Apoptin: Therapeutic Potential of an Early Sensor of Carcinogenic Transformation

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apoptosis, gene therapy, drug targets, tumor-specific sensor, apoptin kinase, anaphase promoting complex

Abstract

The avian virus-derived protein apoptin induces p53-independent apoptosis in a tumor-specific way. Apoptin acts as a multimeric complex and forms superstructures upon binding to DNA. In tumor cells, apoptin is phosphorylated and mainly nuclear, whereas in normal cells it is unphosphorylated, cytoplasmic, and becomes readily neutralized. Interestingly, apoptin phosphorylation, nuclear translocation, and apoptosis can transiently be induced in normal cells by cotransfecting SV40 large T oncogene, indicating that apoptin recognizes early stages of oncogenic transformation. In cancer cells, apoptin appears to recognize survival signals, which it is able to redirect into cell death impulses. Apoptin targets include DEDAF, Nur77, Nmi, Hippi, and the potential drug target APC1. Apoptin-transgenic mice and animal tumor models have revealed apoptin as a safe and efficient antitumor agent, resulting in significant tumor regression. Future antitumor therapies could use apoptin either as a therapeutic bullet or as an early sensor of druggable tumor-specific processes.

INTRODUCTION

Apoptosis not only plays an important role in physiological processes such as tissue and organ formation during embryogenesis (1, 2) but also during adult life, in, for example, tissue renewal, the regulation of the immune system, and the elimination of derailed and/or cancer cells (3). A decreased propensity for apoptosis contributes to tumor formation (4). For instance, the tumor-suppressor protein p53 plays an important role in the prevention of oncogenic transformation. It is generally assumed that tumor suppression is at least in part due to p53-induced apoptosis and that during tumor formation, a selection for p53 loss of function takes place (5). The antiapoptotic protein Bcl-2 and related proteins such as Bcl-X_L are also involved in tumor development. These antiapoptotic proteins are often overexpressed as a result of chromosomal translocations in various tumors, e.g., leukemia, lymphoma, and breast cancer (6, 7).

Apoptosis-suppression is essential for tumor growth, but apoptosis can still be exploited for the treatment of tumors. Despite the fact that many tumor cells have a defect in the decision machinery for apoptosis, they usually still retain an intact execution system. Such tumor cells will still die, if they are provided with an effective apoptotic signal (8–10). Unfortunately, various chemotherapeutic agents fail because they require functional p53 for inducing apoptosis in tumor cells. More than 50% of the human tumors, including melanoma, lung cancer, or colon carcinoma, contain mutated p53. Patients with such tumors have a very low chance of responding to (chemo-)therapy (11). Likewise, overexpression of the antiapoptosis proteins Bcl-2 and Bcl-X_L, or caspase inhibitors, negatively influences the chemotherapeutic treatment of a large number of lymphomas (6, 12).

Considerable efforts are currently being invested in the development of new anticancer therapies, which are based on the induction of apoptosis and are not hampered by the lack of functional p53 and/or overexpression of antiapoptotic genes (13–15). Here, we describe the chicken anemia virus (CAV)-derived protein apoptin, which induces apoptosis specifically in tumor/transformed cells. Its promises for the development of novel antitumor therapies are discussed below.

HISTORY

CAV causes aplastic anemia in young chickens owing to destruction of erythroblastoid cells and immunodeficiency owing to severe depletion of thymocytes (16). We have determined that CAV induces apoptosis in the thymocytes of young chickens, or in transformed chicken cells under culture conditions (17). DNA isolated from infected cells reveals the apoptosis-specific laddering pattern, which is not observed in DNA from noninfected cells. Electron-microscopic analysis shows cells containing condensed chromatin adjacent to the nuclear membrane and apoptotic bodies in the cytoplasm of neighboring epithelial cells.

The CAV DNA was isolated and cloned as a circular double-stranded infectious replication intermediate of 2319 base pairs (18). From this double-stranded DNA intermediate, early after infection, a single polyadenylated polycistronic mRNA is transcribed, which encodes three distinct genes (19, 20). The three genes code for

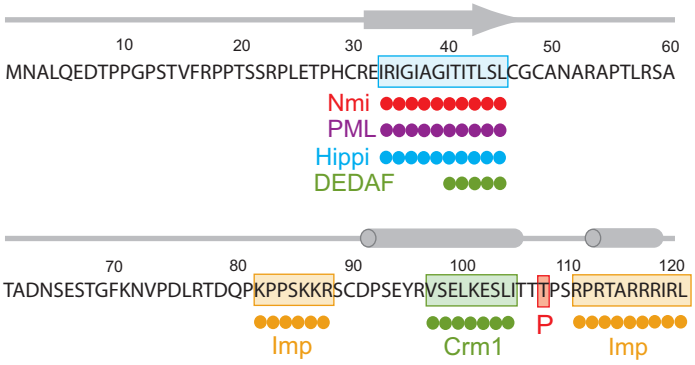

Figure 1

Schematic representation of the apoptin protein. A putative β-strand and two α-helices, as predicted by the PSIPRED software (37), are indicated. The following protein domains are boxed: (iso)leucine-rich region (*blue*), bi-partite nuclear localization sequences (*yellow*), nuclear export sequence (*green*), and phosphorylation site at T108 (*red*). Binding sites for Nmi, Hippi, DEDAF, importin β1 (Imp), Crm1, and the region targeting apoptin to PML bodies are indicated.

the following proteins: VP1 [capsid protein (21)], VP2 [protein phosphatase and scaffold protein (21–23)], and VP3. The products show no homology to each other or to any other known proteins. Later after infection, splicing of the CAV mRNA results in several other RNA products. It is not known whether these RNAs result in the production of functional proteins (24).

To establish which CAV protein is responsible for the induction of apoptosis, plasmids encoding VP1, VP2, or VP3 were transfected into cultured chicken transformed cells. The cells were analyzed by indirect immunofluorescence with specific antibodies and propidium iodide, which stains DNA strongly and evenly in intact cells, but weakly and/or irregularly in apoptotic cells. Expression of VP3 alone was sufficient for induction of apoptosis in a similar way as observed during CAV infection. Therefore, VP3 was renamed apoptin (25). Early after transfection, apoptin is present in the cytoplasm and dispersed throughout the nucleus. Later after transfection, apoptin becomes aggregated in the nucleus and the cells undergo apoptosis. At this point in time, nucleosomal laddering can be seen specifically in the DNA of apoptin-expressing cells (25). Preliminary results indicate that VP2 also has some apoptotic activity, although much weaker than apoptin. Surprisingly, VP2 enhances apoptin-induced apoptosis in transformed cells. Expression of VP1 alone did not result in apoptosis (M.H.M. Noteborn, unpublished results). Apoptin consists of 121 amino acids and is rich in proline, serine, threonine, and basic amino acids (18). The basic regions of the protein may allow interaction with nucleic acids (**Figure 1**).

APOPTIN

The apoptosis-inducing activity of apoptin in transformed chicken cells formed the cradle of a series of unexpected and intriguing observations in human (tumor) cells, which have opened a novel field within fundamental and applied tumor biology.

