Viral elements inducing tumor-related apoptosis

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CHAPTER 8

SUMMARY AND DISCUSSION

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Viruses and the multi-step pathway of cancer

Chromosomal aberrations and genomic instability play a major role in the development and multi-step progression of cancers. It has been estimated that a series of five to six independent genetic events are required for normal cells to become transformed [Gupta *et al.*, 2005; Stewart and Weinberg, 2006; Marguiles *et al.*, 2006]. There are several types of cellular events occurring in tumors: single or small nucleotide sequence changes, altered chromosome numbers, chromosomal translocations and gene amplification. All these modifications have been identified in a broad range of, if not all, virus-associated cancers and are strongly related to the expression of their viral elements.

Although mutations are probably the most important cellular event leading to neoplastic transformation, oncogenic viruses have evolved ways to alter host gene expression without changing the gene structure of the host cell. Generally, (DNA) viruses encode one or several non-structural transcription regulators that ensure optimal host gene expression to create a favourable cellular environment for virus replication. Viral elements can achieve transcription by interfering directly with the host transcription machinery, interacting with specific transcription factors, or influencing upstream signalling pathways, resulting in loss of cellular proliferation control.

Mammalian cells have evolved mechanisms to maintain genomic integrity by inducing cell cycle arrest in response to DNA damage. Such checkpoint mechanisms allow cells sufficient time to repair the DNA damage before the cell commits to DNA replication and cell division, or induces apoptosis in case the DNA damage is too severe. Through the functions of their oncoproteins, viruses have developed several strategies to subvert key regulatory factors leading to cellular DNA replication, proliferation and survival. Reprogramming of the cell cycle benefits the virus by promoting efficient replication of the viral genome in the host cell. In addition, viral oncoproteins support this reprogramming by abrogating negative growth signals and checkpoint controls. Essentially, viral oncoproteins convert into constitutively proliferating cells resembling tumor cells, of which some may be genetically unstable and may contain neoplastic transforming mutations [Gatza *et al.*, 2005].

There are striking similarities between the pathways deregulated by viruses and the deregulated pathways found in several tumors. In general, deregulation of tumor pathways can be divided in the following categories:

1. Deregulation of proliferation control
2. Cell cycle regression
3. Genomic instability resulting in chromosomal aberrations

4. Blocking apoptosis (or promoting cell survival)
5. Angiogenesis and metastasis

Viral proteins in cancer research

In general, viruses possess intriguing features which can be exploited in cancer research. They can have the ability to transform cells and/or to interfere with cell cycle arrest or the programmed cell death machinery of the host cell. Inhibition of apoptosis and growth stimulation are essential for DNA virus replication (in non-dividing cells DNA replication enzymes are lacking). But induction of apoptosis is often used to release the progeny virus particles in the late state of the virus replicative cycle [Talbot and Crawford, 2004; White, 2006]. Viruses have developed these various strategies of cell transformation and/or apoptosis interference with a limited set of genes encoded by their genome. These viral proteins can interfere with the critical cellular checkpoints that play roles in the control of cell proliferation and in the decision to undergo apoptosis. These features have made DNA and RNA virus proteins important tools to study the development of tumors and also to develop novel anti-cancer approaches [Kaletja, 2004].

In an attempt to broaden our understanding of tumor formation and to contribute to novel cancer therapies, the transforming SV40 proteins and the tumor-specific viral apoptosis-inducing protein apoptin and the comparable viral protein TAIP (TTV-derived apoptosis-inducing protein) were investigated.

Transforming activity of viral proteins

Tumor viruses have been extensively used as model for studying tumor development. Although recent results suggest that SV40 virus is linked to human tumor formation, it has been mainly used as a model for human tumor development for many decades [Carbone and Bedrossian, 2006; Saenz-Robles *et al.,* 2001]. SV40 and especially its viral transforming proteins large T antigen (LT) and small t antigen (st) have made significant contributions to the identification of tumor-related pathways [Ahuja *et al.,* 2005]. For instance, studies on the SV40 st protein have contributed to the identification of the tumor suppressor PP2A and its inhibitory role in cell cycle progression and cell survival, by inhibiting either directly or indirectly cdc2 kinase, MAP kinase and PK-B/Akt kinase, respectively. PP2A is a major target for viruses in order to manage host cell regulatory pathways for their own profit, which results in reprogramming of cells into a malignant state. SV40 targets the PP2A complex specifically by substituting the regulatory subunit PR61/B'γ with st antigen [Van Hoof and Goris, 2003]. Experiments with SV40 st have revealed that inactivation of PP2A complexes play an important role in transformation of human cells, and have suggested that also stabilization of c-Myc and activation of Akt contribute to cell transformation mediated by st [Arroyo and Hahn, 2005]. In addition,

PP2A is involved in the regulation of anti-apoptotic Bcl-2 family members, which are closely linked to tumor formation [Jansens et al., 2005]. In this thesis, we have described our experiments with both SV40 LT and st proteins to unravel novel tumor-related pathways (**chapters 3 and 4**).

