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Morphological and molecular features of apoptosis and its role in colorectal cancer prevention

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Apoptosis is a form of cell suicide that is essential for the control of cell growth in multicellular organisms. Apoptosis has a very important biological significance in mammals, both during embryonic life and adulthood. Defects in the apoptosis machinery cause several human diseases, including cancer. The induction of apoptosis is now recognized as a major strategy for the treatment and prevention of cancer. Colorectal cancer is a leading cause of cancer death, with the highest prevalence in North America, Europe, Australia, and New Zealand. Aim of the present article is to review the mechanism and biological significance of apoptosis and highlight the efficacy of natural agents that target apoptosis in colorectal cancer prevention.

KEY WORDS: Apoptosis - Colorectal neoplasms, prevention and control - Cell growth processes.

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Apoptosis: definition and biological significance

Apoptosis is a finely regulated process through which the cell activates a series of lethal mechanisms for its survival. The term is therefore synonymous with "cell suicide".[1] It is an essential mechanism for the control of cell growth in multicellular organisms. The term is derived from the ancient greek: apo = out and ptosis = falling, in analogy to the falling of the leaves from the trees in autumn. This analogy emphasizes that the death of living matter is an integral and necessary part of the life cycle. Apoptosis is also called "programmed cell death", to emphasize the not accidental feature of this process. Indeed apoptosis is characterized by a controlled sequence of events that require gene transcription and protein synthesis and which lead to self-destruction of the cell.

Apoptosis has a very important biological significance in the mammal, both during embryonic life and adulthood. In the embryo, the cells that form the organs are produced in excess and must be destroyed in order to give the right form and function to organs (*e.g.* mesenchymal interdigital folds are reversed for the formation of gaps between the fingers). In humans, about the half of fetal neurons dies during the development of the central nervous system,[2] and this massive neuronal apoptosis is very important to avoid malformations that may be lethal. During ontogeny of the immunocompetent system, the death of 90% of lymphocytes in human fetal thymus is essential for the elimination of self-reactive cells. The persistence of these lymphocytes, which do not recognize the body as their own, can cause diseases so-called "auto-immune".

In adult humans, the physiological role of apoptosis is particularly in the control of the immune response, in the tissue renewal, elimination of pathogen-infected cells and in the prevention of tumors. Ninetyfive% of activated lymphocytes, among the millions produced every day, die in the blood and peripheral tissues. This serves to maintain constant the number of lymphocytes. The drop in hormone levels in certain organs generates apoptosis and tissue regeneration (*e.g.* cyclical slump estrogen causes endometrial exfoliation). Apoptosis can be, also, a cellular response to DNA damage caused by radiation, viral infections, environmental carcinogens, when repair mechanisms fail. This prevents the expansion of transformed cells and the development of tumors.

A malfunction of apoptosis machinery can cause disease. Defects in apoptosis cause diverse pathologies, including autoimmune diseases, cancer and chronic persistently infections, while, neurodegenerative syndromes are an example of diseases caused by excessive apoptosis.

Morphological features of apoptosis

The apoptotic cell has morphological features quite different from the cell who dies by necrosis (caused by an

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external insult, such as trauma, burns, chemical agent). The morphological changes of apoptotic cells are caused by a precise sequence of biochemical and molecular events that lead to activation of proteolytic enzymes. These enzymes are responsible for the destruction of a multitude of protein substrates important for the integrity of the cell. The morphological characteristics of apoptosis are:

— cell shrinkage;

— deformation and loss of contact with neighboring cells;

— condensation and margination of chromatin to the nuclear membrane;

— plasma membrane blebbing;

— expulsion of apoptotic bodies, that is, pieces of the nucleus surrounded by the cell membrane.

The membrane of apoptotic cell exposes phosphatidylserine residues that represent a booster for phagocytes. The apoptotic bodies are removed by phagocytosis by macrophages, without causing inflammation.

Differently, necrotic cell loses integrity of the cell membrane, which results, firstly, in cell swelling, due to loss of the osmotic barrier, followed by total rupture of the membrane. Leakage of cell contents into the microenvironment causes tissue inflammation.

