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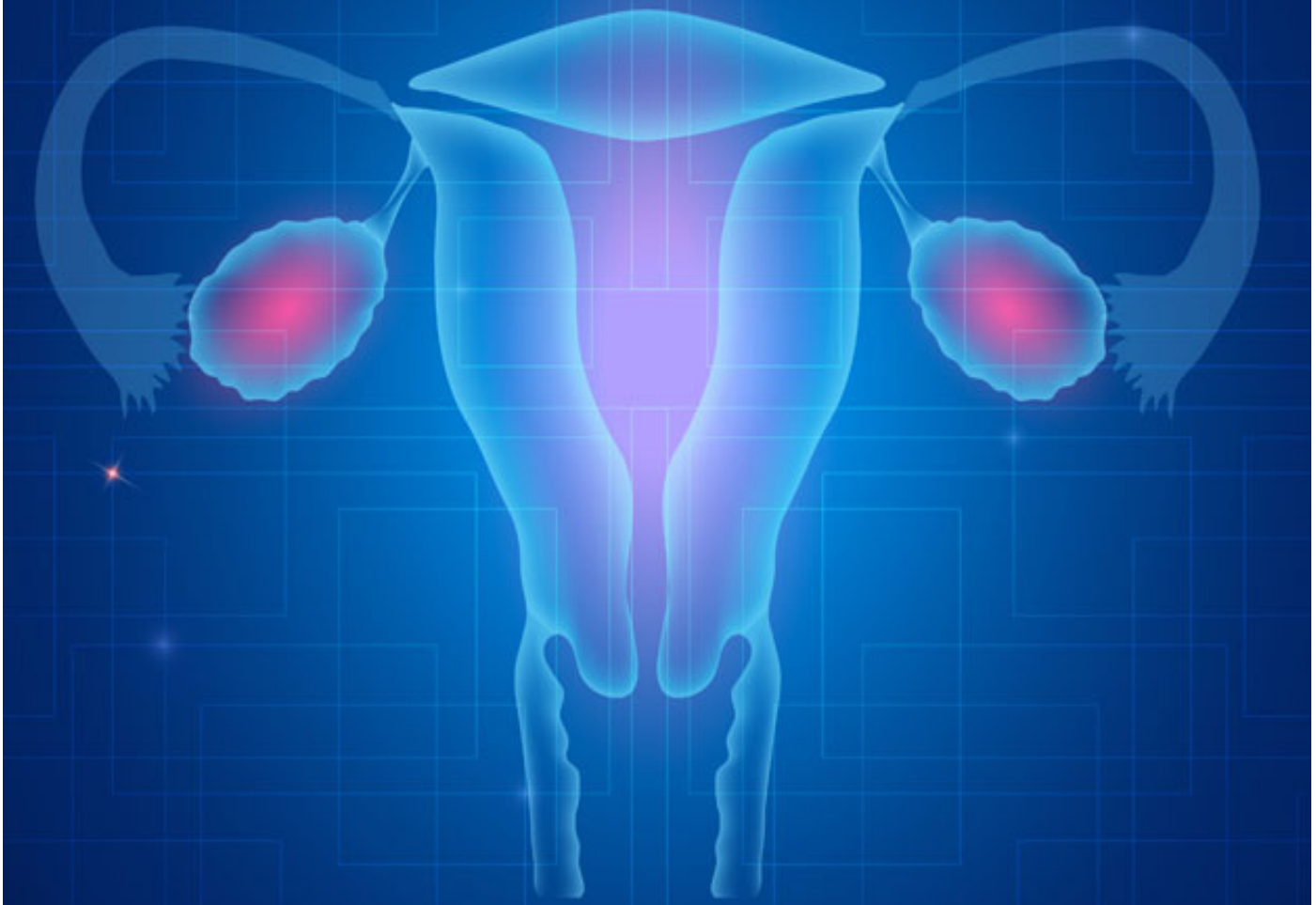

CERVICAL CANCER

Autophagy and Resistance Therapies in Cervical Cancer

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Abstract

Autophagy is a conserved intracellular degradation process that is activated under condition of stress. During this event, diverse intracellular contents since proteins until organelles as mitochondria are degraded. The study of autophagy has spurred renewed interest because it functions not only as a cytoprotective mechanism, but also as a programmed process of cell death. The role of autophagy in cell elimination has been proposed as a strategy for combatting cancer cells that do not respond as expected to the induction of cytotoxic or apoptotic processes. The dual role of this mechanism in tumor processes must be analyzed in greater detail, since research has shown that levels of autophagy can play a decisive role in the fate of such resistant cells.

Abbreviations: ATG: Autophagy-related Genes; Atg8/LC3: Microtubule-associated protein light chain 3; Bcl-2, B: Lymphoma 2; Bcrp: Breast Cancer Resistance Protein; HDAC: Histone Deacetylase; HPV: Human Papillomavirus; Hsc70: Hot Sock Protein 70; Lrp: Lung Resistance-Related Protein; LRP: Resistance-Related Protein; miRNA: MicroRNAs; MOMP: Mitochondrial Outer Membrane Permeabilization ; mRNA: Messenger RNA; Mrp: Multidrug Resistance-Associated Protein; mTOR: Mammalian Target of Rapamycin; MVP: Major Vault Protein; O6 AGT: O6-Alkylguanine- DNA-Alkyltransferase; PARP: Poly (ADP-ribose) polymerase; PE: Phosphatidylethanolamine; Pgp: P-glycoprotein; PI: Phosphatidylinositol; PI3K: Phosphatidylinositol 3-kinase; ROS: Reactive Oxygen Species; SiRNA: Small interfering RNA; SMAC: Second Mitochondria-erived Activator of Caspases; ULK: Unc-51-like Kinases; Vps38/UVRAG: Ultraviolet Irradiation Resistance-Associated Gene; XIAP: X-linked Inhibitor of Apoptosis Protein

Introduction

During the autophagic process, the mitochondria and other cellular elements are conducted toward the lysosomes, and degraded by lysosomal enzymes. The cytoplasmic components to be degraded are encapsulated in double-membrane vesicles called autophagosomes. The autophagosomes are channeled towards the lysosomes, where they degrade the contents by means of lysosomal acid hydrolases.

Recently, autophagy has been described as a process of programmed cell death in both normal cells and those found in pathological conditions. It has been shown that autophagy is not only a cytoprotective process, but also a mechanism for eliminating cells when other types of cell death fail. Strategies for treating cancer include designing drugs that function by eliminating cancer cells, but these intense efforts to find tools that successfully eradicate modified cells while leaving healthy cells unaffected led to the finding that cancer cells employ diverse strategies at the molecular level to elude cell death. Traditionally, necrosis and apoptosis have been the primary options for eliminating cancer cells, but there is now ample evidence that in diverse conditions these processes fail because of the kinds of modifications that the target cells have acquired (a phenomenon known as therapy resistance). It is in cases such as these that autophagy, as a third process of cell death, may come to play an important role, since it is known that under certain circumstances it can induce cell death and so may be able to control cancer progression.

Autophagy

Eukaryotic cells have a highly-organized internal membranous system that allows them to perform their activities correctly. One important process that entails extensive reordering of this system is the autophagy; a conserved system of intracellular degradation found in the eukaryotic cells of organisms ranging from yeast to humans. Autophagy is a self-digestion process in which portions of the cells' cytoplasmic contents are sequestered inside double-membrane structures (Figure 1) that, in turn, are conducted toward lysosomes where they are degraded [1-3]. Lysosomes are the main cellular organelles involved in the degradation that occurs in eukaryotic cells [4]. They have the capacity to degrade all types of cytoplasmic contents by means of the acidic hydrolases they contain [5]. The autophagic process involves eliminating such cytosolic elements as undesirable proteins and damaged organelles, including mitochondria, peroxisomes and ribosomes, among others [6], in order to maintain cellular homeostasis.