Viral proteins sensing tumor-specific pathways

Besides studies on the application of SV40 virus transforming proteins, this thesis mainly focuses on viral proteins that specifically sense transformation-specific pathways, which results in induction of apoptosis. The studies include the adenovirus-derived protein E4orf4 and the chicken anaemia virus-derived protein apoptin as well as the novel apoptin-related TT virus-derived protein TAIP is discussed.

In the introduction (**Chapter 1**) an overview is presented of the adenovirus E4orf4. This protein has the ability to sense tumor-specific pathways when expressed in transformed and tumor cells, and induces a p53-independent cell death program. Although this cell death program shares several apoptotic hallmarks, it clearly differs from the classical apoptosis pathway.

Chicken anemia virus protein apoptin

This thesis describes a study on the transformation-specific apoptosis inducing protein apoptin. We report that, to exert its cell killing behaviour, apoptin needs to be phosphorylated by a transformation-specific kinase, after which it translocates to the nucleus and induces apoptosis. In normal cells apoptin remains in the cytoplasm and us inactive.

The behaviour of apoptin in normal cells has been studied using a recombinant fusion protein of maltose-binding protein and apoptin (MBP-apoptin) (**Chapter 2**). When MBP-apoptin is produced in bacteria, a stable multimeric complex is formed consisting of 30 to 40 monomeric subunits [Leliveld *et al*., 2002]. When apoptin is microinjected into the cytoplasm, MBP-apoptin protein translocates to the nucleus and induces apoptosis in tumor cells, whereas in normal cells the protein remains cytoplasmic and does not bring about apparent toxic effect. In normal cells MBP-apoptin is observed first as fine particles, later as increasingly larger organelle-sized globular bodies, which are not identified as acidic organelles like lysosomes. Finally, apoptin protein becomes epitope shielded and degraded. In normal cells apoptin appears to be non-functional and therefore represents a safe, effective candidate for cancer therapy.

Apoptin is activated in tumor cells and transformed cells, where it is specifically phosphorylated [Rohn *et al*., 2002]. Intriguingly, apoptin becomes activated in normal

cells upon the transient transfection with the transforming SV40 LT, which rapidly induces apoptin's phosphorylation, nuclear accumulation and the ability to induce apoptotic cell death (**Chapter 3**). Activation of apoptin occurs not via direct association of LT with apoptin but is mediated by a transient transforming signal induced by LT. Transient expression of LT in normal cells activates apoptin-specific kinase as demonstrated by both *in vitro* and *in vivo* kinase assays, indistinguishable from the apoptin-specific kinase activity revealed first in tumor cells. These data clearly indicate that the kinase capable of phosphorylating apoptin is constitutively active in stably cancerous state and can be rapidly induced in the early stage of cellular transformation (**Chapter 3**). Analyses with LT mutants revealed that apoptin activation is a function of the N-terminal sequence in LT, the J domain which is largely shared with SV40 st. Interestingly, the shared J domain in st, which lacks a nuclear localization signal (nls), can activate apoptin when it is located in the nucleus. The fusion protein nls-st is localized in the nucleus of normal cells and activates the tumor-specific properties of apoptin. Because no evidence of association or co-localization of LT with apoptin has been demonstrated, it seems likely that the kinase activity is up-regulated by the action of the J domain of LT or of nls-st, both after transient transfection and stable transformation. In both conditions, inhibition of p53 and Rb by LT is dispensable for the activation of apoptin by LT. This is in agreement with the observation that inactivation of p53 and RB by LT (mutants) is not essential for its transforming abilities [Sachsenmeier and Pipas, 2001].

In VH10 human primary fibroblasts, LT rapidly entered into the nucleus and caused activation of apoptin, whereas in human primary mesenchymal stem cell (MSC) cells apoptin maintained its normal-cell-specific properties (aggregation, epitope-shielding and/or degradation), while nuclear trafficking of LT was delayed at early time points after micro-injection. At later time points post injection, when LT appeared in the nucleus, apoptin translocated to the nucleus to induce apoptosis (**Chapter 4).** The delayed LT nuclear trafficking might result in the postponed triggering of the apoptin-specific kinase. These studies clearly show that apoptin reacts on the induction of (early) transformation-related processes.

In addition to the activation of apoptin in the transiently transformed and the stable transformed states, apoptin can be activated in other circumstances. For instance, upon UV-C irradiation apoptin is triggered to induce apoptosis in UV-C-treated skin fibroblasts from individuals with heredity cancer-prone syndromes, whereas fibroblasts from healthy individuals were resistant to apoptin, after the same radiation treatment [Zhang *et al*., 1999]. Fibroblast-like synoviocytes (FLS) from patients with rheumatoid arthritis (RA) show sensitivity to apoptin-induced apoptosis when transduced with MBP-apoptin fusion protein, which agrees with a transformed phenotype of these cells. However, no phosphorylation of apoptin was observed in RA cells by *in vitro* kinase assays. Although RA-FLS display the transforming characteristics and the predicted apoptotic cell death by

apoptin, the mechanism of apoptin's action seems to differ between tumor cells and RA-FLS (Tolboom et al., 2006).

