Chapter 4

Techniques Used for Cytotoxicity Evaluation of Nanoparticles

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Introduction

There have been many comprehensive studies on the use of nanoparticles in biomedical applications over the past decade. Some of these include drug and gene delivery, diagnosis and treatment of cancer and biosensors. The use of nanoparticles helps improve the delivery of anticancer drugs to cancer cells. One of the underlying problems of anticancer drugs is the narrow therapeutic index, accompanied by severe cumulative and acute toxicities in healthy tissues. (1-5) There are also nanoparticles which are used in preclinical and in vitro studies in order to enhance the delivery of cytotoxic chemotherapeutic agents to cancer cells and to decrease toxicity by restricting drug exposure to healthy tissues. For a better management of chemotherapy-induced cytotoxicity in cancers (lung, colon, squamous, pancreas), hydroxycamptothecin, 5-fluorouracil, docetaxel and gemcitabine encapsulated nanoparticles have been used in preclinical studies. (1)

Besides their therapeutic use, nanoparticles might improve cancer detection and diagnosis. Magnetic iron oxide nanoparticles have proved to improve the performance of magnetic resonance imaging (MRI) in diagnosing cancers, as compared to the current cancer imaging contrast agents. (1, 6-9) Protein-targeted antibody-conjugated magnetic iron oxide nanoparticles expressed on the surface of human cancer cells may further improve the accuracy of MRI for the early detection of cancer. Models of

fluorine-18-deoxyglucose encapsulated carbon and polymeric nanoparticles have been examined in preclinical studies in order to increase tumor diagnosis and detection rates using positron emission tomography. (10-12) Carbon nanotubes have been used for protein and gene delivery via non-specific endocytosis in cancer cells. (13-19) As other cytotoxic chemotherapy agents, gold and other metal nanoparticles have proved to increase the therapeutic efficacy of external beam therapy in preclinical models. There will be further studies on the use of nanoparticles as vectors for the delivery of biological and pharmacological agents. (20-28)

Besides their use in the delivery of cytotoxic and biological agents, nanoparticles may also be used as anticancer therapeutics. Gold nanoparticles with a diameter of 5–10 nm have innate antiangiogenic properties. These gold nanoparticles bind to pro-angiogenic heparin- binding growth factors, such as VEGF165 and bFGF, and inhibit their activity. In a preclinical model of ovarian cancer, gold nanoparticles demonstrated to reduce ascites, inhibit the Proliferation of multiple myeloma cells and induce apoptosis in *B-cell chronic* lymphocytic *leukemia*. (29)

Semiconductor quantum dots surface modification, known for their emission of fluorescence when excited at the appropriate wavelength, is investigated for a better detection of lymph nodes and other sites of metastases during surgical procedures. Tumor-specific peptide or antibody-conjugated quantum dots may enhance tumor targeting and increase the diagnostic accuracy of this optical imaging technique. There are various studies on the potential of imaging techniques using selective targeted fluorescent nanoparticles to allow in vivo localization of cancer cells. There is hope that such imaging techniques will increase the accuracy of different types of diagnostic imaging modalities used for cancer detection and treatment. TNF-alpha- coated colloidal gold

nanoparticles are used in early phase cancer clinical trials. (30) Preclinical studies showed that the delivery of tumor necrosis factor-alpha (TNF-α) to malignant tumors was improved by the use of the nanoparticle-based drug delivery system, preventing systemic toxicities that usually restrict the clinical utility of this biological agent. The conjugation of nanoparticles to targeting agents is also researched to deliver gene therapy payloads into cancer cells. (30)

Lastly, immunocomplexes of antibody-coated gold nanoparticles have proved to enhance the detection of certain serum tumor markers, such as carcinoembryonic antigen (CEA), carcinoma antigen 125 (CA125), and carbohydrate antigen 19-9 (CA19-9), more rapidly and more accurately than currently available techniques. There will definitely be extensive use of nanoparticles to enhance cancer detection. (31)