Figures

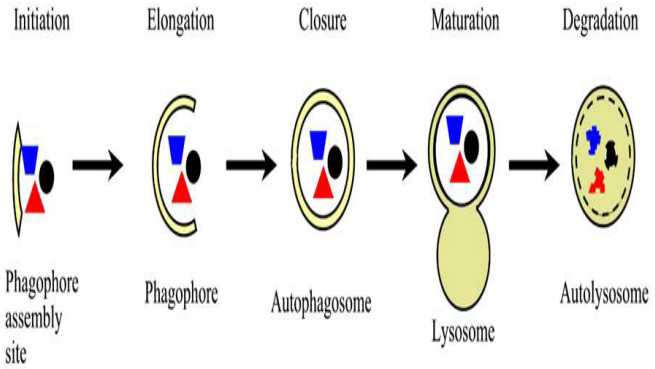

Figure 1: The process of autophagy. The process begins with the formation of the isolation membrane in the assembly site, to form the phagophore. The continuosly growing of the phagophore allows the closure of the vesicle to conform the autophagosome. The fusion of the autophagosome with the lysosome leads the degradation of the cytoplasmic content into the autophagosome.

Autophagy as a physiological process

Autophagy has been recognized as a cytoprotective mechanism that is triggered under conditions of stress, such as starvation, amino acid limitation and hypoxia, etc. This process is conceived to recycle the intracellular contents until the normal conditions are restored [7]. Under these conditions, autophagy is considered a physiological event that is essential to cell survival, and there is evidence that organisms with autophagy-deficient systems do not survive more than one day after birth [8]. These observations underline the importance of autophagy in the processes of cellular homeostasis. Moreover, impairment of autophagy has been related to several neurodegenerative diseases [9] that operate by disrupting this recycling process and allowing the accumulation of the protein aggregates or damaged organelles that cells must eliminate to remain healthy.

Autophagy is performed by three different intracellular mechanisms, depending on the route that the undesirable molecules/structures follow to reach the lysosomes and be degraded. These mechanisms are called: macroautophagy (commonly denominated autophagy), microautophagy, and chaperone-mediated autophagy [10]. The macroautophagy –autophagy– involves the formation of double membrane vesicles called autophagosomes that, once establishes, fuse to lysosomes where the vesicular contents is degraded, and finally some macromolecules are reused. During microautophagy, in contrast, the lysosome actually engulfs the cytoplasm directly by means of self-invagination; while chaperone-mediated autophagy involves the participation of the Hsc70 chaperone which has the ability to recognize the substrate proteins that contain a KFERQ sequence, after this labeled contents is introduced into the lysosomal space (Figure 2).

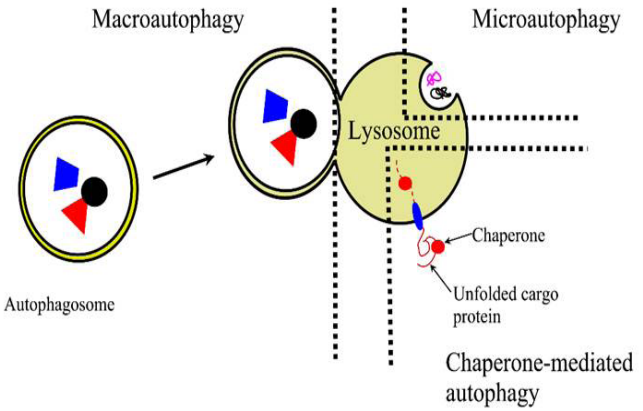

Figure 2: Autophagy mechanisms. Macroautophagy (autophagy) involves the formation of a double membrane vesicle with cytoplasmic content. Microautophagy is conducted by lysosomal invagination to sequester cytoplasmic proteins. Chaperone-mediated autophagy requires the Hsc70 chaperone to recognize the unfolded proteins, and then these labeled proteins are introduced into the lysosome to be degraded.

Growing interest in studies of autophagy has led to the discovery of Autophagy-related Genes (ATG) in yeast and their homologues in mammals [11,12]. At present, over 35 of the ATG genes identified in yeast have been shown to be highly-conserved in mammals as well [12]. Starvation and selective autophagic processes are known to be performed by 15 ATG genes (ATG 1-10, 12-14, 16 and 18) [12]. In addition to these Atg proteins, several other protein-protein interactions have been described that participate during the formation of autophagic vesicles.

As mentioned above, autophagy is triggered by certain stimuli, including starvation, amino acid limitation and hypoxia, among others, all of which lead to the formation of autophagic vesicles and their elongation. Pro-autophagic stimuli affect various intracellular proteins associated with autophagic event. One of these is the evolutionarily-conserved protein kinase TOR/mTOR (Target of Rapamycin) [13], which is considered an important autophagy-regulating protein, since its phosphorylating activity inside pro-autophagic proteins –specifically ATG13– inhibits the onset of autophagy. In yeast and mammals, TOR can form two complexes: TORC1 and TORC2. TORC1 is the most important complex involved in regulating autophagy.

Pro-autophagic stimuli propitiate, first, the formation of autophagosomes, followed by the sequestration and degradation of the cytoplasmic contents. The entire process is mediated by the formation of different Atg protein complexes that, in turn, act to regulate the different steps involved in the autophagic process. Several different complexes have been identified during this process, including one that responds to the initial signals; namely, the Atg1/unc-51-like Kinase (ULK) complex, Atg13, and Atg17. The complexes that mediate vesicle expansion are Atg6, Atg14, Vps34 and Vps15, while the systems that mediate vesicle expansion are Atg8/LC3 (microtubule-associated protein light chain 3) and Atg12 (Figure 3). All the complex or conjugation systems formed during autophagy are highly-regulated. Today we know that there are orthologues of Atg proteins in mammals; however, many of them have not yet been identified.

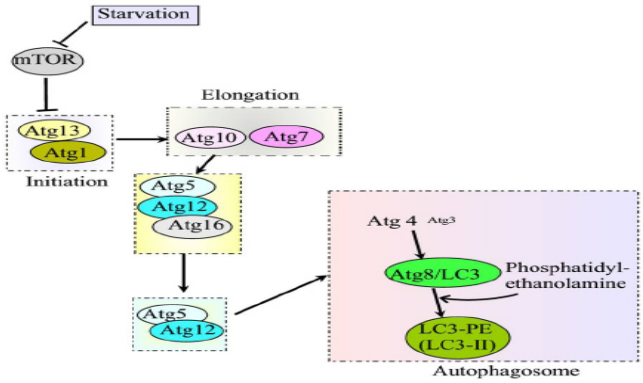

Figure 3: Mammalian autophagy is conducted by sequential steps, regulated by the Atg proteins. The starvation stimulus stops the mTOR activity, allowing the initiation of the autophagy by Atg13 dephosphorilation. Subsequently, diverse Atg complexes are formed until the complete autophagosome formation.

The Atg1/ULK complex is the initial Atg protein response to starvation stimuli. Under normal conditions, the protein serine/threonine kinase complex, TORC1/mTORC1, inhibits the formation of autophagosomes by phosphorylating a group of highly-conserved, Autophagy-Related Proteins (Atgs) [11,12]. Under normal conditions, TORC1 phosphorylates the Atg13 protein to maintain autophagy at a basal level [14], but under conditions of nutrient deprivation TORC1 activity decreases, resulting in the non-phosphorylation of Atg13, which allows Atg1-Atg13 to be incorporated into the Atg17-Atg29-Atg31 complex [14,15].

In mammals, expansion of the autophagic vesicles requires the formation of the PI3K/Vps34 complex, which is made up of Vps34, Beclin1/Atg6 and P150/Vps15 [16]. The lipid kinase Vps34 catalyzes the phosphorylation of Phosphatidylinositol (PI) to produce PI3K which, in turn, acts as a signaling molecule to recruit the proteins involved in membrane tethering and fusion [17,18]. In yeast, autophagy requires the formation of the so-called complex I, which includes Vps34, Vps15, Atg6, and Atg14. Orthologues of Atg14/Atg14L, or Barkor, and Vps38/UVRAG (Ultraviolet Irradiation Resistance-Associated Gene) have also been identified [19-21]. Under normal conditions, a sub-population of Atg14L localizes to the endoplasmic reticulum, but this disposition is altered under conditions of nutrient limitation, allowing the appearance of Atg14L in the phagophore (where it co-localizes with Atg16L) and in the autophagosomes (where it co-localizes with LC3-positive structures) [19-21]. These events indicate the role of Atg14L in diverse phases of the formation of autophagosomes.

