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Programmed Cell Death Mechanisms and Pheocromocytomas: Recent Advances in PC12 Cells

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1. Introduction

The endocrine system is a network of endocrine glands and nerves throughout the body. Endocrine glands produce and release hormones that circulate around the body in the blood. Hormones keep an even balance of chemicals and fluid within the body, and help us respond to changes in the environment. The endocrine system is made up of several glands, including the adrenal glands.

1.1 The adrenal medulla and its hormones

There are two adrenal glands in our body that produce a number of vital hormones essential for survival. The adrenal glands, located at the superior poles of the two kidneys, are composed of two distinct layers, the adrenal cortex and the adrenal medulla. The outer adrenal cortex, which develops from the abdominal mesothelium, surrounding the medulla during embryogenesis, synthesizes and secretes the adrenocortical hormones, the mineralocorticoids and glucocorticoids, as well as the adrenogenic (sexual) hormones.

The adrenal medulla, which comprises the central 20% of the gland, originates from the neural crest, and does not become distinct and compact until the adrenal cortex atrophies during the first few weeks postnatally. The adrenal medulla is a modified sympathetic ganglion which secretes in the bloodstream the catecholamines epinephrine (adrenaline) and norepinephrine (noradrenaline) in response to sympathetic neural stimulation to the medullae. Depending on the physiological conditions, this secretion averages 80% epinephrine to 20% norepinephrine. Although dopamine is present in the adrenal and serves as a precursor for norepinephrine and epinephrine, minimal dopamine secretion occurs and the role of adrenal dopamine is not well understood.

Cells of the medulla are known as pheochromocytes or chromaffin cells, referring to the dark color produced by the polymerization of oxidized catecholamines when these cells are exposed to chromium salts. The early medulla also contains neuroblasts and developing sympathetic ganglion cells, but these populations decrease with proliferation and maturation of the chromaffin cell population during the first years of life in the human. The pheochromocytes are arranged in nests and cords and contain abundant membrane-bound dense granules, in which the catecholamines are stored. On stimulation, these granules are transported to the cell surface via the microtubular system, and the neurotransmitter

contents of the vesicles released by exocytosis. Although circulating epinephrine is derived entirely from the adrenal medulla, only about 30% of the circulating norepinephrine comes from the medulla. The rest is released from nerve terminals and hence the adrenal medulla is not essential for life.

1.2 Functions of medullary catecholamines

The physiologic effects of epinephrine and norepinephrine are initiated by their binding to adrenergic receptors on the surface of target cells. These receptors are prototypical examples of seven-pass transmembrane proteins that are coupled to G proteins which stimulate or inhibit intracellular signaling pathways. Complex physiologic responses result from adrenal medullary stimulation because there are multiple receptor types which are differentially expressed in different tissues and cells. The alpha and beta adrenergic receptors and their subtypes were originally defined by differential binding of various agonists/antagonists and by analysis of molecular clones.

In general, circulating epinephrine and norepinephrine released from the adrenal medulla have the same effects on target organs as direct stimulation by sympathetic nerves, except the effects last five to ten times as long because these neurotransmitter hormones are removed too slowly from peripheral circulation. Additionally, of course, circulating hormones can cause effects in cells and tissues that are not directly innervated. The physiologic consequences of medullary catecholamine release are justifiably framed as responses which aid in dealing with stress. These effects are important in helping the body to react to emergency situations, thus norepinephrine and epinephrine are sometimes called the hormones of "fight or flight". A listing of some major effects mediated by medullary catecholamine are: increased rate and force of contraction of the heart muscle; constriction of blood vessels (this causes widespread vasoconstriction, resulting in increased resistance and hence arterial blood pressure); dilation of bronchioles (assists in pulmonary ventilation); stimulation of lipolysis in fat cells (this provides fatty acids for energy production in many tissues and aids in conservation of dwindling reserves of blood glucose); increased metabolic rate (oxygen consumption and heat production increase throughout the body as well as the breakdown of glycogen in skeletal muscle which provides glucose for energy production); dilation of the pupils; inhibition of certain "non-essential" processes: an example is inhibition of gastrointestinal secretion and motor activity. Common stimuli for secretion of adrenomedullary hormones include exercise, hypoglycemia, hemorrhage and emotional distress.

1.3 Tumours of the adrenal medulla: pheochromocytomas

A tumour cell is part of a tissue that is abnormally growing. It may be either malignant or benign in nature. "Tumour" originally meant "swelling" because, with unchecked cellular reproduction, the tissue affected swells to sometimes grotesque proportions. Tumour cells that are malignant are generally referred to as cancer cells, and have the ability to metastasize, or spread to neighboring tissues and grow tumours there. Benign tumour cells do not invade neighboring tissues, but may grow to great size and cause other problems: breathing, mobility, circulatory. While a malignant tumour might not be eradicated by surgically removing it, a benign tumour generally is.

Tumours of the adrenal gland can develop in either the cortex or the medulla. Benign tumours of the cortex are called adrenal cortical adenomas. Malignant tumours are called

adrenal cortical carcinomas. The World Health Organization reserves the term pheochromocytoma for tumours arising from chromaffin cells in the adrenal medulla. A small number of phaeochromocytomas start outside the medulla part of the adrenal gland and are known as extra-adrenal phaeochromocytomas or paragangliomas. A pheochromocytoma is an intra-adrenal sympathetic paraganglioma. This arbitrary nomenclature emphasizes important distinctive properties of intra-adrenal tumours, including an often adrenergic phenotype, relatively low rate of malignancy, and predilection to occur in particular hereditary syndromes. Although pheochromocytomas are extremely rare in humans, with an annual incidence of less than 1 per million, occult germline mutations characteristic of familial syndromes are now found in more than 20% of patients with apparently sporadic tumours, bringing the percentage of tumours with a known genetic basis close to 30% (Karagiannis et al., 2007). In addition, tumour location and risk of malignancy vary with the underlying genetic defect.

