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REVIEW ARTICLE

APOPTOSIS IN HEALTH AND DISEASE AND MODULATION OF APOPTOSIS FOR THERAPY : AN OVERVIEW

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ABSTRACT

Apoptosis a physiological mechanism that eliminates excessive, damaged or unwanted cells, is a highly regulated pathway important for maintaining homeostasis in multicellular organisms. It can be initiated through various signals via the extrinsic pathway which involves death receptors, or via the intrinsic pathway which is initiated by intracellular damage and involves the mitochondria and release of cytochrome c from it to further activate caspases. The Bcl-2 family of proteins is situated upstream to the irreversible damage of cellular constituents and is an important checkpoint in the fate of a cell. The pro-apoptotic members, BH3 only members include BID, BAD and BIM. They directly or indirectly activate multidomain BAX/ BAK that constitute the requisite gateway to the intrinsic pathway which operates at the mitochondrial surface and endoplasmic reticulum. In contrast, antiapoptotic members such as Bcl-2, Bcl-XL bind and sequester activation. Downstream of mitochondria, the apoptosome involvement is seen to generate caspase activity. Post mitochondria regulation involves IAPs, and their inhibitors. The pathogenesis of several diseases such as cancer, neurodegenerative disorders, autoimmune disorders, heart disease, infectious diseases including AIDS is closely related to aberrant apoptosis. Consequently interest has emerged in employing various therapeutic approaches such as gene therapy, antisense therapy, recombinant biologicals, organic and combinatorial chemistry, to specifically target apoptosis signaling pathways such as death receptors FAS/TRAIL, Bcl-2, p53, IAPs, SMAC and caspases, etc. and are now advancing from preclinical to clinical phase.

KEY WORDS

Apoptosis, Mitochondria, Caspases, Death receptors, Apoptosis based therapies.

INTRODUCTION

Apoptosis is a highly organized, evolutionary conserved, genetically regulated pathway for maintaining homeostasis in multicellular organisms and was coined by Kerr, Wyllie and Curie (1). A genetic program for developmental cell death emerged from the study of the nematode Caenorhabditis elegans by Horvitz who received the Nobel prize for this pioneering work (2). All cell types are capable of undergoing apoptosis, a mechanism that eliminates excessive, damaged or unwanted cells from an organism without damaging surrounding cells. The human body is composed of nearly 10^{14} cells. Everyday billions of cells are produced by mitosis and a similar number die by apoptosis to maintain tissue homeostasis. The cell death appears to be fixed developmentally and is thus programmed and hence also called PCD. Normal cells become unwanted, either because they are superfluous, have served their function, are diseased, have turned senescent or are a threat to the developing organism. Physiologically, all multicelluar organisms use apoptosis/PCD during development, homeostasis, defence, metamorphosis, terminal differentiation, immune response, cellular response to growth factors and hormones. It plays an important role in the immune system, in removing self reactive T cells by negative selection. Lymphocytes can induce apoptosis in target cells: T cells, NK cells have been described to do so. Withdrawal of hormones results in atrophy of the

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hormone dependent tissue and apoptosis has been found to be responsible for this phenomenon in prostate, adrenal cortex, endometrium, and in mammary glands. Apoptotic cell death has also been observed during gut development, in epidermal skin cells and remodeling of cartilage and bones and antiviral defence. It may also be used to minimize the risk in cells frequently exposed to mutagenic chemicals or radiations.

A violation of cellular homeostasis may play a role in various human diseases. Apoptosis dysregulation contributes to half of all human diseases. Excessive apoptosis occurs in neurodegenerative disorders such as Alzheimers, Parkinsons, autoimmune disorders, heart disease, infectious diseases including AIDS (3). Apoptosis prevents malignant transformation whereas abnormal apoptosis can predispose to cancer. Diverse chemotherapeutic agents kill sensitive cells by apoptosis.Apoptosis usually affects scattered individual cells, has distinct morphological features such as cell shrinkage, chromatin condensation, membrane blebbing, formation of apoptotic bodies and shows no inflammatory response. Apoptotic bodies are phagocytosed by neighbouring parenchymal cells or macrophages. Phagocytes recognize "eat me" signals on the apoptotic cell surface which activate cellular engulfment machinery. Phosphatidyl serine exposure on the target cell surface and the phosphatidyl serine receptor on the phagocyte are essential for phagocytosis (4). Cells may also be eliminated by a number of other mechanisms such as autophagy (self digestion), paraptosis, mitotic catastrophe, chondroptosis, etc. These types of cell death differ morphologically and biochemically (most are caspase independent) and each type depends on the type of cell system, kind and intensity of stimuli. In contrast to apoptosis, necrosis represents a passive, non-physiologically induced cell death frequently initiated by damage to the plasma membrane and accompanied by autolysis of the cell (5).

