Review

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Apoptins: selective anticancer agents

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Therapies that selectively target cancer cells for death have been the center of intense research recently. One potential therapy may involve apoptin proteins, which are able to induce apoptosis in cancer cells leaving normal cells unharmed. Apoptin was originally discovered in the Chicken anemia virus (CAV); however, human gyroviruses (HGyV) have recently been found that also harbor apoptin-like proteins. Although the cancer cell specific activity of these apoptins appears to be well conserved, the precise functions and mechanisms of action are yet to be fully elucidated. Strategies for both delivering apoptin to treat tumors and disseminating the protein inside the tumor body are now being developed, and have shown promise in preclinical animal studies.

The discovery of apoptin

CAV, also known as the chicken anemia agent or chicken infectious anemia virus, was first described in Japan in 1974. CAV is a gyrovirus that infects dividing cells, causing anemia, growth retardation, abnormal feathers, leg paralysis, intramuscular hemorrhages, and destruction of bone marrow and lymphatic tissues [1]. Apoptin (see Glossary), is a protein encoded by the *VP3* gene of CAV [2], and is a crucial mediator of the generalized lymphoid atrophy and anemia that leads to the mortality of CAV-infected chicken. Interestingly, apoptin also selectively induces apoptosis in multiple transformed avian and mammalian cell lines, leaving primary and nontransformed cells unharmed [3,4]. Much research has been undertaken to detect the pathways of apoptin-induced apoptosis and its selectivity towards transformed cells. In addition, human viruses similar to CAV have recently been identified, and some of these related apoptins have been preliminarily characterized. In this review, we summarize current knowledge on the effects and molecular mechanism of action of apoptins.

Insights into the natural role of apoptins

The natural role of apoptin has not been completely elucidated. It has been proposed that apoptin induces apoptosis to escape from the host; however, no tangible proof of this has been reported. CAV triggers apoptosis in thymocytes from the thymus cortex and erythroblastoid areas of bone marrow *in vivo* [5]; however, it is also found in several other tissues [6]. CAV expresses three proteins from three genes [2,7]; *VP1*, which encodes the capsid protein with a hypervariable region [8]; *VP2* encodes a dual-specificity protein phosphatase (DPS), which is believed to contribute to signaling events important for viral replication [9], and may also act as a scaffold for capsid assembly [10]; and *VP3*, which encodes apoptin and triggers apoptosis. The first proteins expressed during infection are from *VP2* and *VP3* at 12-h post infection, whereas protein from *VP1* is expressed at 30-h post infection [7].

Apoptin-deficient CAV is unable to replicate [11]. In addition, when the apoptin phosphorylation site T^{108} is mutated to isoleucine, CAV replication ability is strongly reduced [11], likely due to the lack of apoptotic activity of the mutant protein. A similar $T^{108}A$ mutation in apoptin partially impairs the apoptotic activity in tumor cells [12], and completely inhibits the rudimentary apoptotic activity in some primary cells [13]. $T^{108}E$ mutated apoptin retains some ability to trigger apoptosis in primary skin fibroblasts [13]. Interestingly, the apoptin-deficient CAV regains replicative activity when CAV-apoptin is replaced by Torquetenovirus (TTV)-derived apoptosis-inducing protein (TAIP); a protein that shares some amino acid similarities with CAV-apoptin (Figure 1) [11].

Recently, a human gyrovirus was discovered that also encodes an apoptin protein. This human gyrovirus apoptin, which has been detected in samples of skin [14], blood [15],

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Glossary

Akt (also known as protein kinase B): a serine/threonine kinase involved in the stimulation of key anabolic cellular processes, including protein synthesis and proliferation. It has pro-survival and antiapoptotic effects.
Apoptins: small (~13 kDa) proteins encoded by the Gyrovirus *VP3* gene. Two apoptin proteins have been described: CAV-apoptin and HGyV-apoptin. Apoptins are able to induce apoptotic cell death selectively in transformed cells, independent of the p53 pathway.
Apoptosis: a process of programmed cell death induced by external or internal stimuli. The hallmarks of apoptosis include cell membrane blebbing, DNA fragmentation, chromatin condensation, and nuclear fragmentation. Resulting apoptotic bodies are usually engulfed by phagocytes and, therefore, apoptosis does not result in tissue damage.
Bcl2 family: a family of apoptosis regulator proteins that comprises antiapoptotic (including Bcl2 and Bcl-xL) and proapoptotic (such as Bax, BAD, Bak) members. They govern mitochondrial apoptosis through direct interactions between pro- and antiapoptotic members. They control induction of mitochondrial outer-membrane permeabilization, which results in the release of cytochrome c.
Caspases: cysteine-dependent aspartate-directed proteases. Some caspases have a crucial role in apoptosis. Initiator caspases (caspases 2, 8, 9, and 10) proteolytically activate effector caspases (caspases 3, 6, and 7), which in turn, via limited proteolysis, activate numerous proteins involved in the execution phase of apoptosis.
CDK2 (cyclin-dependent kinase 2, or cell division protein kinase 2): a serine/threonine kinase involved in the control of the cell cycle. CDK2 is activated by cyclin E during the early stages of DNA replication, and by cyclin A during the late stages of this process. CDK2 is involved in the inhibition of mitosis upon DNA damage.
Cytochrome c release: an early marker of apoptosis. Cytochrome c released from the mitochondrion triggers caspase cascade and execution of apoptotic cell death.
DNA fragmentation: a late marker of apoptosis. Caspase-activated DNase cleaves DNA at linker regions between nucleosomes, fragmenting the DNA.
Dendrimers: repetitively branched nanoparticles, such as polyamidoamine. Some dendrimers are considered to be promising vehicles for drug delivery.
Gyroviruses: circular, single-stranded DNA viruses belonging to the Circoviridae. The best-known species is chicken anaemia virus (CAV).
Oncolytic virus: a natural or genetically engineered virus that preferentially infects and destroys cancer cells as well as stimulating an antitumor immune response.
PP2A (protein phosphatase 2A): a ubiquitous serine/threonine phosphatase with broad substrate specificity involved in the balancing of several phosphorylation-based signaling cascades.
Proteasome: a multiprotein complex responsible for proteolytic degradation of damaged, misfolded, and surplus proteins tagged by a polyubiquitin chain.
Receptor mediated endocytosis: a process of endocytosis induced by the binding of a natural ligand or another molecule (e.g., agonist, antagonist, antibody, viral, or bacterial cell surface protein). The process can be utilized to deliver cargo inside the cell as long as it is bound to, or incorporated into, the receptor-interacting item.
Virus-mediated gene delivery: an approach of gene delivery to the targeted cells that utilizes the ability of a virus to inject its genetic material into the host cell. Targeted gene delivery exploits specific or preferential interactions of certain viruses with receptors present on the cells of interest.