Pheochromocytoma develops as a single tumour or as more than one growth. Only one adrenal gland is usually affected. Rarely, tumours affect both adrenal glands; these are known as bilateral adrenal tumours. The tumours may occur at any age, but they are most common from early to mid-adulthood. In patients with pheochromocytomas, there is a release of excessive amounts of catecholamines. If the diagnosis of a pheochromocytoma is overlooked, the consequences could be disastrous, even fatal; however, if a pheochromocytoma is found, it is potentially curable. Most pheochromocytomas are benign (noncancerous), and 90% of cases can be successfully treated by surgery (Petri et al., 2009).

Many cardiac manifestations are associated with pheochromocytomas. Hypertension is the most common complication. Cardiac arrhythmias, such as atrial and ventricular fibrillation, may occur because of excessive plasma catecholamine levels. Other complications include myocarditis, signs and symptoms of myocardial infarction, dilated cardiomyopathy, and pulmonary edema, either of cardiac or noncardiac origin. Different neurologic complications may also occur. A pheochromocytoma-induced hypertensive crisis may precipitate hypertensive encephalopathy, which is characterized by altered mental status, focal neurologic signs and symptoms, or seizures. Other neurologic complications include stroke due to cerebral infarction or an embolic event secondary to a mural thrombus from a dilated cardiomyopathy. Moreover, intracerebral hemorrhage may originate because of uncontrolled hypertension.

2. Pheochromocytoma cell lines

Pheochromocytoma cells rapidly cease proliferating in primary culture. In most instances, proliferation of neoplastic chromaffin cells ceases within two weeks and does not resume, although the cells persist in cultures maintained for many months (Tischler et al., 2004). In addition, variable proportions of the tumour cell populations undergo spontaneous neuronal differentiation. Propensity for neuronal differentiation may in part reflect underlying genetic abnormalities. Establishment of pheochromocytoma cell lines is therefore a challenging task. Routinely, different immunocytochemical staining provides a means for distinguishing neoplastic chromaffin cells from other cell types in primary cultures and for rapidly assessing the success of attempts to establish cell lines (Powers et al., 2000).

The development of experimental applications of animal models of pheochromocytoma has, to a large extent, been initially driven by intriguing observations made with human tumour

tissue. Individual animal models have subsequently found their own applications, while at the same time contributing, in different ways and varying degrees, to understanding of human pathology. The rarity of pheochromocytomas is notable across species, with the exception of the rat. In some strains of laboratory rats, upwards of 30% of males spontaneously develop pheochromocytomas. In contrast, the lifetime frequency of the tumours is typically around 1% in wildtype laboratory mice, but much higher in several genetically engineered mouse models (Ohta et al., 2006; Tischler et al., 2004). Similarities and differences between the rat and mouse models suggest both parallel and unique applications for each and also raise questions of which model is more relevant to various aspects of human tumour biology. Advantages of the murine models include genetic resemblances to human pheochromocytomas (Eaton & Duplan, 2004; Molatore et al., 2010). Disadvantages include an apparently less stable phenotype.

At present, a variety of continuous adrenal medullary cell lines have been established (Eaton & Duplan, 2004). Initially, continuous chromaffin cell lines were derived from spontaneous pheochromocytoma tumours of the medulla, either from murine (i.e., rat PC12 cells) or human sources. In particular, the first continuous cell line derived from a sporadic benign human adrenal pheochromocytoma is the KNA cell line (Pfragner et al., 1998) although earlier attempts by these same researchers resulted in four similar human cell lines with finite lifespans of up to one year in culture. However, there is a heterogeneity among KNA subclones. In addition, KNA cells detach early from the tissue culture dish and grow as large multicellular spheroids with loose cell-cell adhesion. A similar medullary adrenal cell line derived from a human pheochromocytoma is the KAT45 cell line (Venihaki et al., 1998). Such cell lines have revealed both the unique characteristics of oncogenic adrenal medullary tumours, as well as similarities in catecholamine regulation to normal chromaffin adrenal tissue. However, both human cell lines have only provided *in vitro* data and it is generally accepted that there are currently no adequately documented human pheochromocytoma cell lines, despite the attempts to establish them.

Over the last few decades, more sophisticated molecular methods have allowed for induced tumourigenesis and targeted oncogenesis *in vivo*, where isolation of specific populations of mouse cell lines of endocrine origin have resulted in model cells to examine a variety of regulatory pathways in the chromaffin phenotype (Eaton & Duplan, 2004; Tischler et al., 2004). Although these cells are attractive experimental models by virtue of their genetic and functional similarities to human pheochromocytomas, a drawback compared to other models is a greater tendency to phenotype drift. This may in part be due to the same factors that cause mouse pheochromocytomas to appear polymorphous in histologic sections or it may reflect cell culture artefact. Finally, conditional immortalization with retroviral infection of chromaffin precursors has provided homogeneous and expandable chromaffin cells in animal models. This same strategy of immortalization with conditionally expressed oncogenes has been expanded to create the first disimmortalizable chromaffin cells (Eaton & Duplan, 2004). However, these promising lines have not been extensively studied, yet.