MOLECULAR MECHANISMS OF APOPTOSIS

There are three different mechanisms by which a cell commits suicide by apoptosis –

1. From signals arising within the cell
2. By death activators binding to death receptors at the cell surface i.e. TNF-α, lymphotoxin, Fas Ligand (Fas L)
3. Can be triggered by reactive oxygen species (ROS).

Apoptosis can be initiated by various external and internal signals and executed through receptor or mitochondrial and several interrelated signaling pathways controlled by the regulated expression of apoptosis-associated genes and proteins. The death receptor pathway exemplified by Fas ligand binding to an extracellular receptor, causes the formation of the Death Inducing Signaling Complex (DISC) that results in the activation of caspase-8. The mitochondrial pathway is activated by most cellular stresses, resulting in the release of cytochrome c into the cytosol. Cytochrome c binds to apaf-1 and pro-caspase-9 to form the apoptosome and catalyses the activation of caspase-9. Initiator caspases, such as -8 and -9, activate effector caspases that cleave multiple cellular proteins. Bcl-2 a proto-oncogene prevents apoptosis by blocking the release of cytochrome c from the mitochondria (6). There is significant cross talk between these two pathways (7,8).

Increase in free intracellular Ca^{2+} has been observed in several instances of apoptosis, but apoptosis in human CEM lymphocytes and in nerve cells dying in the absence of Nerve Growth Factor (NGF) do not require calcium influx to die. Alterations in intracellular calcium may lead to disruption of cytoskeleton organization and in turn blebbing. Several agents that increase c-AMP levels by independent mechanisms induce apoptosis accompanied by DNA fragmentation and the effects appear to be mediated by protein kinase A. Abnormal phosphorylation of cytoskeleton protein is associated with neural cell death in Alzheimer's disease and Down's syndrome. Thus protein phosphorylation and dephosphorylation might be important aspects of cell death (9). Exposure of cells to Reactive Oxygen Species (ROS) leads to apoptosis by inducing DNA strand breaks . Several proto-oncogenes are transiently expressed during early period of apoptosis such as myc, fos, jun. The activation of these genes are also associated with mitosis and differentiation, besides apoptosis. C-myc may play a part in regulating the choice between proliferation and apoptosis (10). Our own study has shown the involvement of c-Jun/AP-1 transcription factor in TNF-α induced apoptosis (11). The tumor suppressor gene p53 has been described to be a mediator of apoptosis; therefore, it appears that cell proliferation and apoptosis may be subjected to coordinated but inverse regulation. Another gene that plays a role in metamorphosis is polyubiquitin. Ubiquitin may be covalently linked to cellular proteins to mark them for degradation. Fas is a gene whose product is a membrane spanning protein homologous to TNF and NGF receptors (12). In a cell which expresses Fas naturally or by transfection, cross-linking by antibody to Fas induces apoptosis.

Bcl-2 Family of proteins

Pro and antiapoptotic family members have been identified. BCl-2 family members possess upto four conserved α-helical

domains, designated BH1, BH2, BH3 and BH4 (13). The oncogene Bcl-2 (B-cell leukemia/ lymphoma) was the first member to be discovered of the expanding Bcl-2 family. Bax and Bak are pro-apoptotic and have BH1, BH2 and BH3 domains. BH-3 only members can be sequestered by multidomain antiapoptotic members Bcl-2, Bcl-XL. Bax and Bad serve as a critical gateway in the intrinsic pathway of apoptosis operating at the mitochondria and endoplasmic reticulum level (14,15). Many pro-apoptotic members, especially the BH-3 only subset, are localized in the cytosol or cytoskeleton and undergo post translational modification, after a death signal, which allows them to target and integrate into the mitochondrial membrane.For eg. Bax translocates from cytosol to mitochondrial outer membrane where it oligomerizes and leads to permeabilization of the mitochondria outer membrane resulting in release of cytochrome c and second mitochondrial activator of caspases (SMAC)/ direct inhibitor of apoptosis binding protein with low PI (DIABLO) from the intermembrane space. BH3 only protein BAD is inactivated by phosphorylation on two serine residues in presence of survival factors (16). PI-3 kinase and AKT can phosphorylate BAD.

Caspases

They are a family of proteases which function in inflammation and in initiation of apoptosis and in the subsequent effector pathway to disassemble the cell.They are synthesized as a single chain of inactive zymogens, differ in the length and amino acid sequence of their NH_2 – terminal prodomain, and are activated by proteolytic cleavage after a death stimulus. Caspases possess a common structural design consisting of four domains: an amino-terminal pro-domain, a large subunit, and a small subunit, separated by caspase cleavage consensus sites, and a linker region connecting these catalytic subunits. After cleavage of the proenzyme, the large and small subunits associate to form a heterodimer and contribute residues that are important for substrate binding and specificity. The two catalytic sites of the tetramer appear to function independently (17). The catalytic site contains a conserved pentapeptide, QACXG, surrounding the active site cysteine residue. Caspases all prefer an aspartic acid residue at P_1 position of their cleavage site. Specificity of individual caspase substrate binding pockets varies, which dictates the preferred amino acids at the P_2-P_4 sites, amino terminal to the substrate cleavage site. They are separated into initiator caspases, which link death signals to the cellular death program, and effector caspases that carry out a coordinated program of proteolysis, resulting in the destruction of critical structures involved in homeostasis and repair. 12 mammalian caspases are known and are numbered in the chronological order of their identification. (18).