The formation of Atg12 and Atg8/LC3 conjugation systems is important for the elongation and expansion of the phagophore's membrane. Sequential steps are required to constitute this complex, as the Atg12-Atg5 system conjugation [22] interacts with Atg16 to form the Atg12-Atg5-Atg16 complex, which is located in the nascent autophagosome [23,24]. Then, Atg7 and Atg10 catalyze the Atg12-Atg5-Atg16 conjugation system to allow formation of the Atg12-Atg5 complex. This complex stimulates lipidation of the Atg8/LC3 protein (microtubule-associated protein light chain 3) with Phosphatidylethanolamine (PE). LC3 is a key regulator of autophagy, as its activity initiates the biogenesis of autophagosomes. The lapidated form of LC3 (LC3-II) is associated with the expanding double membrane of the autophagosome [25,26] that is present in both the inner and outer membrane of vesicles.

Autophagy is a highly-regulated process that, as mentioned above, requires a series of ordered steps in order to form vesicles with cytoplasmic contents. The importance of autophagy as a physiological process resides not only in its role as an important route for eliminating altered or undesirable proteins that cannot be disposed of through the proteasomal route, but also in its participation in survival strategies implemented under stressful conditions.

Role of autophagy as programmed cell death

Autophagy is generally considered as being beneficial to cells. In addition to its contribution to maintaining intracellular homeostasis by eliminating intracellular damaged components, it takes on even greater importance because this process coincides with the recycling of cellular contents for which the amount of nutrients available are limited. In this way, autophagy increases the efficiency of system [see 10 for a review]. Defects in this process have been associated with tumor development, neurodegenerative diseases, cardiac hypertrophy, diabetes and pathogenic infections, as well as abnormal differentiation in many cell lines [10]. The wide variety of possible alterations during the autophagic process are related to the high accumulation of autophagic vesicles, which intensify the degradation process, producing conditions that are adverse to the correct cell functioning, and suggest that autophagy may play a role in cell death.

Cells undergo a process called constant-basal autophagy, an event that allows the recycling of amino acids. However, observations from several studies strongly suggest that autophagy also has a role as a process of cell death [27-32], though this proposal has been the subject of intense debate. In addition, diverse evidence supports the notion that autophagy is also an important factor during such tissue-remodeling processes as differentiation and development. To differentiate basal –or physiological– autophagy from the autophagy of cell death it is important to examine the issue of the levels of autophagy. In this regard, the increased amount of autophagic vesicles has been correlated with the cellular dysfunction that leads to cell elimination (Figure 4).

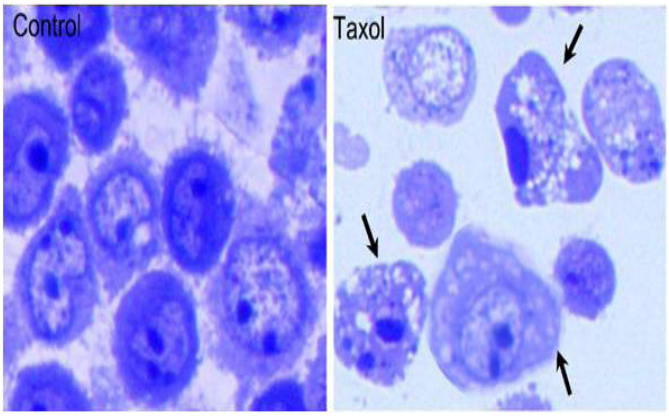

Figure 4: Cervical cancer cells (CaSki) stained with toluidine blue. The control cells have a normal morphology, evidencing large nucleus, homogenous cytoplasm. After the treatment with taxol, a high quantity of vesicles is present in the cytoplasm of several cells (arrows), evidencing an increased autophagic activity.

Autophagic cell death is known as programmed cell death type 2 [33]. Its specific morphological characteristics include: the ultrastructural presence of double-membrane autophagic vesicles with cytoplasmic contents in different degrees of degradation, and the absence of shrinkage of the cytoplasm and DNA fragmentation. High amounts of lysosomes have also been associated with autophagic cell death, since they are responsible for degrading the autophagic cargo.

At the biochemical level, autophagy as an event of cell death is characterized by the participation of the Atg proteins, which contribute to the different steps that conclude with the process of effective autophagy. As mentioned above, TOR maintains autophagy at a basal level under normal conditions by phosphorylating the Atg13 protein [15]. Meanwhile, along the signaling pathways regulated by mTOR [34] lysosomes play an interesting role marked by the presence of amino acids in the lysosomal lumen that allow mTORC1 activation [7,35,36], which confers certain properties to the lysosomes that allow them to act not only as degrading organelles but also as autophagic regulator.

The diverse steps that lead to the formation of complete double-membrane vesicles are highly-regulated by the Atg proteins which, in turn, are regulated by diverse stimuli, such as starvation, amino acid limitation and hypoxia, among others. Cells exposed to sustained, pro-autophagic stimuli are susceptible to the increasing autophagic degradation of their cytoplasmic contents, which leads, eventually, to a condition of general failure that ends in cell death.

Autophagic cell death has been evidenced in several different processes. In Drosophila melanogaster, this form of programmed cell death plays important roles as metamorphosis progresses during organ regression [37]. Also, the degradation of the salivary glands of these insects reflects not only the apoptotic process but also a massive accumulation of lysosomal vesicles [38] that reveals the functioning of autophagy as a process of cell degradation.

The participation of autophagy as programmed cell death has also been demonstrated in mammals; for example, during the regression of mammary glands after lactation in mice and rats [39]. Evidence of this route of elimination in ovaries has been observed during follicular atresia in oocytes taken from both young and adult Wistar rats [32,40,41]. Moreover, experimental models in which apoptosis is inhibited, or the machinery required for apoptotic cell death is made incomplete, have shown that autophagy is capable of eliminating cells destined for death [42]. As a result of all this accumulated evidence, autophagy has been defined as a recently-confirmed route that functions to eliminate undesirable or damaged cells, thus contributing to tissular homeostasis.

Autophagy and cancer

Autophagy has been implicated in both tumor suppression and the progression of certain cancers. The effect of the autophagic process on each of these processes depends on cell type and the stage of advance of the disease. In normal –i.e., non-tumor–cells, autophagy functions as a cytoprotective system, since under certain stressful stimuli cells respond by seeking to ensure their survival. The absence or inefficiency of autophagy in normal cells gives rise to diverse responses, including the formation of benign tumors, the accumulations of enlarged mitochondria, genomic damage, or cancer prone [reviewed in 10]. This suggests that failures in the autophagic process may lead to pro-cancer events, while at the same time revealing the role of autophagy in maintaining proper intracellular homeostasis.

But in tumor cells, this cytoprotective pathway of autophagy changes and this process begins to function distinctly. It is important to note that cancer cells are characterized by a process of constant proliferation that increases cell populations and generates conditions of reduced nutrient availability. Clearly, this involves an increased metabolic demand which entails that the cells must recycle in order to take advantage of the scarce cytoplasmic resources in their microenvironment. As a result, they use autophagy to survive under these stressful conditions, and this is where we can observe the role of autophagy in promoting cancer progression.

Normal tissue architecture is programmed to provide all the resources that cells need through the proper blood irrigation system. However, the high rates of cell proliferation during tumor progression no longer correlate with the process of angiogenesis, conditions that lead to decreased vascular leakage. Under such hypoxic conditions, tumor cells use autophagy to survive. Studies have verified that the angiogenesis process plays crucial roles in tumor growth, wound healing and tissue restoration [see 43, for a review]. The fact that autophagy rescues tumor cells from cell death under hypoxic stimuli, means that this process actually promotes cancer development. On the other hand, this property of autophagy has been used to conduct cells towards the programmed cell death type autophagic by suppressing tumorigenesis, since under the sustained hypoxic stimuli provided by a variety of drugs, some cells suffer as a consequence of the up-regulation of autophagy [reviewed

in 44].