The development of nanoparticles for use in nanomedicine is also in progress and has grown significantly over the past years. The National Science Foundation (NSF) estimated that between 2010 and 2015, the market for pharmaceutical nanoproducts will be of approximately US$ 180 billion per year. (32) Moreover, nanoparticles are already largely dispersed in the air and in hundreds of nanoparticle-containing products on the market, including cosmetics, printer toners, varnishes, drugs and even food. However, there is little knowledge on the risks and toxicity of these nanomaterials. Because of the extensive use of nanoparticles in various fields, there is also a growing concern on their unexpected adverse effects, both academically and socially. Some studies have examined the toxicity of nanoparticles based on their shape, size, surface chemistry, chemical composition, surface activity and solubility. The use of nanoparticles in various fields has been recommended as a result of these initial toxicity studies. Still, it is necessary to rigorously evaluate their toxicity in order to legislate the safe use of all types of nanoparticles. Even if nanoparticles and

their effects on the human body are of great scientific interest, there is no standardized procedure framework for the evaluation of their toxicity. (33-36)

Cytotoxicity

Cytotoxicity is the property of a chemical compound (food, cosmetics or pharmaceuticals) or mediator cell (cytotoxic T cell) to kill cells. (37)

Compared to necrosis and apoptosis, cytotoxicity does not indicate a distinct cell death mechanism. T cell-mediated cytotoxicity or natural killer cell-mediated cytotoxicity incorporate aspects of both necrosis and apoptosis.

There has been an exponential growth of apoptosis publications over the past years. About 30 new molecules have been discovered, all related to the initiation and regulation of apoptosis. Other 20 molecules, related to DNA signalling to DNA replication, transcription or repair, have been demonstrated to affect apoptosis regulation. (38)

Both necrosis and apoptosis can cause cell death. Moreover, certain chemical compounds and cells are toxic to cells and cause their death. (39)

Necrosis and Apoptosis

Necrosis (unprogrammed cell death) is the pathological process due to cell exposure to exposed to acute physical or chemical events.

Apoptosis (programmed cell death) is the physiological process resulting in the elimination of unwanted or useless cells during their development or other biochemical events. (40)

Some of the main morphological features are cell shrinkage, with violent bubbling and surface blebbing, resulting in cell separation into clusters of membrane-bound bodies.

Organelle structure is usually unharmed, but the nucleus shows chromatin condensation, initiated at sublamellar foci and often generating heavily heterochromatic regions.

Changes in cell surface molecules determine the instantaneous recognition and phagocytosis of apoptotic cells by their neighbours. Therefore, many cells can quickly disappear from tissues without much evidence provided by conventional microscopic samples. This process accounts for cell death, normal tissue homeostasis, endocrine atrophy, negative selection in immune system and considerable T-cell death. It is also responsible for extensive cell death after exposure to cytotoxic compounds, hypoxia or viral infection. It is an important factor in the kinetics of tumor cell growth and regression. (41)

Many effects of cancer therapeutic agents are displayed through initiation of apoptosis and carcinogenesis itself seems to depend on selective, critical failure of apoptosis that allows cell survival after DNA damage and mutagenesis.

Caspase gene CED3 is the prototype of the family of cysteine proteases necessary for mammalian apoptosis, known for their predilection for cutting adjacent to aspartate residues. Mammalian caspases appear as autocatalytic cascades and some members (caspase 8 or FLICE) are "apical" and more susceptible for endogenous regulatory protein changes, while others (caspase 3 – also known as CPP32, Yama and apopain) achieve final death. Studies on caspase substrates give details on how cells destroy their structure and function. (42)

Examples of such substrates include cytoskeletal proteins - actin and fodrin

and the nuclear lamins, but also a multitude of regulatory and chaperone-like proteins with cleavage and functional alteration. A good example is *ICAD* (inhibitor of caspase-activated deoxyribonuclease), the nuclease chaperone, whose cleavage allows distinctive apoptotic nuclease, responsible for chromatin cleavage into oligonucleosomal fragments. (43)

Caspases seem to appear as inactive proenzymes in most if not all cells, undergoing activation by cleavage.

