Viral Elements Sense Tumorigenic Processes: Approaching Selective Cancer Therapy

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Abstract: Viruses can produce viral oncoproteins that drive multiple genetic alterations as the consequence of neoplastic transformation. Viral proteins encoded by onco-related viruses such as polyomavirus SV40 or Epstein-Barr virus are involved in cellular processes resulting in imbalance between proliferation and cell death, knowledge of which continues to be crucial for combating cancer. On the other hand, viruses also generate viral components that, from a cold viral protein, can become a tumor-selective killer by sensing cellular tumorigenic hallmarks. For instance, the avian virus derived apoptin protein has been proven to induce tumor-regression in various pre-clinical animal models without showing detectable side effects. In particular, apoptin-interacting protein partners such as components of the anaphase promoting complex were identified as potential anticancer drug targets. The adenovirus-derived protein E4orf4, another viral protein with tumor-specific apoptosis characteristics, has been proven to interact with the tumor-suppressor protein phosphatase 2A. This review aims to describe recent studies with representative viral elements that have contributed to our understanding of critical tumorigenic processes and have conferred an impact on the development of novel anti-cancer therapies.

Key Words: Anti-cancer therapies, apoptin, apoptosis, drug targets, E4orf4, Simian virus 40 (SV40), large tumor antigen (LT) and small tumor antigen (st), tumorigenesis.

1. IMBALANCE OF RESCUE AND APOPTOSIS

In general terms, cancer cells are derailed cells: death inducers are inhibited while survival pathways are stimulated. This imbalance allows tumor cells to survive. However, it may also prove to be their Achilles' Heel, as inhibiting survival and/or enhancing death in tumor cells are well known therapeutic strategies – even though our understanding of the underlying effects of these approaches is often sketchy [1].

The function of rescue pathways in cells, which are stressed, but otherwise normal, is to temporarily stall alarm-induced programmed cell death (apoptosis), allowing attempts to repair intermediate damage. When the damage cannot be repaired, death pathways ensure the safe elimination of the impaired cell. Nevertheless, tumor cells survive despite high intrinsic levels of death alarm because of lesions within their death pathways. In view of these high levels of apoptotic signal, rescue pathways tend to be up-regulated in tumor cells [2].

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 [3]. Other tumor-suppressor genes, such as the one encoding retinoblastoma protein (Rb), can similarly reduce tumor growth *via* induction of apoptosis [4, 5]. Contrarily, the anti-apoptotic protein Bcl-2 and some Bcl-2-related proteins such as Bcl-X_L are involved in tumor development. These anti-apoptotic proteins are often over-expressed as a result of chromosomal translocations in various tumors, for instance, leukemia, lymphoma, and breast cancer [6, 7].

Apoptosis suppression is a prerequisite for tumor growth, whereas apoptosis induction can still be exploited for the treatment of tumors. Despite that many tumor cells have a defect in the decisive machinery for apoptosis, they usually still retain an intact executive system. Such tumor cells will still die, if they are provided with an effective apoptotic signal triggering alternative cell death pathways [8]. Chemotherapeutic agents may fail, for they act only *via* functional p53, which is mutated in more than 50% of the human tumors [3]. Over-expression of the anti-apoptosis proteins such as Bcl-2, or BCR-ABL, negatively influences the chemotherapeutic treatment of a large number of lymphomas [9, 10]. Understanding of the functional role and the protein structure of the anti-apoptosis protein Bcl-2 resulted in the development of novel anticancer agents [11, 12]. In the future, a complete understanding of the rescue/death pathways underlying tumor formation and the identification of alternative death routes in cancer cells will enable the discovery and development of novel anticancer drug targets.

Viruses share similar features for controlling host cell machinery, necessary for a successful viral infection. On one hand, stimulation of cell proliferation allows replication of viral DNA and assembly of virions. On the other hand, the capability of inducing apoptosis after finalization permits the release of the new viruses and benefits viral pyrogenicity.

Here, we describe several viral elements that sense tumor-related processes. Elucidation of their tumor selective sensibility may help understand pathogenic tumorigenesis

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and develop novel anticancer therapies. On one hand, we will discuss virus-derived proteins causing tumor formation; on the other hand, we will illustrate viral proteins inducing tumor-specific apoptosis in human cancer cells.

2. TUMOR VIRAL ELEMENTS IDENTIFY CELLULAR PROTEINS THAT PARTICIPATE IN TUMORIGENESIS

Transforming viruses, such as Polyoma viruses (Simian virus 40, BK virus, JC virus) [13], Herpesviruses (Epstein-Barr virus – EBV) [14], Kaposi's sarcoma-associated herpesvirus (KSHV) [15], Herpesvirus saimiri (HVS) [16], Adenoviruses (AdE2, AdE5) [17], Human Papilloma virus (HPV) [18], Hepatitis B virus [19], Hepatitis C virus [20], and Human T-cell lymphotropic virus type-1 (HTLV-1) [21], have been proven to be important tools for identifying cellular proteins that participate in tumorigenesis. Each of these viruses encodes viral elements capable of disordering key cellular regulatory proteins that forbid viral replication. Over-expression of these viral oncoproteins exerts dominant action by associating with key cellular targets and thus altering cellular pathways that play crucial roles in tumorigenesis.

Therefore, understanding the molecular basis for the transformed phenotype by these viral oncoproteins may facilitate the elucidation of the key cellular players regarding transformation, immortalization and tumorigenesis. Some of the cellular proteins are involved in growth regulation. Next to growth regulation, blocking of apoptosis is essential for viral replication and also essential for tumor development. All these processes harbor potential for developing novel anti-cancer drug targets.