Hypoxic stimuli also generate higher levels of Reactive Oxygen Species (ROS). Under these stressful conditions, autophagy is activated to rescue cells from genotoxic stress. This strategy involves eliminating damaged mitochondria to avoid DNA damage, since the ROS known as OH can directly break the backbone of DNA structures [45,46]. Strategies developed to control the cancer process include inducing DNA damage to produce conditions in which autophagy can function as a process of cellular rescue by means of recycling the intracellular components such that they can provide the energy required to support the process of repairing the DNA and thus avoid cell death [27]. This point indicates autophagy's contribution as a mechanism that develops chemoresistance in cancer cells. On the other hand, the deficiency of certain autophagic genes, such as UVRAG, Atg5 and Atg7, promotes DNA damage and stimulates tumorigenesis. These controversial, and apparently contradictory, functions of autophagy are the focus of much ongoing research. The fact that autophagy functions as a cytoprotective mechanism could provide an opportunity to take advantage of this first autophagic response to substantially increase the process of intracellular degradation and so induce the up-regulation of autophagy in order to change its function from that of a cytoprotective mechanism to a tumor suppressor factor.

Diverse experimental assays have made it possible to determine-or at least propose-new treatments to control cancer progression, therapies that take advantage of the role of autophagy as a process of cell death. Mouse models using the lysosome-inhibitory reagent chloroquine combined with conventional chemotherapy have produced increases in tumor cell death [47], and those pioneering studies led to the design of new autophagy-inhibiting drugs.

The role of autophagy in cancer processes is still in debate because of its cytoprotective functions in stressful environments. However, its characteristics could be improved in order to induce the up-regulation of autophagy, which would lead to a general intracellular collapse due to the increased presence of organelles and the degradation of the cytoplasmic contents (Figure 5). The fact that stimulating the autophagic process can guide cells towards regulated cell death is an advantage that must be taken into account, especially in apoptotic-resistant cancer cells.

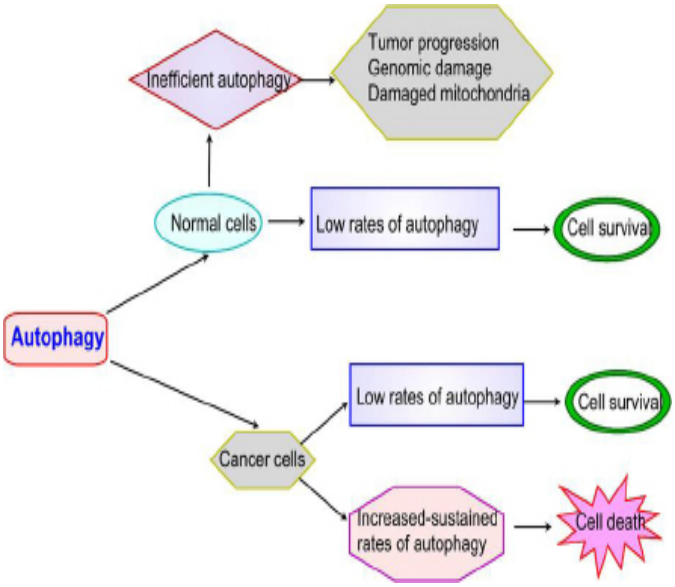

Figure 5: The autophagy process induces different cell responses. In both normal and cancer cells, the autophagy can promote the cell survival, however its characteristics could be improved under increased and sustained stimulus, the autophagy can to conduce to the cells toward the cell death.

Autophagy vs. Apoptosis

Traditionally, the term programmed cell death has been used to refer only to apoptosis, but we now know that other types of programs designed to eliminate cells exist; among them, autophagy. Each route of cell degradation has its own morphological and biochemical characteristics (Figure 6). Looking first at apoptosis, we know that this type of cell death is characterized morphologically by cell shrinkage, DNA fragmentation, condensation of the nucleus and, finally, the formation of apoptotic bodies [48] (Figure 7). This process is effectuated biochemically by proteases called caspases (cysteine proteases) that have the ability to break down the different constituent elements of the cell, including the caspases themselves (auto-catalysis), cytoskeleton proteins, and DNA, among others. Caspases are present in the cell as zymogens, an inactive form known as procaspases [49]. Two groups of caspases work together to conduct the apoptotic process: initiator caspases (such as -8, -9, and 10), and executioner caspases (including -3,-6,& -7) [50]. The inactive proteases can be activated along two pathways: one extrinsic, the other intrinsic (Figure 8). The extrinsic route is mediated by a receptor present in the cytoplasmic membrane that, once activated, causes the recruitment of a group of proteins to activate and dimerize the aforementioned initiator caspases. Once activated, these initiator caspases activate the executioner caspases, which are responsible for the morphological characteristics of the apoptotic process. The intrinsic pathway, in contrast, involves Mitochondrial Outer Membrane Permeabilization (MOMP), which releases pro-apoptotic factors such as cytochrome-c [51] that promotes the formation of apoptosomes. The apoptosome is a complex protein structure made up of cytochrome-c, the Apaf-1 protein, and caspase-9 [52]. Once formed, it is capable of activating the executioner caspases. Apoptosis is regulated by the B lymphoma 2 (Bcl-2) family of proteins, a family that is sub-classified into three different groups according to the specific properties of these proteins in terms of inhibiting or promoting apoptosis. These classifications are: the anti-apoptotic domain (Bcl-2, Bcl-XL, Mcl-1), the pro-apoptotic multi-domain (Bax, Bak), and the pro-apoptotic BH3-only domain (Bid, Bim, Bad, Noxa). These Bcl-2 proteins act at different levels along the apoptotic pathway; in fact, the Bax and Bak proteins are responsible for allowing the MOMP to deliver cytochrome-c from the mitochondrial space, thus promoting the formation of apoptosomes. In contrast, higher levels of the anti-apoptotic Bcl-2 impede the apoptotic process, thus promoting cell survival. During disruption of the mitochondrial membrane, the Second protein Mitochondria-derived Caspase Activator (SMAC) can be delivered in addition to cytochrome-c. This protein blocks the X-linked caspase Inhibitor of the Apoptotic Protein (XIAP) [reviewed in 53]. The relationship between increased levels of IAP or XIAP expression and cancer progression has been amply demonstrated [54,55].

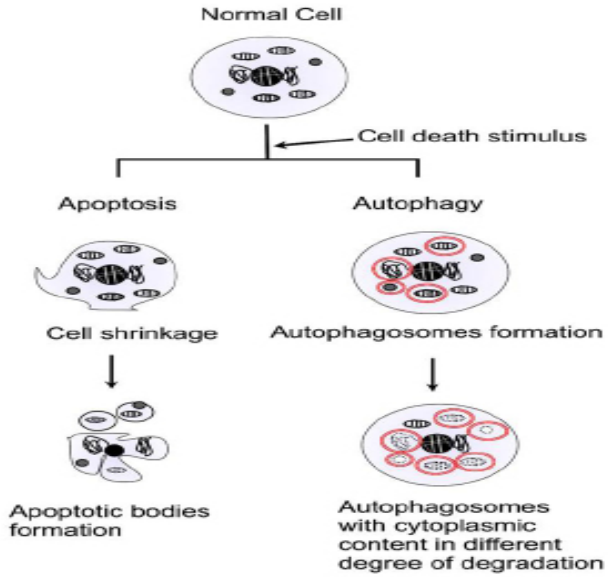

Figure 6: Programmed cell death processes: apoptosis and autophagy. Apoptosis is morphologically characterized by the cell shrinkage, and the formation of apoptotic bodies. During the autophagic cell death an increases quantity of autophagic vesicles in different degree of degradation are present in the cytoplasm.