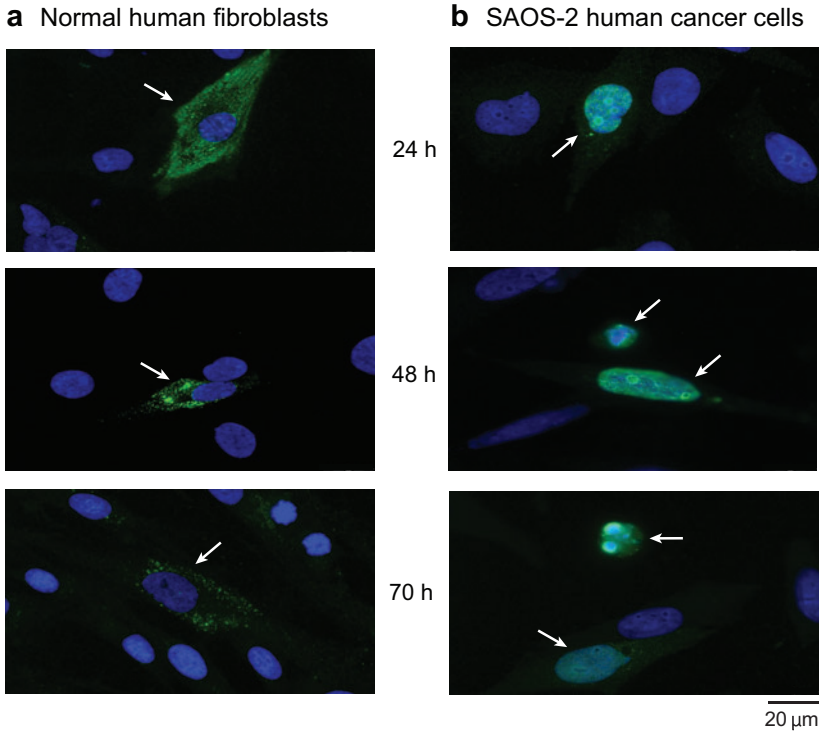

Figure 2

Cellular localization of apoptin in normal cells and cancer cells. (*a*) In normal cells, apoptin is located in the cytoplasm (24 h) and becomes aggregated at 48 h and 70 h. (*b*) In cancer cells, apoptin enters the nucleus (24 h) and induces apoptosis (48 h and 70 h) as shown by chromatin condensation. Normal human fibroblasts and human osteosarcoma (SAOS-2) tumor cells were transfected with a plasmid encoding flag-tagged apoptin. Flag-apoptin was stained with antibodies directed against the Flag-tag (*green*) and cellular DNA was stained with DAPI (*blue*). Transfected cells are indicated by arrows.

Apoptin Induces Apoptosis in Human Tumor Cells

Apoptin can induce apoptosis in cell lines derived from a great variety of human tumors. To date, more than 70 different human tumor cell lines have been shown to be sensitive to apoptin-induced apoptosis (26, 27). On the other hand, apoptin does not induce apoptosis in normal, nontransformed human cells, such as primary fibroblasts, keratinocytes, smooth muscle cells, T cells, or endothelial cells. Long-term expression of apoptin in normal human fibroblasts revealed that it has no toxic or transforming activity in these cells and that apoptin does not interfere with cell proliferation. Zhang et al. demonstrated that primary human hepatocytes and human bone marrow–derived mesenchymal stem cells, which are often affected during conventional chemotherapy and responsible for harmful side-effects, are also resistant to apoptin-induced apoptosis (28). A possible cause for apoptin's tumor-specific behavior is that, in tumor cells, apoptin is located in the nucleus, whereas in normal cells it is found in the cytoplasm (29) (**Figure 2**).

Apoptin Senses Early Stages of Oncogenic Transformation

Whereas normal primary human fibrobasts or keratinocytes are resistant to apoptin-induced apoptosis, the same cell types become sensitive to apoptin following immortalization and stable transformation by SV40 (29). More interesting, certain human

cell lines that are immortal, but not clearly oncogenically transformed (in the sense that they would produce tumors in immunodeficient animals), are also susceptible to apoptin. Such an example is HaCaT cells, spontaneously immortalized human epidermal keratinocytes, which are sensitive to apoptin-induced apoptosis (29). Although these cells show early aspects of cellular transformation (loss of senescence barrier and anchorage-independent growth), they do not form tumors in animals, but rather, when grafted onto nude mice, induce the formation of a well-differentiated epidermis (30). Furthermore, primary fibroblasts from patients with a cancer-prone genetic condition, cells with the so-called ER^+ phenotype (31), are sensitive to apoptin, but only after UV irradiation, in contrast to cells from healthy donors, exposed to UV irradiation in the same way (32). Another relevant observation is the finding that SV40-transformed human fibroblasts become sensitive to apoptin before they have undergone crisis, indicating that apoptin can actually become activated in cells that are not yet truly and/or stably immortalized (29). Even more compelling is the finding that transient transfection of normal human diploid fibroblasts with apoptin, together with the SV40-transforming large-T antigen, results in apoptin-induced apoptosis. Immunofluorescence analysis showed that, in these cells, apoptin moved from the cytoplasm to the nucleus. Apparently, the transient expression of a transforming protein is sufficient to render normal cells susceptible to apoptin, and the establishment of a stably transformed or immortal state is not required (33). These observations strongly indicate that apoptin senses relatively early stages during the multistage process of oncogenic transformation. As these early changes are likely to determine the subsequent path of tumor progression (34), apoptin might target the Achilles' heel of cancer cells.

Apoptin is Neutralized in Normal Cells

Apoptin undergoes specific biochemical changes in normal nontransformed cells (28). These experiments used purified recombinant MBP-apoptin protein, which resembles wild-type apoptin in many aspects, such as tumor-specific induction of apoptosis. Early after microinjection, MBP-apoptin is distributed as fine particles and accumulated into increasingly larger bodies, often marginalized to the plasma membrane, and eventually disappears. In parallel to these visual changes in intact cells, biochemical analyses revealed that MBP-apoptin is still present in cells where it is undetectable by antibodies and is only physically deteriorated at later stages. The analysis revealed that in normal cells, apoptin becomes completely antibody-shielded under native conditions on a C-terminal apoptin epitope as well as partially on an MBP epitope, with the shielding of the former appearing to be more rigorous and rapid than that of the latter. This epitope shielding was totally absent in tumor cells, suggesting that apoptin exhibits a normal cell–specific alteration in conformation and/or accessibility.

Another line of research also illustrates apoptin's instability in normal cells. Pietersen et al. (35) generated transgenic mice expressing apoptin under the regulation of the $H2\text{-}K^b$ promoter to investigate whether apoptin remains inert in normal lymphocytes during development, activation, and proliferation of these cells. There was no difference in B-, helper T-, or cytotoxic T-cell numbers derived from transgenic

spleens compared with spleens from wild-type littermates. Moreover, stimulation of B cells with lipopolysaccharides or stimulation of T cells with interleukin-2 and concavalin A did not result in a growth disadvantage for the transgenic lymphocytes, nor did it lead to increased cell death. Nevertheless, robust apoptin RNA expression was detected in thymus and spleen. Apoptin protein, however, could be detected only after concentration by immunoprecipitation. The amount of apoptin protein detected in transgenic splenocytes was significantly increased by inhibition of the proteasome.

