The art and science of low energy applications in medicine: pathology perspectives

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Abstract

Applications of low energy non-ionizing irradiation result in non-lethal and lethal effects in cells, tissues and intact individuals. The effects of these applications depend on the physical parameters of the applied energies, the mechanisms of interaction of these energies on the target and the biologic status of the target. Recently, cell death has been found not to be a random accident of situation or age but a range of complicated physiological responses to various extrinsic and intrinsic events some of which are genetically programmed and/ or physiologically regulated. Therefore, cell death has been classified into three general groups: 1) Programmed cell death including apoptosis and necroptosis, cornefication and autophagy; 2) Accidental (traumatic) cell death due to the direct, immediate effects of the lethal event and 3) Necrotic cell death which is, by default, all cell death not associated with programmed or accidental cell death. Lethal low energy non-ionizing application biologic effects involve mechanisms of all three groups as compared to high energy applications that predominantly involve the mechanisms of accidental cell death. Currently, the mechanisms of all these modes of cell death are being vigorously investigated. As research and development of new low energy applications continues, the need to understand the mechanisms of cell death that they produce will be critical to the rational creation of safe, yet effective instruments.

Key Words: thermal damage, death mechanisms, ultrasound damage, necroptosis, apoptosis, traumatic (accidental) death, cell membranes, photoreceptors, acoustic cavitation, mitochondrial retrograde signaling

Introduction

Energy and Work: Definitions

Energy is defined generally as the capacity of an entity for doing work Energy can be defined by association with a 1) material body (mass) in motion (kinetic energy) or in a potentially unstable position (potential energy) and 2) as existing "independent" of mass as in the case of electromagnetic radiation. The physical units for energy are the same as those for work, the joule J.

Work W, signifies a force multiplied by the distance that a mass moves in the direction of the applied force

$$W = \text{force} \times \text{distance}$$

The unit of work in the mks system is the joule [J] where force is in newtons [kg-m/sec^2]and distance in meters [m]

$$J = \text{newton} \times \text{meter} = \text{kg}(\text{m/s})^2 \times \text{meter}$$

Frequently, in biological applications, power P, that is, the rate at which work is performed is an important concept in considering instrument design and clinical applications and results.

$$P = J/\text{sec}$$

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Work effects of low and high energy non-ionizing irradiation on biologic targets: General Considerations

The work effects of low and high energy non-ionizing irradiation medical applications are the result of direct interaction of the energy with the target.. These effects are usually mediated by mechanical, thermal or chemical mechanisms that lead to both non-lethal and lethal work effects. Low energy applications in medicine imply application of low powers, low energies and/or short time application intervals. However, the work done upon biological targets by various energy sources also depends on the mechanisms of the energy interactions with the cells and tissues. The total work or effects of the irradiations result from immediate, primary mechanisms of interaction but also the numerous delayed mechanisms that generally reflect the biologic complexity of the targeted cells and tissues. [1-7]

Recent advances in molecular and cellular biochemistry, biophysics, and pathology have revealed new information and insights into cell and tissue physiology that need to be explored relative to the better understanding of mechanisms of the effects or "work" of high and low energy non-ionizing irradiations in biological systems. In particular, mechanisms of cell injury and death have been shown to be much more complicated than considered before. [8-16]

Cell life and death

Dynamic equilibrium of life: General concepts

The basic unit of life and death is the cell. The cell can be defined as the least structural aggregate (mass) of living matter (protoplasm) bound by a semi-permeable membrane, the plasma membrane. [3, 8, 17]. This membrane contains numerous protein complexes that function as receptors, ion and water channels and signal transducers thus connecting the internal workings of the cell with the outside environment and vice versa. [18, 19] The plasma membrane is composed of a bilayer of polar phospholipid molecules that generally will not allow transport of the numerous small and large molecules needed for life across it. Ions and water are passed in and out of the cell via passive and metabolically active channels scattered in the membrane. A major life function of the cell is the delicate regulation of flow of these substances through these channels to generate the appropriate membrane electrical potentials and intracellular salt and water balance. Receptors are specific attachment sites for numerous substances generally categorized as ligands including nutrients, hormones, antigens, drugs and poisons. Frequently, these receptors have a direct, chemical connection with intramembrous and cytoplasmic molecular complexes that allow passage of these ligands into the cell. Other ligands do not necessarily enter the cell but their attachment to certain specific membranous protein complexes (signal transducers) stimulate the formation of second (or even more) compounds (secondary messengers) that signal a series of biochemical reactions that can lead to general cell responses. A few examples of these responses important in low energy applications are the initiation of cell proliferation and growth, synthesis of specific cellular substances for internal or external use and programmed cell death.

