhibition of autophagy with 3-MA is therefore generally associated with the disappearance of MLBs. 3-MA treatment results in the accumulation of inclusion bodies which morphologically resemble AV_i demonstrating that 3-MA is blocking autophagy in the GlcNAc-TV transfectants at an early stage of

autophagic vacuole biogenesis. In the hepatocyte, 3-MA blocks the initial sequestration event in autophagic vacuole biogenesis and is associated with an approximate two-fold reduction in autophagic sequestration (Kopitz *et al.*, 1990; Seglen and Bohley, 1992) which is consistent with the reduction (between 40% and 83%) in cytoplasmic area covered by both MLBs and inclusion bodies in 3-MA treated M1 and M9 cells observed here (Table 2).

The demonstration that cytoplasmic FITC-dextran can be incorporated into the LAMP-2 positive MLBs provides a direct illustration that autophagic sequestration is involved in MLB biogenesis (Figure 11). The fact that this sequestration process is inhibited by 3-MA clearly shows that 3-MA is inhibiting autophagy in these cells and that inhibition of autophagy is directly responsible for the decreased expression of MLBs in 3-MA treated cells. A similar approach has been recently been used to demonstrate that the parasitophorous vacuoles of *Leishmania mexicana* acquire cytosolic material via autophagy and, in a similar fashion to our results, this process is inhibited by 3-MA (Schaible *et al.*, 1999). The role of autophagic vacuole biogenesis in MLB formation implicates autophagy not only in the cellular response to stress but also in a normal cellular function, MLB formation and surfactant secretion by the lung type II alveolar cell. The necessary role of autophagy in MLB biogenesis in Mv1Lu cells suggests that autophagy and autophagic vacuole maturation are involved in MLB biogenesis in multiple cell types. If so, our data may have significant implications for the mechanism of MLB accumulation in lysosomal storage diseases.

The formation of the multiple membrane lamella of the MLB requires a vast amount of cellular lipids and autophagy may constitute the most efficient means of accumulating the necessary molecules within a single organelle. Select resistance of the contents of the autophagic vacuole to lysosomal degradation, possibly due to ß1-6 branched N-glycans in this GlcNAc-TV transfected cellular model and to other mechanisms in various cell types and pathological states, results in the localized formation of membrane lamellae which give rise to the concentric membrane whorls of the MLB.

2.6. ACKNOWLEDGEMENTS

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3. SUPPLEMENTARY RESULTS

3.1. MATHERIAL AND METHODS

3.1.1. Antibody

Polyclonal rabbit anti-sheep SP-A was kindly provided by Dr. Jo Rae Wright, Dept. of Cell Biology, Duke University Medical Centre, which was used at the concentration of 1/100 for fluorescence and 1/250 for western blot. The antibody was revealed by Texas Red and HRP conjugated anti-rabbit secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA).

3.1.2 Fluorescence microscopy

Cells were rinsed with PBS/CM and fixed with 3% paraformaldehyde. All the procedure is the same as explained in Materials and Methods of the article (page 31).

3.1.3. Preparation of lysates

Lung tissue was kindly provided by Dr. Mark Pelletier, homogenized with homogenization buffer (130mM NaCl, 1mM Tris, 5mK KCl, 1mM $MgCl_2$, 2mM EDTA, pH 7.4) containing protease inhibitors (1mM PMSF, 10mg/ml leupeptin, pepstatin A and aprotinin, and 1mM PNGB) at 4°C using a Dounce homogenizer for 50 strokes. The homogenate was passed through a 22G needle to shear DNA.

Cells were washed and rinsed with cold PBS, detached from the plates by scraping and collected by centrifugation for 2 min. at 1600 rpm. The pellet was resuspend in lysis buffer consisting of PBS containing 1% SDS, 1mM EDTA and protease inhibitors (1mM PMSF, 10mg/ml leupeptin, pepstatin A and aprotinin, and 1mM PNGB) at 4°C. After vortexing and sonication, the solution was centrifuged at 15000 rpm for 10 min.

