Research Article

Nucleofection of Rat Pheochromocytoma PC-12 Cells with Human Mutated Beta-Amyloid Precursor Protein Gene (APP-sw) Leads to Reduced Viability, Autophagy-Like Process, and Increased Expression and Secretion of Beta Amyloid

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Pheochromocytoma PC-12 cells are immune to physiological stimuli directed to evoke programmed cell death. Besides, metabolic inhibitors are incapable of sensitizing PC-12 cells to extrinsic or intrinsic apoptosis unless they are used in toxic concentrations. Surprisingly, these cells become receptive to cell deletion after human APP-sw gene expression. We observed reduced cell viability in GFP vector + APP-sw-nucleofected cells (drop by 36%) but not in GFP vector − or GFP vector + APP-wt-nucleofected cells. Lower viability was accompanied by higher expression of Aβ1-16 and elevated secretion of Aβ1-40 (in average 53.58 pg/mL). At the ultrastructural level autophagy-like process was demonstrated to occur in APP-sw-nucleofected cells with numerous autophagosomes and multivesicular bodies but without autolysosomes. Human APP-sw gene is harmful to PC-12 cells and cells are additionally driven to incomplete autophagy-like process. When stimulated by TRAIL or nystatin, CLU protein expression accompanies early phase of autophagy.

1. Introduction

Pheochromocytoma (PCC) is a rare neuroendocrine tumor located in adrenal medulla which secretes massive quantities of catecholamines with malignant hypertension as the fatal outcome. The treatment of choice is surgery associated with high-risk complications (refractory hypertension). In laboratory conditions, pheochromocytoma cells undergo differentiation to neural cells upon treatment with nerve growth factor (NGF) [1]. Alternatively, pheochromocytoma cells could be manipulated with selected gene insertions/silencing leading to additional phenotypic modifications (neural) that cease disproportionate endocrine activity. Accordingly, rat pheochromocytoma cells (PC-12 cell line) are frequently used in in vitro studies as a cellular model of neurodegenerative diseases. Alzheimer’s disease (AD) is the most prevalent neurodegenerative disease. The hallmark is the extracellular deposition beta amyloid (Aβ) accompanied by dementia and progressive loss of cognitive processes. Familial form (early onset associated with mutations in beta-amyloid precursor protein gene, APP) is rare, whereas sporadic form prevails (late onset, 95% of cases). Notably, the molecular mechanisms of neuronal decay in hippocampus and prefrontal cortex in AD remain ambiguous despite extensive studies carried out for more than a century [2]. Formation of Aβ is associated with the activity of membrane bound γ-secretase complex including catalytic role of presenilins (PS-1 and PS-2). This complex attacks remaining of APP just after proteolytic α-secretase (nonfibrillogenic) or β-secretase (fibrillogenic) processing. Shorter isoforms of APP (695 amino acids and less) are specific substrates for α-secretase (ADAM10) while others (751 amino acids and more) are specific for β-secretase.
(BACE1). Protein products of mutated APP gene (amino acid substitutions) are main targets of BACE1 regardless of APP length [3]. Key players in Aβ formation are located in lipid rafts (LR), nanodomains formed by deposition of cholesterol in membrane lipid bilayers [4, 5]. Lipid rafts are cogitate platforms for several signaling pathways including death receptor ligands (DRL) widely known in etiology of AD [6]. Brain is unique in both high self-sufficient cholesterol (CHOL) metabolism, macroautophagy (autophagy), and DRL (TNF-α, TRAIL) secretion by microglia [7] as either process is independent from peripheral regulation. Thus, autophagy in neurons is constitutive rather than cellular adaptation to nutritional stress as brain is specially protected tissue with constant supply of nutrients even under starvation [8]. Aging is known to differently affect brain CHOL metabolism, autophagy, and DRL activity. Age-dependent onset of neurodegenerative diseases most likely correlates with age-dependent increase of brain cholesterol and DRL secretion [9] as well as age-dependent decline of autophagic activity. The former is associated with altered CHOL metabolism and the latter with unknown changes in control of autophagy and DRL [10]. With the progress of aging, the average concentrations of DRL become elevated [11]. Clusterin protein (CLU) is the rare extracellular ATP-independent chaperone present in both body fluids and tissues where it acts either as cytoprotective (glycosylated-secreted form) or cytotoxic (unglycosylated-intracellular form) protein [12–15]. CLU is upregulated in the brains of individuals affected by neurodegenerative diseases [16, 17], via most likely outcome of ER stress-induced calcium depletion and debilitated N-glycosylation resulted in intracellular accumulation of N-glycan deficient proteins [18]. Although neurodegeneration accompanying cell death mechanism (apoptosis, necrosis, necroptosis (aponecrosis), paraptosis (cell death type 3), or autophagic cell death) is still a matter of debate, ER stress and unfolded protein response (UPR) are frequently associated with impaired autophagy [19, 20].