The apoptotic signal

Much of what is known about apoptosis originates from genetic studies on the nematode Caenorabditis elegans that identified a number of genes responsible for apoptosis. Such genes serve to eliminate 131 of the initial 1090 somatic cells that are generated during hermaphrodite development.[3] Four key genes have been identified in C. Elegans: Ced-3, Ced-4, Ced-9 and Egl-1. Ced-3 is a cysteine-protease which is activated for self-proteolysis after a proper aggregation of many molecules. This oligomerization is driven by activator protein Ced-4. Ced-9 and Egl-1 are, respectively, an inhibitor and a stimulator of the complex Ced-3/Ced-4.[1]

Classically, in mammals, we distinguish two pathways of apoptosis: the "extrinsic", which starts from the cell membrane and the "intrinsic" which originates from the mitochondria. The extrinsic pathway is activated by molecules or "ligands" that bind specific membrane receptors called death receptors.[5] The death receptors belong to the superfamily of receptor gene of Tumor Necrosis Factor (TNF-R) and include: TNFR-1, Fas/CD95 and TRAIL (TNF-related apoptosis inducing ligand).[6] These receptors are characterized by the extracellular domains rich in cysteine residues that allow the receptor to recognize the ligand in a highly specific fashion. After binding with the ligand, death receptor forms a complex with two other receptors. This allows the intracytoplasmic portion recruiting caspase 8, by means of an adapter protein, which acts as a bridge between the receptor and the caspase. The family of TRAIL receptors is very interesting because of its implications in the field of oncology. So far, 5 types of receptors have been identified, of which only two are able to trigger apoptosis. Interestingly, the death receptors expressed on tumor cells are able to transduce death; differently, receptors expressed on normal cells are unable to induce death. For this reason, TRAIL ligand is toxic to the tumor but not normal cells and is a good candidate for the treatment of certain types of cancer.

The intrinsic pathway can be activated by stimuli external to the cell (*e.g.*, ionizing radiation, chemotherapy drugs, lack of nutritional intake or growth factors and hormones) and intracellular (DNA damage, altered cell cycle, misfolded proteins etc.). In the intrinsic pathway, mitochondria play a central role in the integration and propagation of death signals originating inside and outside the cell. The mitochondria of cells affected by apoptotic stimuli lose the transmembrane electrical potential, negative inside of about 180-200 mV, that is generated by the electron transport chain.[1] Subsequently, formation of pores on the membrane determines the passage of solutes, sized up to 1.5 KDa, that are normally retained, such as cytochrome c[1]. These pores are generated by the fusion channel adenine nucleotide translocator (ANT) andvoltage-dependent anion channel (VDAC). The first are the most abundant proteins on the inner mitochondrial membrane and are responsible for ATP/ADP antiport. The latter, also called porins, are the most abundant proteins on the outer mitochondrial membrane where they form non-selective pores. The mitochondria of apoptotic cell is no longer able to realize the respiratory chain; this causes ATP depletion, reduced coenzymes and glutathione oxidation, formation of oxygen radicals that cause oxidation of lipids, proteins and nucleic acids. The cytochrome c is a component of the respiratory chain and is the only diffusible because it is not anchored to the membrane but in equilibrium between the inner membrane, and the intermembrane space mitochondrial cristae. Its exit from the mitochondria, ultimately, activates apoptosis.[4]

The extrinsic and intrinsic are not completely separated, because mitochondria are also recruited in the extrinsic pathway, causing an amplification of the signal.

Both pathways, extrinsic and intrinsic, converge in the activation of a common effector, represented by a particular class of proteolytic enzymes called caspases, that are the counterpart of mammalian Ced-3.

Caspases: initiators and executors of apoptosis

The term caspase means "cysteine- dependent and aspartate -specific protease".

The catalytic activity depends on a cysteine residue within the pentapeptide QACRG (glutamine, alanine, cysteine, arginine, glycine) of the active site, highly conserved during evolution of the species. The caspase specifically cuts the substrate immediately after an aspartic acid residue.

In Drosophila, 7 members of this family of proteases have been identified, in mammals at least 14. Their nomenclature referes to the order of publication, *e.g.* caspase-1 (ICE or interleukin-1 converting enzyme) has been the first to be identified in 1993. Not all caspases are involved in the execution of apoptosis. Caspase 3, 8 and 9 are crucial for development of mammalian organisms, as demonstrated by the study of mouse models in which a selective deletion of the genes coding for at least one of these three caspases has been operated. The mice die during embryonic devel-

opment or at birth with severe developmental disorders of the central nervous system.

Caspases are synthesized as inactive zymogens called pro-caspase.[4] These zymogens bear at the N-terminal a prodomain followed by a large and a small subunit, sometimes separated by a peptide junction (linker). The maturation of caspases requires proteolytic processing of procaspase between the large and small subunits, which are therefore separated. The active caspase is derived from the processing of two pro-caspases, it is a hetero-tretramero composed of four subunits: two large and two small. The prodomain serves to caspases to be recruited during the activation process.