Below, we describe more in detail the transforming proteins derived from Simian virus 40 (SV40) involved in tumorigenesis. Their various cellular activities contributing to the imbalance between apoptosis and growth of transformed cells and possibly assisting in identifying drug targets will be discussed.

2.1. The Role of SV40 in Tumorigenesis

For decades, SV40 has been one of the main models for studying human tumor development [22]. Although several reports have linked SV40 to human tumor formation, there is no adequate evidence to support increased tumor incidence in those individuals who received SV40-contaminated poliovirus vaccine [23]. SV40 is a DNA tumor virus with a genome of approximately 5 kb and is one of the best studied members of the polyomavirus family [24]. The ability of SV40 to immortalize 'normal' cells is essential for preventing apoptosis or overcoming senescence state in order to achieve a successful infection [25].

When SV40 has entered the nucleus, virus DNA is released and the early promoter is transcribed, which results in the expression of the large tumor antigen (LT) and small tumor antigen (st) proteins. These viral proteins drive the cell into the S-phase by blocking several cellular proteins [26]. With a very low frequency, the viral DNA becomes integrated in the host genome by non-homologous recombination. If this integration occurs in such a way that the early coding sequences are intact and express both LT and st, the cell and its subsequent descendents are transformed [26].

Expression of the SV40 oncoproteins in transgenic mice resulted in cellular transforming effects ranging from hyperplasia to invasive carcinoma accompanied by metastasis. The consequences of the effect of the SV40 oncoproteins are dependent on the cell types in which they are expressed [27]. To transform human hepatocytes into liver tumor in nude mice, next to LT and st, the expression of oncogenic Ras is necessary, whereas other human cell types require additional expression of human telomerase reverse transcriptase subunit (hTERT) [22].

2.2. Interaction of SV40 LT with Cellular Chaperones

The roles played by the SV40-encoded LT and st in cellular transformation have been examined extensively [27]. It is suggested that the chaperone activity of LT protein is responsible for the first step in the process of *in vitro* cellular immortalization, *i.e.* the extension of life span of the transfected cells. LT induces an up-regulation of selected molecular chaperones such as heat shock proteins (hsp), hsp70 and hsp40, resulting in an unlimited cell division potential [28]. LT associates directly with hsp70. Over-expression of hsp70 restores the structural stability and functional defects of a temperature-sensitive mutant of LT at non-permissive temperature. These observations illustrate that both LT and hsp's have a beneficial effect on each other presence and functionality [29]. Furthermore, LT studies have shown that heat shock proteins are potential drug targets. Recently, Rossi *et al.* have shown that down-regulation of hsp's, by means of small interfering RNA silencing heat shock transcription factor 1 (HSF1), results in massive apoptosis of cancer cells [30].

2.3. Identification of Tumor-Suppressors by Use of SV40 Elements

Many other studies have shown that inhibition of p53 and Rb tumor-suppressors by SV40 LT as well as interference of protein phosphatase 2A (PP2A) by SV40 st, are of importance for SV40 induced transformation. However, it is not yet conclusive, if Rb, p53 and PP2A proteins are the only targets through which LT and st exert cellular transformation, or if additional targets are involved [27]. Nevertheless, these SV40 studies have generated huge progress in understanding tumor formation and contributed to the development of novel anticancer strategies.

Recently, the crystal structure of LT in complex with p53 was determined. The structure reveals an unexpected hexameric complex of LT binding six p53 monomers. Structure-guided mutagenesis of LT and p53 residues supported the p53-LT interface defined by the complex structure. The structure also shows that LT binding induces dramatic conformational changes at the DNA-binding area of p53. In the complex structure, LT occupies the whole p53 DNA-binding surface. All these LT-induced changes within p53 result in the dysfunction of p53 [31]. Most clinical anticancer drugs target DNA, resulting in DNA damage-triggered signaling and execution of apoptosis mediated *via* p53. Unfortunately, approximately half of the tumor cells lack p53, which explains the failure of these anticancer therapies. Therefore, increasing knowledge on DNA-triggered signaling leading to cell death is needed to provide new strategies for therapeutic interventions [32].

All-trans-retinoic acid (ATRA) has been show to arrest the growth of ovarian carcinoma cells in G0/G1 and significantly elevate the level of Rb2/p130, a member of the Rb family of tumor suppressors. As ATRA treatment leads to a significant increase in the amount of Rb2/p130 protein but not mRNA, the elevated levels of Rb2/p130 protein is likely the result of increased stability. In ATRA-treated ovarian carcinoma cells, PP2A binds to Rb2/p130 and dephosphorylates the nuclear location signal of Rb2/p130, leading to the interaction with importins. Rb2/p130 enters the nucleus where it acts to arrest ovarian cancer cells in G1 and suppress proliferation [33].

The replacement of B subunits of PP2A by st inhibits the serine/threonine PP2A phosphatase activity and prolongs phosphorylation-dependent signalling [34, 35]. PP2A represents a large family of highly conserved heterotrimeric enzymes. Their critical importance in cell homeostasis is not only indicated by their interaction with SV40 st but also by the fact that they are the targets of natural toxins such as the tumor promoter okadaic acid [36]. Mutated or lower expression levels of PP2A have been found in certain cancers. PP2A regulates multiple signalling pathways, suggesting many possible signalling pathways and involved targets for cell transformation [37]. Genetic manipulation of particular PP2A subunits has confirmed a role for specific complexes in transformation, and recent work implicates the perturbation of the phosphatidylinositol 3-kinase/Akt pathway and proto-oncoprotein c-Myc stability in transformation by st and PP2A. PP2A mediates its effect on c-Myc by dephosphorylating a conserved residue that normally stabilizes c-Myc, and in this way, PP2A enhances c-Myc ubiquitin-mediated degradation [38]. These results reveal a critical interconnection between a potent oncoprotein, c-Myc, and the tumor-suppressor, PP2A. Rodriguez-Viciana *et al.* reported that SV40 st protein *via* interference with PP2A stimulates MAP kinase cascade, resulting in unbalanced cell proliferation [39]. PP2A was also shown to participate in deactivation of extracellular signal-regulated kinase 1/2 (ERK 1/2), resulting in the inhibition of tumor cell proliferation. Up-regulation of PP2A by conjugated linoleic acid (CLA) has been proven to harbour protective properties in breast cancer [40]. Unravelling the complexity of PP2A signalling pathways will not only provide further insights into cancer development but may also identify novel targets with promise for therapeutic manipulation.