and feces [16], has shown similar properties to the CAV-apoptin, including a subcellular distribution and the ability to induce apoptosis [17]. However, one human gyrovirus strain isolated from stool specimens lacked a gene encoding apoptin [18]. Given that these discoveries are relatively recent, the natural role of human gyrovirus apoptin still awaits full characterization; however, it is probable that it has the same role as CAV-apoptin, due to the functional and sequence similarities. In addition, because human gyrovirus has only been detected in a small percentage of the human population (~1–3%) using a highly sensitive detection method (PCR), and because the sequence of HGyV is almost identical to avian gyrovirus type 2 (AGV2), it is possible that AGV2 and HGyV are in fact the same virus. AGV2 can be detected in most chickens, which suggests that HGyV is a contaminant from contact with poultry and not necessarily a *bona fide* human infectious agent. This issue will be resolved experimentally within coming months.

The presence of virus-like particles has been reported in CAV-infected epithelial cells, macrophages, healthy cells, and in the cell nucleus, but virus-like particles were difficult to detect in apoptotic bodies or dead cells infected by CAV [5,7,19]. Thus, it cannot be firmly concluded that CAV triggers apoptosis to escape from cells. However, apoptotic activity that contributes to efficient virus release has been reported previously in two nonrelated viruses, Bovine Herpesvirus 1 [20], and Rift Valley Fiber Virus [21]. It has been suggested that apoptin induces apoptosis in lymphocytes to deceive the immune system and keep the virus hidden. This theory may not hold, at least in humans, because human primary lymphocytes isolated from healthy donor blood are completely resistant to CAV-apoptin-induced apoptosis [22]. However, this may still be true in chicken because, once young chickens are infected, the virus spreads rapidly throughout several tissues [23,24], reaching the brain and heart in less than 2 weeks [6]. If the chicken survives the CAV infection, the virus may remain in the brain for more than 49 days [24], and longer than 12 months in reproductive tissues, even after the development of anti-CAV immunity by the chicken [25]. Thus, the natural role of CAV-apoptin still awaits full elucidation.

Apoptosis as a primary cancer-killing mechanism triggered by apoptin

Although the mechanism(s) of action of apoptins are not yet completely understood, it is already well established that apoptin can trigger tumor-selective apoptosis (Box 1). It is clear that, in their tumor cell selectivity, apoptins are able to activate caspases independently of p53 [22,26,27], and this process is also independent of death receptors [22]. Moreover, apoptin-induced cell death is strongly influenced by regulators of the mitochondrial pathway, and involves the loss of mitochondrial membrane potential. Expression of apoptin in transformed cells leads to cytochrome c release, DNA fragmentation, and activation of caspase-9 (Figure 2).

Interestingly, different research groups have come to alternative conclusions as to the role of B cell lymphoma 2 (Bcl2) family proteins in apoptin-mediated cell death. Some groups suggest that coexpression of apoptin and Bcl2 accelerates cell death [28], whereas others have shown that Bcl2 and B cell lymphoma extra large (Bcl-XL) both inhibit apoptosis triggered by apoptin [26]. It has been suggested that the inconsistencies of these results is attributed to the variable expression of nerve growth factor IB (Nur77) in the different cell types that were tested. Nur77, a nuclear orphan receptor, is a member of the steroid/thyroid receptor family that is capable of transmitting the apoptotic signal from the nucleus to the mitochondria [29]. Nur77 may bind to Bcl2, changing its properties from antiapoptotic to a proapoptotic protein [30]. Upon expression of apoptin, Nur77 relocates from the nucleus to the cytoplasm, where it causes cytochrome c release and activation of the apoptosome-dependent death pathway [22]. In addition, Nur77 can modulate apoptosis by activating the transcription of pro- and antiapoptotic genes [31]. The role of Nur77 in apoptin-mediated apoptosis was