Caspase 8 is the first to be activated in the extrinsic pathway (initiator caspase). It is recruited by a complex formed by the membrane receptor and its ligand. The recruitment of many molecules of pro-caspase 8 activation causes self-proteolysis.

Caspase-9 is the initiator caspase of the intrinsic pathway. It is recruited by a complex named apoptosome, consisting of the protein Apaf-1 (or factor protease activation of apoptosis), by the cytochrome c from ATP. Apaf-1, the homologue of CED-4 of C. Elegans, acts as activator protein that allows the proper orientation and oligomerization of caspase 9, required for the proteolytic activation car.[3]

Caspase 8 and 9 give rise to a cascade of reactions involving other caspases, which are downstream substrates. Caspase 3 and 7 are the most important execution caspases, that cut numerous substrates localized in different cellular compartments, including the nucleus, cytoskeleton, cytoplasm. In particular, in the nucleus, the proteolytic action activates endonucleases that cleave DNA at the internucleosomal level and inactivates DNA repair enzymes. This results in the loss of DNA by the cell, which expels it in the form of apoptotic bodies.

Regulation of the apoptotic signal

In 1988, the pathogenetic role of a protein that protects from apoptosis was demonstrated in a particular type of follicular lymphoma. Aberrant expression of such anti-apoptotic protein resulted from a chromosomal translocation $t^{14:18}$, which carried the gene locus of this protein from chromosome 18 to chromosome 14, under the promoter of immunoglobulin gene. This protein was called Bcl-2, from B-cell lymphoma.[7] The discovery of Bcl-2 showed for the first time that tumorigenesis not only depends on the cell's ability to escape proliferation control, but also depends on the cell's ability to resist death. Subsequently other proteins structurally similar to Bcl-2 were discovered to form a family. The peculiarity of these proteins lies in the presence of 4 domains that are highly conserved in evolution called BH (Bcl-2 homology domain).[1-4] The BH4 domain has a protective function on the membranes because it prevents the formation of those pores that are responsible for the release of cytochrome c from mitochondria. The BH3 domain serves to dimerization, important for the function of these molecules. In addition to anti-apoptotic members possessing all four domains BH1, BH2, BH3 and BH4 (*e.g.* Bcl-2), there are pro-apoptotic members that have the only domains BH1, BH2 and BH3 (*e.g.* Bax), endowed with the ability to form pores on mitochondria, with lethal effect. Finally, Bcl-2 family members include also smaller proteins that have only the BH3 dimerization domain. BH3 members are pro-apoptotic. The members of the Bcl-2 family control the release of cytochrome c from mitochondria. In the absence of apoptotic stimulus, anti-apoptotic members of the Bcl-2 family ensure the integrity of mitochondria by neutralizing Bax. Following apoptotic stimulus, the production of BH3-only molecules impairs the protective function of Bcl-2 and activates Bax. This leads to alterations of the inner mitochondrial membrane with activation of the intrinsic pathway of apoptosis.[1, 8]

Diet, apoptosis and chemoprevention of colo-rectal cancer

The synergistic effect of a potent proliferative stimulus together with a defect in apoptosis is widely recognized as a mechanism of carcinogenesis. The induction of apoptosis is now recognized as an important strategy for the treatment and prevention of cancer.[9] Many natural substances in the diet have shown an effective anti-cancer action, both in vitro and in vivo. Among these compounds, the most promising are: epigallocatechin gallate contained in green tea, quercetin in onions and tomatoes, resveratrol in grapes, curcumin, isothiocyanates in cruciferous vegetables, organo sulfur in garlic, lycopene in tomatoes. For many of these agents, the ability to inhibit carcinogenesis, impair cancer progression, or even induce cancer regression, has been associated with induction of apoptosis.

Curcumin has demonstrated robust activity in colorectal cancer.[10, 11]

Colorectal cancer is the third leading cause of cancer death in the United States.[12] The incidence worldwide can vary greatly, with the highest prevalence in areas such as North America, Europe, Australia, and New Zealand. The lowest incidence is in India and in less developed areas of South America and Africa. Epidemiological studies suggest that economic development and eating habits are implicated in colo-rectal cancer. A low dietary fiber, a high fat and low in calcium and micronutrients diet increase the risk of this cancer.