Various crucial signal transduction pathways have been identified to be involved in transformation and cancer development by means of SV40-derived proteins (Fig. **1**). Owing these features, viral oncoproteins have been widely employed as invaluable experimental tools for the identification of several key families of cellular regulators, which have become approachable to newly developed anticancer therapies.

3. ADENOVIRUS-DERIVED PROTEIN E4ORF4

3.1. E4orf4 Protein Induces Alternative Tumor-Specific Apoptosis Processes

Evidence indicates that a limited set of common genetic alterations is responsible for tumor regression and cancer cell resistance to current anti-cancer therapies. The ability of tumor cells to escape apoptosis induction, which normally occurs in response to deregulated oncogenic signaling, is an essential one. However, alternative physiological death programs seem to be effective in cancer cells [41].

Adenovirus-derived protein E4orf4 interacts with these alternative tumorigenic death pathways, for it can induce p53-independent apoptosis in human transformed cells but not in normal cells [42-44]. E4orf4 protein is a multifunctional viral regulator, which is derived from the open reading frame of the adenovirus early transcription region 4 (E4), consisting of 114 residues in the case of Ad2 or Ad5. Wolkersdorfer *et al.* have shown in human melanoma xenograft nude models that adenoviral vectors expressing E4orf4 are suitable vectors for oncolytic malignant melanomas [45]. Treatment of murine B16(F10) tumors with therapeutic DNA expressing E4orf4 showed a distinct tumor growth inhibition [46]. Therefore, one may assume that E4orf4 triggers a novel death program that bypasses normal apoptotic pathways in human tumor cells. Thus far, E4orf4 studies have indeed revealed novel insights in tumor-related pathways and generated potential drug targets [47].

3.2. E4orf4 Acts *via* the Tumor-Related Src-Mediated Signalling

Next to the cell membrane and cytoskeleton accumulation, E4orf4 is largely expressed in nuclear regions before the onset of apoptosis [48]. The specific localization of E4orf4 to the cytoplasm, cytoskeleton, and the nucleus illustrates the complexity of E4orf4 signalling with a number of potentially important cellular targets. Robert *et al.* have provided strong evidence that both cytoplasmic membrane-associated and nuclearly-targeted E4orf4 protein induce specific cell death programs in human transformed cells [47].

In transformed cells, E4orf4 works in part by inducing a Src-mediated cytoplasmic apoptotic signal leading to caspase

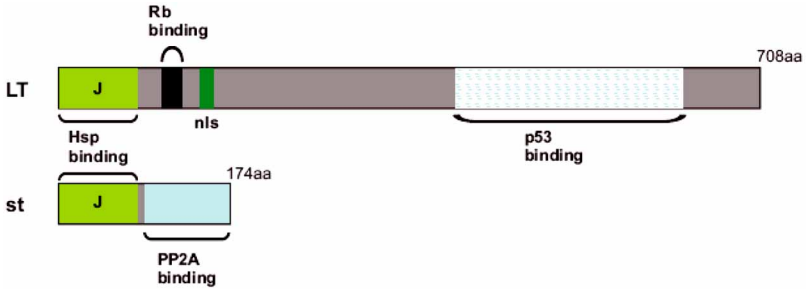

Fig. (1). Schematic model of the direct interactions of SV40 LT and st proteins with distinct cellular proteins, which results in cell transformation.

independent apoptosis. E4orf4 is phosphorlylated by Src family kinases on its tyrosine residues and seems to be critical to promoting the cytoplasmic and membrane localization which is essential for this specific type of apoptosis [49, 50]. The direct interaction has been shown to be between the kinase domain of Src and the arginine-rich motif of E4orf4. This cytoplasmic cell death pathway is induced by the deregulated Src signalling through the binding of E4orf4 to the Src kinase domain which is strongly associated with the E4orf4 accumulation in the cell membrane cytoskeleton. In addition, it has been indicated that the tyrosine phophorylation of E4orf4 involves the calcium regulated cysteine proteases calpains [51].

Recently, Robert *et al.* have shown that E4orf4 triggers the assembly of a peculiar juxtanuclear actin-myosin network that drives polarized blebbing and nuclear shrinkage [52]. E4orf4 activates the myosin II motor and triggers *de novo* actin polymerization in the perinuclear region, promoting endosomes recruitment to the sites of actin assembly. E4orf4-induced actin dynamics requires interaction with Src family kinases and involves a spatial regulation of the Rho GTPases pathways Cdc42/N-Wasp, RhoA/Rho kinase, and Rac1, which make distinct contributions. Inhibition of the actin dynamics per se dramatically impairs E4orf4- induced apoptosis in human cancer cells. The results obtained by Robert *et al.* provide strong evidence for a causal role of endosome-associated actin dynamics in E4orf4 tumor-specific cell killing [52]. As important, E4orf4 studies have generated various potential drug targets, which might be suitable for the development of future novel anticancer drugs (Table **1**) [41].