Autophagy starts when isolation double membrane bilayer (phagophore probably starts off in endoplasmic reticulum) appears in the cytosol, where it engulfs cytoplasmic constituents including organelles. Elongation of the isolation membrane around the region of cytoplasm and closure of the inner and outer bilayers of the isolation membrane leads to formation of double membrane autophagosome. To digest the contents, autophagosome must fuse with lysosome to create autolysosome. Before fusion with lysosome, it may elongate of the isolation membrane and closure of the inner and outer bilayers of the isolation membrane leads to formation of double membrane autophagosome. To digest the contents, autophagosome must fuse with lysosome to create autolysosome. Before fusion with lysosome, it may elongate or both, lack of growth factor signals, oxygen debt, buildup of damaged proteins, or endoplasmic reticulum stress [25]. The fundamental task in cellular homeostasis is played by lysosomes. Whatever, macroautophagy, mitophagy, or chaperone-mediated autophagy the final common path is lysosomal digestion of sequestered substrates. Obviously, lysosome is the target and stand organelle for primary regulator (mTOR complex I, TORC1) which antagonizes release of amino acids. When TORC1 becomes inactive, the lysosomes encourage autophagy through control of substrate sequestration. Although regulatory network of autophagy is complex and far from complete, the outline of basic processes at the molecular level is generally well known.

In this experiment, we hypothesized that mutated APP gene (APP-sw) might be helpful in making pheochromocytoma cells less viable and more susceptible to cell death triggering mechanisms. Foundations of assumption were based on results acquired from former study pointing to higher mortality of PC-12 cells transfected with the mutated APP gene [26–28]. Nevertheless, up to date the research was forced to study apoptosis and/or necrosis. Our preliminary observations with transmission electron microscopy revealed that PC-12 cells nucleofected with mutated APP-sw gene show symptoms of extensive autophagy-like process which is frequently observed in neurodegenerative diseases. The novelty of this study is that it put concern on the process of autophagy widely believed to be survival mechanism, even though its anomalous course leads to cell deletion (cell death type 2). As APP processing is often located in lipid rafts reliant on cholesterol, we tested statins and cholesterol chelator MβCD. Additionally, pheochromocytoma cells are immune to death receptor ligands [29] so alternate use of TNF-α and TRAIL was justified with regard to cell viability, APP processing, and molecular markers of autophagy. Intracellular CLU expression was monitored to check if the protein is accumulated in transgene-bearing cells.
Overall, this study should shed more light on the molecular mechanisms of cell death related to tumor cell differentiation and changes observed in cellular models of neurodegenerative diseases.

2. Materials and Methods

2.1. Materials. Media (Dulbecco’s modified Eagle’s medium (DMEM) low glucose (5.5 mM), F-12K medium, Kaighn’s Modification of Ham’s F-12 medium with Glutamax), PBS (including Ca\(^{2+}\) and Mg\(^{2+}\)), antibiotics, heat inactivated sera (fetal bovine serum (FBS) and horse serum (HS)) were purchased from Gibco Life Technologies (Grand Island, NY, USA). Nerve growth factor (NGF), tumor necrosis factor alpha (TNF-\(\alpha\)), and tumor necrosis factor alpha-related apoptosis inducing ligand (TRAIL) (Sigma Aldrich Chemical Co., St. Louis, MO, USA) were dissolved according to manufacturer. Metabolic inhibitors (atorvastatin: ATOR, simvastatin: SIM, nystatin: NY, methyl-\beta-\(\alpha\)-cyclodextrin: M\(\beta\)CD, rapamycin: RAPA, and chloroquine: CHLOR) if necessary were dissolved in DMSO. All other reagents were cell culture tested, of high purity, and unless otherwise stated they were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Plastics, including BioCoat Collagen IV coated Cellware, were from Becton Dickinson (BD Biosciences, Franklin Lakes, NJ, USA) and tubes for deep freezing from Nunclon (Nunc, Roskilde, Denmark) while syringe filters were purchased from Corning-Costar Inc. (Cambridge, MA, USA).