The initiation caspases include caspases -2, -8, -9, -10 and -11 and the effector caspases include caspases -3, -6 and -7. The effector caspases directly degrade multiple substrates including structural, regulatory proteins in the cell nucleus, cytoplasm and cytoskeleton and their activation can be caused by cathepsins, calpains and granzymes (19). The receptor mediated pathway is initiated by the binding of death ligands belonging to tumor necrosis factor (TNF)/ nerve growth factor (NGF) to their respective death receptor. To date, eight human members of the death receptor family have been identified i.e. TNF-R1, Fas, DR-3, DR-4/ TRAIL-R1, DR-5/ TRAIL-R2, DR-6 and NGF-R (20). All death receptors contain a death domain in their cytoplasmic domain for eg. binding of Fas ligand induces aggregation of Fas upon which this activated receptor recruits the adaptor protein Fas Associated Death Domain (FADD), and the initiator caspase-8 to a multiprotein complex DISC that is essential for the initiation of apoptosis cascade. The receptor independent pathway of caspase activation involves pro-apoptosis signals originating in the nucleus, mitochondria, the ER lysosomes and the golgi bodies (21). P53 is a central link in apoptosis induced by DNA damage. Many apoptotis stimuli eg. stress, etc converge on the mitochondrial pathway. They can influence the permeability of the Outer Mitochondrial Membrane (OMM), leading to release of cytochrome c. Bcl-2 family of proteins are the major regulators of mitochondrial pathways (22).Apaf-1 and cytochrome c in the presence of ATP or dATP lead to formation of apoptosome, which in turn activates caspase-9 and triggers the activation of downstream caspase-3 and -7. Chang et al (23) have shown that the aggregation of multiple procaspase-9 molecules can induce their activation without apoptosome. Increasing the release of intracellular Ca^{2+} from ER also induces apoptosis. It is also reported that on induction of apoptosis, lysosomal enzymes such as cathepsins may enter the cytosol and facilitate cytochrome C release from the mitochondria (24).

Inhibitors of apoptosis proteins (IAPs)

Cells have devised an additional level of caspase control. IAPs are a family of proteins characterized by a zinc-binding region rich in cysteine and histidine residues termed baculoviral IAP repeat (BIR). All IAPs share a specific BIR region of 70 amino acids required to provide the antiapoptotic effect (25). Members of the family include proteins such as XIAI, c-IAP_2 that bind and inhibit caspases. Proteins such as survivin function primarily in the control of cytokinesis and is subject

to cell cycle dependent transcriptional regulation. XIAP binds directly to caspases -3, -7 and -9 for its antiapoptotic function. IAP activity is tightly regulated at transcriptional and post transcriptional levels i.e. at the level of translation, protein degradation, regulatory protein- protein interactions and the ubiquitin – proteosome system of protein degradation (26).

Role of intracellular organelles: Mitochondria and Endoplasmic reticulum (ER)

Many of the critical control steps in the intrinsic apoptosis pathway are located at the surface of intracellular organelles. The mitochondrial dysfunction that occurs in cell death is manifest as altered transmembrane potential ($\Delta \varphi$ m), the release of proteins from the inner mitochondrial membrane and the production of ROS. The localization of Bcl-2 and the translocation of pro-apoptotic proteins to the mitochondrial membrane emphasizes the importance of mitochondrial dysfunction in the action of these molecules. Antiapoptotic Bcl-2, Bcl-XL block apoptosis in a glucose independent manner but do not prevent metabolic decline when growth or survival factors are withdrawn. Proteomic studies indicate an intimate integration of glucose metabolism and apoptosis involving BAD . $VDAC_2$ binds and inhibits BAK at the mitochondria of viable cells (27).

The ER also serves as a critical control point in the intrinsic apoptotic pathway. Bcl-2 and BAX/BAK localize to the ER. Ca^{2+} is regulated at the ER by Bcl-2 family (28). Certain death signals rely on Ca^{2+} ER pathway rather than the mitochondrial pathway, such as Ca^{2+} – dependent lipid second messengers as well as pathologic oxidative stress.