3.3. PP2A and APC/C Complex are Involved in the Tumor-Specific Alternative Apoptosis Pathway Triggered by E4orf4

An essential feature for the E4orf4 tumor-specific apoptosis activity is its interaction with PP2A [43, 53]. PP2A is one of the major protein serine/threonine phosphatases in the cell, and plays a role in multiple cellular processes, including metabolism, transcription, RNA splicing, translation, cell cycle progression, morphogenesis, signal transduction, development and transformation [54]. PP2A consists of a scaffolding A subunit, a catalytic C subunit, and one of several regulatory B subunits. The adenovirus E4orf4 protein associates with PP2A by directly binding to the B-subunits [42, 55-57]. Interestingly, PP2A is also targeted to oncoproteins such as the above described SV40 st [58], which suggests that interaction of oncoproteins with PP2A triggers E4orf4 tumor-specific apoptosis activity.

It remains, however, to be determined whether PP2A is involved in the Src-mediated cytoplasmic and/or the nuclear cell death pathways mediated by E4orf4, and whether the tumor-selective killing of E4orf4 can be assigned to one specific compartmental pathway or both pathways. Nonetheless, both Src and PP2A enzymes are critical targets of E4orf4, which likely cooperate to trigger E4orf4-induced tumor cell killing and whose relative contributions may vary in function of the cellular background [48, 51].

To uncover additional components of the E4orf4 network required for its tumor-related induction of apoptosis, Maoz *et al.* used a yeast genetic system to select gene deletions conferring resistance to E4orf4 [59]. They found that the Cdc55, the yeast PP2A B-subunit and YND1, encoding a yeast Golgi apyrase, are involved in E4orf4-induced apoptosis. Furthermore, they detected that the anaphase promoting complex/cyclosome (APC/C) plays a crucial role in E4orf4 activation. These results indicate that the APC/C is crucial for E4orf4-induced apoptosis in transformed human cells. Indeed, Kornitzer *et al.* have provided evidence that E4orf4 can induce G2/M arrest *via* its association with APC/C in mammalian transformed cells before apoptosis [60]. Therefore, one may regard APC/C complex as a crucial factor in tumor formation as well as partner of alternative apoptosis pathways in tumor cells (see also section 5.4. below).

4. CHICKEN ANEMIA VIRUS DERIVED APOPTIN

4.1. Apoptin Induces Apoptosis in Human Tumor Cells

Another protein with transformation-specific apoptosis characteristics is the apoptin protein. This protein is derived from the chicken anemia virus (CAV), which replicates only in transformed chicken cell lines, indicating that at least a part of the CAV life-cycle requires transformation-like cellular events [61]. In 1994, Noteborn *et al.* have described that in these chicken transformed cell lines, the synthesis of the apoptin protein alone mimics CAV-induced apoptosis [62, 63]. Apoptin is a 121-amino-acid protein, which does not resemble any other sequenced animal or viral protein. It consists of regions rich in proline or basic amino acids and over-

Table 1. Cellular Proteins Involved in E4orf4's Tumor Selectivity

Cellular protein	Characteristics	Citations
APC1	Apoptin associates with APC1 resulting in induction of apoptosis in the absence of functional p53	[109, 110]
PP2A	PP2A is targeted to oncoproteins such as SV40 st E4orf4 seems to pull PP2A from a proliferation into an apoptosis pathway	[42, 56-58]
Src	E4orf4 interacts with kinase domain of Src kinase, which is deregulated in tumor cells	[49, 50]
Calpains	Calcium regulated cysteine proteases calpains are involved in E4orf4 phosphorylation needed for its activity	[51]

all contains a high percentage of serine and threonine residues (Fig. **2**).

MNALQEDTPP GPSTVFRPPT SSRPLETPHC REIRIGIAGI

TITLSLCGCA NARAPTLRSA TADNSESTGF KNVPDLRTDQ

PKPPSKKRSC DPSEYRVSEL KESLITT**T**PS RPRTAKRRIR L

Fig. (2). Amino acid sequence of apoptin. The amino acids of apoptin (1-121) are depicted in the single-letter code, the bipartite nuclear localization sites are underlined and the threonine residue at position 108, phosphorylated by a tumor-specific kinase activity is presented in bold.

Since these early observations, a series of biomedical studies on apoptin have been carried out in human cell systems. Apoptin can induce apoptosis in cell lines derived from a great variety of human tumors. On the other hand, apoptin does not induce apoptosis in normal, non-transformed human diploid 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 apoptin has no toxic or transforming activity in these cells and that apoptin does not interfere with cell proliferation. Primary human hepatocytes and stem cells, which are chemotherapy-sensitive to harmful side-effects of conventional treatments, are also resistant to apoptin-induced apoptosis [64].

4.2. Apoptin *In Vivo* Preclinical Anti-Tumor Studies

In vitro pre-clinical studies on apoptin have clearly shown that apoptin merits further investigation to determine whether an apoptin-based medication will meet the clinical criteria [65-72]. Indeed, a series of *in vivo* experiments carried out in animal tumor-models have justified this assumption. Protein transduction *via e.g.* TAT-peptide [73], systemic delivery of genes *via* e.g. (adeno)viral vectors [74-76] or *via* Asor-apoptin delivery vehicles [77] are examples of clinically relevant methods for apoptin delivery.