Certain experimental conditions may also influence apoptin's tumor-specific selectivity (**Chapter 5**). For instance, certain tags can affect the subcellular localization of apoptin. Green fluorescence protein (GFP)-tagged apoptin enters the nucleus of human primary cells, though no significant death effect was induced. C-terminally Strep-tagged apoptin is not phosphorylated in human tumor cells, whereas N-terminally Strep-tagged apoptin is. The quality of the transduced DNA can influence apoptin's behaviour. Transduction of one apoptin-encoding DNA batch into normal cells by either transfection or microinjection resulted in an unexpected apoptin-killing, whereas parallel microinjection experiments with other apoptin-encoding DNA batches and MBP-apoptin protein preparations retained apoptin's normal cell non-toxicity. Finally, high passage numbers of normal diploid cells may cause features of replicative senescence, while culture media and serum might contain stress-inducing factors. All these results illustrate that the experimental circumstances regarding apoptin studies should be carried with all possible precautions.

Despite the fact that worldwide more and more researchers are studying apoptin as a sensor for transformation-related processes, the tumor-specific mechanism of apoptin-induced apoptosis is not really understood. Besides the tumor-related kinase activity that targets apoptin, apoptin has been found to interact with a number of cellular proteins, including Bcl10, FADD, Hippi, DEDAF, APC/C and Nur77 [Tavassoli *et al*., 2005; Zhang et al. 2007]. Burek and colleagues showed that Bcl2 and Bcl-xl inhibits apoptin-induced cell death under certain conditions. They concluded that apoptin-induced cell death engages a caspase-dependent mitochondrial pathway and is controlled by pro- and anti-apoptotic Bcl-2 family members [Burek et al., 2006]. Recently, Liu *et al.* have clearly demonstrated that Bcl-2, Bcl-xl and other anti-apoptosis factors such as survivin and caspase inhibitors XIAP and CIAP do not interfere with apoptin-induced apoptosis in human tumor cells. Our results on the effect of Bcl-xl on apoptin-induced apoptosis presented in **chapter 6** are in agreement with those of Liu *et al.* and Danen-Van Oorschot *et al.* (1999). Further studies are required to solve this controversy and pinpoint the molecular mechanism of apoptin-induced tumor-specific apoptosis.

TT virus derived apoptosis-inducing protein

The third studied viral protein is the putative protein derived from TT virus. This virus harbours a circular single-stranded DNA genome, which shows striking similarities to chicken anaemia virus (CAV) in its genomic organization (**Chapter 7**). Since CAV encodes apoptin, it was investigated whether TT virus encodes a similar factor.

A detailed comparative analysis resulted in the identification of TTV ORF 3, which encodes a 105 amino acids protein and shows intriguing similarities to apoptin in both sequences and predicted structure. This putative protein was named TTV-derived apoptosis inducing protein (TAIP). TAIP seems to induce apoptosis in a p53-independent manner preferentially in human liver tumor cells.

Further experiments in human hepatocytes are needed to show if TAIP causes cell death in a tumor-specific manner, although the preliminary experiments in normal rat hepatocytes microinjected with plasmid DNA encoding TAIP showed no apoptosis in comparison with the control microinjections. Also of interest is to examine the phosphorylation state or other modifications of TAIP, since phosphorylation is one of the tumor-specific features of apoptin. Our studies suggest that TAIP might be a useful tool for identifying the underlying mechanism of liver-tumor formation.

Concluding remarks

Based on the data presented in this thesis and reported in other studies, it may be concluded that the approach of studying cell transformation processes with viral oncoproteins in combination with viral transformation-specific apoptosis inducing proteins is of relevance. It had led to a better insight into the tumor-specific cell killing behaviour of apoptin, while it also provided information on a protein kinase activity that may be shared by most if not all transformed cells and tumor cells. Ongoing preclinical studies with apoptin are promising and will hopefully enter the clinical phase trials in the very near future.

Much more work is needed to further elucidate the underlying mechanisms of apoptin-induced tumor-specific apoptosis. Future studies in tumor cells or rheumatoid arthritis-derived cells as well as cells transiently transformed by viral oncogenes, will help us to understand further the mechanism of apoptin-induced apoptosis, and will also provide further information on fundamental questions concerning the transformed state of cells and their sensitivity to alternative apoptosis pathways. Finally, these studies will provide approaches for the application of viral proteins that sense transformation-related processes as therapies against diseases as cancer and rheumatoid arthritis.

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