From these studies (28, 35), it can be concluded that apoptin is unstable in normal cells. These normal cell–specific, unstable characteristics of apoptin underline its nontoxic effect on normal healthy tissue and enhance its safety as a potential tumor-specific therapeutic agent.

TUMOR PROCESSES SENSED BY APOPTIN

In an attempt to correlate apoptin's tumor-specific activity with its structural and biophysical properties, Leliveld et al. (36) used the bacterially expressed MBP-apoptin, described above. In vitro, the protein spontaneously formed highly soluble, noncovalent globular complexes comprising 30 to 40 subunits, a process mediated by a domain in the N-terminal half of apoptin [possibly involving the extended β-strand that is predicted by the PSIPRED software (37) (**Figure 1**)]. This multimerization was robust and virtually irreversible; further, the globular aggregates were stable in cellular extracts. Studies of apoptin ectopically expressed in living cells confirmed that apoptin also exists in large complexes in vivo. Cytoplasmic microinjection of human tumor cells with internally, irreversibly cross-linked recombinant apoptin protein complexes resulted in nuclear import and induction of apoptosis, indicating that the multimeric state of apoptin is fully competent to exert its death-inducing function. Further experiments showed that apoptin adopts little, if any, regular secondary structure within the complexes. This surprising result would classify apoptin as the first protein for which, rather than via the formation of a well-defined tertiary and quaternary structure, semirandom aggregation is sufficient for activity (38) (see also Other Proteins with Tumor-Specific Apoptosis Activities, below, specifically, the section on HAMLET).

In tumor cells, but negligibly in normal cells, apoptin protein translocates to the nucleus prior to the induction of apoptosis (28). Immuno-electron microscopy of tumor cells showed that, once inside the nucleus, apoptin predominantly colocalizes with heterochromatin and nucleoli (36). Apoptin's preference for these DNA-dense structures may be explained by the finding that apoptin cooperatively forms distinct superstructures with DNA. In vitro, these superstructures do not grow beyond a diameter of approximately 200 nm, containing up to 20 multimeric apoptin complexes per superstructure and covering approximately 3 kb of DNA. A single apoptin multimer was shown to possess eight independent, aspecific DNA-binding sites that preferentially bind to DNA strand ends, but which could also collaborate to bind longer stretches of DNA. Apoptin's high affinity for naked, undecorated double-stranded and single-stranded DNA and for DNA double-strand breaks suggests that it may also capture such DNA in superstructures in vivo (36).

The consequences of any such binding are currently unclear; while it may be a by-product of some nucleic acid binding function required for viral genome replication that has no cellular ramifications, it is also conceivable that it could actually contribute to the induction of apoptosis. Further studies are needed to understand the physiological consequences of such binding both for the virus and for the infected (or apoptin-expressing) host cell.

Tumor-Related Nuclear Trafficking of Apoptin

To further explore the role of nuclear localization in apoptin-induced apoptosis in tumor cells, Danen-van Oorschot et al. employed a mutagenesis strategy (39). Both predicted basic nuclear localization signals (NLSs) of apoptin, at positions 82–88 and 111–121 (**Figure 1**), were proven to function independently, although both domains were required to obtain efficient nuclear localization. These data imply that the C-terminal region of apoptin contains a canonical bipartite-type nuclear localization signal.

Strikingly, further investigations using constructs encoding N- and C-terminal halves of apoptin showed that the protein contains two different domains that induce apoptosis independently (39). A protein containing only the N-terminal domain (position 1–69) localized in the cytoplasm and induced much less apoptosis than did the C-terminal domain (position 80–121), which translocated to the nucleus of its own accord. However, fusion of the N-terminal region of apoptin to a heterologous NLS resulted in visible translocation to the nucleus and a more robust killing activity. Reciprocally, C-terminal constructs lacking either NLS exhibited reduced apoptosis. Therefore, a correlation exists between nuclear localization and killing activity in both domains. Guelen and coworkers also showed that the nuclear localization of full-length apoptin in tumor cells is required for its ability to induce apoptosis. The authors showed that targeting apoptin to the endoplasmic reticulum or mitochondria, thus inducing an extranuclear tethering, greatly reduced its cytotoxicity (40).

The data described above raised the possibility that apoptin's tumor-specificity might be mediated solely by compartmentalization. In other words, if only enough apoptin could access the nucleus of normal cells, would that be sufficient to activate apoptosis? This simple hypothesis proved not to be true, as enforced nuclear location of apoptin in normal cells by means of a heterologous NLS did not enable apoptin to induce cell death (39). This result indicates that, in addition to nuclear localization, one or more additional events are required for apoptin to become fully active—a hypothesis that turned out to be true (see section below on Tumor-Specific Modifications of Apoptin).

The fact that apoptin binds to naked DNA in vitro (36), together with the importance of nuclear localization for its activity in tumor cells (39), opened the possibility that apoptin might play a role in the regulation of gene expression. Nevertheless, Danen-van Oorschot et al. reported that de novo gene transcription and translation were not required for apoptin-induced apoptosis, which implies that all upstream effectors and downstream targets are already present in the cell (39). Apoptin binding to DNA could also conceivably result in repression of transcription, especially

as apoptin is known to colocalize in tumor cells with heterochromatin (36), which is predominantly transcriptionally inactive. Nevertheless, apoptin itself did not act as a transcriptional repressor in a simple reporter assay (39).

Apoptin-Mediated Ceramide Signaling and Tumor Cell Killing

Recently, Liu et al. have proposed a possible mechanism for apoptin-mediated tumor cell death (41). They showed that apoptin modulates the sphingolipid-ceramide pathway, leading to increased concentration of ceramide in human prostate tumor cells. The resulting cell death is directly related to the amount of ceramide in the treated cell, as downregulation of ceramide, either by increased expression of acid ceramidase (ACDase) or by inhibition of acid sphingomyelinase (ASMase), decreased apoptin-induced apoptosis (41, 42). Ceramide is considered to be a tumor suppressor lipid (43). Ceramide metabolism is regulated by a complex biochemical pathway, and the molecular dissection of this pathway has identified several new targets for drug development. Interestingly, ceramide can be generated in multiple cellular compartments, including endoplasmic reticulum, mitochondria, nucleus, lysosomes, and plasma membrane, with key regulatory enzymes showing distinct compartmentalization. It has been inferred that the ceramide pathway has many similarities to the p53 signaling pathway, and both serve as comprehensive sensors of cellular stresses (44). Apoptin increases ceramide by activation of ASMase, resulting in increased hydrolysis of sphingomyelin to ceramide. Furthermore, apoptin was shown to inhibit ACDase, which deacylates ceramide to sphingosine. Liu et al. (42) found that in approximately 65% of human prostate tumors, the ACDase activity was significantly upregulated, making acid ceramidase a target for therapeutic intervention. In support of this view, the same authors showed that the combination of apoptin with the acid ceramidase inhibitor LCL204 increased cell killing. This was also shown in vivo in a mouse model, where cotreatment significantly increased regression of prostate cancer–derived xenografts (42).