Transduction of the apoptin gene by replication-deficient adenoviruses to a subcutaneous human hepatoma grafted on nude mice resulted in considerable demise of the tumor cells. Apoptosis and disruption of the tumor integrity were apparent in the transduced regions [74]. Serial adenovirus-mediated intratumoral transfer and expression of the apoptin gene instigates regression or complete remission of human hepatomas grown as xenografts in immune-deficient mice, and significantly increases the long-term survival of the treated mice [75].

Current cancer therapies are largely limited by their toxicity to normal tissues [78, 79]. Innovative strategies designed to circumvent this problem aim at tumor-specific delivery of a toxic substance, or alternatively, at systemic delivery of a tumor-specific substance. Apoptin is one of a few proteins suited for the latter approach. Peng and colleagues convincingly showed that systemic delivery of Asor-apoptin is a safe anti-tumor treatment [77]. *In vivo* systemic delivery of 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 clearly were not affected.

Recently, Lian *et al*. [76] have reported that combined administration of IL-18 and apoptin results in an even higher induction of an effective anti-tumor immune response and tumor regression. Growth of established Lewis lung carcinoma (LLC) tumors in C57BL/6 mice immunized with plasmid DNA encoding apoptin in conjunction with plasmid encoding interleukin-18 (IL-18) was significantly inhibited, compared to growth of LLC tumors in mice immunized with plasmid encoding IL-18 or plasmid encoding apoptin alone.

Liu *et al.* showed that treatment of human prostate cancer cells with an adenoviral vector containing green fluorescent protein-tagged apoptin (AdGFP-apoptin) *in vitro* resulted in the inhibition of cell proliferation and induction of apoptosis [80]. Furthermore, they demonstrated that the apoptin-mediated apoptosis was independent of various apoptotic regulators, including p53, caspase-3, survivin, FLIPs, XIAP and CIAP. In addition, they demonstrated that intratumoral administration of adenovirus synthesizing GFP-tagged apoptin suppressed tumor growth *in vivo*. In addition, combined treatment of AdGFP-apoptin with the acid ceramidase inhibitor LCL204 enhanced the anti-tumor activity, resulting in increased animal survival (see also section 4.2). Liu *et al.* stated that the observed anti-tumor activity was greater than predicted and proposed that a bystander tumor killing effect was occurring. Furthermore, a potential role for anti-angiogenic activity is indicated.

4.3. Chemotherapy Combined with Apoptin

Resistance to apoptosis can be a direct consequence of mutations in certain tumor-suppressor genes like p53 or certain anti-apoptotic proto-oncogenes such as Bcl-2 and Bcl-xl. Therefore, new cancer therapies have to be developed that bypass the resistance to chemotherapy of tumors. Besides its tumor-specific apoptosis-inducing characteristics, apoptin has some other relevant features making it suitable for cancer therapy. Apoptin acts independently of p53 [81-83] and is in certain tumor cell lines stimulated by Bcl-2 or insensitive to BCR-ABL and Bcl-xl [84-86], which suggests that apoptin can induce apoptosis in cases where (chemo-)therapeutics might fail. The fact that apoptin induces apoptosis in hepatocarcinoma HepG2 cells but not in normal healthy liver cells, implies that side effects of apoptin treatment are expected to be minor [85].

Recently, it has been reported that chemotherapeutic agents combined with apoptin treatment of human tumor cell cultures results in enhanced cytotoxicity [87]. 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 non-small lung carcinoma A549 cells, p53-negative osteosarcoma Saos-2 cells, and p53-mutant prostate cancer Du145 cells. Finally, apoptin was proven to be co-effective when combined with chemotherapeutic agent methotrexate [88]. 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 anti-tumor therapy.

All these various preclinical anticancer studies with apoptin clearly demonstrate its potency and indicate that apoptin therapy combined with other drugs can be exploited for treatment of various cancer types.

5. APOPTIN SENSES TRANSFORMATION-RELATED PROCESSES

Besides the development of a gene or protein therapy strategy against cancer based on (viral) vectors expressing apoptin, one should also envisage the therapeutic strategy that apoptin seems to interact with tumor-related elements. Because apoptin, as a death effector, is active in all tumor cells tested so far, it is possible that apoptin reveals a tumor essential survival pathway, probably linked to quite downstream apoptosis events. Systematic investigation of the known cancer hallmark pathways where apoptin is specifically activated should provide important information. If apoptin responds to a currently unknown cancer pathway, this could yield new insights in the process of carcinogenesis as well as new targets for cancer treatment. Below, we describe the current understanding of the tumor-related elements by means of apoptin as a tumor-specific sensor (Table **2**).

5.1. Apoptin Interferes with Tumorigenic Nuclear Modules

A striking difference in the cellular localization of apoptin was observed in human normal diploid cells versus tumor cells [89]. In tumor cells, apoptin is located mainly in the heterochromatic regions of the nucleus, whereas in normal cells it is present in perinuclear structures [90-92].

Poon *et al.* have postulated that apoptin's nuclear accumulation is modulated by a CRM1-recognized nuclear export signal that is active in normal but not in tumor cells [93]. *In vitro* studies revealed that apoptin acts as a multimeric complex and interacts with DNA, resulting in distinct superstructures of approximately 20 multimeric complexes of apoptin [94].

In normal cells, apoptin multimers are nontoxic, become epitope-shielded and eventually degraded in a normal-cell-specific fashion [95]. Future studies on the tumor-cell-related interaction of apoptin with (hetero)chromatin and normal-cell-specific-neutralization pathways will shed light on tumor-specific processes and also provide tumor-related drug target candidates.