2.2. Rat Pheochromocytoma PC-12 Cell Cultures and Treatments. The rat PC-12 cell line was obtained from European Collection of Animal Cell Cultures (ECACC). Cells were initially suspended in growth media (GM) containing DMEM with Glutamax supplemented with 10% (v/v) fetal bovine serum (FBS) + 5% (v/v) horse serum (HS), pen : strep (penicillin : streptomycin solution, 50 IU/mL/50 \(\mu\)g/mL), gentamicin sulfate 20 \(\mu\)g/mL, and Fungizone (amphotericin B, 1 \(\mu\)g/mL) and plated onto plastic noncoated culture flasks. G-418 (400 \(\mu\)g/mL) and ampicillin (100 \(\mu\)g/mL) were additionally present in GM for growth and selection of positively transfected PC-12 cells. They were cultured at 37°C in a humidified 5% CO\(_2\) and 95% air in incubator. After reaching 70–80% confluence, PC-12 cell suspensions were subcultured, and the same quantity of cells was seeded onto 60 mm Petri dishes, 96-multiwell plates (Becton Dickinson, BD Biosciences, Franklin Lakes, NJ, USA) depending on the experimental protocol. For differentiating and differentiation states when PC-12 cells reached 80% confluence, growth medium was switched to differentiation medium (DM) containing DMEM with Glutamax supplemented with 4% (v/v) fetal bovine serum (FBS) + 2% (v/v) horse serum (HS) + NGF (50 ng/mL) and the same antibiotic: antimycotic mixture. Cells were allowed to differentiate for 48 h and the DM was replaced by freshly prepared serum-free reference medium (RM) containing 0.1% BSA (w/v) + NGF (50 ng/mL) with or without experimental factors for another 48 h (48 h/48 h). When the experimental factors were dissolved in DMSO, the equivalent volume of vehicle (0.1% v/v) was added to the control cells. If necessary the same quantity of differentiated cells with neural phenotype was removed from the culture plates using 0.5% (w/v) trypsin-EDTA (harvesting), centrifuged in DM at 200 ×g for 5 min, media were aspirated, and cell pellets were resuspended in RM. Media were changed every other day. Cell monolayers were harvested for Western blots or PCR. Floating dead cells were removed during media change or washed with PBS and were not included in these experiments.

2.3. Cloning and Expression Vectors of the Homo sapiens Beta-Amyloid Precursor Protein Gene 1-695 (Transcript Variant 3, APP-wt), Swedish Mutation in Homo sapiens Beta-Amyloid Precursor Protein Gene APP-KM670/671NL Double Mutation in the APP Gene Resulting in Amino Acid Substitutions of Lys to Asn (Codon 670) and Met to Leu (671), and Precision Shuttle Mammalian Vector with C-Terminal Tag GFP (pCMV6-ACGFP). Cloning and expression of the Homo sapiens beta-amyloid precursor protein gene 1-695 (transcript variant 3, APP-wt), Swedish mutation in Homo sapiens AP precursor protein gene APP-KM670/671NL double mutation in the APP gene resulting in amino acid substitutions of Lys to Asn (codon 670) and Met to Leu (671), and Precision Shuttle mammalian vector with C-terminal tag GFP (pCMV6-ACGFP) carrying resistance genes to antibiotics (\(\text{Neo}^+\) and \(\text{Amp}^+\)) were ordered in OriGene Technologies, Inc., Rockville, MD, USA, and purchased from STI Bartosz Czajkowski (Poznań, Poland). Both genes (APP-wt and APP-sw) were expressed under GFP promoter (Supplementary material Figure 1 available online at http://dx.doi.org/10.1155/2014/746092). Mock-nucleofected cells (reference, “M”) underwent complete procedure except for no vector was added prior to nucleofection.