Apoptotic cell clearance

Regional loss of mitochondrial membrane potential is rapidly followed by externalization of Phosphatidyl Serine (PS) during apoptosis (29). A spatio-temporal relationship may exist between the drop in mitochondrial ATP production and inhibition of aminophospholipid translocation, thus yielding localized externalization of PS(30). Dying cells may also use modified sugars as recognition signals as in the case of macrophages which are endowed with an array of phagocytic receptors such as PSR, the class A, B, D scavenger receptors (SRA) CD36, the integrin receptor and bacterial LPS receptor CD14 (31). There is also molecular cross-talk between distinct cell surface receptors that form a phagocytic synapse to amplify signals for engulfment (32). Importantly, apoptotic cells may provide a source of auto antigens (33) and loss of certain engulfment receptors on dying cell may lead to autoimmune diseases, suggesting a crucial role for specific receptor-ligand interactions during programmed cell clearance. It is suggested that most macrophage receptors do not bind to PS directly, but rather via soluble bridging proteins that bind to both the signal on the apoptotic cell and the receptor on the engulfing cell such as Gas b, β_2-microglobulin and milk fat globule epidermal facton-8 (MFG-8) (31). Thus differential macrophage usage of bridging molecules could confer an additional mechanism for tissue-specific regulation of cell clearance. DNase II, a lysosomal acid endonuclease expressed in phagocytes has an important role in degrading DNA after engulfment of apoptotic cells. In addition macrophages after ingestion of apoptotic bodies, secrete anti-inflammatory cytokines that serve to further downregulate the inflammatory response (34). They also instruct neighbouring cells to survive, through the secretion of growth factors such as VEGF.

Apoptosis and the immune system

Apoptosis is the most common form of death in cells of the immune system and regulates lymphocyte maturation, receptor repertoire selection and homeostasis (35), and occurs when the antigen – specific receptors of the lymphoid cell are not stimulated or the lymphocytes are deprived of trophic cytokines, involving the death receptor/death ligand systems. CD 95 plays a significant role in the immune system. Its expression can be boosted by cytokines such as IFN γ, TNF and by the activation of lymphocytes . CD -95 mediated apoptosis is triggered by its natural ligand CD 95L which is seen on killer cell – derived vesicles and can also be cleaved from the membrane by metalloproteinase. Cytotoxic T lymphocytes (CTLs) kill their targets cells by the death receptor pathway, others use perforin and granzyme B to eliminate infected cells. Downstream of CD95, caspase – 8 cuts Bid, this truncated Bid activates the mitochondrial pathway which activates caspase -9 and further downstream caspases leading to apoptosis.

The T- cell repertoire in the thymus is shaped by apoptosis and survival signals, without signs of inflammation (36). Molecules such as glucocorticoid hormones, cytokines, co-stimulating cell surface receptors, signaling molecules, transcription factors and nitric oxide modulate the survival and apoptosis of thymocytes. Besides T lymphocytes, apoptosis is also observed in mature peripheral T cells, to downregulate the number of reactive cells and to terminate the immune response which serves as a second line of defence against autoimmunity by deleting auto reactive cells in the periphery. Three cell surface molecules are key elements in the regulation of B-cell life and death i.e. B – cell receptor (BCR), CD 40 and

CD95. BCR activation induces apoptosis by the mitochondrial pathway. BCR- activated B cells can be rescued from apoptosis by co- stimulation by way of CD40 that has been activated by CD 40L expressed on T cells and macrophages. There are interactions between antigen presenting cells (APCs), T cells and B cells. T and B cells influence each other and persistence clonal expansion and apoptosis of other cells, but it is the APCs that prime the T cells and initiate T-cell dependent immunity (37). APCs engulf apoptotic and necrotic cells and present their antigens to T cells. Activated APCs synthesize CD 95L, TRAIL, TNF and other factors that modulate the activity and functions of T cells. In turn, activated T cells influence the APC function and thereby affect the course of the immune response. Thus the immune system requires that the cells give and receive life and death signals at the same time and it is the cellular context that determines which signal dictates the cellular response.