3.1.4. Western Blotting

Protein content of the lysates was determined using the BCA assay (Pierce, Rockford, Illinoise, USA) and 75 μg of protein was loaded onto the SDS-PAGE gel and transferred to nitrocellulose blotting paper. Nitrocellulose blots were

blocked with 5% milk powder in PBS and incubated with primary antibody to SP-A, followed by anti-rabbit secondary antibodies coupled to horseradish peroxidase (Jackson, West grove, Pennsylvania) and visualized by chemiluminescence (ECL, Amesterdam, Oakville, Ontario) using Kodak XRP X-ray film.

3.2. RESULTS

3.2.1. Mv1Lu cells express SP-A

GlcNAc-TV transfected Mv1Lu cells express MLBs. MLBs are the organelles responsible for storage and secretion of surfactant (1, 2, 4, 59, 81). Mv1Lu transfected cells may therefore be an excellent model of alveolar type II cells in culture. In order to investigate whether Mv1Lu transfected cells express surfactant associated proteins, and due to the unavailability of antibodies against other surfactant proteins, we used a rabbit polyclonal antibody against sheep SP-A. By immunofluorescence, labeling of GlcNAc-TV transfected Mv1Lu cells with anti-SP-A was detected indicative of the expression of SP-A in Mv1Lu cells (Fig.10). In order to confirm this fluorescent microscopy observation, western blots were also performed with anti-SP-A. To demonstrate the specificity of the antibody, we also probed tissues derived from mink and rat lung. The rabbit anti-sheep SP-A antibody recognized SP-A in both rat and mink lung, demonstrating that this antibody recognizes mink SP-A. By western blot, the appearance of a faint band of approximately 30 kDa confirmed the expression of SP-A in untransfected and GlcNAc-TV transfected Mv1Lu cells (Fig. 11). It is obvious that there is greater expression in lung tissue, which is probably due to the larger number of type II cells that contain surfactant, and the greater production, and accumulation of surfactant in tissue compared to cells in culture. The presence of a band for SP-A in Mv1Lu cells (nontransfected cells) may be due to the storage of SP-A outside of MLBs.

Figure 13: Expression of SP-A in fluorescent microscopy analysis: M9 cells were immunofluorescently labeled with anti-LAMP-2 (A) and anti-SP-A (B). LAMP-2 positive vacuoles representing MLBs (A) are not labeled with anti-SP-A, showing that SP-A is not present in MLBs.

Bar = 10 μm.

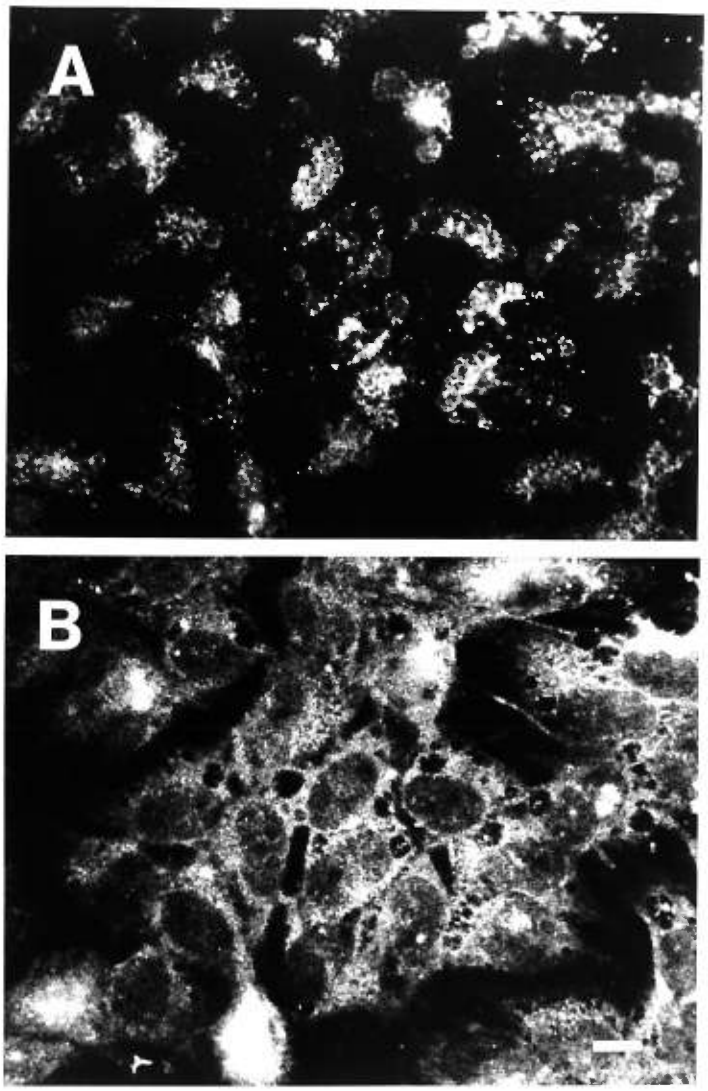
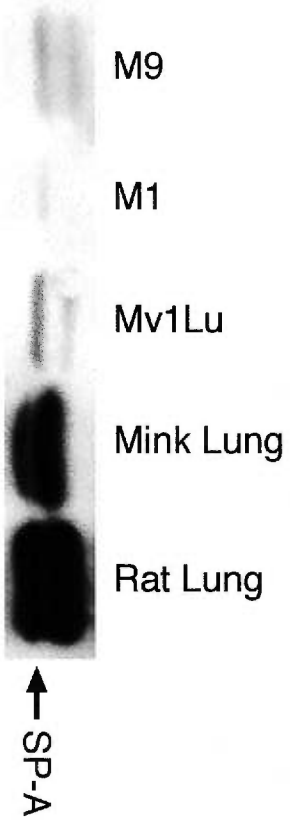