2.4. Transfection of PC-12 Cells with the Homo sapiens Beta-Amyloid Precursor Protein Gene 1-695 (Transcript Variant 3, APP-wt), Swedish Mutation in Homo sapiens Beta-Amyloid Precursor Protein Gene APP-KM670/671NL Double Mutation in the APP Gene Resulting in Amino Acid Substitutions of Lys to Asn (Codon 670) and Met to Leu (671), and Precision Shuttle Mammalian Vector with C-Terminal Tag GFP (pCMV6-ACGFP). The procedure for PC-12 cell transfection was based on the method described by the manufacturer (Amaza Cell Line Nucleofector Kit V, Lonza Cologne AG, Cologne, Germany). Program was used for Nucleofector 4D. In short, 6-multiwell plates were prepared by filling appropriate number of wells with 1.4 mL of media (Ham’s F-12 with 15% HS + 2.5% FCS with Glutamax, 1.5 g/L sodium bicarbonate without antibiotics). Plates were preincubated/equilibrated in a humidified 37°C/5% CO\(_2\) incubator. To obtain single cell suspension the PC-12 clusters were passed through a 22-gauge needle (10–15 times). The required number of cells (2 × 10\(^6\) cells per sample) was centrifuged in sterile Eppendorf tubes (1.5 mL) of molecular purity at 200 ×g for 10 minutes at room temperature. Supernatant was removed completely, and cell pellets were resuspended carefully in 100 \(\mu\)L room-temperature complete nucleofector solution. An aliquot of 100 \(\mu\)L of cell suspension was mixed with
2 μg DNA of appropriate vector. Then, cell/DNA suspension was transferred into certified cuvette and closed with a cap and after selection the appropriate program nucleofection was performed. The cuvettes were left for 15 min in room temperature; afterwards 500 μL of the preequilibrated culture medium was added to the cuvette and samples were gently transferred into the prepared 6-well plate (final volume 1.9 mL, media per well). Cells were analyzed 24 hours after nucleofection using light (A) and fluorescence microscopy (B) (supplementary material Figure 2). Average transfection efficiency of PC-12 cells after 24 hours after nucleofection was analyzed by flow cytometry. Viability was measured by using MTT assay. One day after nucleofection, cells were used to carry out the experiments.

2.5. Determination of Cell Viability. Cell viability was based on the ability of cells grown on 96-multiwell plates to convert soluble MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] into an insoluble purple formazan reaction product with minor modifications to protocol described [30]. Briefly, cells were uniformly seeded in 96-multiwell flat bottomed plates and grown in GM. Confluent cultures were washed with PBS and then exposed to DM for 48 h. Afterwards, DM was replaced by RM with or without experimental factors for another 48 h. Relative viability (percentages of mean control value) was evaluated. To do this, media were removed and cells were washed with PBS and were further incubated with MTT for 1 h at 37°C in a humidified 5% CO2 and 95% air in incubator. Next, MTT solution was removed and water insoluble formazan was immediately dissolved in DMSO. Alternatively, cell viability was determined on the basis of modified lysosomal uptake of neutral red dye [31]. Viable cells will take up the dye by active transport and incorporate the dye into lysosomes, whereas nonviable cells will not take up the dye. PC-12 cells were grown in 96-multiwell flat-bottomed plates in GM. After reaching confluence, cells were switched into postmitotic status by incubation in DM (differentiation) for 48 h. Next, wells were immersed with RM (CTRL) and experimental media for another 48 h. To do this, media were removed, cells were washed with PBS and were further incubated with YO-PRO-1 (1 μM), and propidium iodide (10 μg/mL, PI) dissolved in PBS for 0.5 h at 4°C on ice in the dark. After incubation, YO-PRO-1 and PI accumulated in apoptotic and necrotic cells, respectively. The validity of the method was verified by observations in fluorescent microscope (Olympus IX71 Series, Osaka, Japan). For YO-PRO-1, the fluorescence was measured using the optimum wavelengths of 485 nm (λex) and 530 nm (λem). Simultaneously, the fluorescence of PI bound to核酸 acids was measured using the optimum wavelengths of 590 nm (λex) and 630 nm (λem).

2.7. Antibodies, Immunoblotting, and Microscopic Imaging. For analysis of protein expression, 30 μg of protein isolated from whole-cell lysates and wide-range molecular weight standards (Precision Plus Protein Kaleidoscope, Bio-Rad Polska, Warsaw, Poland) was electrophoresed on a 7.5, 10, or 12% acrylicamide SDS-PAGE gels and immunoblotted onto polyvinylidene difluoride Immulon-Blot PVDF membranes (Bio-Rad Polska, Warsaw, Poland). The membranes were blocked for 1 h in room temperature either with 5% nonfat dry milk (NFDM) in TBST (NaCl 137 mM, KCl 2.7 mM, and Tris base 19 mM) or in 5% BSA in TBST (depending on the antibody used). Cells were cultured with or without experimental factors indicated in figure legends, harvested, washed, and lysed with RIPA lysis buffer (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) was added. To lyse the cell pellets, cells were broken up by repetitive triturating with the syringe with attached needle (21 G, 0.8 mm diameter). Cell suspension was then left on ice (4°C) for 30 min and centrifuged for another 5 min (4°C, 8,000 xg). Next, viscous solution was divided into smaller volumes and transferred to fresh Eppendorf tubes and stored at −80°C until being used. For protein quantification in the whole-cell lysates, a protein-dye-binding method [33] with commercial reagent was used (Bio-Rad Laboratories, Hercules, CA, USA).