Apoptosis and the nervous system

Neuronal cell death by apoptosis occurs during brain development. Like all cells, neuronal survival requires trophic support (38). Neuronal apoptosis is regulated by the Bcl-2 protein family, the adaptor protein Apaf-1 and caspases. However, different types of neurons at different development stages, express different combinations of Bcl-2 and caspase family members, thereby providing specificity of regulation. Overexpression of Bcl-2 can override the death signal induced by withdrawal of trophic factor. The expression of Bcl-2 is high in the CNS during development and is downregulated after birth, whereas the expression of Bcl-2 in the peripheral nervous system is maintained throughout life, suggesting that Bcl-2 is crucial for the maintenance of neuronal survival. Bcl-XL is critical for survival of immature neurons and its expression continues to increase into adult life. Pro-apoptotic Bax is widely expressed in the nervous system. Apaf-1 is indispensable in the apoptosis of neuronal progenitor cells. The two major caspases involved in neuronal cell death are caspase-3 and -9. The latter activates the former . Thus caspase inhibitors are able to block neuronal cell death. PI (3)K-AKT pathway also plays a central role in neuronal survival and PI(3)K inhibitors block the survival effect of NGF. Neuronal survival pathways are induced by the binding of NGF to its receptor Trk A, a cell surface receptor with intrinsic tyrosine kinase activity. NGF induces the autophosphorylation of TrkA which provides docking sites for signal transduction molecules such as phospholipase $C\gamma$ phosphoinositide 3-Kinase [PI(3)K] and the adaptor protein ShC, and these coordinate neuronal survival. Activated PI(3)K present in cytosol induces the activation of Akt. The phosphorylation of transcription factor cAMP- response element binding protein (CREB) and IKK stimulates the transcription of pro- survival factors, whereas the phosphorylation of pro-apoptotic Bad, forkhead and caspase-9 inhibits the pro-apoptotic pathway. In parallel another pathway by the interaction of ShC- Crb2 and SoS activates the Ras-Raf-MEK-ERK pathway. Both PI (3)K and MAP kinase pathways converge on the same set of proteins Bad and CREB to inhibit apoptosis. The removal of NGF results in a decrease in MAP kinase and PI(3)K activities, followed by a series of early metabolic changes including the increased production of ROS, decreased glucose uptake and decreased RNA and protein synthesis. The release of cytochrome c from mitochondria induces the activation of caspases in sympathetic neurons and inhibition of caspase activity might be sufficient to block neuronal cell death under certain pathological conditions. Physiological apoptosis in the developing brain and pathological apoptosis in the adult brain share similar molecular mechanisms in the effector phase. Toxic insults resulting from biochemical or genetic changes trigger neurodegenerative diseases by co-opting apoptotic signaling pathways, for eg. through free-radical generation or caspase activation.

Apoptosis and Human disease

Apoptosis dysregulation contributes to nearly half of all human diseases (39). Derailment of apoptosis in the regulation of immune system leads to several diseases with either too much or too little apoptosis. Dysfunction of CD 95 leads to autoimmune lymphoproliferative syndrome (ALPS). Inability of the immune system to eliminate self reactive lymphocytes by apoptosis causes autoimmunity. Lack of apoptosis plays role in generation of tumors such as follicular lymphomas. Tumors develop multiple mechanisms to evade elimination by the immune system such as lack of expression of co-stimulatory or MHC molecules, production of immunosuppressive cytokines. AIDS, characterized by a depletion of CD_4^+ T helper cells is a disease with too much apoptosis. Immune homeostasis and maintenance of immune tolerance are dependent on apoptosis induction and rapid clearance of apoptotic cells in peripheral and central lymphoid organs (40). Autoimmune disease arise from either defective elimination of autoreactive T or B cells, resulting in tissue destruction, or from defective clearance of apoptotic cells displaying autoantigen on their cell surface as in the case of Systemic Lupus Erythematosis (SLE) subjects (41). The BH-3 only protein Bim dependent apoptosis constitutes a critical barrier to autoimmunity. B-cell activity factor (BAFF) is a ligand required for peripheral B-cell survival and homeostasis (42). Mice transgenic for BAFF have increased numbers of mature

B and T cells and develop autoimmune- like manifestations, such as high levels of rheumatoid factors, circulating immune complexes, anti-DNA autoantibodies and Ig deposition in kidneys. In contrast, ingestion and presentation of self-antigen from apoptotic cell corpses may serve to induce immune tolerance (43). Cell clearance plays an active role in the resolution of inflammation through macrophage production of anti-inflammatory cytokines such as TGF-β and downregulation of proinflammatory mediators such as TNF-α. A study has indicated that defective airway clearance of apoptotic cells in cystic fibrosis and bronchiectasis patients may be due to elastase-mediated cleavage of the PSR on phagocytes which may contribute to persistent airway inflammation in these individuals (44). Elastase also cleaves the receptor CD14 on the surface of macrophages, and thereby reduces engulfment of apoptotic cells (45). IL-1β is one of the key mediators in response to microbial invasion in the body, inflammation and tissue injury. Defective control of inflammasome (a NALP- driven platform for caspase oligomerization) due to a mutation in NALP3 gene results in constitutively elevated IL-1β maturation and secretion , causing serious auto inflammatory disease in humans.