Another important component of the internal structure of the cell is its cytoskeleton comprised of protein macromolecules that provide 1) internal scaffolding supporting and connecting the various functioning organelles, 2) structural connections to other cells and the outside connective tissue and 3) the basic mechanical structures for a) cell motion, b) intracellular transport of organelles and packets of secretory products and c) cell division (mitosis). [18, 20, 21]

Life is characterized by the dynamic equilibria among the cellular functions of metabolism, growth, reproduction, communication and movement. These life functions are responsible for the responsiveness and adaptability of the cell to the constant changes in its environment. However, disruptions of these equilibria can lead to morbidity and/or mortality of the cell.

Dynamic equilibrium of life: Disruptive effects of applied energies

In general, mild disruptions of the dynamic equilibrium of life produce illness (morbidity) in the cell but not death. The factors governing these sickening but non-lethal disruptions are 1) the insults are mild and, usually, of low intensity, 2) the insults are short-lived and transient, 3) the physiological defects are small relative to the total functioning volume of

the cell, 4) time and metabolic resources are available for regeneration and repair and 5) the cell has protection against the effects of the insult.

On the other hand, the lethal disturbances of the dynamic equilibrium of cellular life 1) are more severe and global or 2) signal more specific death mechanisms (programmed cell death), 3) produce more extensive physiologic damage, 4) overwhelm available repair resources, 5) occur when there is no time for completion of the repairs and 6) the cell has no protective mechanisms in place to resist the insult and its effects.

The rest of this paper will focus on the lethal effects or " work" of low energy non-ionizing irradiations with emphasis on mechanisms of cell death that could be activated by these irradiation exposures.

Mechanisms of Cell Death: Overview

Recently, an international committee proposed a classification of several pathways of cell death .[9] This classification was compiled based on the numerous recent scientific findings of consistent biochemical and anatomical features that seem to be common to certain different cell death pathways. [Tables 1 & 2]

Apoptosis and necrosis

Originally, apoptosis and necrosis were defined as characteristic morphological changes that occurred as the result of the processes of cell death.[3, 8, 9] Morphologically, apoptosis involves an orderly progression of 1) cell shrinkage, 2) chromatin margination and DNA fragmentation , 3) plasma membrane and cytoplasmic blebbing and 4) cell fragmentation leading to the formation of apoptotic bodies which are bound in plasma membranes. The apopototic bodies retain some energy producing metabolic and signal transduction functions including expression of specific membrane ligands to activate phagocytosis by adjacent cells or macrophages in tissues. Cells undergoing apoptosis initially show increased production of ATP and continued functioning of the respiratory enzymes in the mitochondria. The morphological events of apoptosis can be seen within hours of the onset of the process and are complete within 24 to 48 hours. Since the time of its first histopathological definition, additional physiologic studies of apoptosis have revealed that it is a form of cell death that follows characteristic, genetically regulated, very complicated biochemical and biophysical processes involving both intracellular and extracellular initiation pathways.

Theoretically, apoptosis is a individual cell phenomenon that does not leave any cellular debris to initiate inflammatory or immune responses. However, as is described below, many mechanisms of cell death are interrelated and can be initiated by the same event. In addition, prolonged stimulating exposures of low energy non-ionizing irradiations can produce different cell death mechanisms that will be activated over time with different cells dying at different times. Even in cell cultures, not all cells are programmed to die simultaneously.

Necrosis involves diffuse cellular swelling, diffuse rupture of cellular membranes, loss of vital respiratory mitochondrial function and gradual anatomical and biochemical disintegration of all cell contents. The morphological features of necrosis first appear histologically at 12-24 hours at the earliest and, depending on the cell type, tissue and species, require 2-5 days to fully develop in tissues. The biochemical signatures of necrosis are secondary to the early decrease of ATP production by the damaged mitochondria and the subsequent loss of metabolic enzymatic functions particularly those of the respiratory chain. The frequently used "vital stains" that reveal areas of cell death are based on the loss of the oxidation-reduction functions of certain respiratory enzymes in the mitochondrial