Antibodies against listed proteins were used: actin, clust-erin (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), APP, sAPPα, beta amyloid 1-16 (6E10) (Covance, Inc., NY, USA), APP (Merck Millipore, Darmstadt, Germany), MAP LC3 (Novus Biologicals, Cambridge, UK), and VPS34 (Thermo Fisher Scientific, Pierce Biotechnology, IL, USA). Working antibody concentrations (from 1:200 to 1:2000) varied depending on the protein detected and were applied according to the manufacturer’s recommendation. Secondary polyclonal antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) raised against respective species and conjugated to horseradish peroxidase were used for detection, followed by enhanced chemiluminescence assay (Amersham International, Aylesbury, UK). After exposure and processing the films were scanned and analyzed using Kodak EDAS 290/Kodak ID 3.5 system.
Morphological changes and cell survival were monitored under an inverted phase-contrast and fluorescent microscope, respectively (Olympus CK40, model ICD703WP, and Olympus IX71 Series, Osaka, Japan). The formation of neural cells was monitored by obtaining photographs using digital camera (supplementary material Figure 2, Olympus Camera, Tokyo, Japan).

Demonstration of the presence and intracellular location of certain modifications (autophagosomes, autophagic vacuoles, and multivesicular and multilamellar bodies) was based on ultrastructural studies (transmission electron microscopy (TEM)). Cells were fixed in 2.5% paraformaldehyde and 2% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) for 2h at 4°C. Cells were washed with the same buffer and postfixed with 1% OsO₄ in 0.1M sodium cacodylate buffer for 1h. Next, cells were dehydrated in a graded ethanol alcohol series and embedded in Epon 812. Ultrathin sections were mounted on copper grids, air-dried, and stained for 10 min with 4.7% uranyl acetate and for 2 min with lead citrate. The sections were examined and photographed with a JEOL JEM 1011 electron microscope (Jeol, Tokyo, Japan).

2.8. Cellular Aβ 1-40 Assays. Production of Aβ 1-40 was measured in PC-12-transfected cells expressing wild-type human APP (APP-wt, W), Swedish mutated APP (APP-sw, S), empty vector (GFP only, G), and complementary DNA (cDNA). Cells were seeded overnight at 3 × 10⁴ cells per well in a 96-multiwell plate. Cells were incubated in DM for 48 h and washed with PBS, and fresh DM media were added for another 48 h with or without experimental factors. Next, cellular media were harvested and assayed for the presence of Aβ 1-40 with an Aβ 1-40 homogenous time resolved fluorescence (HTRF) assay (CisBio) according to manufacturer’s instructions. For all cell types (W, S, and G), Aβ 1-40 values were normalized for cell viability, as determined with the MTT assay.

2.9. Statistical Analysis. Each experiment was repeated at least three times. The data are expressed as the means ± SEM. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Kruskal-Wallis, Tukey’s, Newman-Keuls, or Benferroni multiple range test. Regression analysis (linear) was carried out to draw appropriate standard curves. P values of less than 0.05 were considered statistically significant. Statistical differences from nontreated control cells were indicated by different lower case letters (P < 0.05, bar charts). Statistical analyses were performed using GraphPad Prism version 5.0 software (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Viability of Nucleofected PC-12 Cells Is Markedly Reduced by APP (APP-sw) but It Is Not Affected by TNF-α, TRAIL, Atorvastatin, Simvastatin, Nystatin, or MβCD. Nucleofected PC-12 cells (GFP vector, GFP vector + APP-wt, or GFP vector + APP-sw) after two days of differentiation in DM were left untreated or treated with TNF-α (10 ng/mL), TRAIL (10 ng/mL), atorvastatin (5 μM), simvastatin (5 μM), nystatin (1 μM), or MβCD (0.2 μM) for another two days in RM. Cell viability (MTT and NR assays) expressed as percent of control value (untreated cells) was significantly reduced after APP-sw cell nucleofection (Figures I(a) and I(b), supplementary material Figure 4). No effect of the above-mentioned experimental factors was observed irrespectively to nucleofection (GFP vector, GFP vector + APP-wt, or GFP vector + APP-sw) (P > 0.05). In turn, percentage of YO-PRO-1 and PI-positive cells in relation to nontreated cells (% control) did not differ markedly between the transgene or type of treatment (data not shown). It points to other than apoptosis and necrosis cell death mechanism responsible for lower cell viability after APP-sw cell nucleofection.

