Apoptin : oncogenic transformation & tumor-selective apoptosis

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Chapter 3

Family at last: highlights of the first international meeting on proteins killing tumour cells

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Abstract

Recently, a select number of viral and cellular proteins sharing a unique property have been identified, namely apoptin, HAMLET, TRAIL, MDA7, E4ORF4, NS1 and Brevinin-2R, where all have the remarkable ability to selectively kill transformed and/or tumour cells, while causing no or only minor cytotoxicity in normal and non-transformed cells. Research worldwide is now starting to unravel the molecular signalling pathways by which each of these proteins exerts its tumour-selective function, and the first (pre)clinical studies are very promising. The workshop 'Proteins Killing Tumour Cells' brought together, for the first time, researchers working on this novel class of intriguing proteins. Participants from 14 different countries shared their views on (possible common) mechanisms behind tumour-specific apoptosis induction and strategies to implement this knowledge in the clinic.

Cellular Transformation and Apoptosis

The ability of certain viral and cellular proteins to induce tumour-selective apoptosis implies the existence of a common set of characteristics, shared by all tumour cells, and recognized by these tumour-killing proteins (see Table 3-1). Mathieu Noteborn highlighted the delicate balance between survival and death pathways in normal and transformed cells, thereby effectively setting the theme of the meeting. Michael Green (University of Massachusetts, USA), using a genome-wide RNA interference screen, implicated secreted IGFBP7 (insulin-like growth factor binding protein 7) in the induction of senescence and apoptosis by oncogenic Braf. Paradoxically, overexpressing both proteins in normal melanocytes induced senescence, whereas in melanoma cells this resulted in apoptosis. James Pipas (University of Pittsburgh, USA) discussed the role of different domains of the SV40 large T antigen in oncogenic transformation. For instance, the N-terminal J-domain, in combination with the LXCXE domain, induces cellular proliferation by inactivating Rb and liberating E2Fs from repression. Additionally, microarray analysis revealed a new 'detoxification' function for the C-terminal domain of the large T antigen via the p450 pathway. Jason Arroyo from the Hahn group (Dana Farber Cancer Institute, USA) explained how the SV40 small T antigen contributes to the induction of transformation by inhibiting the protein phosphatase 2A (PP2A) family. Specific subunits of these heterotrimeric proteins are implicated in cell transformation: RNAi knockdown of the B56γ-subunit and mutation of the Aβ-subunit both promote transformation, whereas deletion of the Aα-subunit results in apoptosis. A common target of both transforming and tumour-selective apoptosis-inducing viral proteins appears to be the anaphase-promoting complex (APC). As illustrated by Jose Teodoro (McGill University, Canada), knockdown of the APC1 subunit by siRNA resulted in G2M cell cycle arrest and apoptosis in human tumour cells. Strikingly, this effect is mimicked by APC inactivation through interaction with either apoptin

or adenovirus E4orF4, two proteins discussed during this meeting, thus revealing a common pathway.

Proteins Killing Tumour Cells

HAMLET

Catharina Svanborg (Lund University, Sweden) introduced HAMLET (Human α-lactalbumin made lethal to tumour cells), a complex between the milk protein α-lactalbumin and oleic acid. HAMLET kills tumour and immature cells but not healthy differentiated cells by a mechanism independent of caspase activation, Bcl-2 and p53. Svanborg showed that macroautophagy is involved in HAMLET-induced cell death, as siRNA downregulation of the Beclin1 and Atg5 markers rescued HAMLET-treated cells. In clinical trials with bladder cancer patients, HAMLET exerted a direct and selective cytotoxic effect on the cancer tissue, whereas in patients with skin papillomas it resulted in complete resolution of 29 out of 35 treated papillomas. Christel Rothe Brinkmann discussed a bovine variant of HAMLET with similar cytotoxicity as the human homologue.

Brevinin-2R

Brevinin-2R-triggered cell death also involves autophagy and does not require caspase activation. The 25-aa peptide, presented by Marek Los (University of Manitoba, Canada), is a non-haemolytic defensin isolated from the skin of the frog *Rana ridibunda* and induces cell death in several cancer cell lines, with little toxicity towards primary cells.