Tumor-Specific Modifications of Apoptin

Apoptin is considered to be activated by a general and early tumor-specific pathway because it induces apoptosis in many different human tumor cells, immortal and/or transformed cells, or cells transiently transfected with specific transforming proteins. Apoptin is inactive in the normal healthy counterparts of the above-mentioned cells. Rohn et al. (45) have reported that apoptin is phosphorylated robustly in a broad panel of tumor cells but negligibly in normal cells. The tumor-specific phosphorylation was mapped to threonine 108. A gain of function point mutant (T108E) enabled apoptin to accumulate in the nucleus and kill normal cells, implying that phosphorylation is a key regulator of apoptin's tumor-specific properties. Analyses of human tissue samples confirm that apoptin kinase activity is detectable in primary malignancies but not in tissue derived from healthy individuals. Because of the remarkably high number of diverse human transformed or tumor cell types tested that are susceptible to apoptin-induced apoptosis, it seems likely that these cells must share a common lesion that is highly selected, and possibly essential, in tumor development or maintenance.

Apoptin kinase (Apop-K) is a strong candidate for such a pathway. The kinase seems to be deregulated/activated quite early in the development of a "transformed" phenotype because soon (20–30 h) after transient transfection of normal cells with SV40 large T antigen, apoptin becomes phosphorylated, accumulates in the nucleus, and is able to induce apoptosis (45).

As mentioned above, apoptin contains a bipartite nuclear localization signal (positions 82–88 and 111–121) (39), which flanks the relevant phosphorylation site at T108. This structural organization is suggestive of a direct effect of threonine phosphorylation on nuclear targeting via the bipartite NLS. Poon and coworkers showed that the whole region (position 74–121) constitutes a highly specialized NLS that mediates the targeting of foreign proteins (e.g., green fluorescent protein) specifically to the nucleus of transformed cells (46). However, from a mechanistic point of view the situation appears to be more complicated. Evidence indicated that besides the bipartite NLS, the 74–121 domain of apoptin also contains a nuclear export sequence (NES) regulated by CRM1 (47) (see **Figure 1**). Phosphorylation of threonine T108 directly affects nuclear export of apoptin by inhibiting CRM1 action. The following model has been proposed: In normal cells, apoptin is readily exported out of the nucleus via its NES in a CRM1-dependent fashion, whereas in cancer cells, due to T108 phosphorylation, the NES is inactivated and nuclear export is prevented (47). We would, however, like to add a note of caution here as all experiments focusing on apoptin's NES function have been performed with GFP-fusion constructs. Whereas apoptin-GFP fusions seem to function similarly to native apoptin in cancer and immortal/transformed cells, the same cannot be said for normal cells, where very often (but also not always) relatively high amounts of GFP-apoptin can be found in the nucleus (48, 49).

Taken together, the identification of apoptin kinase activity constitutes the first evidence indicating that apoptin's tumor-specific activities result from direct activation by a cancer-associated cellular pathway. Apparently, activation of this kinase constitutes a possibly essential change associated with the cancerous or precancerous state. The elucidation of both upstream and downstream effectors in this pathway should further improve our understanding of the complex process of malignant transformation and thereby may lead to the development of novel therapeutic approaches for its treatment.

We have mentioned above that apoptin harbors 2 different independent death domains constituted by positions 1–69 and 80–121, respectively (39). A mutagenic approach, using alanine substitution, indicated that only the C-terminal domain (which actually contains T108) relies on phosphorylation for triggering apoptin-dependent cell death (48). This experiment provides an explanation for the finding that when T108 is mutated in the context of the complete protein, the induction of apoptosis in cancer cells is severely hampered but not completely abolished (49).

A nice example of this duality is provided by the analysis of fibroblast-like synoviocytes (FLS) from patients with rheumatoid arthritis (RA). It is well-known that these cells present features of transformation and derailed apoptosis, which is reflected by elevated gene expression of proto-oncogenes such as c-Myc, c-Ras, and c-Jun and apoptosis inhibitors such as Bcl-2 and Mcl-1 (50, 51). Simultaneously, RA synovial

fibroblasts contain mutations in tumor suppressor genes such as p53 (52). Isolated FLS from patients with RA, grown under tissue-culture conditions for 2–3 passages, were significantly more sensitive to apoptin-induced apoptosis than FLS from trauma patients (53). These data are in agreement with the transformed-like nature of FLS in patients with RA (54). However, the mechanism of apoptin's action on FLS seems to be different from that on tumor cell lines, given the fact that apoptin is not phosphorylated at amino acid residue T108 in RA FLS as is the case in tumor cell lines. Hence, it is very likely that the N-terminal death domain of apoptin is responsible for the death effect on RA FLS. This is a very interesting observation, implying that wild-type apoptin senses at least two different transformation/activation-dependent processes in human cells, where only one relies on apoptin phosphorylation.

In a transgenic apoptin-mouse model established by us (35), the apparent instability of apoptin in various organs can be blocked by proteasome inhibitors. Our subsequent pilot experiments have shown that apoptin can indeed become ubiquitinated. Recently, others have reported that apoptin is subject to modification by sumoylation (55). These results indicate that apoptin is a substrate for the ubiquitin-proteasome pathway; however, sumoylation does not appear to be essential for apoptin's tumor/transformation-specific activity (55).

Apoptin Tumor-Related Protein Partners

The identification of apoptin-associating proteins will also guide us to appreciate the pathways underlying early stages of tumor formation. Regarding the above-mentioned complexity of apoptin's tumor-related induction of apoptosis, one might expect a large panel of cellular proteins interacting with apoptin in a tumor-specific manner. Recently, various research groups have identified several apoptin protein partners showing tumor-related characteristics.

The N-Myc interaction (Nmi) protein has been identified independently by Sun and coworkers (56) and by our own laboratory as an apoptin-associating protein. Nmi is an interferon inducible cytoplasmic protein whose exact cellular function is not yet fully understood. Nmi can shuttle to the nucleus, for instance, by binding to the high-mobility group transcription factor Sox10 (57). The interaction domain of apoptin with Nmi has been mapped to amino acids 33–46 (56).

Danen-van Oorschot and coworkers (58) have reported that apoptin interacts with DEDAF, a protein previously found to associate with the proapoptotic death effector domain (DED)-containing DNA-binding protein DEDD. DEDAF (Rybp in the mouse) has a predominantly nuclear location and colocalizes to some extent with apoptin in cancer or transformed cells but not in normal cells. More interestingly, and similarly to apoptin, DEDAF displays cell death activity when overexpressed in tumor cells and appears not to induce apoptosis in normal nontransformed cells. DEDAF is a member of a larger, well-conserved protein family, which includes the Yaf2 protein. Recent work in a zebrafish system has indicated that in contrast to the cell death–promoting function of DEDAF, Yaf2 has cell survival–promoting properties (59). In our opinion, these data make the DEDAF/Yaf2 signaling pathway an attractive focus for future apoptin studies.