Although the available cell models have already been used for several additional novel applications, they are best regarded as complementary systems of human pheochromocytomas. Data indicates that caution is warranted in drawing general conclusions from any single cell line, but also suggest that understanding of factors that permit pheochromocytoma cells to proliferate might itself provide important insights for tumour biology.

2.1 PC12 cells

Among chromaffin cell lines, the earliest example is PC12 rat cell line. It was first established from a representative rat pheochromocytoma in 1976 (Greene & Tischler, 1976) and has become an important workhorse in many disciplines. PC12 cells arose from animals that had been irradiated postnatally, probably with resultant genetic damage that permitted the lines to be established. The phenotype of the PC12 line has been remarkably stable during almost 35 years of propagation. However, a somewhat variability of different desired traits has occurred in some laboratories, emphasizing the importance of freezing and storing early passages of any cell line. The characteristics of the cells have also been affected by culture conditions, most notably a switch made in some laboratories early in the history of the cell line from RPMI 1640 medium to Dulbecco's modified Eagle's medium, which increases cell flattening and cell-substratum adhesion.

The popularity of PC12 cells is mainly because of their extreme versatility for pharmacological manipulation, their ease of culture and the large amount of background knowledge on their proliferation and differentiation. Like adrenal chromaffin cells, PC12 cells synthesize and store dopamine and sometimes noradrenaline, which are released upon depolarization in a Ca^{2+} -dependent way. A common application of PC12 cells concerns the study of the cellular and molecular aspects of cell death, involving normal and neoplastic conditions. Also, a notable characteristic of PC12 cells is that they can readily be induced to differentiate in culture with the NGF, whereby cells cease to multiply, assume a neurite-bearing phenotype that resemble mature sympathetic neurons and exhibit firm attachment to the substratum.

3. Apoptosis in pheochromocytoma PC12 cells

The fate of a cell is determined by a balance of survival or promoting signals. While survival signals mediate cell maintenance, promoting signals induce cells to proliferate, differentiate, transform or apoptose. The natural occurrence of cell death has long been appreciated and was widely studied by nineteenth century biologists. While multiple modes of cell death have been described, undoubtedly the most renowned process is the programmed form of cell death known as apoptosis. In many diseases, aberrant regulation of apoptosis is the central abnormality. For example, resistance of cells to apoptosis is thought to be responsible for many types of cancer, while on the other hand in many neurological disorders an excessive neuronal death is a central feature.

As NGF can readily induce PC12 transition from a naïve, actively proliferating to a quiescent, differentiated phenotype, the responsiveness to NGF by PC12 cells has allowed them to be used for a great variety of studies concerning not only PC12 cells as a purported model of adrenal medullary cells, but as a model of neuronal differentiation and pluripotency. These features are possessed by primitive progenitors from the medulla which can differentiate into either chromaffin cells or sympathetic neurons, depending on the local microenvironment.

A search of the PubMed database in June 2011 yields almost 1800 papers published from 1990 describing apoptosis in PC12 cells. Indeed, PC12 is considered a suitable and reliable model to study (neuro)apoptotic mechanisms as well as for predicting (neuro)cytotoxicity of experimental drugs and natural compounds. Although caution is warranted in drawing general conclusions from any single cell line, understanding of factors that modulate PC12 cell proliferation/death might itself provide important insights in cell biology.

In this section, we will concentrate on the programmed cell death mechanisms in PC12 cells, considering primarily the recent insights achieved in the last years. We will also consider these mechanisms under a pharmacological point of view, as for instance in the context of (neuro)cytotoxicological studies and/or the developing of promising compounds with potential therapeutic applications.

3.1 Cellular machinery

Apoptosis, also known as programmed cell death, is characterized by distinctive stereotyped morphological and biochemical alterations, such as exposure of phosphatidylserine on the outer leaflet of the plasma membrane and blebbing, cell shrinkage, chromatin condensation, and DNA fragmentation. This process leads to the formation of apoptotic bodies that are subsequently eliminated by phagocytosis. Key event in the apoptotic process is the activation of caspases, a family of cysteinyl aspartate-specific proteases. They are constitutively expressed in almost all cell types as inactive proenzymes (zymogens) that became processed and activated in response to a variety of pro-apoptotic stimuli. Evidence for the sequential activation of caspases has led to the concept of a caspase cascade: during apoptosis, apoptogenic stimuli induce the autocatalytically activation of initiator caspases; subsequently they cleave and thereby activate downstream effector caspases that carry on the cleavage of specific proteins in order to “dismantle” the cell.