Apoptin

Los also reported on the chicken anaemia virus-encoded protein apoptin, which induces apoptosis in numerous cancer and transformed cells, but leaves normal healthy cells unharmed. Los identified the nuclear orphan receptor Nur77 as a crucial component

of apoptin-induced cell death. In addition, he found that apoptin interacts with and activates Akt kinase, leading to nuclear localization of Akt.

Apoptin's tumour-selective cytotoxicity appears to be associated with nuclear localization and phosphorylation on T108. The nuclear targeting signal within apoptin comprises a bipartite nuclear import signal flanking a nuclear export signal. David Jans (Monash University, Australia) proposed a model where, selectively in cancer cells, NES function is inhibited by phosphorylation of apoptin on Thr108, resulting in nuclear accumulation. Dongjun Peng (Huazhong University, China) showed, in collaboration with the Leiden group, that transient expression in normal fibroblasts of the SV40 large/small T antigens is sufficient to induce apoptin's nuclear localization, phosphorylation and apoptosis. Interestingly, suppression of PP2A activity by the small T antigen (see Arroyo, above) appears to play a major role in this process. Paola Bruno from the Tavassoli group pointed out that low levels of apoptin phosphorylation can be detected in normal early passage fibroblasts infected with adenovirus expressing apoptin fused to GFP. Apparently, apoptin kinase activity is present in normal cells but has very low activity, whereas in tumour cells it is highly activated.

Rhyenne Zimmerman introduced a number of apoptin-interacting partners (AIPs) identified by the yeast two-hybrid technique. Strikingly, these AIPs are all known or putative tumour-suppressor genes or oncogenes, likely to be implicated in the differential behaviour of apoptin in normal and cancer cells. Poramaporn Klanrit showed for the first time (in collaboration with the Melino group (University of Rome, Italy) that apoptin is able to activate TAp73 and its downstream pro-apoptotic target PUMA, independently of p53 function. Joseph Cheng from the Norris group (University of South Carolina, USA), reported on the implication of the ceramide pathway

in the tumour-selective activity of apoptin. In prostate cancer cells, apoptin expression results in ceramide upregulation and acid ceramidase inhibitors can significantly enhance apoptin cytotoxicity both in prostate cancer cell lines and tumour xenografts.

Several groups discussed the use of a protein transduction strategy to deliver apoptin via the TAT protein transduction domain from HIV-1. Joop Gäken (King's College London, UK) investigated the potential of transduced apoptin to purge haemopoietic stem cells of malignant leukaemia cells, for use in autologous transplantation. His-tagged TAT-apoptin protein was shown to induce apoptosis in leukaemic cells but not in normal blood lymphocytes. Marcella Flinterman presented a novel mammalian secretable delivery system, using a modified TAT-tag. Jun Sun (Huazhong University, China) reported topical application of PTD4-apoptin onto the tumour bearing skin of immune-deficient mice. Apoptosis and disruption of the tumour integrity were apparent, whereas the surrounding normal tissue was not affected.

E4orf4

Similar to HAMLET and apoptin, over-expression of the adenoviral E4orf4 protein triggers p53-independent cell death in transformed and cancer cells, without necessitating caspase activation or mitochondrial dysfunction. However, as explained by Josée Lavoie (Laval University, Canada), E4orf4 killing requires Src tyrosine kinases and RhoGTPase-dependent perturbations of actin dynamics on endosomes. The traffic of recycling endosomes to the *trans*-Golgi is deregulated in a Cdc42- and Rab11a-dependent way, contributing to cell death. Furthermore, E4orf4 inhibits, by direct binding, v-Src-induced morphological transformation. These data support a key function for Src tyrosine kinases and actin-regulated endosome traffic in the tumour-selective killing activity of E4orf4.