Granzyme B is a protease delivered to T cell-target cells which triggers these latent proenzymes and constitues one of the killing mechanisms of cytotoxic T cells. (44)

There are endogenous triggers as well, such as C. elegans CED4 and its protein homologue, of mitochondrial origin, possibly initiating apoptosis in mammalian cells, inhibiting the cellular energy metabolism, causing critical cell injury and affecting mitochondrial respiration. Thus,

CED4 may interact with agents associated with mitochondrial injury, such as calcium and reactive oxygen species, and initiate apoptosis. (45)

Another mitochondrial protein of great significance in initiating apoptosis is the *mammalian CED-9 homologue* BCL-2. BCL-2 has the tertiary structure typical for a bacterial pore-forming protein, targeted to the mitochondrial outer membrane. It abrogates apoptosis, probably by binding CED4 and the Bcl-2–associated X *protein* (BAX) and forming heterodimers. Like CED4, this is another killer protein. BCL2 and BAX have structurally and functionally similar homologues and are also inserted into the outer nuclear membrane (ONM) and the endoplasmic reticulum (ER). (46)

There are other examples of death receptor signal transducers.

P53 is a tumor suppressive protein activated by DNA damage which triggers apoptosis. This can occur by transcriptional activation of BAX. (47)

Ceramide, found in cell membrane, can lead to the activation of acid sphingomyelinase in the cell, signaling plasma membrane damage. (48)

Tumor necrosis factor receptors (fas/apo-1/CD95, TNF receptor I) mediate the activation of caspase. (49)

When these receptors bind to a ligand, they receive a death stimulus and initiate a series of protein-protein interactions, forming the death inducing signalling complex (DISC) that recruits and activates caspases.

These mechanisms connecting cell injury to apoptosis are determined by the activation of preformed proteins. Transcriptional mechanisms can also initiate apoptosis, but not much is known about them.

A good example is cell killing is that induced by the Drosophila gene reaper, transcriptionally activated two hours before injury-induced death in the organism. Apoptosis in Drosophila can occur without reaper transactivation, but with increased stimuli, showing the existance of a *threshold* for *reaper*-induced apoptosis. (50)

Another death initiating gene is the immediate-early gene, c-myc. Transcriptional activation of c- myc induces DNA synthesis and, when lacking concurrent cytokine support, c-myc activation initiates apoptosis. This can be seen as a threshold regulatory mechanism, as c-myc expression increases the need for insulin-like growth *factor* 1, *required for survival*. (51)

Studies on cell transformation by viruses prove the significance of these apoptosis pathways. These strong survivors have found many ways of escaping

cell death. Therefore, papovavirus SV40, adenovirus type 12, Human Papilloma Virus type 16 and Epstein-Barr Virus, all express proteins that inactivate apoptosis by p53 inactivation or BAX binding. (52) Lytic viruses also possess death postponing mechanisms, such as the cowpox virus serpin crmA and the baculovirus p35 caspase inhibitors. (53)

Differences Between Necrosis and Apoptosis

Necrosis and apoptosis are different in terms of morphology and biochemistry. (54) Necrosis is induced by cell exposure to extreme physiological factors (hypothermia, hypoxia) which may result in plasma membrane damage. Under physiological conditions, direct plasma membrane damage is induced by high doses of nanoparticles. Necrosis brings along some major morphological changes, such as cell swelling, cytoplasmic vacuoles, distended endoplasmic reticulum, cytoplasmic blebbing, condensed, swollen or ruptured mitochondria, ribosome disaggregation and detachment, organelle disruption, swollen and ruptured lysosomes, and finally cell membrane disruption. (55) There is no inflammatory reaction as apoptotic cells do not deliver their chemical constituents into the surrounding interstitial tissue and rapidly undergo phagocytosis by macrophages or adjacent healthy cells. (56) Necrosis is determined by cell exposure to severe deviation from physiological conditions, resulting in plasma membrane damage. It is characterized by cell swelling and organelle disruption, with little initial change in chromatin. Due to final plasma membrane rupture, cytoplasmic contents including lysosomal enzymes are delivered into the extracellular fluid. Thus, there is a correlation between in vivo necrotic cell death and extensive tissue damage, determining a strong inflammatory response. (57)