3.2. Production of β-Amyloid 1-40 Is Markedly Elevated in GFP Vector + APP-sw Nucleofected Cells Comparing to GFP Vector and GFP Vector + APP-wt-Nucleofected Cells. The concentration of immunoreactive β-amyloid 1-40 (nonfibrillogenic form) in supernatants of GFP vector + APP-sw-nucleofected cells collected from 96-multiwell plates used to determine cell viability increased significantly to 53.58 pg/mL (Figures 1(c) and 1(d)). No changes in β-amyloid 1-40 concentration were found in supernatants collected from remaining untreated cell cultures (GFP vector and GFP vector + APP-wt, P > 0.05). Neither administration of TNF-α, TRAIL, atorvastatin, simvastatin, nystatin, or MβCD led to significant elevation of β-amyloid 1-40 concentration in supernatants collected from GFP vector + APP-sw nucleofected cells as the observed increase was at the cut-off line for HTRF method (according to manufacturer β-amyloid 1-40 detected in supernatants at concentration of 30 pg/mL and below has to be neglected) (Figures 1(c) and 1(d)).

3.3. GFP Vector + APP-sw-Nucleofected PC-12 Cells Show Symptoms of Excessive Formation of Autophagosomes and Multivesicular Bodies but Not Autolysosomes. Ultrastructural examination of PC-12-nucleofected cells (GFP vector, GFP vector + APP-wt) with TEM revealed little evidence of autophagy. As shown in respective micrographs, the symptoms of autophagy-like process were observed regardless of nucleofection (GFP vector, GFP vector + APP-wt, or GFP vector + APP-sw); however, the intensity of autophagic phenotype was considerably advanced in GFP vector + APP-sw-nucleofected PC-12 cells (Figure 2). There is scarce evidence for ER abnormalities in micrographs representing APP-sw-nucleofected PC-12 cells (polysomes and reticular tubules are regularly distributed). Nevertheless, as some tubules seem to be distended we suggest that it may represent phagophores in the elongation phase.

3.4. Expression of Clusterin Protein Is Not Affected by Nucleofection (GFP Vector, GFP Vector + APP-wt, or GFP Vector + APP-sw) Although It Is Markedly Increased after TRAIL or Nystatin Administration; Additional Treatment with Rapamycin (1 μM) or Chloroquine (30 μM) for 1 Hour Revealed the Highest Expression of MAP LC3-I, LC3-II,
and VPS34 Proteins in GFP Vector + APP-sw-Nucleofected Cells. Immunoblotting carried out with PVDF membranes loaded with proteins separated by SDS-PAGE from six subsequent nucleofections (1-6) unraveled that APP genes had no significant effect on the expression of secretory clusterin (sCLU, Figure 3(a)). Expression of clusterin protein rose, however, after treatment with TRAIL or nystatin (Figure 3(b)). The sAPPα protein (α-secretase product) was exclusively observed in GFP vector + APP-wt-nucleofected cells, except lane 5 representing mixture of GFP vector + APP-wt plus GFP vector + APP-sw-nucleofected cells (positive control) (Figure 3(a)). In turn, total APP protein expression (unprocessed and processed form) was exclusively detected in APP gene-nucleofected cells (GFP vector + APP-wt and GFP vector + APP-sw, Figure 3(a)). In contrast, the expression of immunoreactive β amyloid 1-16 (any form) was absent in GFP vector and GFP vector + APP-wt and found solely in GFP vector + APP-sw-nucleofected cells (Figure 3(a)). Markers of autophagy (VPS34, MAP LC3-II, and Beclin 1) were also monitored with WB. Functionally active MAP LC3-II (LC3-II) was detected in GFP vector, GFP vector + APP-wt-, and GFP vector + APP-sw-nucleofected cells (Figure 3(a)). Interestingly, expressions of early autophagy marker VPS34 (class III phosphoinositide 3-kinase, PI3K III) and LC3-II were significantly elevated after TRAIL, nystatin, simvastatin, or MβCD administration (Figure 3(b)).

**Figure 1:** Bar charts (means ± SEM) represent cell viability (MTT assay, upper panel) or Aβ 1-40 concentration in supernatants (HTRF assay, lower panel) normalized for cell viability as determined with the MTT assay measured in the same wells of multiwell plates. Values are expressed as % of control (untreated PC-12 cells nucleofected with GFP, or GFP + APP-wt, or GFP + APP-sw). ((a)–(c)) The effect of atorvastatin (10 μM), simvastatin (10 μM), or MβCD (0.2 mM); ((b)–(d)) the effect of TNF-α (10 ng/mL), TRAIL (10 ng/mL), or nystatin (1 μM). Different lower case letters indicate statistically significant differences between means (P < 0.05).
Figure 2: Micrographs (TEM) show ultrastructure of PC-12 cells nucleofected with GFP ((a)–(c)), or GFP + APP-wt ((d)–(f)), or GFP + APP-sw ((g)–(i)). Symptoms of autophagy: black arrows indicate multilamellar bodies and black arrowheads indicate autophagosomes, multivesicular bodies, and lysosomes. Abnormal autophagy in GFP + APP-sw-nucleofected cells is manifested by buildup of autophagosomes with double membrane (g) and multivesicular bodies (h); large autophagosome with double membrane contains smaller autophagosomes (i) and there is scarce evidence for single-membrane autolysosomes. Bars represent 100 μm.