The induction of apoptosis can be mediated by two pathways: the death receptor-dependent or the mitochondria-dependent pathways, also known as the extrinsic and intrinsic apoptotic pathways, respectively (Jin & El-Deiry, 2005). The extrinsic apoptotic pathway begins at the cell surface where death receptors bind their ligands, such as TNF family members, including FasL (or CD95L). Ligand binding to the death receptor triggers the oligomerization of the death receptor and the aggregation of the characteristic intracellular motif, known as “death domain” able to recruit adaptor proteins containing death domains, such as FADD. These adaptor proteins function to activate initiator caspase-8 and/or caspase-10, resulting in the formation of the death-inducing signaling complex. Activated initiator caspases in turn process and activate the downstream executioner caspases, including caspase-3, -6, and -7, which execute the destruction of the cell (Degterev et al., 2003). The study of death receptors-induced apoptosis in PC12 cells has been particularly important in understanding the pathogenesis of neurodegenerative diseases. For instance, it is of interest to note that amyloid β peptide toxicity (a model of Alzheimer disease *in vitro*, see also below) may involve the CD95 pathway while TNF is strictly linked with excitotoxic mechanisms. The intrinsic apoptotic pathway involves the mitochondria in response to diverse cellular stress such as UV and gamma irradiation, heat and the activation of some oncogenic factors. The critical event in the mitochondria-mediated apoptotic pathway is the mitochondrial outer membrane permeabilization, that prompts the release of various proteins of the mitochondrial intermembrane space into the cytosol. In addition, the mitochondrial proteins of Bcl2 family (i.e., Bcl2 and Bax) play a fundamental role as negative or positive modulators. Of importance, cytochrome c, released from mitochondria, binds to Apaf-1 to form a complex, the “apoptosome,” that recruits procaspase-9 and facilitates its oligomerization and activation. Activated caspase-9, in turn, processes and activates the executioner caspases-3, -6, and -7, which drive the execution of the cell.

The homeostasis of healthy tissues is maintained by the survival signaling pathways responsible for the control of cell proliferation. In this respect, one of the best-studied

mechanism responsible for apoptosis in PC12-derived neurons is the serum or growth factor withdrawal leading to the classical apoptosis. In contrast, the best-characterized survival pathway in PC12 cells is that involving NGF. NGF belongs to a family of structurally related neurotrophin proteins, including BDNF, NT-3, and NT-4/5 that function to support the growth and survival of many populations of neurons. NGF binds the TrkA neurotrophin receptor, whereas BDNF and NT-4/5 bind the TrkB neurotrophin receptor, and NT-3 primarily binds the TrkC neurotrophin receptor. These Trk neurotrophin receptors are able to protect neuronal cells from apoptosis, and they can stimulate neuronal regeneration in different model systems. NGF activates a variety of signaling cascades, including the protein kinases ERK1/2 and PI3K-Akt pathways, that are dynamically linked to the apoptotic machinery in a complex cellular signaling network. Their activation by survival signals serves to block apoptotic signaling. The proto-oncogene protein kinase Raf-MEK-ERK1/2 pathway is the better characterized Ras (a GTPase) effector pathway that plays a key role in PC12 cell proliferation. Once activated, ERK phosphorylates and thereby regulates the activities of a number of substrates, including multiple transcription factors inducing alteration in gene expression directly linked with the extracellular signal from the cell surface receptors and thus preventing apoptosis. It is very recent the discovery that, among all the targets of NGF-activated ERK in PC12 cells the two microRNA 221 and 222 are involved in NGF-dependent neuronal survival. (Terasawa et al., 2009). In PC12 cells, the PI3K-Akt pathway, the other downstream mediator of NGF, includes, among its several targets, the FoxO family of transcription factors which play a particularly important role in the regulation of cell death, proliferation, and survival through modulation of the expression of cell-cycle inhibitory genes and pro-apoptotic genes. The kinase Akt also controls the activity of mTORC1, another key mediator of cell growth, proliferation, and survival that can function to inhibit PC12 cell apoptosis.

Another intriguing issue, recently demonstrated in several models including PC12, is that cell cycle activation often precedes neuronal death, indicating that neurons attempt to divide before dying (Bianco et al., 2011). Multiple pathways and stimuli, including NGF deprivation, have been demonstrated to participate in the regulation of cell cycle events during death of differentiated PC12. These aspects may have a significant clinical interest. Indeed, different papers have reported the re-expression of proteins of the cell cycle in neurons from patients with Alzheimer disease, Parkinson's disease, ataxia telangiectasia, stroke, and other neurodegenerative conditions.

On the other hand, controversial is the use of PC12 as model of apoptosis induced by the release of the excitatory neurotransmitter glutamate. For many years PC12 cells have been used to investigate NMDA receptor mediated excitotoxicity and potential neuroprotective mechanisms (Lee et al., 2006). In a key paper, Casado and coll. (Casado et al., 1996) demonstrated an increase in NR1 protein (a NMDA channel subunit) expression levels in PC12 cells in response to NGF, and their ability to evoke NMDA and glutamate induced currents. However, other studies are not entirely consistent with these results. Among them, Edwards and coll. (Edwards et al., 2007) demonstrated the absence of functional NMDA receptors in PC12 cells thus suggesting that the mechanism by which NMDA or glutamate induces excitotoxicity in PC12 cells cannot be assumed to occur by direct actions on NMDA receptors but, instead, non-NMDA receptor mechanisms, including oxidative stress, may drive cytotoxic response to high glutamate concentrations.