On the other side, apoptosis occurs under normal physiological conditions,

the cell being an active participant in its own programmed death. It mostly appears during normal cell turnover and tissue homeostasis, embryogenesis, induction and maintenance of immune tolerance, development of the nervous system and endocrine atrophy. (58) There are typical morphological and biochemical features of cells undergoing apoptosis. Morphological changes occuring during apoptosis have been observed by light and electron microscopy. Cell shrinkage and pyknosis can be identified by light microscopy in the early phases of apoptosis. (59) Cell shrinkage is represented by smaller cells, dense cytoplasm and tightly packed organelles. Pyknosis results from chromatin condensation, which is the most typical characteristic of apoptosis. Histologic examination of samples stained with hematoxylin and eosin shows that apoptosis involves both individual cells and small cell clusters. The image of the apoptotic cell is that of a dark round or oval eosinophilic mass with dense purple fragments of nuclear chromatin. (57) Subcellular changes can better be identifed by electron microscopy. In the early phase of chromatin condensation, the electron and nuclear dense material tipically accumulates peripherally under the nuclear membrane, with possible nuclei of uniform density. Macrophages or adjacent epithelial cells quickly recognise and phagocytose these apoptotic bodies in vivo. This efficient mechanism of in vivo removal of apoptotic cells does not evoke any inflammatory response. Apoptotic bodies and remaining cell fragments ultimately swell and lyse in vitro. This final phase of in vitro cell death is called secondary necrosis or apoptotic necrosis. Apoptosis can be initiated by both transcriptional and non-transcriptional pathways which have similar effector mechanisms mediated by caspases and regulated by BCL2 family members. Low doses determine a variety of harmful stimuli. Nanoparticles, heat, radiation, hypoxia and cytotoxic anticancer drugs can initiate apoptosis but can also induce serious necrosis. (59-64) The coordinated energy-dependent process of apoptosis implies the activation of caspases, a

family of cysteine proteases, together with a series of events that correlate initial stimuli with final cell death.

Cell Proliferation Assay MTT Assay

Introduction

Cell viability and proliferation assays are the ground for many in vitro studies on the response of cell populations to external factors. Cell growth determination is conventionally accomplished by counting viable cells after vital dye staining. There have been different approaches. Trypan blue staining is a simple method of assessing cell membrane integrity (estimating cell proliferation or cell death) but it is not sensitive and cannot be adapted for high-throughput screening (HTS). A radioactive uptake assay, such as *thymidine incorporation assay*, is accurate but also time-consuming and requires handling of radioactive substances. (65) The reduction of tetrazolium salts is a widely accepted procedure for measuring cell proliferation. (66) Yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide), a well-known tetrazole, is reduced to purple formazan in living cell mitochondria. The absorbance of this colored solution can be assessed by spectrophotometry (between 500 and 600 nm wavelength). The absorption maximum depends on the solvent employed. This reduction only occurs when mitochondrial reductase enzymes are active, being directly related to the number of viable (living) cells. When the amount of purple formazan produced by treated cells is compared with that produced by untreated cells, the effectiveness of the death-inducing agent can be understood by creating a dose-response curve. MTT solutions absorbed in tissue culture media or balanced salt solutions without phenol red, had a yellowish color. Mitochondrial dehydrogenases of viable cells are

converted to purple MTT formazan crystals by cleavage of the tetrazolium ring, insoluble in aqueous solutions. The crystals can be solubilized in acidified isopropanol. The resulting purple solution is spectrophotometrically measured. An increase in cell number leads to an increase in the amount of MTT-formazan formed and in absorbance. (67)

The MTT assay measures the cell proliferation rate and the reduction of cell viability when metabolic events induce apoptosis or necrosis. There small number of phases in this assay facilitates sample processing. The MTT reagent used yield low background absorbance.