Table 1

Classification of Cell Death Pathways

Death Pathway	Characteristics
Programmed Cell Death	*Morphologically and biochemically regulated cellular death pathways*
Apoptosis	Sequence of morphological and biochemical changes mediated by either intrinsic or extrinsic signals that lead to characteristic morphological features and biochemical reactions in dying cells that are genetically predisposed to this death mode. The entire pathophysiological events of cellular apoptosis occur over a few - 24 hours for cells in cell culture and up to 48-72 hours for susceptible cells in the tissues of surviving individuals.
Necroptosis	Regulated characteristic morphological disintegration of cells initiated by the interaction of various membrane and intracellular receptors with introduced factors or triggers of necrotic cell death. These "death receptors" can be intrinsic to the cell or induced by the presence of particular extrinsic pathologic entities such as certain bacteria or viruses. Characteristic protein (enzyme) complexes called necrosomes may be hallmarks of necroptosis
Cornification	Regulated sequence of post-mitotic maturation followed by death of superficial squamous epithelial cells in the stratified sqamous epithelium of skin, hair and certain well-differentiated squamous cell carcinomas.
? Programmed Cell Death	*?Cell Death Mechanism*
Autophagy	Death in cells which contain large numbers of autophagosomes. Autophagosomes are intracellular, membrane bound organelles containing remnants of cellular debris, phagosomes (ingested material) and lysosomes. These are the "garbage sacks" of cells. It is not clear if the cell death is due to the stresses and strains that lead to the accumulation of the autophagosomes or if the death is produced by other mechanisms in cells that contain a large number of autophagosomes.
Non-Programmed Cell Death	*Cell death not regulated by intrinsic genetic expression or cellular regulatory pathways*
Necrosis	Cell death and post-mortem disintegration of cell structure and function NOT due to the characteristic cellular death mechanisms of apoptosis or necroptosis.
Accidental (Traumatic) Cell Death	Cell death, usually immediate, due to the direct, usually global, effect of some extreme (high energy) chemical or physical disturbance to the cell

Modified from: Kroemer et al. Cell Death and Differentiation 16: 3-11. 2009; Vandenabeele et al. Nature Biol Rev 11:700-714. 2010, & Thomsen, SPIE Proceedings 7181: 718102-1-15 2009.

Table 2

Accidental (Traumatic) Cell Death: Mechanisms

Types of Interactions	Direct Effects
Thermal	Thermal Denaturation of Proteins: Heat Fixation, Extensive Thermal Denaturation of Enzymes, Thermal Denaturation of Cytoskeletal Proteins Water Vaporization and Desiccation,
Mechanical	Membrane Rupture (Mechanical Cell Lysis) Ballistic Impact Sonic Cavitation (Membrane rupture, Generation of Reactive Oxygen Species Frictional Heat Energy Generation
Osmotic Stress	Rapid, Non-reversed Exposure to Hypotonic or Hypertonic Environments such as in Freeze-Thaw Cycling
Chemical	Extreme pH Changes, Cellular Exposure to Lipid (Cell Membrane) Solvents, Rapid Generation of Reactive Chemical Species.