PC12 cells have been also shown to be useful models to characterize the molecular mechanisms that determine cellular commitment to cell death following oxygen-derived

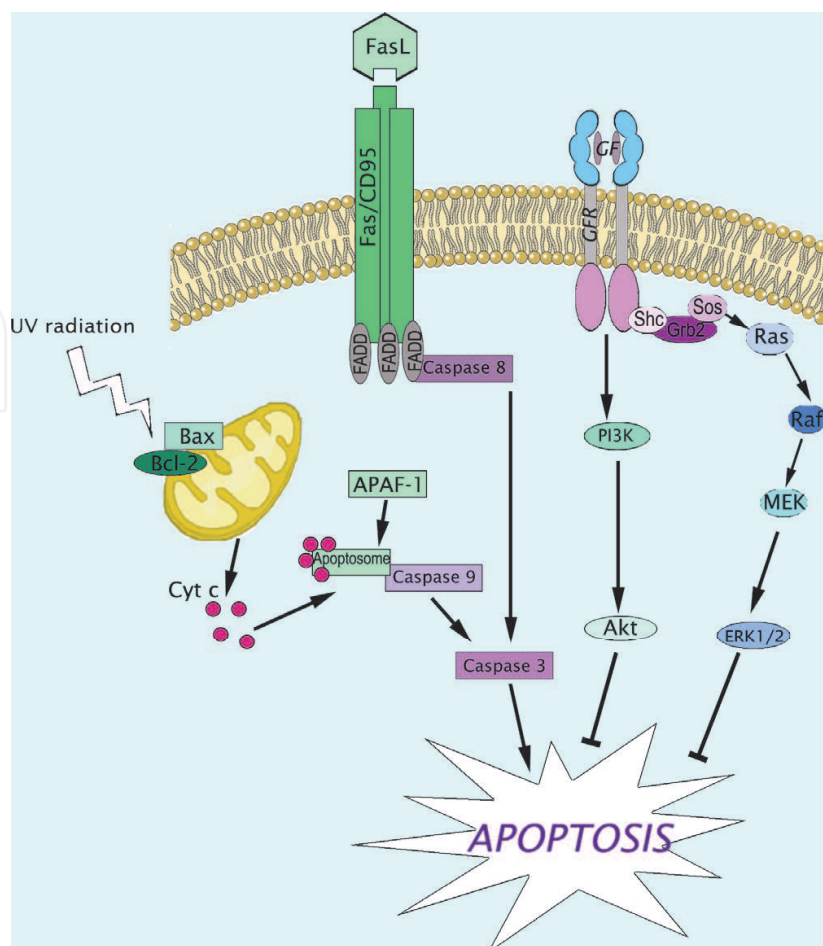


Fig. 1. Scheme of some apoptotic mechanisms achieved in PC12 cells (GF: Growth factors; GFR: GF receptors; Shc/Grb2/Sos: classical protein complex involved in Ras activation).

free radicals including superoxide, peroxynitrite, peroxy radicals, and the hydroxyl radical. The toxicity of oxygen-derived free radicals arises from the presence of one or more unpaired electrons that extract electrons from macromolecules, ultimately inactivating them. At high level reactive oxygen species induce the oxidation of protein, lipid and DNA/RNA, increase the leakage of lactate dehydrogenase and reduce the intracellular glutathione level. In neuron and in neuronal cell lines (e.g., PC12) accumulation of H_2O_2 can activate the stress sensor JNK/p38 MAPK pathway (Cho et al., 2008) and eventually the tumour suppressor gene p53 (Reuter et al., 2010). A source of the hydroxyl radical is also the nitrogen-derived radical, nitric oxide, which can combine with superoxide to produce peroxynitrite. Peroxynitrite is capable of directly damaging proteins, lipids, and nucleic acids and can generate hydroxyl radicals and NO_2 in PC12 cells (Pytlowany et al., 2008; Shacka et al., 2006).

In the last decade increasing attention has been focused on alternative signaling pathways leading to cell death, as for instance autophagy and parthanatos. In PC12 cells serum or growth factors withdrawal can lead to autophagy instead of apoptosis. Autophagy is an evolutionarily conserved lysosomal pathway involved in the turnover of long-lived proteins and organelles. Functions of autophagy include: remodeling during development and differentiation and elimination of unwanted or damaged organelles and molecules. These functions are important for maintenance of cytoplasmic homeostasis. Autophagy can be

stimulated in response to different situations of stress, such as starvation, changes in cell volume, oxidative stress, accumulation of misfolded proteins, hormonal signaling, irradiation, xenobiotic or the pro-apoptotic ligand TRAIL treatment. Following the induction of autophagy, autophagic vesicles or autophagosomes are formed through the assembly and expansion of double-layered, membrane-bound structures of endoplasmic reticulum around whole organelles and isolated proteins. The autophagosome encapsulates the cytosolic materials, then docks and fuses with lysosomes or other vacuoles, causing degradation of the autophagosomal contents. At the molecular level, the signaling pathway that leads to autophagy seem to involve the activities of PI3K and mTOR. In PC12 cells, two key autophagy genes, ATG7 and beclin 1, have been recently reported to be involved in this non-apoptotic death pathway.

In traumatic brain injury, excitotoxicity, ischemia, and, in many neurodegenerative disorders, PARP-1 activation is an early biochemical cell death event. PARP-1 activation leads to a unique form of cell death that is in large part mediated via accumulation of PAR and nuclear translocation of AIF from mitochondria that induces large-scale DNA fragmentation, chromatin condensation, and cell death. PARP-1-dependent cell death is caspase independent as caspase inhibitors are ineffective in limiting it and is termed parthanatos. In several neuronal model including PC12, parthanatos has been recently studied together with its players (Kondo et al., 2010). The intriguing aspect emerging from parthanatos is that the nuclear-mitochondrial cross talk with PAR is important player for cell death initiation. Indeed, PAR is generated mainly in the nucleus and localizes to cytosol and interacts with mitochondria to induce cell death. Parthanatos is a unique form of cell death that occurs across organ systems, which is primarily mediated by toxic accumulation of PAR in cytosol from overactivation of PARP-1. Although the mechanism of parthanatos includes PAR as a signaling molecule to induce AIF release from mitochondria, the mechanistic aspects of the AIF-releasing capacity of PAR remain unclear.