A
B

Figure 14: Expression of SP-A in cell lines and lung tissue: Western blotting of lysates from mink and rat lung, untransfected Mv1Lu cells and GlcNAc-TV transfected Mv1Lu cells (M1, M9). The band of approximately 32 kDa. corresponds to SP-A. Significantly increased expression of SP-A was found in lung tissue compared to the cell lines.


M9
M1
Mv1Lu
Mink Lung
Rat Lung
SP-A

4. DISCUSSION

4.1. GlcNAc-TV TRANSFECTED Mv1Lu CELLS AS A MODEL FOR TYPE II ALVEOLAR CELLS IN VITRO

4.1.1. GlcNAc transfection of Mv1Lu cells :

Mv1Lu is an immortalized cell line derived from mink lung. This cell line was transfected with GlcNAc-TV, the enzyme responsible for initiation of polylactosamine glycosylation. After transfection, there was an increase of about 2.5-18 times of GlcNAc-TV activity, which correlated with increased β1-6GlcNAc branching of oligosaccharides of LAMP-2 proteins, and increased expression of L-PHA reactive β1 branched N-glycans [14]. Transfection of the contact-inhibited lung epithelial cell line with GlcNAc-TV resulted in the expression of a partially transformed phenotype including loss of the contact-inhibited phenotype, tumorigenicity in nude mice, reduced substratum adhesion, motility, and an increase propensity to apoptosis [14]. The amazing fact about these cells is that they maintain the morphological character of type II cells. They contain lots of multilamellar bodies in culture, which resemble the same organelles in isolated alveolar type II cells from lung.

4.1.2. Transfected Mv1lu cells as a model for type II cells:

In vitro studies of type II alveolar cells and secretion of surfactant is limited because type II cells in culture rapidly lose their cell type specific characteristics or functional differentiation [18, 44, 56, 64, 70, 82, 88]. Characteristics like visible MLBs and a cuboidal shape disappear after 3 to 4 days in culture. Disappearance of type II cells is associated with the progressive appearance of type I cells, and so it is thought that type II cells may be the progenitor of type I cells [70]. MLBs easily disappear in type II cells after a short time in culture. The incorporation of radiolabeled palmitate and acetate incorporated into PC and PG, the major phospholipids of surfactant, falls as early as 1 day in culture [19].

A lot of attempts have been made to maintain the morphological and biological differentiation in primary cells. Most of the efforts have focused on the matrix and conditioned medium of primary cultures of type II cells [44, 64]. Kawasa et al. [44] developed a serum-free, hormonally defined medium for maintenance of a more differentiated phenotype of type II cells reflected in the relative increase of incorporation radiolabeled acetate into PG, and PC, and the expression of MLBs. Runnels et al. [64] showed that a matrix rich in fibronectin maintains the morphological characteristics of type II cells in culture. Despite all these efforts, few of them can be considered as a real model for type II cells maintaining the morphologic and functional characteristics of type II cells and none of them has reached an immortalized cell line.