Necroptosis

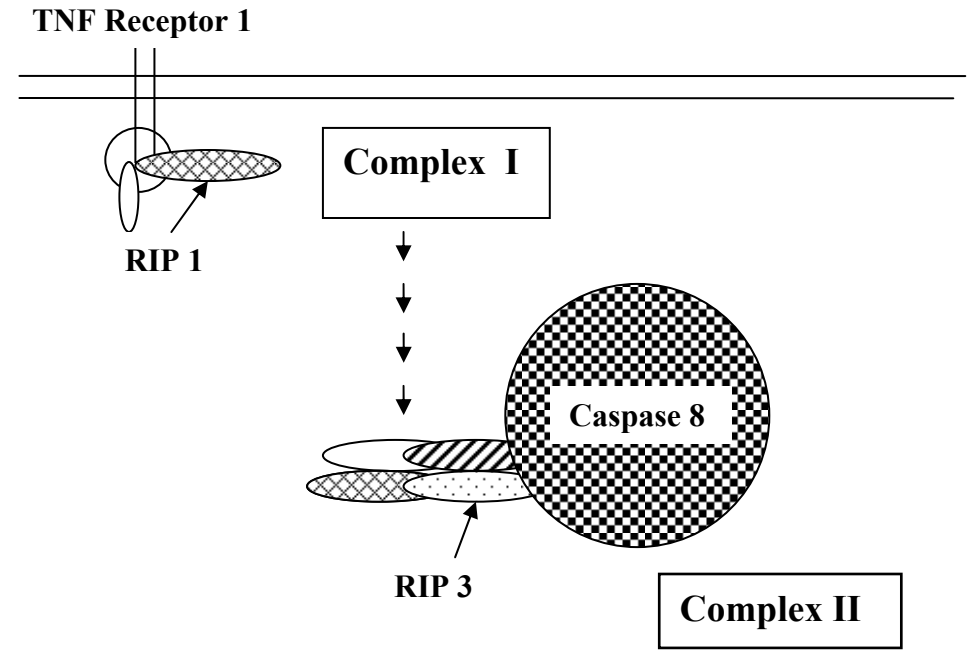
More recently some physiological features of necrosis have been found to indicate that, at least in some situations, necrosis can be initiated by ligand-receptor binding signal transduction (or other specific initiators) and follow characteristic, sequential regulated physiological pathways just like apoptosis. [10] Indeed, some biochemical complexes and physiological changes have been found to be common to both mechanisms of cell death. Recently, the label, necroptosis, has been introduced to distinguish this regulated necrotic cell death from unregulated, accidental necrotic cell death. [10, 22] It is possible that future studies will show that many instances of necrotic cell death will be found to be initiated by some form of specific signal or metabolic event that induces a cascade of sequential pathophysiological events therefore the death will be classified as necroptosis, a type of programmed cell death.

The literature contains numerous, but isolated examples of necroptosis in bacteria, fungi and eukaryotic cells. These examples of necroptosis are associated with different initiating mechanisms involving ligand-receptor binding signal transduction, presence of intracellular caspase inhibitors, production of reactive oxygen species, activation of the mitochondrial membrane permeability transition pore complex, and many, many more. [10]

A comprehensive formulation of the necroptosis pathway(s) has not been sorted from these numerous isolated examples. However, studies of activation of necroptosis by binding of the ligand, tumor necrosis factor (TNF), to a specific plasma membrane receptor, TNFR 1, have suggested some consistent features of necroptosis, including 1) a specific initiating event that signals 2) a sequence of protein complex formation and 3) subsequent activation of biochemical cascades that lead to the characteristic degenerative changes generally associated necrosis. TNF binding to the TNF receptor

1(TNFR1) found on many different kinds of eukaryotic cells can induce cell death either by apoptosis or necroptosis. [Figures 1-3]

Binding of TNF to the TNFR1 receptor signals the formation of an intracytoplasmic, membrane-associated protein complex, Complex I, containing among other proteins, the receptor interacting protein kinase, RIP 1. [Figure 1] After several reactions, Complex I is modified and coupled with RIP 3 and Caspase 8 to form a second complex, Complex II. Caspase 8 is one of the several enzymatic proteins that are characteristic of apoptosis but as is shown in Figure 2 the Caspase 8- containing Complex II can either intitiate a cascade of biochemical reactions that lead to cell death by apoptosis or a separate cascade that leads to cell death by necroptosis. Which pathway is chosen depends on the presence or absence of a Caspase 8 inhibitor in the cell: necroptois occurs if the inhibitor is present and apoptosis occurs if the inhibitor is absent.



Modified from Vandenabeele et al. 2010

Figure 1. Ligand-receptor binding mediated signal transduction initiating apoptosis/necroptosis.

The initiation of apoptosis depends on the activation of Caspase 8 that results in proteolytic inactivation of RIP1 and RIP3 and starts the apoptotic cascade. [Figure 2] On the other hand, inhibition of the reactive site of Caspase 8 then leads to the assembly of a multiprotein complex containing RIP 1 and RIP 3 called a necrosome. [Figure 3] Necroptosis depends on the regulated mutual interations of RIP 1 and RIP 3 kinase (phosphorylation) activities. Several studies have indicated that this necrosome interacts in many ways with several different biochemical pathways and with the mitochondria to initiate the pathophysiologic changes associated with necroptotic cell death. Thus, at the current stage of understanding, the presence of this necrosome, the combined RIP 1 RIP 3 molecule, can be considered a biochemical hallmark of cellular necroptosis.signaled by TNF binding to the plasma membrane receptor, TNFRP 1. In the future, other initiators may be found to be associated with formation of other necrosomes in necroptosis.