Cheng and colleagues (60) have identified another apoptin-associating protein, human Hippi, the protein interactor and apoptosis comediator of huntingtin interacting protein 1 (Hip-1). Interaction between Hippi and Hip-1 results in caspase-8 recruitment, which is believed to be the molecular trigger for increased apoptosis in Huntington's disease (61). Hippi clearly colocalizes with apoptin in the cytoplasm of normal human cells, but in transformed cells it stays in the cytoplasm, whereas apoptin is transported to the nucleus (60). These features would support a model where Hippi suppresses apoptin activity in normal cells. On the other hand, Hippi can induce apoptosis on its own when overproduced in, e.g., HeLa cells (62).

Maddika et al. have reported that apoptin triggers the cytoplasmic translocation of Nur77 (also known as TR3 or NGFI-B) in human tumor cells (63). Nur77, a nuclear orphan receptor known to transmit apoptotic signals from the nucleus to mitochondria, controls both survival and death of cancer cells (64). A wealth of recent experimental data demonstrates that Nur77 activities are regulated through its subcellular localization. In the nucleus, Nur77 functions as an oncogenic survival factor, promoting cell growth. In contrast, Nur77 is a potential killer when it migrates to mitochondria, where it binds to Bcl-2 and converts its survival phenotype into an apoptotic stimulus via release of cytochrome c (63). Interestingly, downmodulation of Nur77 by siRNA protected MCF7 cells against apoptin-induced cell death (63).

The group of Michael R. Green has shown that in human H1299 cells (metastatic lung carcinoma) apoptin associates with APC1, a subunit of the anaphase-promoting complex/cyclosome APC/C (65). This interaction was not found in normal human fibroblasts. In cancer cells, apoptin multimerization and APC1 interaction are mediated by domains that overlap with sequences of the bipartite nuclear localization signal (position 80–121). Furthermore, apoptin expression in transformed cells induces the formation of PML nuclear bodies and recruits APC/C to these subnuclear structures (66). Evidence was provided demonstrating that apoptin expression inhibits APC/C function in p53-minus cells in a way similar to depletion of APC1 by RNA interference. APC1 inhibition leads to APC/C disruption, whereafter the cells will undergo cell death following G2/M arrest. The various interactions of apoptin with cellular partners in both cancer and normal cells are schematically represented in **Figure 3**.

Other Proteins with Tumor-Specific Apoptosis Activities

Besides CAV-derived apoptin, which is the main focus of this review, other viral or cellular proteins that harbor tumor-specific apoptotic activities have been described. We briefly describe these various proteins here and compare, whenever possible, the mechanistic properties of these tumor-specific death effectors with those of apoptin.

TRAIL (67) is a member of the tumor necrosis factor (TNF) superfamily that, upon binding to its receptor (DR4 or DR5 in humans), induces the classical extrinsic, p53-independent death receptor pathway (68). Interestingly, while both tumor and normal cells express the appropriate TRAIL receptors ubiquitously, normal cells are generally more resistant to TRAIL-induced killing, for reasons that remain controversial (69, 68). In any case, whereas TRAIL uses the extrinsic cellular death pathway,

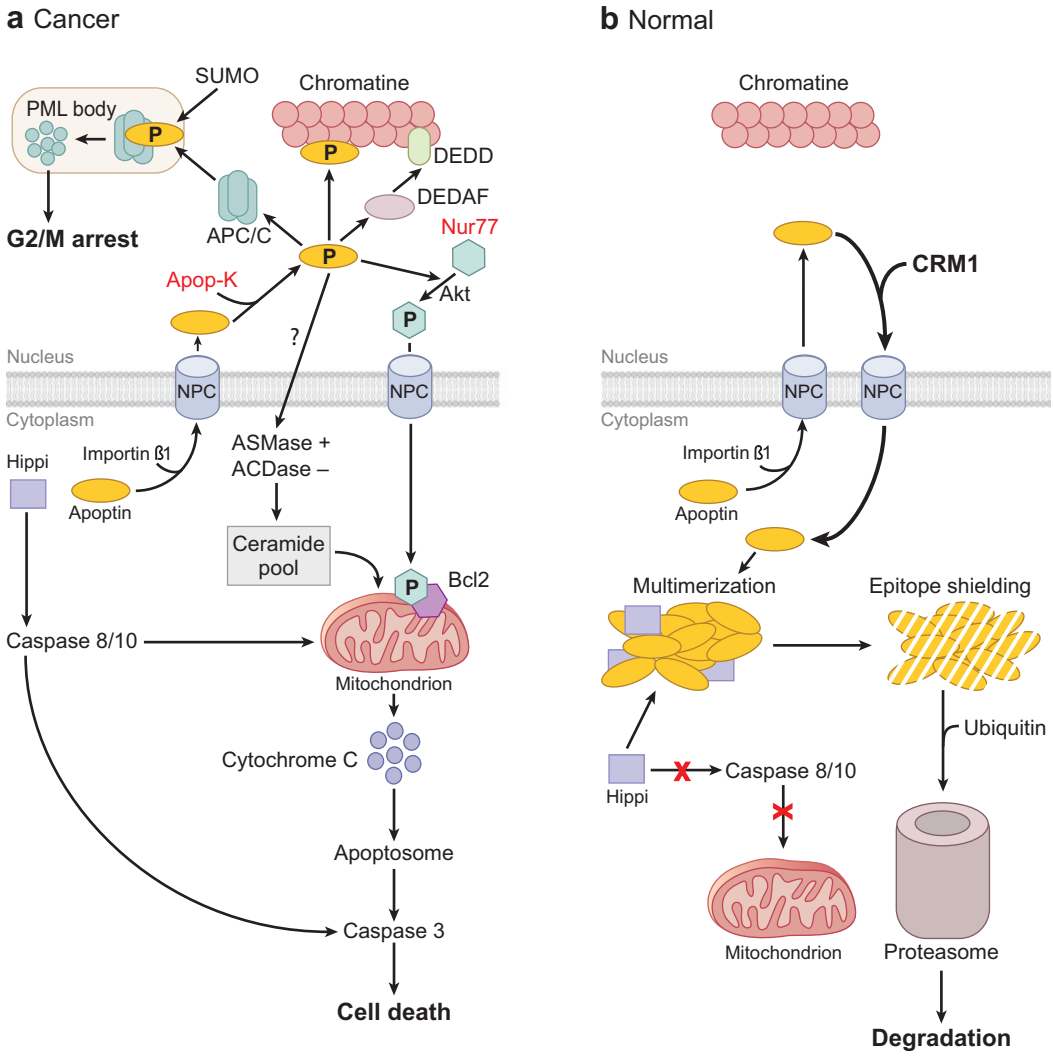

Figure 3

Hypothetical model of the cellular associations of apoptin with various binding partners and their differential impact on signal transduction pathways in (*a*) cancer cells and (*b*) normal cells.

apoptin was shown to function independently of this pathway and appears to target directly the intrinsic mitochondrion-born death pathway via the Nur77 intermediate (63).