The correlation between cell number and produced signal is assessed for each type of cell, thus allowing an accurate evaluation of changes in cell proliferation rate. (66)

Protease Activity Assays

Caspase activation is a unique characteristic of early stage apoptosis. Members of the ICE/CED-3 family of aspartate-*specific cysteine proteases* are important intermediaries of the complex biochemical events accompanying apoptosis. Caspase cleavage sites are marked by three to four amino acids followed by an aspartate residue. Caspases are normally synthesized as inactive precursors (procaspases). Caspases are activated by ihibitor release or cofactor binding through cleavage at internal aspartate residues determined by either autocatalysis or the action of another protease. There is a wide selection of fluorogenic caspase substrates to choose from. (68)

Caspase-3 (CPP32/apopain) plays a dominant role in the apoptosis pathway, amplifying the signal from initiator caspases (such as caspase-8) and showing full commitment to cell disaggregation. Besides cleaving other caspases in the

cascade, caspase-3 has proven to cleave other proteins, such as poly (ADP-ribose) polymerase (PARP), DNA-dependent protein kinase, protein kinase Cδ and actin. (69)

CellEvent Caspase-3/7 Green detection reagent (C10423) is a new generation of caspase substrates, very useful for the study of apoptosis. The cell-permeant CellEvent reagent consists of the four–amino acid peptide DEVD (containing the recognition site for caspases 3 and 7) conjugated to a nucleic acid–binding dye. As DEVD inhibits the ability of the dye to bind to DNA, CellEvent Caspase-3/7 Green detection reagent is basically nonfluorescent. In the presence of activated caspase 3/7, the dye is cleaved from the DEVD peptide and binds DNA, producing a bright green-fluorescent signal (absorption/emission maxima ~502/530 nm) suggesting apoptosis. This powerful assay is highly specific for caspase 3/7 activation and it detects an almost total inhibition of the CellEvent Caspase-3/7 Green detection reagent signal in the cells pretreated with a caspase 3/7 inhibitor. (70)

Assays based on CellEvent Caspase-3/7 Green Apoptotic Detection Reagents can be accomplished easily. Cells are incubated with the CellEvent reagent in complete culture medium for 30 minutes and then evaluated using fluorescence microscopy (high-content screening). While apoptotic cells showing activation of caspase 3/7 exhibit bright green-fluorescent nuclei, cells without activation of caspase 3/7 exhibit minimal fluorescence. Cleaved reagents identifiy caspase 3/7–positive cell nuclei and thus, the stain can supply data on nuclear morphology, including condensed nuclei typical of late‐stage apoptosis.

One of the main benefits of assays based on CellEvent caspase-3/7 green detection reagent is that there is no need for washing, which results in the protection of sensitive apoptotic cells that are typically lost during these rinses. Apoptotic cell loss during washing can generate the underestimation of the

extent of apoptosis in the sample, resulting in poor assay accuracy. Moreover, formaldehyde fixation and detergent permeabilization do not harm the fluorescent signal resulting from the cleavage of CellEvent Caspase-3/7 detection reagent, giving flexibility for conducting endpoint assays and investigating other proteins using immunocytochemical methods.

One of the first methods employing flow cytometry was cell cycle analysis by quantitation of DNA content. Various DNA binding dyes can be used to stain the DNA of mammalian, yeast, plant or bacterial cells. These dyes are stoichiometric meaning that they bind according to the amount of DNA present in the cell. Thus, cells in S phase will have more DNA than cells in G1 phase. They will take up equivalently more dye and will be of brighter fluorescence until they have doubled their DNA content. The cells in G2 phase will be approximately twice as bright as cells in G1 phase. Apoptosis is a classical form of programmed cell death in eukaryotes, of great importance during embryogenesis, in the homeostatic control of tissue integrity, tumor regression and immune response development. When receiving specific signals, several distinctive biochemical and morphological changes take place inside the cell. A family of proteins known as caspases, and perhaps other proteases, are activated in the early stages of apoptosis. These proteins divide key cellular substrates that are required for normal cellular function, including structural proteins in the cytoskeleton and nuclear proteins. Caspases can also activate other degrading enzymes such as DNases, which begin to cause DNA fragmentation at the linker regions between oligonucleosomes. These biochemical events result in morphological changes inside the cell and extensive DNA fragmentation. The products of DNA fragmentation are nucleosomal and oligonucleosomal DNA fragments (180 bp and multiples of 180 bp), generating a characteristic ‘‘ladder’’ pattern during agarose gel electrophoresis. Due to the partially damaged DNA from apoptotic cells, the fraction of low-molecular-weight DNA can be extracted, whereas the