Although programmed cell death is well accepted as a common homeostatic property of all tissues, its frequency is underestimated. Dying cells are rarely observed in normal tissues, thus illustrating the effectiveness of the clearance process. Indeed, a key event in the apoptosis programme is the swift clearance and phagocytosis of dying cells by wandering or resident scavengers and neighbouring phagocytes (Gregory & Pound, 2011). However, the vast majority of research on apoptosis has focused on the stages leading up to the clearance phase, or indeed has been carried out under artificial conditions lacking the clearance phase such as in model populations of non-phagocytic cells *in vitro*. In normal or pathological tissues, apoptotic cells are invariably found in association with macrophages and neighbouring cells of various lineages which actively engage in the apoptotic cell clearance process. In the developing brain, multipotential cells phagocytose their dying neighbours prior to the appearance of the specialist phagocytes of the nervous system, the microglia. Furthermore, in tumours, clearance of apoptotic cells is mediated by macrophages, non-macrophage stromal cells, and tumour cells themselves. These features need to be taken into account in elucidating the finer mechanisms of apoptotic cell death, as for instance those underlying the interactions of the apoptotic cell with its environment.

3.2 Factors and compounds involved in apoptosis regulation

Much current work with *in vitro* systems for (neuro)cytotoxicity/(neuro)cytoprotection testing lies in maximizing their potential for yielding valid mechanistic response. Among the

advantages of *in vitro* models, i.e., PC12 cells, are the option to study a single cell type of interest in the absence of other cell types, ease of direct observation and measurement of cellular responses to chemicals, a defined extracellular environment, and direct interactions of the chemical with test cells. Until recently, it was thought that cytotoxic/cytoprotective drugs affected target cells directly by interfering with some life-maintaining function. However, of late, it has been shown that exposure to several drugs with disparate mechanisms of action modulates apoptosis in both pathological and normal cells.

Among ubiquitous toxic environmental contaminants, cadmium, manganese, copper (an essential trace element contained in common foods), methylmercury, and bisphenol A may induce apoptosis in PC12 cells. Recently, it has been demonstrated that melamine, which has been used in milk powder as an additive to raise the measured protein content, and monocrotophos, a widely used organophosphate pesticide, also displayed apoptotic effects. In contrast, the phenol tert-butylhydroquinone, a synthetic food grade antioxidant, that is used to stabilize foods, fats and vegetable oils against oxidative damage, prevents apoptosis in differentiated PC12 cells. Similar effects are achieved by pyrroloquinoline quinone, a novel redox cofactor which exists in various foods.

Current evidence in the literature supports a cytoprotective role of NGF in PC12 cells (see also above) such as against apoptosis induced by TNF α . The transition of cells from a proliferative to a differentiated, quiescent stage is associated with acquisition of a highly reduced intracellular environment, which confers cytoprotection against oxidative challenge. The reason as to why PC12 cells acquire a reduced intracellular status during differentiation is probably due to the fact that neuronal cells are highly oxidative, which could render cells vulnerable to reactive oxygen species-induced injury. Therefore, adaptation of differentiated neuron-like PC12 cells to a reduced environment and a higher expression of redox enzymes would permit cells to function optimally under oxidizing conditions, and increase the likelihood for survival against oxidative challenges.

Oxidative stress has been widely believed to be an important pathogenetic mechanism of neuronal apoptosis, occurring in different brain diseases, as for instance Alzheimer disease and Parkinson's disease. It is thus not surprising that neuron-like PC12 cells are extensively used in the study of brain disorders. In this respect, amyloid β peptide, dopamine, 6-hydroxydopamine, and MPP⁺ are shown to induce apoptosis in PC12 cells. Amyloid β peptide toxicity is a well-established pathway of neuronal cell death which play a role in Alzheimer's disease. In addition, 6-hydroxydopamine is a neurotoxin used by scientists to selectively kill dopaminergic and noradrenergic neurons. The main use for 6-hydroxydopamine in scientific research is to induce Parkinsonism, as well as the neurotoxin 1-methyl-4-phenylpyridinium. Similarly, dieldrin, which may selectively destroy dopaminergic neurons, induces apoptosis in PC12 cells. It is a chlorinated hydrocarbon originally produced as an insecticide and has been reported to be one of the environmental factors correlated with Parkinson's disease. The effects of some of these substances can be reverted by different anti-apoptotic compounds. Among them, some novel substituted bisphenol A derivatives, bone morphogenetic protein 7, and galantamine have protective effects against amyloid β peptide-induced PC12 apoptosis. Galantamine is an acetylcholinesterase inhibitor widely used for patients with Alzheimer's disease. In addition, granulocyte-macrophage colony-stimulating factor, a hematopoietic cytokine that has the potential for clinical application, significantly reduces 1-methyl-4-phenylpyridinium-induced PC12 apoptosis. Some interesting results may also account from gene therapy strategies. For instance, the anti-apoptotic herpes simplex virus type 2 gene

ICP10PK has been reported to protect neuronally differentiated PC12 cells from apoptosis death caused by MPP⁺. Finally, other substances which display somewhat activity in brain have been shown to modulate apoptosis in PC12 cells: the opioid morphine induces apoptosis, while lithium chloride, the antipsychotics citalopram and trifluoperazine, and serotonin exert opposite effects.