Several reports have shown that the melanoma differentiation-associated gene-7 (mda-7) (recently renamed IL-24) is a novel tumor suppressor that has tumor-specific growth inhibitory and proapoptotic potentials in a broad range of human cancer cells (70). Similar to TRAIL, mda-7 acts via the extrinsic receptor mediated death pathway

[Fas in the case of mda-7 (71)], followed by downregulation of survival factors such as Bcl-2 and Akt (72). Molecular effectors that are involved in mda-7-mediated tumor killing include the caspase cascade and the induction of G2/M cell cycle arrest through the inhibition of the Cdc25 pathway (73). In vivo experiments have revealed that adenovirus-mda-7-mediated tumor killing shows combinatorial synergy with various drugs (72, 74, 75).

Adenovirus type 2 early region 4 ORF 4 (E4orf4) has multiple functions during viral infection, but when expressed ectopically in cultured human cells, it induces cell death in transformed or tumor cells but not in healthy cells (recently reviewed in 76). Studies into the mechanism of action of E4orf4 have been fruitful, revealing at least two independent death pathways that are p53-independent, and in some cell lines, caspase-independent. One pathway involves rapid induction of apoptosis via calpains and Src from the plasma membrane (77), whereas a slower autonomous pathway was recently identified that may stem from as yet unknown execution activities in the nuclear compartment (77–80). This duality in harboring two independent death domains is also found in apoptin, although in that case both domains appear to function via nuclear channels. For the membrane-dependent pathway of E4orf4, Src-mediated tyrosine phosphorylation of E4orf4 is needed: Mutation of a number of tyrosine residues in the viral protein results in nuclear location and a significant drop in death-promoting activity (79). Interestingly, normal cells can be sensitized for E4orf4-dependent apoptosis by transient transfection of oncogenes [e.g., E1A/myc or E1A/activated Ras combinations (81)]. This situation is very reminiscent of what we have described for apoptin and suggests that in the case of E4orf4, early steps in the oncogenic transformation of human cells are sensed. From this point of view, it is also important to mention that E4orf4 binds to the Bα subunit of protein phosphatase 2A (PP2A) and that this binding is directly linked to cell death induction in tumor cells (82). This is intriguing because the interaction of PP2A with several DNA tumor virus proteins is required for virus-mediated cell transformation (83, 84). Another striking similarity between E4orf4 and apoptin is the finding that E4orf4 also targets the APC/C, which results in G2/M arrest, both in a heterologous yeast system and in mammalian cells (85). Apparently, both E4orf4 and apoptin act via the APC/C complex, resulting in their tumor-specific induction of apoptosis, which makes the anaphase-promoting complex/cyclosome a very attractive target for future anticancer therapeutics.

Another intriguing protein able to specifically kill tumor cells was isolated from a specific fraction of human milk and was called HAMLET (Human α-lactalbumin made lethal to tumor cells). The protein was shown to kill tumor cells by a mechanism resembling apoptosis (86). Many different types of tumor cells are susceptible to HAMLET, whereas healthy cells are resistant. The activity was shown to reside in a complex between the human milk protein α-lactalbumin and oleic acid, which is the most abundant fatty acid in human milk. HAMLET is a partly unfolded/molten globule-like complex under physiological conditions (87), which is reminiscent of apoptin's unstructured properties, as discussed above. Caspase inhibition or Bcl-2 overexpression did not affect cell survival and the caspase response was Bcl-2 independent. In cancer cells, HAMLET translocates to nuclei and binds directly to

chromatin, as does apoptin, and the death response was unrelated to the p53-status of the tumor cells. Similarly to apoptin, p53 deletions or gain of function mutations did not influence the HAMLET sensitivity of tumor cells (88).

In conclusion, the studies carried out with these various tumor-specific death proteins, including apoptin, will eventually result in finding the bits and pieces of the puzzle underlying specific stages in the multistep process of tumor cell development.

POTENTIAL THERAPEUTIC APPLICATIONS

The remarkable ability of apoptin to induce apoptosis specifically in tumor cells creates high potential for therapeutic applications in two ways. First, the described and unknown cellular partners of apoptin might proof targets for novel antitumor therapies. Second, apoptin-treatment shows promising results in murine tumor models.

Sensing Druggable Targets by Using Apoptin

Apoptin is active in all tumor cells tested so far and therefore it likely senses a tumor essential pathway. Identification of cellular partners of apoptin can, therefore, point to new targets for antitumor therapies. If apoptin and related proteins respond to a presently unknown cancer pathway, this could yield new insights in the process of carcinogenesis, new targets for cancer treatment, and the design of combination therapies.

A first proof of concept that tumor-related drug targets can be identified based on apoptin studies is provided by apoptin's interactions with the APC/C complex (15, 65, 66). Similar to apoptin treatment, depletion of the apoptin-associating protein APC1 by siRNA leads to apoptosis, also in the absence of functional p53 (65). Remarkably, as already mentioned above, Kornitzer et al. (85) have described that the APC/C complex also plays a crucial role in the tumor-specific induction of apoptosis by the tumor-specific apoptosis inducer E4orf4 protein. The APC/C complex is involved in the coordination of key events during mitosis. Microtubuli interfering agents, such as the antitumor drug taxol, are believed to disrupt microtubuli dynamics and thereby deregulate the APC/C-controlled mitotic spindle checkpoint. Indeed, both apoptin and microtubuli-interfering drugs result in G2/M arrest followed by late apoptosis. In addition, acid ceramidase, overexpressed in several tumor cells and inhibited by apoptin, has recently been recognized as a next drug target. Studies on apoptin may thus provide new insight in the mechanisms and reveal new druggable targets.

Apoptin-Treatment of Solid Tumors in Preclinical Models

Apoptin has several features making it well suited for cancer therapy. Tumor-resistance to apoptosis can be a direct consequence of mutations in tumor-suppressor genes such as p53 or overexpression of antiapoptotic proto-oncogenes such as Bcl-2 and Bcl-X_L (89). Apoptin acts independently of p53 (15, 65) and is insensitive to BCR-ABL and Bcl-X_L (26), which suggests that apoptin can induce apoptosis in cases where (chemo)therapeutics might fail.

Recently, it has been reported that chemotherapeutic agents combined with apoptin treatment of human tumor cell cultures results in enhanced cytotoxicity (90). Combined treatment of recombinant adenovirus AdAptVP3-expressing apoptin with different concentrations of etoposide clearly showed an additive cytotoxic effect in human osteosarcoma U2OS cells. Paclitaxel combined with apoptin expression acted additively in p53-positive human osteosarcoma U2OS and nonsmall cell lung carcinoma A549 cells, p53-negative osteosarcoma Saos-2 cells, and p53-mutant prostate cancer Du145 cells. Finally, apoptin was proven to be coeffective when combined with the chemotherapeutic agent methotrexate (91). The fact that the additive cytotoxicity, achieved with a combined apoptin-chemotherapeutic agent treatment, does not depend on a functional p53 tumor suppressor, illustrates the power of this potential combinatorial antitumor therapy.