non-damaged DNA remains in the cell nucleus. Because DNA fragments are lost from apoptotic nuclei and nuclear DNA content can be easily measured by flow cytometry, after nucleic acid staining with specific fluorochromes, there are several methods to assess apoptotic nuclei from a quantitative point of view. When staining apoptotic cells with PI and analyzing them with a flow cytometer, they exhibit a broad hypodiploid (sub-G1) peak, which can easily be differentiated from the narrow peak of cells with normal (diploid) DNA content in the red fluorescence channels. This method has a few advantages. It provides (i) a rapid, reliable and reproducible estimation of apoptosis, (ii) a simultaneous analysis of cell-cycle parameters of surviving cells and (iii) when necessary, a simultaneous analysis of cell surface antigens recognized by fluorescein isothiocyanate- or Alexa 488-conjugated monoclonal antibodies and the extent of apoptosis. However, there are many types of apoptosis and the extensive DNA fragmentation and the loss of DNA fragments are not universal in apoptotic death. (71) Moreover, necrotic cells sometimes exhibit certain degrees of DNA fragmentation that might result in hypodiploid nuclei. (72) In addition, besides apoptotic cells, the 'sub-G1' peak can display nuclear fragments, clumps of chromosomes, micronuclei or nuclei with normal DNA content but different chromatin structure and diminished accessibility of fluorochrome to DNA (i.e., cells undergoing differentiation). In conclusion, the presence of a hypodiploid DNA peak is not an authentic proof of apoptotic death. In order to confirm apoptosis, use morphological (microscopic observation of apoptotic bodies), biochemical (DNA ladder in agarose gel) or specific demonstration of DNA breaks (terminal deoxynucleotidyl transferase assay) before the quantitative analysis by flow cytometry. Another major issue in the quantitative assessment of apoptotic cells by flow cytometry is the differentiation between true apoptotic nuclei and nuclear debris. An appropiate determination of acquisition parameters (volume of particles, usually measured as forward scatter (FSC) and of diploid

DNA peak by using a calibration standard (DNA check beads), and negative and positive cell controls is essential before using the method for a cell line that has not been evaluated before. Keep in mind that apoptosis is a dynamic process and that there is a short ‘‘time-window’’ when apoptotic cells display their characteristic features. Therefore, different methods can produce different results according to the time of the apoptotic process. For example, in early phases of apoptosis, terminal deoxynucleotidyl transferase can be positive for DNA breaks and cell membrane can display Annexin-V positive phosphatidylserine. (73) However, morphological observation can be negative for apoptotic bodies and flow cytometric analysis can be negative for the sub-G1 peak, as DNA fragments are still maintained in the nucleus. Correspondingly, the DNA ladder cannot be detected by agarose gel electrophoresis. Still, when used properly, the propidium iodine (PI) flow cytometric assay is a rapid and easily reproducible method that can be adjusted for assessment of apoptosis in various cell types. (74)

Lysed mitotic cells, micronuclei and chromosome aggregates can be mistakenly recognized as apoptotic cells, especiallly when using hypotonic propodium iodine solution (quick method). A better exclusion of objects/events with minimal DNA content is obtained if using a linear rather than logarithmic scale in the PI emission histogram. (74) If cell debris still strongly influences the percentage of hypodiploid nuclei, evaluate the samples by fluorescence microscopy, and in case of extensive cell lysis, use an alternative method. Flow cytometric analysis that cannot indicate a hypodiploid peak despite the presence of apoptosis as demonstrated by other methods (morphological observation and/or Annexin-V positivity) can be related to absent or very low DNA loss from apoptotic nuclei because of the presence of large DNA fragments. In this situation, use a specific extraction procedure as shown above. (75)

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