5.2. Apoptin-Mediated Ceramide Signaling and Tumor Cell Killing

Recently, Liu *et al.* have reported a possible mechanism for apoptin-mediated tumor cell death [96]. They showed evidence that apoptin modulates the sphingolipid-ceramide pathway, leading to increased concentration of ceramide (Fig. **3**) in human prostate tumor cells and consequently resulting in cell death [97]. Ceramide is considered to be a tumor suppressor lipid [98, 99]. 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. Therefore, one can conclude that the ceramide pathway has many similarities to the p53 signaling pathway, and both serve as comprehensive sensors of cellular stresses. Apoptin increases ceramide by activation

Table 2. Cellular Proteins Involved in Apoptin's Tumor Selectivity

Cellular protein	Characteristics	Citations
Tumor-related kinase activity	Apoptin is phosphorylated in human tumor cells but not in normal cells SV40 LT induces apoptin-related kinase activity	100,101, 103
Acid ceramidase	Apoptin inhibits acid ceramidase resulting in tumor-specific induction of apoptosis	80, 96
APC1	Apoptin associates with APC1 resulting in induction of apoptosis in the absence of functional p53	109, 110
DEDAF	Over-expression of DEDAF induces apoptosis specifically in human tumor cells	105
Nur77	Translocation of Nur77 due to to apoptin synthesis results in tumor cell death	107
Nmi	Apoptin seems to change the Nmi-mediated proliferation activity into an Nmi-mediated apoptosis activity	104
Hippi	Associates with cytoplasmic apoptin in human normal cells Apoptin is neutralized in a normal-cell-specific manner by an unknowm mechanism	95, 106

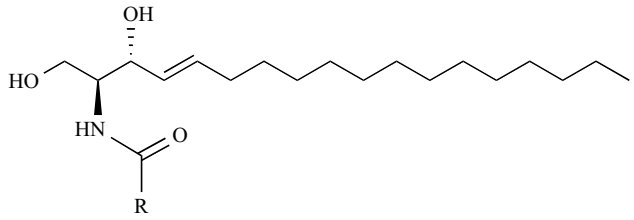

Fig. (3). The chemical structure formula of ceramide is given, which is involved in apoptosis induced by the viral death effector apoptin.

of acid sphingomyelinase, resulting in increased hydrolysis of sphingomyelin to ceramide. Furthermore, apoptin was shown to inhibit the enzyme acid ceramidase, which deacylates ceramide to obtain sphingosine. Liu *et al.* [96] found that in approximately 65% of human tumor prostates, the acid ceramidase activity was significantly up-regulated, making acid ceramidase as a target for therapeutic intervention.

5.3. Tumor-Specific Apoptin Kinase Activity

Rohn *et al*. [100] and Poon *et al*. [93] have, independently, reported that apoptin is phosphorylated extensively in a broad panel of tumor cells but negligibly in normal cells. The tumor-specific phosphorylation of apoptin was mapped to the 108 threonine at the C-terminal position (108T). 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 activation. Studies with alanine mutants of full-length apoptin that could not be phosphorylated at 108T showed a significant impairment in the ability to induce apoptosis in human tumor cells [100, 101], though not in a complete abolishment. This is due to the fact that apoptin contains two apoptosis domains [89]. The domain within the N-terminal region seems to function in a phosphorylation-independent fashion, whereas the domain situated within the C-terminal region appears to be regulated by phosphorylation. Indeed, Rohn *et al.* [101] showed that truncation of the C-terminal 80-121 amino acid region of apoptin where the 108T phosphorylation site is alanine-substituted results in a complete abolishment of its apoptotic activity.

The identification of the apoptin kinase activity implies that apoptin's tumor-specific activities result from direct activation by a tumor-associated cellular pathway, and suggests that activation of this apoptin-kinase constitutes a possibly essential aberrance associated with the cancerous or pre-cancerous cellular state. Ben *et al.* [102] have reported that in human lymphoma U937 cells the c-Jun N-terminal kinase plays a role in apoptin-induced apoptosis. Particularly, Zhang *et al.* have observed that transient co-expression of transforming SV40 LT is sufficient to activate the apoptin kinase and its apoptotic ability in human normal cells [103], implying that the tumor-specific apoptin kinase is signaling at the early stage of the tumorigenic process.

Apoptin's action has been described as p53-independent and expression of apoptin in LNCaP cells (p53 wild-type) did not induce detectable levels of p53 Ser15 phosphorylation [80, 96]. However, apoptin expression in DU145 cells (mutant p53) consistently increased p53 phosphorylation at Ser15 in a time-dependent manner compared to control Ad-GFP-infected cells. Apoptin expression strongly induced elevation in p53 Ser15 protein in DU145 cells 18–36 h after treatment, while p53 protein was elevated only at the 6-h time point. Elevation of protein levels of MDM2, a p53-negative regulatory protein, and the p53-responsive gene Noxa, was also delayed until 18 h post infection. There was some response of p53 protein in control GFP cells at 21 h, but no substantial changes were observed in Noxa or MDM2 over this period.

A further understanding of the molecular events that regulate the phosphorylation and binding of apoptin to DNA may provide insight into the molecular mechanism by which apoptin is localized to the nucleus of tumor cells while it is still able to activate sphingomyelinase in the cytoplasm. As important, the elucidation of both upstream and downstream effectors in the apoptin-kinase signaling pathway should further improve our understanding of the complicated process of malignant transformation and thereby may lead to the development of novel clinical therapeutic approaches with apoptin. The future development of chemical compounds interfering with the apoptin-kinase signaling pathway will result in more efficient anticancer drugs harboring fewer side effects, for it only interferes with a tumor-related pathway.