Several groups have now examined the effect of apoptin in vivo in mouse tumor models (**Table 1**). Pietersen and colleagues developed a strategy for the use of apoptin in cancer therapy based on adenoviral vectors (92, 93). A single injection of the apoptin-producing adenovirus in xenografted hepatomas generated by subcutaneous injection of human HepG2 hepatocytes in nude mice, resulted in a delay in tumor growth (92). Tumor histology showed loss of tissue integrity and increase of interstitial space. The number of proliferating cells as detected by BrdU-labeling was dramatically decreased in the apoptin-transduced regions versus control-treated tumors (94). Importantly, the apoptin-producing adenovirus did not have appreciable toxic effects when injected intraperitoneally, intravenously, or subcutaneously into healthy rats (**Table 2**).

To increase the transduction efficiency, further studies using these and other nonreplicative viruses, including a fowlpox virus–based vector, used a regimen of multiple intratumoral injections during several days. These approaches resulted in a significant overall survival benefit for the apoptin-treated mice and, in some cases, resulted in complete regression of the established tumor (41, 93–95). In general, independent of the viral vector used, the apoptin-treated tumors can be divided into three distinct groups: those with a complete response, those with a significant delay in tumor growth, and those with tumor growth kinetics similar to tumors treated with control vector. Because no replicating virus can be produced by either the adenovirus or the fowlpox virus–based vectors, any tumor cell that escaped viral infection during the treatment will stay alive and proliferate, provided that there is not too much surrounding damage. Indeed, the characteristic lobule structure of hepatoma tumors prevents an even distribution of the apoptin gene (92).

Recently, apoptin has also been combined with other treatments in vivo. As discussed above, apoptin expression modulates the ceramide-sphingolipid pathways leading to enhanced ceramide levels (41). The majority of prostate tumors have elevated acid ceramidase levels compared with neighboring normal prostate tissue (42). In vitro, upregulated acid-ceramidase protected cells from apoptin-induced apoptosis, whereas cotreatment with the acid-ceramidase inhibitor LCL204 sensitized cells for apoptosis. In vivo, combined treatment enhanced the antitumor activity of apoptin in xenografted prostate tumors in mice, resulting in significantly reduced tumor growth and increased animal survival (42) (**Table 1**).

Table 1 Apoptin antitumor activity after in vivo treatment

Administration (doses)[1]	Preclinical model[2]	Outcome	Reference
Adenovirus (1x)	Hepatoma	• Reduced tumor growth • Nonequal tumor penetration of nonreplicating virus	(92)
Adenovirus (1–5x)	Hepatoma	*After 1 injection, tumor histology showed* • Decreased tumor integrity • Loss of cell-cell contact • Reduced proliferation (BrdU-labeling) *After 5 injections* • Prolonged survival for 60% of the treated mice • After 6 months, 30% of the treated mice were tumor free	(94)
Adenovirus (8x)	Breast carcinoma	• Reduced tumor growth • No/minimal weight loss	(93)
Fowlpox virus (3x)	Hepatoma	• Prolonged survival for 70% of the treated mice • After 3 months, 30% of the mice were tumor free	(95)
Plasmid DNA (3x)	Lewis lung carcinoma	*Apoptin treatment* • Reduced tumor growth • Decreased tumor integrity • Prolonged survival for 60% of the treated mice • No effect on the immunocellular response *IL-18 treatment* • Similar effect on tumor growth as apoptin alone • Increased percentage of CD3/CD4+, CD3/CD8+ cells, and Th1 helper cell cytokine levels • Isolated cytotoxic T cells were LCC cells specific *Apoptin + IL-18 treatment (compared with single treatments)* • Enhanced effect on tumor response • Prolonged survival for 80% of the treated mice • Enhanced effect on immumocellular response	(96)
Adenovirus (4x)	Prostate cancer	*Apoptin treatment* • Reduced tumor growth • Prolonged survival for 60% of mice *LCL204 (acid ceramidase inhibitor) treatment* • No significant effect on tumor growth • Slightly prolonged survival *Apoptin + LCL204 treatment (compared with single treatments)* • Enhanced effect on tumor response • Enhanced survival	(42)
Asor-plasmid systemic delivery (1x)[3]	Hepatoma in situ	• Reduced tumor growth after 5 days • Tumor histology: decreased tumor integrity • Apoptin DNA and mRNA observed in liver only	(98)

[1]Intratumoral delivery unless stated differently.

[2]Xenografts in nude mice unless stated differently.

[3]Plasmid DNA was chemically conjugated to the Asor-peptide, which specifically targets to asioglycoprotein receptor present only at the cell surface of hepatocytes and hepatocarcinoma cells.

Table 2 Effect of apoptin on normal tissue after in vivo treatment

Administration	Preclinical model[2]	Outcome	Reference
Adenovirus, subcutaneous Intravenous Peritonal	Rat	*No significant differences compared with empty vector in* • Weight • Tissue histology • Amount of apoptotic cells	(92)
Chromosomal integration under H-K2 promotor[1]	Transgenic mice	• No effect on survival, gross morphology, or behavior • Mendelian birth ratios • High mRNA levels, mainly in lympoid tissues. • Low protein levels in vivo, but high in isolated cells after proteasome inhibition • No effect on T and B cell during development or after in vitro stimulation	(35)
Adenovirus systemic delivery	Prostate cancer	• No morbidity of any type between GFP and apoptin-GFP expressing virus suggesting low systemic toxicity	(42)
Asor-plasmid systemic delivery[2]	Hepatoma	• Apoptin DNA and mRNA detected in tumor and normal liver only, not in other tissues, including spleen, lung, kidney, and heart • No overt pathological changes in any tissue, including liver	(98)

[1]H-2K promoter, homolog of human MHC class I, starts expression late in embryogenesis.
[2]Plasmid DNA was chemically conjugated to the Asor-peptide, which specifically targets to asioglycoprotein receptor present only at the cell surface of hepatocytes and hepatocarcinoma cells.

Lian and colleagues (96) combined apoptin treatment with interleukin-18 (IL-18) and reported that combined administration results in an even higher induction of an effective antitumor immune response and tumor regression. Lewis lung carcinoma (LLC) xenograft tumors in C57BL/6 mice were immunized with plasmid DNA-encoding apoptin in conjunction with plasmid-encoding IL-18 by intratumoral injection. IL-18 and apoptin appear to affect tumors via complementary pathways. Whereas apoptin directly targets the tumor cells, IL-18 treatment appears to act via enhancing the immune response toward tumor cells, as treatment with IL-18 alone effectively reduced tumor growth in vivo, but had no effect in cell culture experiments. Indeed, the number of immunoreactive cells, such as CD3+CD4+ and CD3+CD8+ cells, was increased in the IL-18-treated mice. Furthermore, cells freshly isolated from the spleen showed increased activity of cytotoxic T-cells toward LCC cells specifically, and isolated T-cells excreted higher levels of Th1-type cytokines, such as IL-2 and IFN-γ. Cotreatment with apoptin slightly enhanced the effects on immunoreactive cells and resulted in a higher degree of tumor growth suppression and prolonged survival.