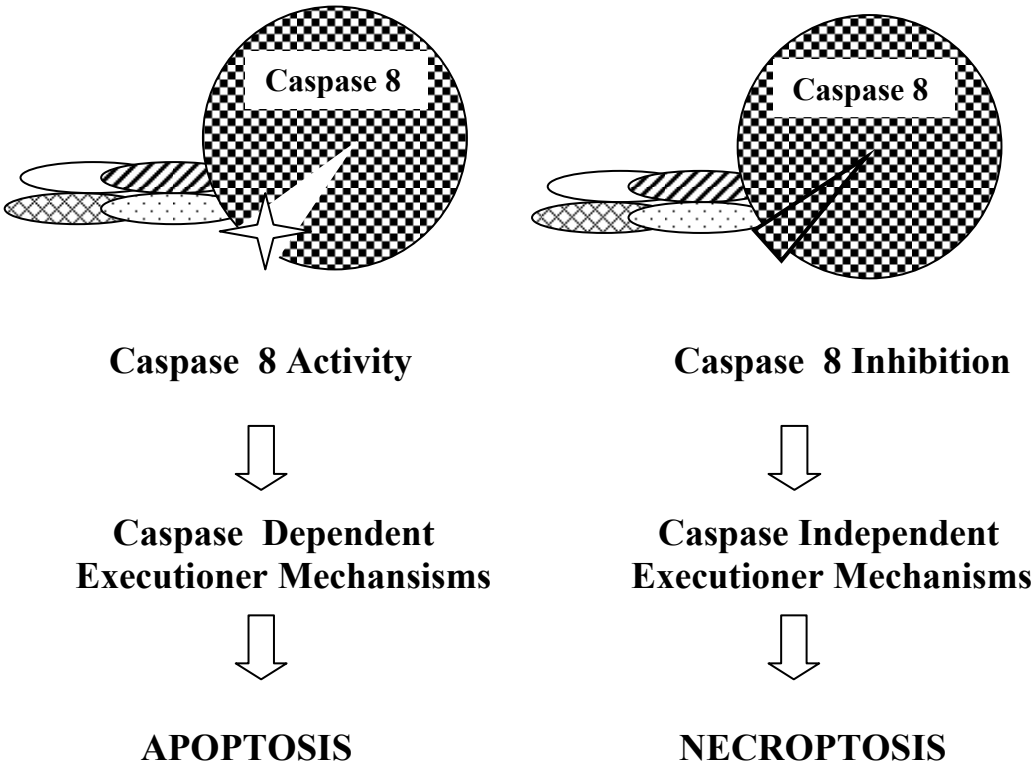


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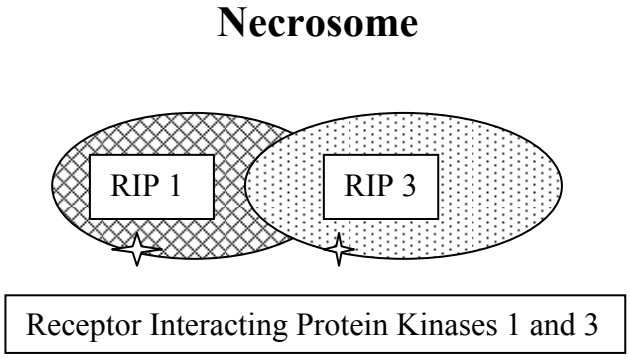
Figure 2. Proposed relationships of the apoptosis/necroptosis pathways of cell death in the TNF receptor binding signal trandsuction mechanisms of cell death



Modified from Vandenabeele et al. 2010

Figure 3 Necrosome of TNF receptor binding signal transduction mechanism of necroptotic cell death.

In summary, the current literature supports a working hypothesis for the mechanisms of necroptosis to involve 1) a specific extracellular or intracellular initiating or triggering event that signals 2) the production of complexes that, depending on the presence of inhibitors or other initiators will lead to either apoptotic or necroptotic pathways and 3) the formation of specific multiprotein molecules, necrosomes, that initiate the numerous metabolic and degenerative events of necroptotic cell death. However, other features of necroptotic cell death will probably be identified as investigation of cell life and death continues.