GlcNAc-TV transfected Mv1Lu type II cells, maintain morphological characteristics of alveolar type II cells in situ such as a rounded apical surface covered by short microvilli, a cytoplasm rich in mitochondria, and rough and smooth reticulum endoplasmic, and the expression of multilamellar bodies (MLBs), which contain the base material for lung surfactant. GlcNAc-TV transfected Mv1Lu cells represent the first immortalized type II alveolar cell line which exhibits stable expression of MLBs. How MLBs are stabilized in these cells is not really clear, but their expression following GlcNAc-TV transfection suggests that polylactosamine glycosylation has some role in this process.

In order to evaluate the utility of Mv1Lu cells as a model of type II cells, the most important characteristic of type II cells, which should be investigated is the secretion of surfactant. Alveolar type II cells have a unique ability to synthesize a profile of phospholipids, which are major components of surfactant. Investigation of the production of phospholipids by these cells is therefore one of the tools that can be used to evaluate the value of Mv1Lu cells as a model of lung type II cells. This can be done by incorporation of radiolabeled precursors such as [^{3}H] acetate or [^{14}C] glycerol, into phospholipids [53, 70]. In addition to phospholipids, investigation of the expression of surfactant associated proteins is of importance. Due to the unavailability of antibodies against SP-B and SP-C, we could not study

the expression of these proteins, but the presence of SP-A in Mv1Lu cells was confirmed by both fluorescence microscopy and by immunoblotting (Fig.13, 14). Low levels of SP-A in cultured cells compared to real tissue may be due to the greater ability of type II cells in situ to secrete surfactant due to hormonal effects or other factors that are absent in cell culture. By fluorescent microscopy, the large LAMP-2 labeled vacuoles are not labeled for SP-A suggesting that there is no SP-A in MLBs (Fig 10). However, isolation and purification of MLBs from Mv1Lu cells and analysis of SP-A expression by western blotting are necessary to confirm this idea. Whether SP-A is present in MLBs remains controversial [66], but its secretion from type II cells is certain. It seems that the expression of SP-A by Mv1Lu cells indicates that they may have retained differentiated characteristics of type II alveolar cells, and can be considered as a model of type II cells.

The expression of SP-B and SP-C, in Mv1Lu cells could also be investigated by immunofluorescence and western blotting. Since Mv1Lu cells contain MLBs, it is possible that they secrete SP-B and SP-C. It has been shown that knockout of the unglycosylated SP-B disrupts the formation of MLBs in transgenic mice [11].

In order to determine whether the MLBs of Mv1Lu cells resemble those of the type II cells, the presence of enzymes like acid phosphatase, aryl sulfatase B, β-glucuronidase, β-N-acetylglucosaminidase, and esterase whose existence has been demonstrated in MLBs of type II cells [17, 38, 70], can be investigated. It has been shown that there is some alteration, increase or decrease, in the level of these enzymes with time in culture [79]. Considering the fact that MLBs of GlcNAc-TV transfected Mv1Lu cells have lysosomal character (they express LAMP-2 proteins on their membrane), it is probable that MLBs in Mv1Lu cells also contain these enzymes. Levels of these enzymes in MLBs from GlcNAc-TV transfected Mv1Lu cells compared to MLBs from isolated alveolar type II cells, is worth investigating.

4.1.3. Is the expression of MLBs a result of transformation or increased glycosylation?

Transfection of Mv1Lu cells with GlcNAc-TV resulted in an increase of GlcNac-TV activity and the expression of a partially transformed phenotype, which is tumorigenic in nude mice [14]. A question that remains to be addressed is whether the expression of MLBs in GlcNAc-TV transfected Mv1Lu cells is related to the expression of the characteristics of precancerous cells, or rather to increased polylactosamine glycosylation in these cells.