TNF-α and atorvastatin apparently diminished clusterin and LC3-II expressions, even though they did not affect VPS34 expression levels (Figure 3(b)). No effect of treatment was found in the expression of Beclin 1, protein important at the sequestration stage of autophagy (Figure 3(b)). To make the issue of autophagy clearer, additional “flux” experiment was performed. After the experiment ended, the cells were additionally treated with rapamycin (1μM) or chloroquine (30μM) for 1 hour. As it is presented in supplementary material Figure 5, the highest expression of LC3-I, LC3-II, and VPS34 proteins was observed in GFP vector + APP-sw-nucleofected cells.

4. Discussion

Incompetent autophagy causes decline of cell viability resulting from accumulation of nonfunctional organelles, proteins, and protein aggregates. Progressive changes inevitably end up in cell death but the precise link mechanisms and type of cell death beneath are unknown. We could not demonstrate that reduced viability of nucleofected cells was caused by apoptotic and/or necrotic cell death as percent of YO-PRO-1 and PI-positive cells did not differ significantly irrespectively of transgene or treatment (data not shown). Discussion about any other types of cell death (necroptosis and/or paraptosis) that could account for drop in percentage of viable cells in this experiment would be highly speculative. The paradox of autophagy is that it is essential for keeping cellular homeostasis, and consequently any disruption of this homeostasis results in severe effects [34]. Autophagy impairment as a method of tumor cell elimination provides strong rationale for developing strategies other than apoptosis induction. Lysosomal membrane permeabilization (LMP) which is known to occur in lysosome storage diseases (LSD) represents large group of disturbances where autophagy went wrong. LMP is frequently observed in AD where it is associated with elevated lysosomal pH [35]. Definitely, nucleofection of PC-12 with mutated APP gene was confirmed at genomic (PCR) and translational levels (WB) (Figure 3(a), supplementary material Figure 3). It led to significant drop of
cell viability (by 36%) accompanied by prevalent autophagy-like process. Possible ultrastructural modifications of ER including excessive formation of phagophores might result from the expression of mutated form of APP and impaired lysosome fusion with autophagosomes. Why the APP-sw-nucleofected PC-12 cells have nonfunctional modifications in cellular organelle essential for autophagy is not known, although numerous papers emphasize the importance of this process in pathogenesis of Alzheimer’s disease [19, 20]. Moreover, the autophagy could not reverse lower cell viability suggesting that it could go awry. Cautious analysis of micrographs obtained in TEM provided evidence of extensive formation of autophagosomes as well as numerous multivesicular bodies with little presence of autolysosomes in APP-sw-nucleofected PC-12 cells (Figures 2(g)–2(i)). Neither experimental factor affected cell viability confirming extreme resistance of PC-12 cells to death receptor ligands (TNF-α, TRAIL) or cholesterol inhibitors (atorvastatin, simvastatin, nystatin, and MβCD), (Figure 1(a)). Previously, statins were reported to induce autophagy and inhibit viability of cancer cells [36–38]. Apparently, in our study lower cell viability had predominantly something to do with changes induced by the expression of APP-sw gene (P < 0.001 by two-way analysis of variance). Actually, significant rise of human Aβ1-40 secretion was solely noticed in APP-sw-nucleofected PC-12 cells (from undetectable levels to 53.83 pg/mL, P < 0.05). Elevated concentration of Aβ in supernatants collected from APP-sw-nucleofected PC-12 cell cultures was substantiated by the results of WB. The expression of Aβ1-16 peptide (any form: Aβ1-40, Aβ1-42, and Aβ1-43) was detected in APP-sw-nucleofected but not in GFP vector- or GFP vector + APP-wt-nucleofected cell cultures (Figure 3(a)). It is important to stress that each form of Aβ evokes distinct effects in affected cells. In general, AD pathology and neuronal death are

|   | M | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|
| G |  |   |   |   |   |   |
| W |  |   |   |   |   |   |
| S |  |   |   |   |   |   |