Importantly, this study also showed that the immunoresponsive cells of animals treated with apoptin alone did not differ from controls. This is of relevance because infection of chicks with the natural apoptin-producing chicken virus CAV results in a reduction of erythroid and lymphoid progenitor cells (97). Destruction of precursor

T cells results in depletion of mature cytotoxic and helper T cells, which were clearly present in the apoptin-treated mice. It should be noted, however, that here a single, local vaccination was given and that the responsive cells may have entered the tumor region only after treatment and were not expressing apoptin themselves. Even so, this is an indication that local treatment supports, rather than damages, the immunoresponsive cells. A more direct indication that apoptin outside its context of CAV infection in mammalian cells is not toxic for immune cells is provided by transgenic mice in which apoptin was specifically expressed in lymphoid cells by placing it under the H2-Kb promoter. This promoter is expressed in mice during late-embryogenesis (d11), primarily in lymphoid tissue. The apoptin-expressing transgenic mice were born in a normal Mendelian distribution and apparently underwent normal development (35). No study performed so far has indicated any detectable toxic effect of apoptin to normal tissue (**Table 2**).

Peng and colleagues (98) showed that targeting the apoptin gene to the asialoglycoprotein receptor provides a safe antitumor treatment using systemic administration. Delivery of this Asor-apoptin via the tail vein into mice bearing in situ hepatocarcinomas resulted in specific and efficient distribution of apoptin in both hepatocarcinoma cells and normal liver cells. The in situ hepatocarcinomas showed significant signs of regression, whereas the surrounding normal hepatocytes or other tissues clearly were not affected (**Table 1**).

Improving Delivery Strategy

It has been shown that a threshold level of apoptin is needed for inducing apoptosis in tumor cells (40, 99). This observation again points to the requirement of an accurate delivery system of apoptin. The wide variance of tumor effects, ranging from nonresponsive to complete tumor regression after intratumoral injections of nonreplicative viruses, may be related to insufficient transduction, as discussed above. A possible improvement of the local delivery/intratumoral injection of death genes is to generate conditionally replicating (adeno)viral vectors. These vectors can be designed to selectively replicate in tumor cells by placing viral genes that are essential for viral replication under the control of a tumor-specific promoter (100). Importantly, such agents indeed have been shown to preferentially replicate in tumor cells and not in human liver tissue ex vivo (101), whereas efficient penetration into tumor xenografts was achieved (102). Application of these vectors may ensure enhanced spread of apoptin throughout the tumor.

Another efficient way to deliver DNA, and even proteins, is by receptor-mediated endocytosis (103). Transport-receptors have the ability to bind ligands with large cargo and to internalize these. The asialoglycoprotein receptor is specifically and abundantly expressed on hepatocytes (104). As already mentioned above, Peng and colleagues (98) showed that by conjugating an apoptin-expressing plasmid to the ligand of this receptor, asialoglycoprotein (asor), apoptin was specifically and efficiently distributed in both hepatocarcinoma cells and normal liver cells after systemic delivery. Because the asialoglycoprotein receptor is mainly present on liver (tumor) cells, other tumors have to be targeted by applying ligands to other internalizing

receptors. Recently, a receptor has been identified that can target apoptin to brain tumors. The receptor is the membrane-bound precursor of heparin-binding epidermal growth factor (HB-EGF), which is also known as the diphtheria toxin receptor (DTR) (105). This receptor is constitutively present at the blood-brain barrier and is strongly upregulated in many tumors, such as human glioblastoma (106), human melanoma (107), and in inflammatory vascular endothelium present in tumors. Moreover, a human applicable nontoxic ligand (CRM197, a mutant of the diphtheria toxin) is available, which has been used on millions of people in vaccines and in cancer patients (108). Biopharmaceutical drugs have been selectively delivered to the brain by this receptor (109). This opens the opportunity to target the apoptin gene to (brain) tumors with a disease-induced selectivity. Recently, we have been able to force in vitro cultured human glioblastoma cells into apoptosis following nonviral CRM-197 targeted and receptor-mediated delivery of an apoptin-expressing plasmid (de Boer, Noteborn, unpublished data). Therefore, the combination of apoptin antitumor therapy and CRM197-targeting technology provides great opportunities for future tumor treatments.

CONCLUDING REMARKS

Since its discovery in the early 1990s as a viral protein able to induce apoptosis in a subset of CAV-infected cells, apoptin (VP3) has grown out into a specific sensor of the transformed state of human tumor cells and a promising agent for future anticancer therapeutics. From a mechanistic point of view, it is now becoming increasingly clear that this small avian viral protein senses survival signals in tumor cells and manages to redirect these signals into death-inducing apoptotic stimuli. This action radius on the verge of cellular life and death makes apoptin, in addition to its potential as a therapeutic agent and tumor-specific sensor, a promising tool for studying fundamental processes involved in early stages of carcinogenesis.

SUMMARY POINTS

1. Apoptin induces apoptosis in human tumor cells, but not in healthy normal cells. Apoptin's activity in tumor cells does not rely on an active p53 pathway.
2. In tumor cells, apoptin is targeted to the nucleus, resulting in apoptosis, whereas in normal cells, it stays in the cytoplasm where it becomes neutralized.
3. Early stages during the multistage process of oncogenic transformation are sufficient to activate apoptin. As these early changes are likely to determine the subsequent path of tumor progression, apoptin might target the Achilles' heel of cancer cells.
4. Identification of the apoptin kinase activity demonstrates that apoptin's tumor-specific activities result from direct activation by a cancer-associated cellular pathway.

5. Apoptin's interference with ceramide signaling and its effect on Nur77 implicate both sphingolipid metabolism and mitochondrial signaling in apoptin-induced apoptosis.
6. APC/C appears to be a central downstream target of apoptin-induced cell death. Inhibition of APC1 by apoptin results in APC/C disruption, G2/M cell cycle arrest, and p53-independent apoptosis. As APC/C is also involved in E4orf4-induced cell death, the anaphase-promoting complex/cyclosome is an attractive future drug target for anticancer therapy.
7. Preclinical in vivo models based on different human xenografts indicate that apoptin is a safe and efficient potential anticancer drug.
8. In vivo and in vitro combinatorial treatments of tumor cells with apoptin and other (chemo-)drugs enhance tumor regression.

FUTURE ISSUES

1. Which tumor-specific cellular (survival) kinase is involved in the transformation-related phosphorylation of apoptin?
2. What is the exact cellular function of apoptin-associated proteins and what is their role in apoptin-mediated processes both in tumor/transformed cells and in their normal counterparts?
3. Are structural features of apoptin and other tumor-specific death-inducing proteins (e.g., HAMLET) responsible for their tumor-specific apoptotic activity or their normal-cell-specific neutralization?
4. The development of systemic delivery vehicles for accurate gene and/or protein delivery will be indispensable in the future.

DISCLOSURE STATEMENT

A.G. de Boer is the academic partner of to-BBB technologies, which exploits CRM197-drug technologies. He has received grants from Stichting Technische Wetenschappen; Int. Stichting Alzheimer Onderzoek; and Sanfilippo Foundation, USA, and has filed a patent application (Differentially expressed nucleic acids in the blood-brain barrier under inflammatory conditions).

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41. Apoptin modulates ceramide/sphingolipid metabolism as part of its tumor-specific apoptosis action.

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