The level of polylactosamine glycosylation and the level of expression of MLBs is relatively correlated (table 1). In R2 cells that express low level of GlcNAc-TV activity, the area of cytoplasm covered by MLBs is very low. In M9 cells, there is a greater area of cytoplasm covered by MLBs compared to M1, although the level of GlcNAc-TV expression is higher in M1 cells. Interestingly the number of MLBs is higher in M1 cells than in M9 cells. This correlation suggest that polylactosamine glycosylation might have a role in the expression of MLBs. It is known that modified expression of polylactosamine is associated with cellular differentiation of various cell types [58], and also that increased GlcNAc-TV expression is associated with increased polylactosamine glycosylation in oncogenically transformed and undifferentiated cell lines [101]. Furthermore, glycoproteins of the inner surface of lysosomes have been proposed to protect the lysosomal membrane from the contents of lysosomes, and it has been suggested that polylactosamine glycosylation of LAMPs has a role in the resistance of these proteins to lysosomal degradation [30, 48]. Since MLBs have lysosomal character and express LAMP, it may be concluded that polylactosamine glycosylation of MLB glycoproteins may increase their resistance to degradation by lysosomal proteases. In order to determine the role of glycosylation in stabilizing MLBs, blocking agents of glycosylation like swainsonine, castanospermine, deoxymannojirimycine, could be tested. However, since these drugs are also inhibitors of some lysosomal hydrolases, their possible inhibitory effects on the formation of MLBs may be due to the prevention of the maturation of AVs and

not to inhibition of glycosylation [54]. We were therefore, unable to present definitive evidence for the role of glycosylation in MLB formation, and furthere experimentation is required.

On the other hand, GlcNAc-TV expression in Mv1Lu cells is associated with the expression of early events of cellular transformation [14]. There is some evidence which indicates the existence of MLBs in tumor cells [24, 99]. From a benign tumor of human lung, a papillary adenoma of type II pneumocytes, Yamamoto et al. [99] found homogenous dense granules, as well as a number of multilamellar bodies, in the cytoplasm of these cells. They also observed that various transitional forms from dense granules to lamellar bodies, were present in these cells [99]. Based on what has been observed there is a possibility that the expression of MLBs in GlcNAc-TV transfected cells may be due to cellular transformation.

4.1.4. Is the expression of MLBs following transfection with GlcNAc-TV unique for Mv1Lu cells?

In order to see whether the effect of transfection with GlcNAc-TV on cultured cells is unique for type II cells, other cell lines that usually express MLBs in vivo should be transfected. The expression of GlcNAc-TV in the mouse embryo was examined by in situ RNA hybridization. mRNA transcripts of GlcNAc-TV were expressed throughout the E9.5 mouse embryo. GlcNAc-TV transcripts were also expressed prominently in the bronchial epithelium [35]. But there is no evidence, based on my knowledge, of the transfection of any other cells with GlcNAc-TV. Transfection of those cells that express MLBs in normal conditions (other than lung type II cells), such as cornified layer of skin epidermis, oral epithelium, mucosa cell of nose, mesodermal cell layers of joints, with GlcNAc-TV would determine whether the expression of MLBs is unique for lung type II alveolar derived Mv1Lu cells or a general consequence of transfection with GlcNAc-TV.

4.2. LYSOSOMAL DEGRADATION AND AUTOPHAGY IN MLB BIOGENESIS

4.2.1. The Role of Protein Degradation in the Formation of MLBs

We treated Mv1Lu transfected cells with leupeptin, which is known as a protease inhibitor [31, 41, 49, 85, 100] . After treatment of the cells with leupeptin for different times, the gradual transformation of MLBs to AVs in transfected cells was observed (Fig. 4). The appearance of heterologous transforming vacuoles suggests that MLBs are continually fusing with lysosomes, and that the transformation of newly incorporated material into membrane lamellae requires lysosomal degradation. Interestingly, when we washed out leupeptin from the medium, leupeptin-induced AVd transformed into MLBs (Fig.5). This observation demonstrated the essential role of protein degradation in the formation of MLBs.

4.2.2. The role of autophagy in MLBs formation

It has been shown that 3-methyladenine (3-MA) inhibits autophagy at the initial sequestration step [100]. 3-MA treatment of transfected Mv1Lu cells resulted in the disappearance of large vacuoles labeled with LAMP-2 (corresponding to MLBs). According to Seglen [73], 3-MA caused a two-fold reduction in autophagic sequestration. This is consistence with what we observed in electron microscopy, in which there was a significant reduction (not complete disappearance) in the number of MLBs (Table 2). Treatment with 3-MA also resulted in accumulation of inclusion bodies resemble AVi, confirming that 3-MA is blocking autophagy at the initial sequestration step in these cells (Fig.7).