5.4. Apoptin Tumor-Related Partners

The identification of apoptin-associating proteins will also guide us to apperceive the pathways underlying tumor formation. Regarding the 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 revealed several apoptin protein partners showing tumor-related characteristics. One of them is homologous with the N-Myc interaction (Nmi) protein, which might explain partially its tumor-specific activity. Nmi is known to be expressed at low level in normal tissues, but high level in transformed cell lines. It is assumable that apoptin discerns and then switches the Nmi-mediated proliferation (transformation/tumor-formation) activity into an Nmi-mediated apoptotic activity [104].

Danen-van Oorschot *et al.* [105] have reported that apoptin interacts with DEDAF, a protein previously found to associate with the pro-apoptotic death effector domain (DED)-containing DNA binding protein DEDD. Similarly to apoptin, DEDAF displays cell death activity when over-expressed in tumor cells and appears not to induce apoptosis in normal non-transformed cells. On the other hand, Cheng *et al.* have identified another apoptin-associating protein, human Hippi, the protein interactor and apoptosis co-mediator of Huntingtin interacting protein 1, which clearly co-localizes with apoptin in the cytoplasm of normal human cells, but stays in the cytoplasm, while apoptin is transported to the nucleus of tumor cells [106].

Maddika *et al.* have reported that apoptin triggers the cytoplasmic translocation of Nur77 (also known as TR3 or NGFI-B) in human tumor cells. Nur 77 controls both survival and death of cancer cells [107]. A wealth of recent ex-

perimental data demonstrate that Nur77 activities are regulated through its subcellular localization. In the nucleus, Nur77 functions as an oncogenic survival factor, promoting cancer 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 one *via* release of cytochrome c [108].

The group of Green has shown that apoptin associates with APC1, a subunit of APC/C [109]. Apoptin multimerization and APC1 interaction are mediated by domains that overlap with sequences of the bipartite nuclear localization signal. Furthermore, apoptin expression in transformed cells induces the formation of PML nuclear bodies and recruits APC/C to these subnuclear bodies [110]. Green *et al.* have provided evidence that apoptin expression inhibits APC/C function in p53-minus cells, in a way similar to depletion of APC1 by RNA interference. These cells will undergo cell death following G2/M arrest, whereas normal cells do not [109].

The fact that depletion of the apoptin-associating protein APC1 results in a specific tumor kill, makes possible to identify tumor-related drug targets based on apoptin studies. Furthermore, the previously mentioned apoptin partners and the apoptin kinase-related pathways, underline the selective killing of transformed cells by apoptin *via* a complex scenario of tumor-related factors, which all constitute potential drug targets. Remarkably, Kornitzer *et al.* have described that APC/C complex also plays a crucial role in the tumor-specific induction of apoptosis by E4orf4 (see section 3.3).

5.5. Apoptin Induces Apoptosis in Rheumatoid Arthritis-Related Transformed Cells

The relation between cancer and autoimmune diseases such as rheumatoid arthritis (RA) has been realized for several years [111]. Fibroblast-like synoviocytes (FLS) from patients with RA present features of transformation and derailed apoptosis [112]. RA FLS exhibit 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 [113]. Simultaneously, RA synovial fibroblasts contain mutations in tumor suppressor genes such as p53 [114].

Tolboom *et al.* have shown that isolated FLS from RA patients, grown under tissue-culture conditions for 2-3 passages, were significantly more sensitive to apoptin-induced apoptosis than FLS from trauma patients [115]. These data are in agreement with the transformed-like nature of FLS in RA patients [116]. However, the mechanism of apoptin's action on FLS seems to be different from that on tumour cell lines, given the fact that apoptin is not phosphorylated at amino acid residue threonine 108 in RA FLS as is the case in tumor cell lines. Danen-van Oorschot *et al.* [89] have reported that apoptin contains two death domains, which reside respectively in the N- and C-terminus, and are able to induce apoptosis independently. Only the C-terminal death domain is regulated by phosphorylation [100, 101]. Hence, we infer that the N-terminal death domain of apoptin is responsible for the death effect on RA FLS.

The observation that apoptin induces apoptosis specifically in transformed-like RA cells, makes apoptin also a potential drug against RA. The close relationship between cancer and RA becomes clear also by the fact that cancer drugs are approved for RA treatment [117, 118]. Methotrexate therapy, an anti-cancer remedy, is also one of the conventional RA therapies [119]. Treatment of rheumatoid patients with methotrexate in combination with abatacept significantly improved health-related quality of life [120]. However, long-term side effects seem to be related to methotrexate treatment of RA patients [121]. The *in vitro* additive cytotoxic effect of apoptin in combination with methotrexate treatment of human tumor cells, described here, might also be effective for RA-derived transformed-like cells. If so, apoptin treatment will decrease the reported side effects of methotrexate treatment. Future (pre)clinical experiments have to be performed to prove our predictions.

6. CONCLUDING REMARKS

Unbalanced survival and/or apoptosis pathways play a major role in the induction of cancer. Of equal importance, inhibition of apoptosis pathways makes many tumors resistant to treatment using conventional cytotoxic agents.

Studies with viral proteins derived from DNA-oncoviruses, such as SV40 LT and st, have been proven to be valuable for uncovering cellular processes in aberrant proliferation and inhibition of apoptosis. Nevertheless, many aspects of the relevant cancer pathways have to be further deciphered.