name	description	source	effect
hydroxyecdysone	ecdysteroid hormone	different plant sources	anti-apoptotic
apocynum leaf	flavonoid extract	<i>Apocynum venetum</i>	anti-apoptotic
aucubin	iridoid glycoside	<i>Aucuba japonica</i> and other plant sources	anti-apoptotic
camptothecin	alkaloid	<i>Camptotheca accuminata</i>	apoptotic
cEppt		cinnamon extract	anti-apoptotic
decursin	coumarin	<i>Angelica gigas</i>	anti-apoptotic
euplotin C	sesquiterpenoid	ciliate protist <i>Euplotes crassus</i>	apoptotic
gomesin	peptide	tarantula <i>Acanthoscurria gomesiana</i>	apoptotic
ginsenoside Rb1	steroid glycoside	ginseng (<i>Panax</i>)	anti-apoptotic
icariin	flavonoid	<i>Epimedium brevicornum</i>	anti-apoptotic
isochaihulactone	lignan compound	<i>Bupleurum scorzonerifolium</i>	anti-apoptotic
isoflavones	poliphenolic compounds	<i>Fabaceae</i>	anti-apoptotic
kaempferol	flavonoid	different plant sources	anti-apoptotic
lactacystin	cyclic amide	bacteria <i>Streptomyces</i>	apoptotic
MAM	naphthoquinone	<i>Polygonum cuspidatum</i>	anti-apoptotic
olive leaf		olive tree (<i>Olea europaea</i>)	anti-apoptotic
paclitaxel/docetaxel	taxanes	<i>Taxus brevifolia, baccata</i>	apoptotic
paeoniflorin	monoterpene glycoside	<i>Paeonia albiflora</i>	anti-apoptotic
puerarin	isoflavone C-glycoside	<i>Pueraria lobota</i>	anti-apoptotic
quercetin	flavonoid	<i>Quercum</i> and other plant sources	anti-apoptotic
salidroside	phenylpropanoid glycoside	<i>Rhodiola rosea</i>	anti-apoptotic
schisandrin B/C	lignans	<i>Schisandra chinensis</i>	anti-apoptotic
SG-168	acetal skeleton	<i>Dendrobium nobile</i>	anti-apoptotic
silibinin	flavonolignan	milk thistle (<i>Silybum arianum</i>)	anti-apoptotic
tetrahydroxystilbene glucoside	monomer of stilbene	<i>Polygonum multiflorum</i>	anti-apoptotic
trans resveratrol	phenol	grapes	anti-apoptotic
vincristine/vinblastine	vinca alkaloids	<i>Catharanthus roseus</i>	apoptotic
xyloketals		mangrove fungus <i>Xylaria</i>	anti-apoptotic

Table 1. Examples of natural products identified as modulators of PC12 cell apoptosis

Many commercialized drugs have been obtained by the synthesis of new compounds. On the other hand, an alternative source of drugs is natural products (and the structural modification of natural products), which frequently seem to be more effective and/or less toxic. A natural product is a chemical compound or substance produced by a living organism - found in nature that usually has a pharmacological or biological activity. Nature

is an attractive source of new therapeutic candidate compounds (da Rocha et al., 2001) as a tremendous chemical diversity is found in millions of species of plants, animals, marine organisms and microorganisms. For many living organisms, this chemical diversity reflects the impact of evolution in the selection and conservation of self-defense mechanisms that represent the strategies employed to repel or destroy predators. However, the development of novel agents from natural sources presents obstacles that are not usually met when one deals with synthetic compounds. For instance, there may be difficulties in accessing the source of the samples, obtaining appropriate amounts of the sample, identification and isolation of the active compound in the sample, and problems in synthesizing the necessary amounts of the compound of interest. These problems became evident when the tubulin-interacting agent taxol was introduced in clinical use. Antitumour activity was observed in various *in vitro* systems, including PC12 cells, and *in vivo* tumours. It took some years to develop a semi-synthetic analog which is derived from a renewable source. Currently, total synthesis has been achieved drug supply is no longer a problem. Among their various biological activities, natural products can modulate apoptosis signaling pathways (Fulda, 2010). In PC12 cells, many scientific papers report pro-apoptotic and anti-apoptotic activity of different natural products, isolated mostly from plant sources, others from microbes, fungi and different marine organisms. Table 1 shows some examples of agents derived from natural sources which have been studied in 2010-2011 as modulators of PC12 cell apoptosis. Some “classical” apoptotic modulators have been also indicated.

In many studies, PC12 cell models to study apoptotic mechanisms/modulations have been obtained with the use of lipopolysaccharide, the major component of the outer membrane of Gram-negative bacteria, or ethanol. Apoptosis can be also induced by depletion of serum and NGF from the culture medium. Other factors may include glucose deprivation, a high oxygen atmosphere or oxidative agents as for instance hydrogen peroxide, peroxyntirite, the lipid peroxidation product 4-hydroxynonenal, and 7-ketocholesterol, the major oxidation product of cholesterol found in human atherosclerotic plaque. Moreover, many other organic/endogenous compounds, like parathyroid hormone(1-34), corticosterone, and ceramide exert pro-apoptotic effects in PC12 cells. Interestingly, taurine, a free amino acid with antioxidant activity present in high concentrations in a variety of organs of mammals, protects cells from apoptosis induced by hydrogen peroxide. In addition, heparin-binding epidermal growth factor-like growth factor is a member of the epidermal growth factor family that is expressed in many cell types. In PC12 cells, its protective effects against apoptosis induced by oxygen and glucose deprivation has been reported. Recently, different synthetic compounds have been shown to display cytoprotective behaviours against oxidative stress, among them folacin C60, the isothiocyanates 3H-1, 2-dithiole-3-thione, fasudil mesylate, the new 1,2,4-triazine, , 3-butyl-6-bromo-1(3H)-isobenzofuranon and a new synthetic 1,2-diaryl oxazine derivative, 2-ethoxy-4,5-diphenyl-1,3-oxazine-6-one. In this line, the soluble guanylyl cyclase activator YC-1, which inhibits the hypoxia-inducible factor 1, and edaravone, a potent free radical scavenger in clinical use, significantly antagonize the PC12 apoptosis induced by glutamate or prion protein peptides, respectively.