Incorporation of FITC-dextran into the LAMP-2 positive MLBs, and inhibition of incorporation after 3-MA treatment, confirmed the role of 3-MA in blocking autophagy. These results together demonstrated that 3-MA blocks autophagy and also blocks the formation of MLBs, identifying a role for autophagy in the formation of MLBs.

Based on our data a role for autophagy has been described in the biogenesis of an organelle. This means that autophagy is not just implicated in response to cellular stress, but is also involved in a normal cellular function. It would be therefore, of interest to study the possible involvement of autophagy in the secretion of surfactant. Mv1Lu cells are excellent model for studying autophagy and all the organelles that are involved in this process, like AVi, AVd, lysosomes and MLBs.

4.3. LYSOSOMAL STORAGE DISEASES

The knowledge of biogenesis of an organelle is a great help in understanding of the physiopathology of the disorders related to that organelle.

Lysosomal storage diseases (LSDs) are genetically determined metabolic diseases characterized by dysmorphology and dysfunction of the lysosomal system. The ethiology of the diseases can be categorized as follows: 1. the deficiency of a lysosomal enzyme; 2. the deficiency of a protein assisting one or more lysosomal enzymes in their catalytic function by activation and/or stabilization, or by substrate presentation; 3. the deficiency or dysfunction of a lysosomal membrane carrier protein essential for the export of degradation products from the lysosomal interior to the cytoplasm; 4. the defective targeting of lysosomal proteins to lysosomes [65]. These defects cause different types of diseases, which can be categorized based on the pathway affected, and the nature of the accumulated substrate. For instance deficiencies which affect the degradation of mucopolysaccharides are called mucopolyssacharidoses, and in the same manner, glycogenesis, and lipidoses [32]. At the cellular level lysosomes are enlarged, sometimes with abnormal shape, and in some cases like Niemann-Pick and galactosialidosis an accumulation of MLBs is observed. A better understanding of the degradation process, and also the process of formation of MLBs in pathologic condition would be a great help in improving the efforts to treat LSDs. Involvement of autophagy might provide new approaches to treatment of these diseases.

In LSDs there are defects in the degradative system and an accumulation of lamellar bodies. Accumulation of N-acetyllactosamine repeating units in liver glycopeptide fractions in Gaucher's disease has been shown as well as an accumulation of undegraded glucocerebroside within the lysosomes [13]. In galactosialidosis there is accumulation of lamellar bodies at the cellular level which is accompanied with the increased expression of modified oligosaccharides including polylactosamine [2, 60]. Accumulation of polylactosamine and MLBs in some LSDs and the presence of MLBs in GlcNAc-TV transfected Mv1Lu cells suggests that the mechanism of formation of MLBs in the two cases may be similar.

The possible involvement of autophagy and polylactosamine in accumulation of MLBs in these diseases may open new roads to treatment of LSDs using inhibitors of autophagy and polylactosamine. It should be however noted that the accumulation of MLBs is not always accompanied with polylactosamine accumulation. Niemann-Pick diseases, which is a sphingomyelincholesterol lipidosis, is characterized by excessive foam cell formation and accumulation of MLBs. In type I disease there is a deficiency in lysosomal sphingomyelinase, which degrades sphingomyelin to ceramide and phosphocholine. Type II Niemann-Pick disease is not related to a primary deficiency of lysosomal sphingomyelinase, but it has been shown that LDL-cholestrol was abnormally sequestered in mutant fibroblasts, which resulted in the excessive accumulation of lamellar bodies [71]. Nevertheless, the role of autophagy in the accumulation of MLBs in Niemann-Pick disease, and other LSDs is worth defining.

4. CONCLUSION

GlcNAc-TV is the enzyme responsible for initiation of polylactosamine glycosylation. Transfection of Mv1Lu cells, mink lung type II epithelial cells, with GlcNAc-TV, resulted in the formation of multilamellar bodies. Formation of MLBs in GlcNAc-TV transfected Mv1Lu cells implicates protein glycosylation in organelle biogenesis. Using this cellular model, protein degradation and autophagy were shown to be involved in the formation of MLBs. The type II origin of Mv1Lu cells suggests that GlcNAc-TV transfected Mv1Lu cells are a potential immortalized cell model for surfactant synthesis and secretion. However, the relevance of our data on MLB formation to surfactant secretion by lung type II alveolar cells or to MLB accumulation in LSDs remains to be determined.

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