A complete surgical resection of the primary tumor is curative when the tumor is localized to the site of origin. In metastatic cancer, surgery is associated with systemic chemotherapy and possibly radiation therapy, as palliative treatment. Chemotherapy is usually based on combination regimens involving folic acid, 5-fluorouracil and irinotecan. Another treatment option involves the combination of folic acid, 5-fluorouracil and oxaliplatin. In 2004, the FDA approved the use of biologic drug Avastin® (bevacizumab), in combination with fluorouracil containing regimens. This biological therapy aims at the prevention of metastasis through inhibition of the vascular endothelial growth factor (VEGF). An increased expression of cyclooxygenase-2 (COX-2) in approximately 90% of sporadic colon carcinomas and in 40% of adenomas of the colon has also given the boot in clinical studies to test the effect of NSAIDs, and specific inhibitors COX-2 in the chemoprevention of

colon cancer. Curcumin is the main component of turmeric, a spice common in India and surrounding regions, derived from the rhizome of Curcuma longa. Fractions of turmeric known as curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin) are the active compounds. Curcumin, because of its high anti-oxidant potential, is also being tested in the Alzheimer's disease and as an anti-inflammatory. Pre-clinical studies in a variety of tumor cell lines have shown that curcumin has a direct antineoplastic activity in vitro[10,11]. Moreover, curcumin has shown a potentiating effect on the action of conventional drugs such as 5-fluoruouracile (5-FU), the trans-retinoic acid, cisplatin, celecoxib, and doxorubicin.[13-19] The antioxidant potential of curcumin derives from the ability of this natural compound to sequester mutagenic / carcinogenic reactive oxygen species (such as superoxide anion, hydroxyl radicals, peroxides and radicals nitrite).[20] In colon cancer cell lines, curcumin has been shown to activate the caspases 9, 3 and 8.[21, 22] The pro-apoptotic action of curcumin is mainly linked to the inhibition of the transcription factors NF-kappaB and activation of MAPK, in particular cJun N-terminal kinase.[21] Furthermore, it has been demonstrated also an anti-proliferative effect of curcumin in cell lines of colon cancer, mediated by blockade of the cell cycle in G2 / M phase.[23]

Many clinical studies have been conducted in patients with colo-rectal cancer to evaluate the safety, tolerability, pharmacokinetics and pharmacodynamics of curcumin.[24-26] These studies have concluded that curcumin is safe, well tolerated and with minimal side effects. Dosages up to 8 g per day of curcumin are well tolerated.[27] Although, longer term and larger prospective clinical research trials will be necessary to definitely prove that curcumin supplements can directly reduce the risk of colorectal cancer, however, evidence accumulated so far are an important step in that direction.

Conclusions

Discovery of apoptosis and genes involved in this process, yelded the Nobel Prize for researchers Sydney Brenner, Robert Horvitz and John Sulston, in 2002, for the important implications in physiology and medicine. In recent years, a significant improvement in the treatment of cancer, is surely due to the introduction of new drugs that selectively affect molecular targets of the apoptotic signal. The discovery that many natural agents ingested in the diet impair carcinogenesis and can even induce cancer regression by stimulating apoptosis, has opened up new hopes for the chemoprevention of cancer. Colorectal cancer is a leading cause of cancer death in the world. Although ongoing clinical trials with curcumin, this natural compound promises to be an excellent agent to reduce the morbidity and mortality of this cancer, delaying the process of carcinogenesis.

Riassunto

Caratteristiche morfologiche e molecolari dell'apoptosi e il suo ruolo nella prevenzione del cancro del colon retto

L'apoptosi è una forma di suicidio cellulare che è essenziale per il controllo della crescita cellulare negli organismi multicellulari. L'apoptosi ha un significato biologico molto importante nell'uomo, e nei mammiferi in generale, sia durante lo sviluppo embrionario che nell'età adulta per il controllo dell'omeostasi di organi e tessuti. Un difettoso funzionamento di questo meccanismo può causare diverse malattie umane, e, tra queste, il cancro. L'uso di agenti naturali e farmacologici che attivano l'apoptosi è oggi riconosciuto come strategia importante per il trattamento e la prevenzione del cancro.

Il cancro del colon retto rappresenta una delle principali cause di morte per tumori, con la più alta prevalenza in Nord America, Europa, Australia e Nuova Zelanda. Molti studi oggi sono in corso, aventi come obiettivo lo sviluppo di strategie per la prevenzione di questo tumore. Questo articolo si propone di sintetizzare le attuali conoscenze sui meccanismi molecolari e significato biologico dell'apoptosi con particolare riferimento all'efficacia di agenti naturali che attivano l'apoptosi e risultano promettenti nella prevenzione del cancro colo-rettale.

PAROLE CHIAVE: Apoptosi - Tumori colorettali, prevenzione e controllo - Processi di crescita cellulare.

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