The ability to induce apoptosis in many tumoural systems, including PC12 cells, has been reported as key property for different chemotherapeutic drugs or substances with potential therapeutic activity. For instance, the chemotherapeutic drugs fluorouracil, paclitaxel, and enedinyes are potent inducers of apoptosis in PC12 cells. Also, therapeutic concentrations of

cisplatin may cause a hybrid type of cell death characterized by concurrent apoptosis and necrosis in the same individual cells. Interestingly, I-387, a novel synthetic compound that inhibits tubulin action and exhibits potent antitumour activity in various preclinical models, has been shown to display apoptotic activity in different systems, including PC12 cells, suggesting that it may represent a new antimitotic agent for management of various malignancies. It is also of interest the fact that human pheochromocytomas express high levels of the receptor subtype 2 for the neuropeptide somatostatin. Recently, it has been demonstrated that somatostatin agonists as well as somatostatin cytotoxic compounds may induce PC12 cell apoptosis, although no data are available on pheochromocytomas *in vivo* (Ziegler et al., 2009). Generally, the implications of such apoptotic studies in oncology are puzzling. Indeed, often forgotten in the biology of tumours, cell loss in malignant disease is a very significant component of tumour dynamics. One may assume that enhanced apoptosis will retard tumour growth and hence indicate a favourable prognosis compared with tumours showing a low apoptotic activity. However, also the opposite seems to be true: apoptosis is a common process in high-grade malignancy, with high apoptosis indices generally reflecting poor prognosis. Clearly, the balance between cell birth and cell death must favour the former in order for a tumour to grow but, given the properties of apoptotic cells and their capacity to affect their microenvironments and host immune systems, it seems likely that the cell-death programme also provides oncogenic drive (Gregory & Pound, 2011). In this respect, the implications that the homeostatic properties of apoptosis can be hijacked as a sinister, facilitatory mechanism for malignant disease development and progression are clear and the programme may be regarded as 'altruistic' in this context: loss of a fraction of the growing population for the greater good of the whole (but detriment of the host).

4. Conclusion

From a general point of view, dysregulation of apoptosis is associated with many pathologic conditions and may even be central in the pathogenesis of many diseases (Zangemeister-Wittke & Simon, 2001). Apoptosis research is rapidly proceeding making it difficult to keep track of the constant stream of newly identified proteins and molecular interactions involved in cell death regulation. However, there is a general agreement that the suppression, overexpression or mutation of a number of factors which orchestrate the apoptotic process are associated with disease. As outlined above, the study of apoptosis mechanisms in PC12 cells represent one application currently of great potential interest.

In pheochromocytomas as well as in the majority of malignancy there is a need of novel compounds which may act as potent and selective apoptotic modulators. In the clinical practice of benign pheochromocytoma, the most common treatment is surgical removal of the entire affected adrenal gland. On the other hand, there is currently no effective treatment for malignant pheochromocytoma. Radiotherapy provides benefit in some patients with malignant pheochromocytoma. Chemotherapy with cyclophosphamide, vincristine, and dacarbazine may produce partial remission, but again is not curative. In this respect, much work is still to do in order to exploit new chemical space for drug-like molecules.

5. Abbreviations

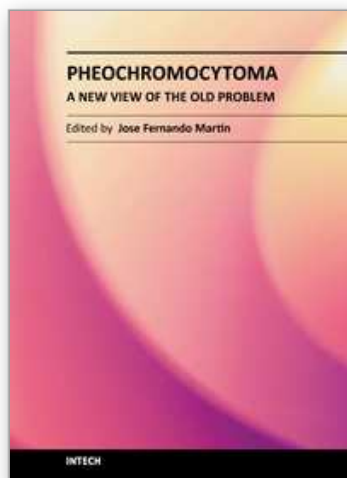
AIF, apoptosis inducing factor; *Apaf-1*, apoptosis protease-activating factor 1; *BDNF*, brain-derived neurotrophic factor; *ERK*, extracellular-regulated kinases; *FADD*, Fas-associated death domain; *FasL*, Fas Ligand; *FoxO*, forkhead box O; *GTP*, guanosine triphosphate; *JNK*, Jun N-terminal Kinase; *MAPK*, mitogen-activated protein kinases; *MEK*, MAPK kinases; *MPP⁺*, 1-methyl-4-phenylpyridinium; *mTOR*, mammalian target of rapamycin; *mTORC1*, mTOR complex 1; *NGF*, Nerve Growth Factor; *NMDA*, N-Methyl-D-aspartic acid; *NT*, neurotrophin; *PARP-1*, poly (ADP-ribose) polymerase-1; *PI3K*, phosphatidylinositol 3-kinases; *TNF*, Tumour Necrosis Factor; *Trk*, transmembrane tyrosine kinase proteins.

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