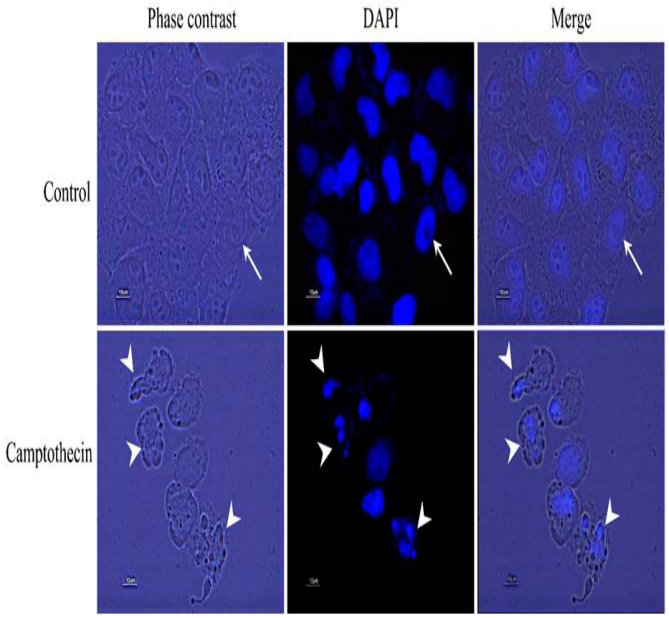

Figure 7: HeLa cervical cancer cells treated with the pro-apoptotic drug camptothecin. The control cells have a polyhedral shape (phase contrast), the DAPI stain evidence the chromatin distributed in the nuclear space. The arrow is pointing a group of normal cells. The morphological changes are evident after the treatment with camptothecin, the cellular shape has changed to a round shape as a consequence of the cellular shrinkage, the chromatin is highly compact, and several apoptotic bodies are evident (arrow heads).

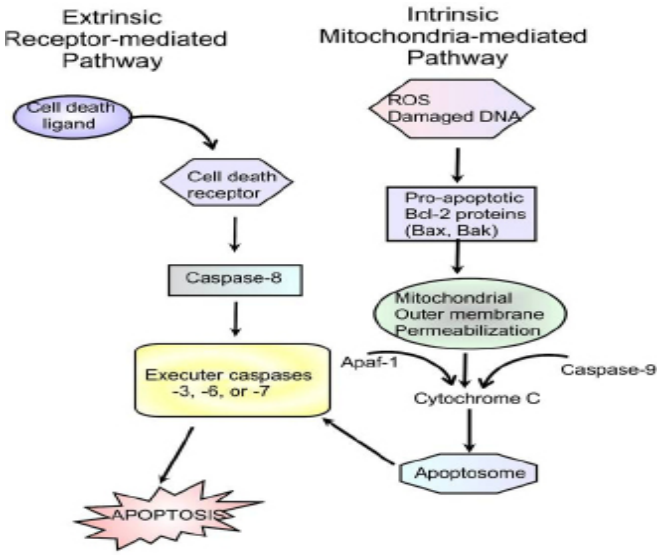

Figure 8: Extrinsic and intrinsic apoptotic routes. Extrinsic route is mediated by a receptor present in the cytoplasmic membrane, the interaction of this receptor with its ligand, allows the activation of the initiator caspase-8, which in turn will activate to the executioner caspases. The mitochondria-mediated intrinsic route, requires the participation of the Bcl-2 pro-apoptotic proteins to induce the MOMP and deliver the cytochrome-C protein to form the apoptosome, which in turn will activate the executioner caspases.

Turning now to autophagic cell death, also known as type 2 programmed cell death; its morphological hallmark is the large quantities of autophagosomes that form in the cellular cytoplasm, though neither cellular shrinkage nor condensation of the nucleus occur. The biochemical process carried out during autophagy involves a significant increase in the activity of the Atg proteins and Atg8/LC3 lipidation, as mentioned above. It is important to mention that autophagic cell death is conducted by a complex molecular mechanism in which the Atg proteins (or their homologues in mammals) are the principle players that produce the increased levels of autophagic vesicle formation. In fact, some of the complexes that form, and are preserved, during autophagy are essential to the process. One example is the Atg-12-Atg5-Atg6 complex, which is required for proper Atg8/LC3 lipidation with Phosphatidylethanolamine (PE). Any disruption of even one of the components of this complex inhibits the formation of autophagosomes [56]. Hence, the two key properties of type 2 programmed cell death are the increased quantity of autophagic vesicles in the cytoplasmic space and, at the biochemical level, the high levels of Atg8/LC3 lipidation during the formation of autophagosomes.

Under cancerous conditions, cells develop the ability to elude cell death. Existing knowledge of different types of cell death makes it possible to design strategies to eliminate these undesirable cells. Obviously, cancer cells behave differently than normal cells, and there is even evidence that in some cases the molecular machinery that performs apoptosis is absent or ceases to function in those cells [57-59]. Under these conditions, apoptosis cannot eliminate the altered cells; hence, it is necessary to design new strategies to control cancer progression, strategies that involve not only the cytotoxic agents that

are known to lead cells towards necrotic death or apoptotic elimination. The difference in the molecular and morphological characteristics of necrosis and apoptosis consists in the fact that the former induces an immunological response triggered by the loss of the integrity of the cytoplasmic membrane in necrotic cells. As a result, controlling pathological cells using necrosis-inducers provokes undesirable side effects in patients so treated. Research and the design of new therapies that guide altered cells to apoptosis has allowed us to discover the deficiencies in the molecular machinery inside these cells. In this regard, autophagy has been proposed as a potential new pathway for eliminating cancer cells that do not respond to pro-apoptotic stimuli. As mentioned above, this is a recent field of research, but the fact that this process has been shown to be involved in metabolic processes that maintain tissue homeostasis –as in rat ovaries– or during development and metamorphosis in insects, justifies exploring the feasibility of inducing autophagy as a process of cell death.

Some reports from in vitro experiments have produced evidence to suggest that inducing autophagy could be an effective method for controlling cancer cells [60]. In addition, the feasibility of simultaneously inducing both routes of cell death –apoptosis and autophagy– has also been evidenced [61]; allowing the proposal that type 2 programmed cell death could be a viable route for the elimination of cancer cells.

Other studies, however, have demonstrated that autophagy can actually promote cancer growth by allowing cancer cells to survive under nutrient-deprived or hypoxic conditions, or despite the DNA damage caused by chemotherapy [62]. This research suggests that resistance to cell death occurs under the aforementioned stimuli, and thus supports the notion that autophagy promotes, rather than inhibiting, the proliferation of cancer cells. In this regard, one key point to consider before choosing autophagy as the stimulus for the control of cancer cell proliferation is the nature of the microenvironment in which those cells develop. It is also important to assess the intensity of the pro-autophagic stimulus required to ensure that it functions as a process of cell death and not as a mechanism that offers cytoprotection.

It is thus clear that while the use of high doses of pro-autophagic stimuli might yield better therapeutic results than pro-apoptotic stimuli, if the doses of pro-autophagic stimuli applied are too low they could have the opposite effect and promote the proliferation of cancer cells. Hence, the role of autophagy in tumor progression must be evaluated very carefully to avoid inadequate conditions in which this process could protect the cancer cells. The realization that autophagy is another, very significant, process of cell death has opened new avenues for designing strategies to control this route of cell elimination that consider autophagy an oncotarget that can be regulated during anti-cancer therapy.

Cervical cancer

Cervical cancer is the second-most common form of cancer in women, and one of the principle causes of death in females, with an especially high impact in developing countries. The main risk factor for developing this disease is infection by the Human PapillomaVirus (HPV). To date, some 100 different types of HPV have been identified, of which at least 40 are capable of infecting the female genital tract. Of these, 13 are classified as “high risk” (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68), while 7 sub-types have been associated with higher incidences of cancer development (26, 53, 66, 67, 70, 73, 82). HPV18 is especially strongly-related to cervical adenocarcinoma, while HPV16 is related to the development of cancer in squamous cells. The first HPV types identified were 18 and 16, the two most often related to cervical cancer development, as demonstrated by the frequency with which they appear in biopsies [63].