Therefore, cell death cannot be defined solely anatomically but requires biochemical and pathophysiological qualifiers to completely identify the precise mechanisms involved. [9] This information and insight is needed 1) to design rational treatment protocols that take advantage of the synergistic effects of death mechanism sinitiated by different drugs and energy applications, 2) to avoid competing or interfering mechanisms that could minimize the beneficial effects of combined therapies, 3) to avoid using low energy applications that could potentially cause harmful effects to susceptible cell populations particularly in diagnostic applications and 4) to improve and standardize communication among investigators and clinicians who want to use different modes of death to improve health care.

Mechanisms of cell death in low energy irradiation exposures

The biologic effects of low energy non-ionizing irradiation are influenced by 1) their particular mechanisms of interaction with their targets, 2) irradiation exposure times, 3) the biological state of the targeted cells and tissues at the time of irradiation and, if the biologic targets survive, 4) their state of repair at various intervals after irradiation. [1, 3] The biologic effects of low energy non-ionizing irradiations can be quite variable and can be non-lethal or lethal. In fact, some types of low energy non ionizing irradiation are thought to produce their therapeutic effects not by killing cells but by stimulation of cell proliferation and cell metabolism . [23, 24, 25]

Prolonged low temperature heating leading to cell death: mechanisms

Prolonged low temperature heating can lead from mild (reversible) to lethal (non reversible) disruptions of the dynamic equilibrium of cells. The very narrow separation of relative susceptibilities of normal cells and their rapidly, dividing cancerous counterparts to heating has lead to the art and science of hyperthermia cancer treatments. Unfortunately, the temperature/time exposure safety margin between effective killing of cancer cells while sparing normal tissues is very narrow in patients unlike the situation of cells in the test tube. A major treatment mechanism of cell death in hyperthermia is induction of apoptosis in susceptible cancer cells. Theoretically, cells that undergo apoptosis in tissues will be phagocytosed by neighboring phagocytic cells thus disappear. Unfortunately, non-cancerous endothelial cells that line blood vessels and some immune cells (lymphocytes) are also susceptible to the apoptotic effect of slightly elevated temperatures. Their death will lead to secondary effects such as thrombosis and hemorrhage and decreased immune responses that could increase the morbidity and risk of mortality of the patient. As a result, hyperthermia treatments involve a delicate balance of maximizing cancer cell kill while saving as many normal cells as possible. Addition to hyperthermic protocols of other treatment regimes based on other cell killing mechanisms has provided a larger clinical safety margin.

Prolonged low energy heating can lead to environmental and cellular desiccation and thermal denaturation of proteins since many thermal effects are not only temperature dependent but also depend on time at temperature.[5-7]

Low intensity light therapies based on specific wavelengths: Mitochondrial retrograde signaling pathway

Over the years, Tiina Karu and others have reported the healing effects of visible red and infrared light irradiation in humans and animals thought to be mediated by the effects of these wave lengths on cell proliferation and metabolism. [23, 26--28] Recently, she has proposed a basic mechanism of effect based on the specific absorption of red and infrared light by the mitochondrial terminal respiratory enzyme, cytochrome c oxidase. [26] She proposes this photosignaling event

initiates the mitochondrial retrograde signaling pathway, recently described as a communication pathway sending information between the mitochondria and the nucleus that influences many cellular functions. [29,30]

In general, the proposed mechanism is as follows:

> Light interacts with the mitochondrial photoacceptors, cytochrome c oxidase, NADH and flavins, that then modulate various mitochondrial metabolic events including, among others, 1) increased production of reactive oxygen species (ROS), 2) changes of mitochondrial membrane potential , 3) changes in Ca++ flux, 4) increased production of nitric oxide (NO) and many, many more. These mitochondrial metabolic products then act as second messengers signaling gene expression and RNA synthesis in the nucleus, activation of apoptotic pathways in the cytoplasm, changes of permeability of the plasma membrane and activation of ligand receptor binding.