Systematic investigation of the known cancer hallmark pathways for the activation of the transformation-related apoptosis proteins *e.g.* E4orf4 will provide important information for the identification of novel drug targets. Like the SV40 st onco-protein, the tumor-specific apoptosis inducer E4orf4 associates directly with the tumor-related protein PP2A, which underlines that PP2A is an essential player in unbalanced processes underlying tumor development. The identification of the interaction of both transformation-specific death inducers E4orf4 and apoptin with the cellular APC/C complex in tumor cells implies the importance of APC/C as a potential drug target for a novel anticancer therapy. Another identified potential drug target is acid ceramidase, which is inhibited by apoptin in tumor cells resulting in apoptosis. The growing panels of E4orf4- and apoptin-associating proteins provide potential drug targets too and the future identification of apoptin-kinase will generate another potential drug target. The interaction and/or functional effects of the here described transforming and transformation-specific apoptosis proteins with identified potential drug targets, is shown in Fig. (**4**).

Besides the identification of several potential drug targets by apoptin, especially its direct expression in human tumor cells seems to be suitable as an anticancer therapy. Apoptin's killing activity in human tumor can be enhanced by different chemotherapeutic agents. In addition, apoptin is also a reliable candidate for unraveling the transformation-related pathways underlying the pathogenesis of rheumatoid arthritis.

Studies based on viral onco-proteins as well as viral proteins harboring tumor-specific apoptosis characteristics are complementary to each other in unraveling the mechanism of transformed-related processes and for developing novel therapies.

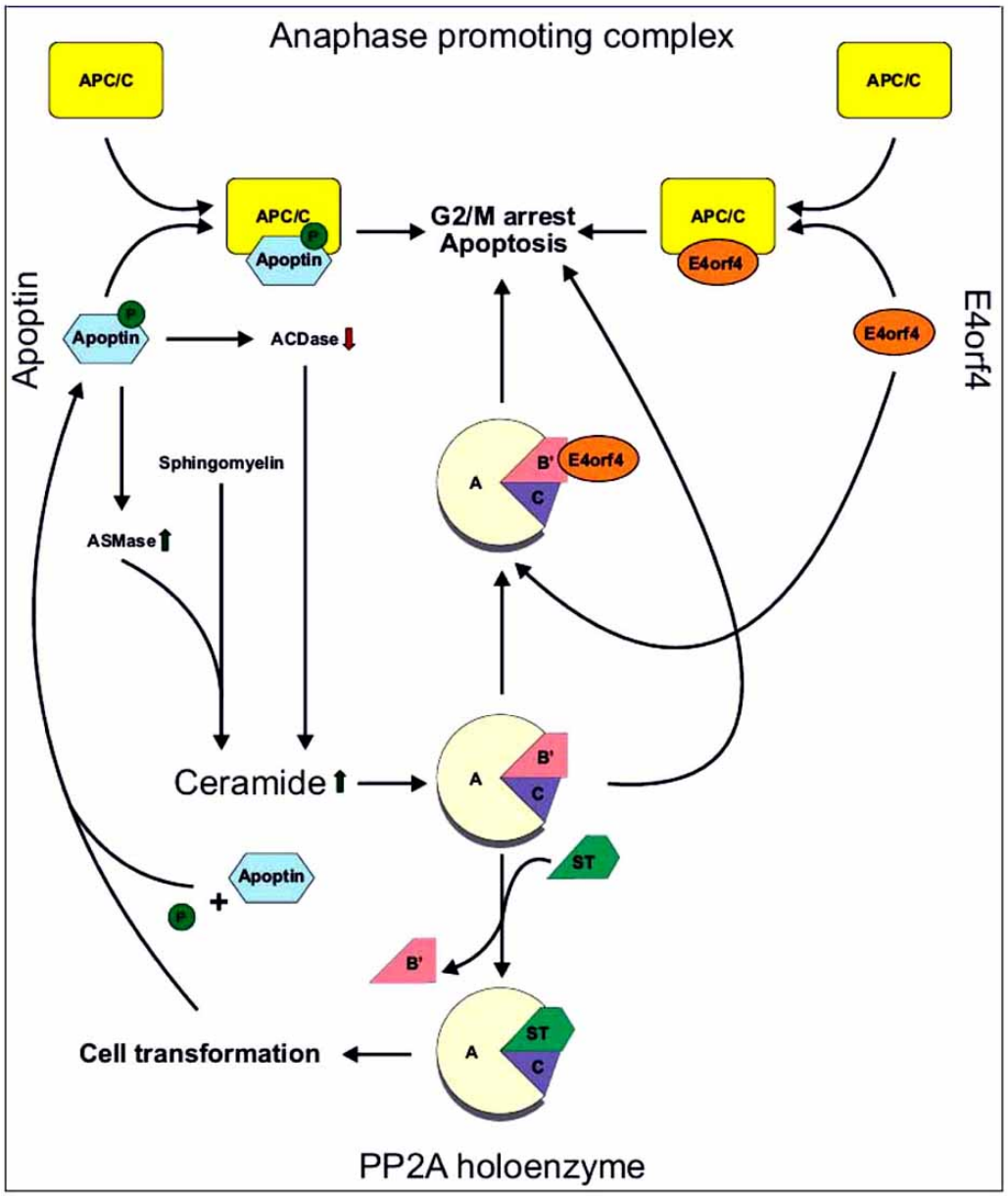

Fig. (4). Schematic representation of potential drug targets identified with transforming and/or transformation-specific apoptosis proteins. The DNA-tumor SV40 virus derived st protein binds to PP2A by replacing its subunit B', which results in cell transformation, whereas binding of E4orf4 to PP2A induces apoptosis specifically in tumor cells. Association of apoptin or of E4orf4 with the anaphase promoting complex/cyclosome (APC/C) generates G2M arrest in tumor cells, subsequently followed by induction of apoptosis. Synthesis of apoptin inactivates acid ceramidase (ACDase) and stimulates its counterpart acid sphingomyelinase (ASMase) resulting in the increase of the tumor suppressor ceramide and *via* PP2A in induction of apoptosis [122].

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