In and of itself, HPV infection is not decisive in the development of cancerous conditions, but it is unquestionably an important risk factor in the generation of tumor processes. It is when an HPV infection becomes chronic and persists for several years that it may lead to cancer development. Because the incidence of cancer progression in HPV-infected patients is below 1%, other factors must be present in order for cancer to occur [64].

HPV completes its infectious cycle by infecting epithelial cells. Once inside those cells, it produces infectious particles that will be spread by those same cells. This process requires the expression of different viral products –encoded in the virus’ genome– that couple to the process of epithelial cell differentiation. This finding allowed researchers to establish a direct relationship between viral progression and morphological changes in infected cells, and this has provided a useful tool for identifying the level of disease progression from the initial (CIN I) to advanced phases (CIN III) [65]. In this regard, cytological evidence shows that cells suffer morphological changes during the transformation to malignancy, and that these changes have made it possible to group cervical intraepithelial neoplasia into three categories: initial (CIN I), moderate (CIN II), and severe (CIN III).

The HPV genome contains a circular, double-stranded DNA approximately 8 kilobase pairs in size. Three distinct regions can be distinguished: The regulator region, whose function is to control replication and transcription (URR); the codifying region for “early” protein expression (E1, E2, E4, E5, E6, and E7); and the codifying region for “late” protein expression, related to viral capsid formation (Figure 9). The replicative cycle of HPV is coupled to the keratinocyte differentiation program. The “early” viral proteins are produced in non-differentiated keratinocytes in the basal layer of the stratified epithelium, while the “late” proteins are produced in the distal epithelial layer where the viral capsid forms. HPV does not infect, or replicate in, the antigen-presenting cells of the epithelium, thus avoiding the possibility that these cells could enclose the virions to activate the immune system, while also limiting the initial immune response. The “early” proteins accumulate first in the nucleus, where they generate a low response by the immune recognition system. The “late” proteins appear several months after infection onset, but their low level of expression substantially reduces the likelihood that they will be detected, so the immunological response elicited will be modest [66].

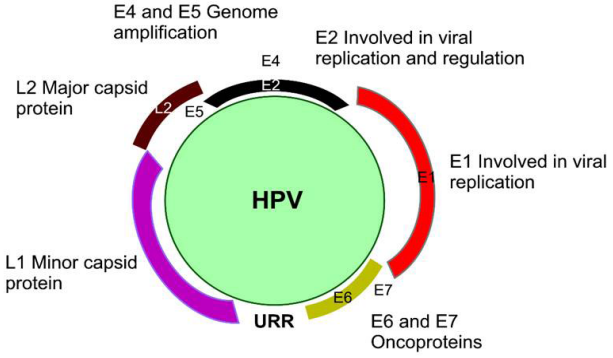

Figure 9: Different regions of the HPV genome. Three distinct regions can be distinguished.

An HPV infection begins when the viral particles reach micro-abrasions in the basal epithelial layer. Once a cell is infected, the viral genome is established as an episomal genome inside it and begins to generate a few copies before integrating its genome with that of the host cell. In this phase, the E1 and E2 proteins are the only ones expressed by the viral genome. These proteins are keys to viral replication and segregation during division in the host cell [67]. E2 has an attachment domain to DNA close to the origin of replication, but E1 is recruited. E2 has attachment sites to several proteins, such as L2 and different host cell proteins. Infection begins in the cells closest to the basal lamina, which are characterized by a high replication rate. At that point, the proto-oncogenic proteins E6 and E7 are not necessary, and are expressed at levels so low that they go undetected by the immune system. Obviously, this allows the virus to continue propagating enclosed within the infected cells while undergoing a continuous proliferation process. These low levels of proto-oncogenic proteins can persist for several years in the form of a chronic infection. During the subsequent phase of infection, the E6 and E7 proteins induce the proliferation of the infected cells, causing cervical neoplasia and, eventually, cancer [68].

It is well-known that inducing cell proliferation is one of the most important functions of the E7 protein, and that this occurs through its interaction with members of the Rb family of proteins. This process of interaction induces the release of E2F transcription factors. Once these are released, they translocate to the nuclear space where they activate cellular progression. Also, E7 is capable of inactivating the cyclin kinase cell-cycle regulators p21 and p27, thus promoting unregulated cell division. p53 is ubiquitinated by the E6 protein so it can be degraded by the proteosome pathway and thus elude cell cycle arrest and evade apoptotic cell death. E5 is a transmembrane protein located in the endoplasmic reticulum and associated with the proton pump responsible for endosome acidification. This affects the recycling of the growth membrane receptors in epithelial cells, causing increased EGF-mediated signaling that propitiates proliferation of both the cell and the virus [69].

The final phase of the HPV infection cycle begins when the replicated viral particles are packaged and released by the host cell. At this point, the L1 and L2 proteins are expressed actively, since they shape the viral capsid. Also, expression of the E4 protein increases; it is stored in the cytoplasm and then expressed on the surface of the infected cells. The E4 protein has been related to the release of the virus from the host cell, so high levels of this protein have led to it being used as a marker that identifies advanced phases of this viral infection [70].

At present, several methods for diagnosing and preventing cervical cancer exist. The one most commonly used is cervical screening (the Papanicolau smear test). Diagnosis is based on morphological analyses of the exfoliated cells [71]. Smears of normal cells show the presence of keratinized cells, but smears of transformed cells reveal large nuclei and regular cytoplasm (characteristics similar to those of cells in the basal layer). The Pap smear test has led to a significant reduction in cases of cervical cancer, but it is important to remember that as a morphological test it has a relatively high level of error. Moreover, it does not allow determination of the degree of disease advance, so constant tissue monitoring is necessary [72].

A test with greater sensibility and precision evaluates the DNA of the HPV. It has the advantage of detecting the degree of tumor advance. The technique most widely-used to detect viral DNA is called PCR (polymerase chain reaction). Unfortunately, this is a very expensive procedure, so access to it is limited, especially in the general population of developing countries where the incidence of this type of cancer is high.

More recently, new means of diagnosing the development of pre-cancer lesions have been introduced, such as E6, E7, and p16 mRNA detection. However, these markers are only capable of detecting cancer processes when they are already in advanced stages.

Not long ago, a vaccine against type 16 and 18 HPV infections was developed. Since them it has been included in vaccination programs in an effort to reduce the incidence of cervical cancer. This vaccine should be administered before young females become sexually active to eliminate the risk of HPV infection. It has been estimated that vaccination could considerably reduce cases of cervical cancer. Two vaccines are currently available: Gardasil (Merk & Co) and Cervarix (Glaxo Smith Kline). Both protect against HPV-16, HPV-18, HPV-31, and HPV-45. In addition to the vaccine, it is important to offer informative programs to prevent HPV infection, since timely detection means a higher probability of controlling cancer development [73].

It is of supreme importance to continue and increase research into the development of new drugs to combat cervical cancer, especially ones with the ability to eliminate cancer cells specifically with fewer-or no-side effects.

Resistance to therapies

Dynamic developments in science have allowed the generation of new strategies for the early detection of cervical cancer. Detecting tumors in the initial phases certainly means better prognoses for patients. Indeed, evidence shows that when treatment begins in the early stages of the disease evolution is almost always favorable. However, despite all the clinical tools available for detecting cervical cancer, this disease continues to be one of the most common causes of death worldwide, in part because most cancers are asymptomatic in the beginning. One important problem associated with cancer in general is that symptoms tend not to be apparent until the late phases of the disease, often when metastasis has already begun. As a result, treatments for cancer in advanced phases are more aggressive than those applied in initial stages. Chemotherapy, radiation therapy, anti-hormonal therapy, and combinations of these approaches are among the most common strategies employed in advanced cases of cancer, but prognoses in such cases are usually discouraging.