Thus, the photoactivation of the mitochondrial retrograde signaling pathway leads to many cellular reactions that not only affect cell growth, cell proliferation and cell metabolism but also initiate cell death. So far, the only effective wavelengths to activate this pathway are found in the red-infrared region because of the absorptive specificity of the mitochondrial photoreceptors. In the future, other cellular photoreceptors such as heme-containing molecules may be found that are activated by other wavelengths to produce both positive and negative cell responses. [23]

Therapeutic ultrasound: enhancing particular mechanisms of cell death by modulation of irradiation parameters

The major treatment effect of ultrasound is based on modulations of frequencies and intensities that elevate the temperature of the tissues in the targeted areas. Focused ultrasound treatments usually produce cell death by 1) accidental (traumatic) cell death mechanisms of thermal denaturation of proteins, heat fixation and membrane disruptions (cell lysis), 2) programmed cell death mechanisms of apoptosis and necroptosis and 3) secondary lethal effects produced by the host responses to the heat including hemorrhage and vascular thrombosis, tissue ischemia and release of lytic enzymes and cytotoxic peptides by invading inflammatory cells.

More recently, low intensity ultrasound applications designed to enhance ultrasound induced apoptosis are being developed for various combination cancer treatments. [31, 32] Current studies are being done *in vitro* on carefully controlled cancer cell cultures under precisely defined irradiation conditions to determine the best parameters for maximizing production of apoptosis without introducing thermal effects. These investigators are using the latest biological techniques including proteonomics to discover the basic molecular mechanisms involved in ultrasound-induced apoptosis. The investigations have shown that cell kill by apoptosis can be induced in the cells but the total cell kill involves 1) the direct cell effect ,that is cell death by mechanical lysis, and programmed cell death by 2) apoptosis and by 3) necroptosis. The results of the studies emphasize that the mechanisms and features of apoptosis and necroptosis are interrelated and that one mechansism cannot be separated from the other. In addition, other studies have shown that in relatively prolonged irradiation exposures, the relatively slow events of apoptosis and necroptosis can be interrupted by the direct mechanical disruption of cellular membranes.[33], [34]

Diagnostic ultrasound with microbubble contrast agents

Diagnostic ultrasound applications are designed not to produce any pathologically significant thermal damage and minimize mechanical effects. However, the need to increase contrast in diagnostic ultrasound images has lead to the development of contrast agents that are suspensions of stabilized gas micro bubbles. [31, 32] Non-thermal mechanisms of ultrasound effect include mechanical disruption of cell membranes and the effects of acoustic cavitation in biologic materials. Acoustic (sonic) cavitation is associated with the production of sonoluminescence, sonochemical reactions that produce reactive oxygen species and mechanical shock waves that can have many effects on tissues including cell death by initiation of apoptosis, necroptosis and cell lysis. The collapse of the microbubbles present in the sonication field produces numerous fragments that can act as cavitation nuclei that can enhance diagnostic contrast of ultrasound and the non-thermal effects cell killing mechanisms of ultrasound. Because of these effects, the use of echo-contrast agents is being explored as a method of enhancing cell killing by low intensity ultrasound. However, the evaluation of the safety of these applications and their mechanisms of cellular effect is a continuing field of interest. [32]

Ultrasound mediated non-viral gene delivery

Modern gene therapy techniques require the delivery of genes into cells. For several years, gene delivery relied on the incorporation of the therapeutic or diagnostic genes into viruses or viral plasmids that were either introduced into the cell by physical or biologic means. In general, this delivery system worked reasonably well in cell cultures but proved to be complicated by inefficient infectivity in targeted cells and by nonspecific and incomplete distribution in intact animals. In addition, the viral DNA was permanently incorporated into the host genome providing potential pathologic and genetic complications in the future. Sonication using stabilized liposomal bubbles containing only the desired treatment genes or drug has recently been investigated for non-viral gene as well as drug delivery. [35, 36] Ultrasound enhanced delivery of the treatment materials will remove the problems of viral carriers but does introduce the potential problems of unwanted excessive sonic cavitation effects in the targeted tissues. Again, a delicate balance between desired treatment effect and the potential of unwanted side effects will be a developmental challenge for the engineer who wants to use sonic techniques to cure cancer or genetic deficiency diseases.

Conclusions

Cell life is the preamble to cell death. Death…..

> "……is result of the severe disturbance of equilibrium that leads to the end of all vital functions without the possibility of recovery either in the whole organism or any parts of it."

Cell death no longer is seen as a random event but frequently follows complicated pathways predetermined by the physiological and genetic characteristics of the cell and the physical and temporal parameters of the applied forces. These pathways and their mechanisms of execution can be exploited to manipulate various treatment goals in intact individuals. However, before cell death mechanisms can be exploited successfully, much research has to be done to not only understand the biology of the mechanisms but also to understand their safe use.

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