Neuronal death occurs in various neurodegenerative disorders. Extensive neuronal loss occurs in the brain of Alzheimer's disease and caspase cleavage of amyloid precursor protein may be involved. The adult onset neurodegenerative diseases caused by proteins with expanded polyglutamine tracts are characterized by selective loss of specific neuronal subpopulations. Ineffective clearance of polyglutamine expansion proteins by the ubiquitin – proteosome pathway might contribute to the formation of intranuclear inclusions that induce apoptosis (46), as seen in Huntingtons disease, spinocerebellar ataxia types 1 and 3 (47). Huntington's disease (HD) results from an aberrant expansion of CAG repeats, which is translated into a polyglutamine repeat in the Huntingtin protein (Htt), which is a substrate for caspase-3. Cytosolic aggregates of polyglutamine repeat proteins may recruit pro-caspase 8, resulting in the activation of caspase –8 (48). Thus caspases play an important role in mediating cell death in human neurodegenerative diseases.Amyotrophic lateral sclerosis (ALS) is associated with death of motor neurons in the spinal cord and brain due to mutations in the gene encoding CuZnSoD . These SOD-1 mutations can activate caspase-1 and caspase-3 and might increase free radical generation, leading to motor neuron apoptosis. Certain VEGF haplotypes that compromise transcription of the VEGF gene in humans are associated with an increased likelihood of developing ALS (49). Low levels or an inability to upregulate VEGF in response to trauma, ischemia, or stress may therefore constitute a common feature in neurodegenerative disorders.

Apoptosis as well as necrosis of cardiomyocytes occurs in congestive heart failure, myocardial infarction, acute transplant rejection (50), via activation of the extrinsic pathway. Apoptosis repressor with CARD (ARC) is an apoptosis-regulating molecule expressed primarily in skeletal muscle and cardiac tissue and its over expression is shown to suppress both intrinsic and extrinsic pathways of apoptosis and thus help in treatment of diseases with excessive apoptosis of myocytes.

Cancer involves a succession of genetic changes/mutations, which transform a normal cell to a malignant one, leading to excessive proliferation and decreased apoptosis or apoptosis resistance (51). Nearly half of human cancers have mutations in p53. A recent study has shown that a protein termed apoptosis – stimulating protein of p53 (ASPP) is able to interact with p53 and enhance p53 – induced apoptosis, but not cell cycle arrest (52). An inhibitory member of ASPP family (i ASPP) has been found which is an inhibitor of p53 and it is up regulated in human breast carcinomas expressing wt p53 and normal ASPP. Apaf-1 and caspase –9 serve as downstream effectors of p53- dependent apoptosis, and their disruption facilitates oncogenic transformation in mice. Survivin, a member of IAP family is found to be expressed in most human cancers (53). Deregulation of Bcl-2 family members is also common in cancer. Elevation of Bcl-2 protein is commonly found in a variety of haematopoietic malignancies. Mutations that inactivate pro-apoptotic Bax have also been observed in several tumors. A recent study suggested that cancer cell cannibalism could have important consequences for tumor growth and genetic diversity within a tumor cell population (54). Cancer cells can engulf apoptotic bodies and reutilize the salvaged DNA, suggesting horizontal transfer of genetic information between somatic cells . Prostate cancer cells have been shown to exchange and propagate drug resistance genes in vitro through engulfment of apoptotic bodies (55). Thus transfer of oncogenes through the engulfment of apoptotic bodies may constitute a novel mechanism for the propagation of genetic instability and/or diversity in tumors.

Apoptosis-based therapies and drug targets

The pathogenesis of several diseases involves deregulated apoptosis (56). Consequently apoptosis based therapeutics has emerged as an important area.Different approaches are being followed employing gene therapy, antisense strategies, recombinant biologicals, classical organic and combinatorial chemistry, to target specific apoptotic regulators.

Because death receptor pathways are involved in a wide array of disorders, hence they are interesting targets for development of new, alternative therapies. TNF is involved in a wide spectrum of biological pathways. Besides its antitumor effects, TNF efficiently destroys tumor supplying blood vessels by apoptosis and improves the permeability of the vasculature to cytotoxic drugs like melphalan and doxorubicin.Anti-TNF antibody adalimumab has shown good results in phase III clinical trials for rheumatoid arthritis (57). CD95/CD95L have been implicated in stroke, multiple sclerosis, graft-versus-host disease and others and thus are an appealing target for therapeutic intervention. But, cancer therapy targeting TNF/ CD95 receptor/ ligand systems showed severe side effects such as hepatotoxicity (58). However, TRAIL seems more promising as it specifically kills tumor cells while sparing the normal cells. TRAIL mediated apoptosis is independent of p53 and can bypass overexpression of Bcl-2 proteins by directly activating the caspase cascade. Chemo and radiotherapy can even sensitize nonresponding tumor cells to TRAIL, thereby showing synergistic effects to both apoptotic pathways. TRAIL agonists have reached clinical phase for combating cancer and other apoptosis related disorders, either as a single agent or with standard therapy. A synergistic effect was seen with TRAIL and 5-FU (59).