Because one of the principle properties of tumor cells is their high rate of cell proliferation, one strategy deployed is to eliminate as many cells as possible to inhibit this accelerated process of cell division. Chemotherapy consists in delivering drugs that arrive in an active state and in concentrations sufficient to induce the process of cell death in the tumor cells. Unfortunately, most tumor cells generate resistance to this kind of therapy, either through genetic predisposition or a process of acquisition. To make matters worse, the drug resistance developed by cancer cells is highly dynamic, a response that can emerge against different drugs with distinct mechanisms of action [74].

Chemo resistance develops in tumor cells as drug treatment advances. We know this because at treatment onset the cancer cells are eliminated efficiently, but –inexplicably– at some point during therapy the tumor mass begins to grow again. Clearly, high rates of selection occur inside the tumor microenvironment, since the tumor cells generate –relatively quickly– diverse mutations to adapt to the surrounding conditions, a situation that triggers intense competence for survival among the different cell populations that develop inside the tumor. Here, the cells that develop more mutations acquire a selective advantage over other cells in the microenvironment. This hypothesis could explain the resistance acquired progressively by tumor cells, since when the pro-elimination stimulus is first applied it eliminates the most sensitive cells, leaving the less sensitive ones to develop new phenotypes that confer resistance and the possibility to continue cell progression. Diverse mechanisms in the cell that promote resistance to chemotherapy have been described, including: low drug accumulation inside the cell, increased inactivation of the drugs or their intermediaries, modification of the target molecules, increased DNA repair rates, and altered mechanisms of programmed cell death [75] (Figure 10).

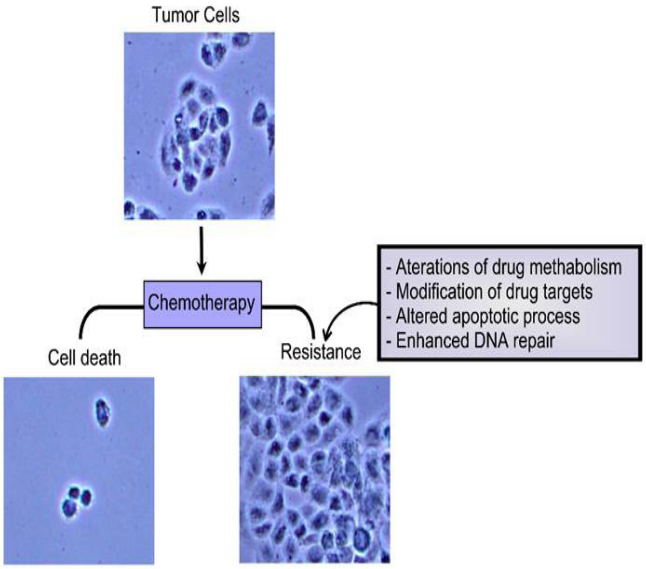

Figure 10: Tumor cells respond in different manner under chemotherapy treatments. The desirable response is that the cells are conducted to the cell death; however, they are capable to develop a resistance by means of different strategies.

In cell cultures, the most frequent mechanism of resistance to chemotherapy is reduced drug accumulation inside the cells, an effect that could be produced by the activation of specific carriers present in the cytoplasmic membrane that actively "pump" drugs out of the cells. Reductions in the amount of the drugs available inside the cells can also be achieved by the accumulation of cytoplasmic vesicles that fill with the active ingredients and thus impede delivery [76].

The existence of the drug resistance-related protein (LRP) was demonstrated by Dano [77], who described the active efflux of daunomycin in treated cells, and showed that those same cells are resistant to doxorubicin. P-glycoprotein (Pgp), a 170 Kda protein implicated in multi-resistance to drugs, was described by Kartner and cols. [78]. Proteins including Mrp (Multidrug resistance-Associated Protein), Bcrp (Breast Cancer Resistance Protein), and Lrp (Lung Resistance-related Protein) are expressed normally in different cell types, such as liver, kidney and lung cells. These proteins are related to normal detoxification routes, but their over-expression derives in tumor processes.

In the liver, kidneys and intestines, the Pgp protein functions as an H+-ATPase proton pump that participates in eliminating toxic substances. But in tumor cells, Pgp is over expressed and has been related to resistance to several drug types: taxans, etoposides, anthracyclines, and extracts of Vinca rosea (C. roseus).

Mrp is a family of transporters that includes Mrp1, Mrp2, Mrp3, Mrp4, Mrp5, and Mrp6. These transporters are present in both the cytoplasmic membrane and the membrane of the endoplasmic reticulum, where they participate in the elimination of toxic substances and the formation of cytoplasmic vesicles that can sequester the chemotherapy drugs administered. They also have the ability to transport anions and to participate in eliminating guttation-conjugated, natural toxins and heavy metals like arsenic, and are related to cisplatin resistance. The protein of this family that has been most intensively studied is 190 KDa Mrp1, which is highly-expressed in normal lung, prostate and thyroid cells, and has a low level of expression in kidney cells [79].

The Brcp transporter protein was isolated from cells exposed to mitoxantrone [80]. Brcp (MXP, ABCP, and ABCG2) is activated by dimerization or multimerization. It has been reported to present high resistance to such drugs as doxorubicin and topocan, and to interfere with topoisomerase I inhibitors. Brcp has been reported in normal mammal epithelium, has a low expression in lung tissue, and is highly-expressed in acute leukemia and lung cancer cells [81].

Lrp/MVP (major vault protein) is a 110 KDa protein, the principle constituent of a group of ribonucleoproteins with a complex, barrel-type structure called vaults. Most of these ribonucleoprotein complexes are associated with cytoplasmic vesicles near the enveloping nuclear pores. They have been related to nucleus-cytoplasm transport. Lrp has been detected in diverse tissues, including the lungs, kidneys, heart, prostate gland, and bone marrow. Lrp over-expression has been associated with resistance to such drugs as vincristrine, cisplatin, adriamycin, and taxol [82].

Multidrug resistance has been related to such altered molecular mechanisms as the topoisomerases. These are a group of proteins with enzymatic activity whose function is to break –in an orderly manner– the double-strand of DNA during the replication process in order to dissipate the mechanical tension inside the double-strand that results from this event. At the end of this process, these same proteins join the strands again. There are two classes of topoisomerases, simply called I and II. Topoisomerase II is an enzyme essential to DNA replication and transcription processes, as well as to recombination and chromosome segregation. Topoisomerase I, meanwhile, facilitates access of the different factors implicated in the aforementioned processes to the DNA, where they break and separate the double-strand. This is the homologue of the bacterial DNA gyrase, which is made up of a 170-KDa homodimer. Topoisomerase I functions similarly to type II, but on the single strand of DNA. Alterations in the structure of topoisomerases I and/or II could modify their sensitivity to the drugs designed to inhibit its activity. Most chemotherapeutics have as their target highly-proliferative cells that are undergoing a continuous replication process. The topoisomerases are targets of several anti-tumor drugs,

including: adriamycin, daunorubicin, ellipticine, actinomicyn D, mitoxantrone, topocan, and irinotecan. These kinds of drugs act by trapping the topoisomerases inside the DNA, a process that leads to cell death. In addition, it has been reported that some of the drugs mentioned above increase concentrations of enzymes associated with DNA breakdown and inhibit cells' capacity to repair the DNA. Significant activity by the topoisomerases has been reported in normal cells in the thymus and spleen, as well as in tumor processes in breast cancer and some sarcomas [83].

Tumor cells acquire the ability to evade the effects of chemotherapeutics by increasing their capacity to repair DNA. This allows the cells to elude programmed cell death and survive to continue proliferating. This increased capacity is due to the over expression of genes that code to DNA repair proteins like PARP (poly (ADP-ribose) polymerase) and O6 AGT (O6-alkylguanine-DNA-alkyltransferase). The results are the survival of the tumor cells and a lessening of the effects of anti-tumor drugs such as cisplatin.

Drug resistance is also related to the malfunctioning of diverse mechanisms that regulate the cell cycle and programmed cell death. In this case, DNA damaged by chemotherapy and then improperly or incompletely repaired can be passed on to future generations of cells in a process that leads to the successive accumulation of genetic alterations that reduce the effects of the drug therapy administered.