**Figure 3:** Analysis of protein expression. Letter “M” indicates mock-nucleofected cells, while G, W, S stand for PC-12 cells nucleofected with GFP vector (G), or GFP vector + APP-wt (W), or GFP vector + APP-sw (S), respectively. (a) Identification of APP, sAPPα, clusterin, LC3-II, Aβ1-16, and beta-actin in whole cell lysates isolated from six subsequent nucleofections; (b) identification of VPS34, Beclin 1, clusterin, LC3-II, and beta-actin in whole cell lysates isolated from cells nucleofected with GFP vector (G), or GFP vector + APP-wt (W), or GFP vector + APP-sw (S).
associated with excessive production of Aβ, but mainly Aβ 1-42 peptide is severely fibrillogenic (the source of fibrilles and senile plaques). In turn, the expression of sAPPα, the protein product of α-secretase which rules out the formation of Aβ, was found entirely in GFP vector + APP-wt-nucleofected cell cultures (Figure 3(a)). The latter observation authenticates APP695 as highly specific substrate for α-secretase activity. Finally, total APP expression levels (both processed and unprocessed form) measured by immunoblotting showed this protein in GFP vector + APP-wt- and GFP vector + APP-sw-nucleofected cells only (Figure 3(a)). These results are consistent with a common view addressing importance of Aβ in etiology and pathogenesis of AD and also advocate assumption that Aβ is harmful to PC-12 cells. How does Aβ affect autophagy is not known at present and needs additional scrutiny. Expression of clusterin (CLU) protein which represents unique ATP-independent extracellular chaperone was measured to find out if this protein is affected by nucleofection, treatment, and autophagy. No changes were detected irrespectively to type of nucleofection in six subsequent nucleofections (Figure 3(a)). The expression of protein markers of initial steps of autophagy (VPS34 and LC3-I, LC3-II, and Beclin 1) did not differ between transgenes or the type of treatment (Figures 3(a) and 3(b)) but the image markedly changed after final 1-hour treatment with rapamycin (1 mM, RAPA) or chloroquine (30 mM, CHLOR). This “flux” experiment demonstrated that autophagy was incomplete and has the highest rate in the GFP vector + APP-sw-nucleofected cells as autophagy inhibitor at the level of autolysosome formation (CHLOR) led to the accumulation of LC3-I/ LC3-II (supplementary material Figure 5). Additionally, autophagy stimulator rapamycin was unable to reverse the effect of chloroquine. Thus, it is essential to monitor autophagy with including stimulators/inhibitors (RAPA, CHLOR). The use of inhibitor (CHLOR) revealed that lack of differences in the expression of VPS34 and LC3-I, LC3-II, and Beclin 1 between the GFP vector and GFP vector + APP-wt-nucleofected cells was probably due to lysosome-dependent degradation. As mentioned earlier the LC3-I is activated by APG7L/ATG7, transferred to ATG3, and conjugated to phospholipid (PE) to form LC3-II. It should be stressed that soluble form of LC3-II is not observed in PC-12 cells and thus LC3-II bands shown in the respective immunoblots represent LC3-II anchored to phagophores. However, similarly to VPS34 and LC3-II, CLU expression levels increased in response to TRAIL or nystatin treatment pointing to possible involvement of this protein in early phase of autophagy. Given that TRAIL or nystatin administration was able to increase expression of incompletely glycosylated 40 and 60 kDa CLU variants (Figure 3(b)) in either type of transgene bearing cells, it remains unclear whether this effect is directly linked to autophagy. It is not possible that clusterin simply is induced by TRAIL and NY in all types of cells and has nothing to do, at least here, with autophagy. As far as we know, this is first report showing that VPS34, LC3-II, and CLU are elevated in parallel when PC-12 cells are challenged with TRAIL or nystatin. No obvious link between these proteins and clusterin was demonstrated so far.

5. Conclusions

Pheochromocytoma PC-12 cells are completely resistant to treatment with death receptor ligands (even two days with 100 ng/mL of TNF-α or TRAIL combined with 1 nM of actinomycin D had no effect on cell viability, data not shown). Nonetheless, cell viability dropped significantly in GFP vector + APP-sw-nucleofected cells but not in GFP vector- or GFP vector + APP-wt-nucleofected cells. At the same time just GFP vector + APP-sw-nucleofected cells expressed Aβ 1-16 and secreted Aβ 1-40. At the same time profound autophagy-like process occurred with numerous autophagosomes and multivesicular bodies but with scarce evidence of autolysosomes. Summing up, human APP-sw gene is apparently destructive to PC-12 cells as cells are driven to incomplete autophagy-like process. It seems that CLU protein accompanies early phase of autophagy (isolation and sequestration).

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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