Bcl-2 proteins are alternative targets for both pro and antiapoptotic therapeutic approaches (60). Different strategies such as antisense approach, applications of BH3 domain peptides or synthetic small molecules which interfere with Bcl-2 family members, have been tried. For eg clinical trials targeting Bcl-2 by Genasense is underway, bispecific antisense oligos effectively killed cancer cells (61). Compared to downregulation of Bcl-2 protein, the interference with Bcl-2 protein by BH3 domain peptides or small drugs is a more direct approach to sensitize cells to apoptosis. Clinical trials in malignant melanoma, follicular lymphoma showed anti tumor response with antisense G-3139 against Bcl-2. Clinical trials are also ongoing for Non- Hodgkins lymphoma along with antisense Bcl-2/ cyclophosphamide. Pre-clinical trials are ongoing with antisense Bcl-2 for breast cancer, leukemia, small cell lung cancer. Antisense survivin is being studied for osteocarcinoma, lung adenocarcinoma, etc. Mimics of BH3 domains that compete for binding pockets of antiapoptotic members such as Bcl-2, Bcl-XL displace pro-apoptotic members, triggering cell death. Peptidomimetics derived from the BH3 domains of pro-apoptotic members, as well as chemical small molecules are under development.

The IAPs are of interest and IAP antagonists help in anticancer therapies by killing cancer cells by themselves or by augmenting the effect of chemotherapeutic agents. Evidence suggests that apart from inhibiting caspases, IAPs participate in other cellular functions, including protein degradation, cell cycle control and signal transduction. Antisense targeting of XIAP has been shown to sensitize a variety of tumor cell lines to radio or chemotherapy (62) and clinical trials are underway with AEG 35156 for solid tumors. Cancer therapeutic approaches employing SMAC peptides or SMAC-mimetic drugs (Oxazoline derivative) are being developed by several companies in order to inhibit IAPs and to restore caspase activity (63). The targeting of IAPs for clinical use is hampered by the limited information on correlations of IAP expression with certain disease, but they have a clear potential for treatment of cancers. However, protein-based therapies have disadvantages as they have to be administered systemically, in high doses, and regularly for chronic disease. In this regard, nonpeptidic agents such as caspase inhibitors might turn out to be better options.

Caspase inhibitors represent modified tetra or tri peptide pseudosubstrates comprising of the cleavage sequence of the caspase target coupled with an aldehyde or ketone group. They consist of an electrophilic group, the P1 aspartic acid and the most variable P2-P4 peptidomimetic region. The electrophilic group targets the active cysteine residue of the caspase and depends on its substituents (aldehyde or ketone) leading to reversible and irreversible caspase inhibition. Ketones are irreversible inhibitors that covalently bind to the cysteine at the active enzymatic center of caspases, whereas aldehydes are reversible/ competitive caspase inhibitors for eg. x-VAD fmk, VX-740 (caspase-1 inhibitor),VX-756,VX799 , IND-1965, MX-1013 (inhibits several caspases), MX 1122 (inhibits caspase-3) (64). The use of these cytoprotective drugs has been widely proven in animal models for various disease conditions associated with inappropriate apoptosis, such as heart and liver injury by alcohol, or hepatitis B and C infection, lung injury, sepsis, brain ischemia, acute myocardial infarction, nephrotoxic nephritis and in arthritis (65). SiRNA-mediated inhibition of caspase-8 gene expression in liver prevents Fas mediated apoptosis and recently potent caspase inhibitors have been designed based on oxamyl dipeptides IDN-6556 for phase III clinical trials (66).

Caspase activators may help to combat cancer, etc. Pharmacological activation of cellular caspases by small cell permeable drugs might provide a more efficient venue to target cancer cells. Small molecule caspase activators such as peptides containing arginine-glycine-aspartate (RGD) motif exhibit apoptotic properties. They can directly bind near the active site of caspase-3 leading to its acute activation. RGD

peptides are already in clinical use as antithrombotic drugs. Another apoptotic triggering approach is overexpression of chimeric immunocaspase-3 or immunocaspase-6 gene. Caspase-3 fused with antibodies directed towards extra or intracellular tumor-specific proteins seems to provide selective induction of apoptosis in tumor cells. Several gene therapy approaches aimed at replacing the defective caspases by their normal counterparts are being tested (67), by development of caspase constructs with inducible caspase-1, -3 or -9 molecules. Inducible caspases have been engineered by fusing them to chemical dimerization domains. After delivery of these chimeric death switches by adenoviral gene transfer, caspases can be activated to trigger apoptosis in tumor cells by cell permeable dimerization drugs . Inducible caspase -9 under the control of an androgen responsive promoter was specifically targeted to prostate cancer cells (68).Caspase-6 under control of human telomerase reverse transcriptase promoter (hTERT) was transferred into glioma cells (69). Amgen are evaluating caspase-3 linked to HER 2-antibody Herceptin as a clinical treatment. Blocking gene expression of caspase inhibitors using antisense oligonucleotides or antisense RNAs is another potential strategy for cancer therapeutics (70). Potentiation of drug-induced apoptosis by various caspase-cascade triggering agents has been demonstrated in various malignancies (67).