Multidrug resistance is thus a consequence of diverse factors, including the interaction between drugs and their targets and a variety of intracellular mechanisms that regulate cell proliferation and the cell death process.

Autophagy as a therapy in cervical cancer

The imperative to find new, more effective, therapies to control the proliferation of cancer cells has led to the design of mechanisms capable of eliminating cells that do not respond to the normal processes of cell death described herein; namely, accidental necrosis and apoptotic programmed cell death. One particularly interesting characteristic of cancer cells is their capacity for proliferation that sidesteps all the checkpoints that normally do not allow such uncontrolled growth. It has been shown that several kinds of tumor cells have specific characteristics that allow them to elude cell death by interrupting or inhibiting some step in the apoptotic mechanism.

Diverse proteins regulate the success of cell progression by preventing damaged information. The tumor suppressor gene p53 is responsible for inhibiting cell proliferation when a genome is altered; in fact, it is known as the "guardian of the genome" [84]. There are reports that down-regulation of p53 is related to over 50% of all cancers in humans [85]. The importance of this protein resides in the multiple activities it performs in cells. For example, it participates in cell-cycle inhibition when DNA is damaged, during apoptosis it can induce increases in the presence of reactive oxygen species, and it also promotes inhibition of blood-vessel formation [reviewed in 86]. There is considerable evidence that anomalies in the availability of p53 occur in diverse types of cancer, and that they inhibit cell elimination by means of apoptosis, thus allowing the cancer to advance. In the specific case of cervical cancer, HeLa cervical cancer cells contain wild-type p53 that is inactivated by expression of the E6 protein from HPV-18. The p53 protein induces cell death through translocation to the nuclear space that activates the transcription of target genes, such as the tumor suppressor TP53INP1 [87]. The fact that the p53 protein is inactivated in HeLa cells confers a certain resistance to apoptosis, though there is evidence that in HeLa cells TP53INP1 bonds to the Atg8/LC3 protein to induce HeLa cells towards cell death of the autophagic type [87].

Since it is evident that HeLa cervical cancer cells contain inactivated p53, recent research has focused on the capacity of p53 to inhibit cell proliferation in a status-independent manner. The D40 gene encodes a kinetochore protein, an important player in cell division, so inhibition of this protein by short inhibitory RNA (siRNA) leads the HeLa cells towards cell death in a p53-independent manner [88]. This suggests that cancer cells with little or no p53 protein that, as a result, do not respond to the apoptotic stimulus, can be led to cell death by means of this mechanism.

Another type of alteration present in cancer cells is marked by low levels of pro-apoptotic proteins, such as caspases [57-59,89,90] or members of the pro-apoptotic Bcl-2 family [91,92], which confer high resistance to the induction of cell death and thus promote proliferation of cancer cells. But also present in cancer cells are increased levels of anti-apoptotic Bcl-2 proteins. Intensive efforts to control cancer cells have resulted in the design of molecular strategies capable of restoring apoptosis. Certain molecular events regulate transcriptional activity in the cell, including Histone Deacetylase (HDAC), which acts at the level of transcription and cell differentiation by regulating the status of histone acetylation, and HDAC inhibitors, which have been reported to inhibit the growth of cancer cells [93] by inducing apoptosis. Surprisingly, however, in HeLa cells with apoptotic defects such as the deletion of the Apaf-1 gene or upregulated anti-apoptotic Bcl2, the HDAC inhibitors induce autophagic cell death [94]. The fact that tumor cells can avoid apoptotic cell death through different mechanisms has stimulated research into means of combating those using new mechanisms. In this case, autophagy emerges as a new potential strategy for eliminating altered cells (Figure 11).

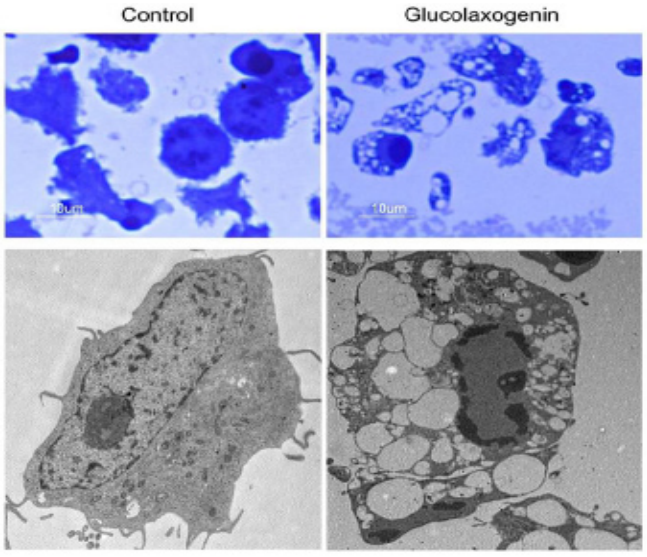

Figure 11: ViBo cervical cancer cells treated with the pro-autophagic drug glucolaxogenin. The upper micrographs are evidencing the general morphology of the cells stained with toluidine blue staining. The down electron micrographs are evidencing the ultrastructural changes provoked after the treatment with glucolaxogenin. Increased quantities of autophagic vesicles are formed conducing to the cells toward the autophagic cell death.

Autophagy as a mechanism of cell control has been evidenced in C33A cervical cancer cells treated with the glycoalkaloid α-solanine by downregulating the phosphorylated Akt/mTOR pathway [60], activating the autophagic process, and channeling the cells to autophagic elimination. Other chemicals that have been shown to induce autophagic cell death in cervical cancer cells are ursolic acid [95] and zoledronic acid [96].

Modulation of genetic expression is another tool employed to control cancer cells. Small interfering RNA (siRNA) is endogenous to the short non-coding RNAs that inhibit mRNA (messenger RNA) translation and so reduce protein expression [97]. Current molecular techniques allow the construction of diverse siRNA to inhibit the proteins involved in the pathways of cell death. For example, beclin 1, which is implicated in the autophagic process, has been silenced in HeLa cells by means of siRNA. This silencing induced cell proliferation, indicating that inhibiting autophagy allows the cancer to progress. In addition, the over expression of beclin 1 induced inhibition of HeLa cell proliferation, evidencing the role of autophagy as a cell death process capable of eliminating cervical cancer cells [98].

MicroRNAs (miRNAs) are about 22-nucleotides long. They act by negatively controlling genetic expression by blocking mRNA translation and/or mediating mRNA degradation. Upregulation of the expression of some ATG genes using miRNA techniques allows autophagic activation [99,100]. In HeLa cells, over expression of the miRNA MIR155 suppresses the activation of mTORC1 and AKTm, leading to a decrease in the number of cells [101]. In this vein, it has been shown that during the silencing of Beclin-1, Atg3, Atg4, Atg5 or Atg12 by means of siRNAs, cervical cancer cells become more susceptible to radiotherapy [102], which contributes to a more successful anti-cancer therapy.

The role of autophagic cell death in cancers is complex, given the fact that it also has the ability to act as pro-survival mechanism. In the case of cervical cancer, several studies have demonstrated that under specific conditions autophagy can be employed to eliminate cells that are unresponsive to apoptosis; but here it is important to consider the intensity of the stimulus to ensure that autophagy is sufficiently intense to propitiate cell failure.

Conclusions

Existing knowledge of the cancer process focuses largely on the question of how to control cancer cell progression. Thus, the process of cell death involving these types of cells has been amply analyzed in efforts to better understand their behavior. Most of the work on designing strategies to eliminate cancer cells has focused on the process of necrosis, but apoptosis has been seen as a more desirable method of elimination, in part because it does not produce the intense side effects associated with necrosis. Identifying the disrupted or incomplete pro-apoptotic players in cancer cells has now shifted research towards developing new strategies to eliminate cancer cells. Today we know that in addition to its cytoprotective function, autophagy at a certain intensity of activity can play a role in programmed cell death, and this has stimulated work on designing new strategies for treating cancer by inducing autophagy in a constant, sustained manner that can eliminate apoptosis-resistant cancer cells.

Acknowledgments

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