Different strategies have been undertaken to target p53 such as restoration of normal function of mutant p53, as p53 mutations are present in more than 50% sporadic human cancers. Strategies to reintroduce p53 include gene transfer of p53, using synthetic peptides to restore wild type p53 function, using monoclonal antibodies to increase transcriptional activation function, stabilizing DNA binding domain using low molecular weight compounds to rescue transcriptional activation function, block p53 turnover by inhibiting p53-Mdm 2 interaction. However, all these strategies although promising have a major obstacle, i.e. of inefficient tumor specific drug delivery. Introduction of small synthetic molecules, which stabilize p53 in a transcriptionally active state by allosteric modification of its conformation, are thought to target especially tumor cells having a high accumulation of mutant p53. Another way of stabilizing p53 is by interfering with the binding of its negative regulator Mdm2 either by employing peptides or synthetic drugs (71) such as chalcones, nutlins (imidazole compounds). Several clinical trials currently evaluate the delivery of wt p53 expressing adenovirus (Ad-p53) by intratumoral injection. INGN2 is currently being tested in phase 3 clinical trial for head and neck cancer. Specific targeting of p53-deficient tumor cells by adenovirus mutant ONYX-015 helps this virus replicate only in cells without wt p53 expression. Synthetic peptide aptamers competing intracellularly for E6 binding with p53 are promising approaches to induce apoptosis in HPV-16 positive cancer cells (72). Targeting of heat shock protein (HSP)-90 by gel danamycin is a strategy to restore the degradation of mutant p53 by the proteosome. This drug has also shown to deplete mutant p53 in various tumor cell lines.

Mitochondria is another target for therapy. Mitochondria, the cell powerhouses, are essential for maintaining cell life, and they also play a major role in regulating cell death, which occurs when their membranes become permeabilized (73,74). Key factors regulating mitochondrial membrane permeabilization (MMP) include Ca^{2+}, the cellular redox state and the mobilization and targeting to mitochondria of Bcl-2 family members. MMP includes either outer or inner membrane permeabilization. The former is regulated by Bcl-2 family and the latter by redox status of mitochondrial protein vicinal thiols and mitochondrial permeability transition (PT) (75). Bcl-2 is overexpressed in many solid tumors and it increases resistance to cell death. Thus strategies are aimed at decreasing Bcl-2 resistance to cell death (76) by using crosslinking agents such as diazenedicarboxylic acid bis 5N, N-dimethylamide (diamide), using ligands of mitochondrial benzodiazepine receptor (BR) using gene silencing by targeting antisense of Bcl-2 by G3139 (77). Alternative approach is to target proapoptotic Bcl-2 peptides to mitochondria for eg. gene therapy employing Bax-delivery vectors, inducible recombinant Bax adenovirus, adenoviral delivering vector chimeras of IL-2 and Bax. Mimics of other apoptosis inducing proteins including Smac/D1ABLO have also been developed. In addition to calcium, the PT is also known to be regulated by the mitochondrial thiol-redox status which is, in part maintained by levels of glutathione. Loss of glutathione leads to mitochondrial protein oxidation, MMP and necrosis (78). The redox-PT is a mitochondrial inner membrane phenomenon. Therefore, targeting the mitochondrial inner membrane and the PT represents an attractive chemotherapeutic modality to eradicate cancer cells, such as the use of lipophilic cations that preferentially accumulate in the mitochondrial matrix of cancer cells compared to normal cells, the use of toxic peptides such as mastoparan that target the $\Delta \varphi$ m, and agents that target and deplete mitochondrial DNA. Conventional anticancer agents such as doxorubicin, cisplatin and paclitaxel cause MMP indirectly by activating pro-apoptotic second messengers i.e. p53, ceramide, Fas/ Fas L pathway. A number of agents that induce cell death independently by acting directly on mitochondrial membranes include arsenite, lonidamine, betulinic acid, and viral protein (79). Mitochondrial DNA

depleting agents such as ditercalinium inhibit respiration, electron transport and oxidative phosphorylation.

CONCLUSION

Recent advances in apoptosis research have paved the way for targeting apoptosis for therapy, using different strategies and pharmacological manipulation. But, there are some technical limitations for techniques such as gene therapy, antisense strategies. We also need to address certain questions i.e. whether apoptosis can be selectively modulated in one organ or cell type without adverse effects on other key systems. If cells are salvaged by inhibiting apoptosis will they remain functional or will cause greater harm in the long run. It needs to be determined to what extent toxicity of normal tissue will limit the application of apoptosis-based therapies in clinical trials. Perhaps, apoptosis-based treatment will need to be tailor made for each patient and one should also take into consideration the emergence of resistance to treatment. Therefore, a combination of current conventional treatment and apoptosis-targeted events seems a more likely successful scenario.

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