TRAIL

TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) selectively induces death receptor-mediated apoptosis in several cancer cell lines. Robbert Cool (University of Groningen, the Netherlands) discussed the binding of TRAIL to the death receptors DR4 and DR5, the decoy receptors DcR1, DcR2 and osteoprotegerin. To avoid sequestering by the decoy receptors, Cool developed DR5 receptor-selective TRAIL variants with improved thermal stability. Henning Walczak (DKFZ Heidelberg, Germany) introduced the TRAIL receptor agonists currently enrolled in phase I and II clinical trials, including TRAIL/Apo2L and antibodies against DR4 and DR5. TRAIL monotherapy is rarely effective, as most primary tumours seem to be TRAIL resistant. Walczak discussed how this might be circumvented by combination therapy with either chemotherapy or ionizing radiation (IR). Jannie Borst showed how TRAIL, in a soluble isoleucine-zippered form, as produced by Walczak and coworkers, enhances indeed the efficacy of radiotherapy. The apoptotic pathway induced by IR is blocked by Bcl-2, indicating that a mitochondrial contribution is required for caspase activation and cell death. Interestingly, pretreatment of Bcl-2 overexpressing cancer cells with IR makes the cells more susceptible to TRAIL-induced cell death.

MDA7

Rajagopal Ramesh (University of Texas, USA) showed that expression of the melanoma differentiation associated gene-7 (MDA7), also known as interleukin-24 (IL-24), in lung cancer and melanoma is inversely correlated with cancer progression and patient survival. MDA7/IL-24 selectively killed human cancer cells both *in vitro* and *in vivo*, with minimal effect on normal cells. In clinical phase I trials MDA7 was well tolerated and showed evidence of significant clinical activity (melanoma and solid tumours).

Parvovirus MVM-NS1

Jean Rommelaere (DKFZ, Heidelberg, Germany) discussed the tumour-specific cell killing properties of parvovirus minute virus of mice (MVM) and its encoded protein NS1, responsible for MVM-induced cytotoxicity. MVM can kill tumour cells resistant to standard cytotoxic agents such as cisplatin or TRAIL. The efficacy of MVM in a rat glioma model was illustrated by the complete remission of intracranial tumours, without side effects, and with no tumour relapse for over 1 year. Investigations of the molecular mechanism of NS1-dependent cell killing revealed that acidic vesicles accumulate in the cytosol of MVM-infected cells, leading to the cytosolic accumulation of cathepsins and activation of cathepsin B, which, in turn, leads to the activation of autophagy. NS1 exerts its toxicity by interacting with casein kinase II-α (the catalytic domain of CKII).

Conclusions

Apoptin, E4ORF4, NS1, HAMLET, TRAIL, MDA7 and Brevinin-2R are part of a new family of proteins, consisting of both cellular and viral proteins that use various mechanisms of cell death, but share one key feature: their killing activity is efficient in transformed and cancer cells and negligible in normal and healthy cells. A potential new member of this tumour killing protein family, IGFBP7, was introduced during the meeting. Besides the obvious applications for tumour targeting, these proteins also constitute very sensitive probes to zoom-in onto essential molecular and cellular changes during carcinogenic transformation. The workshop was unanimously assessed as very stimulating, bringing together for the first time various research groups working on different proteins but all with similar potentials for cancer therapeutics. As one participant stated: 'We all thought we were orphans, but now we know we are family'.

Table 3-1 Direct comparison of various properties of proteins killing tumour cells.

	Apoptin	**HAMLET**	**TRAIL**	**MDA7**	**E4orf4**	**NS1**	**Brevinin-2R**
Protein origin	Chicken anaemia virus	Complex between human milk protein - lactalbumin and oleic acid	TNF family-related apoptosis-inducing ligand	IL-10 cytokine family member	Adenovirus type 2	Parvovirus minute virus of mice (MVM)	*Rana ridibunda*
Subcellular localization	Cytoplasm (normal cells) nucleus (cancer)	Cytoplasm (normal cells) nucleus (cancer)	Extracellular receptor binding	Extracellular receptor binding	Nucleus (early) cytoplasm (late)	Cytoplasm	Cytoplasm (lysosomes)
Mechanism of (tumour-selective) cell death	Apoptosis – intrinsic pathway	Apoptosis, autophagy	Apoptosis – extrinsic pathway	Apoptosis – extrinsic pathway	Apoptosis-like	Autophagy	Autophagy
p53-dependence	No	No	No	No	No	?	?
Therapeutic status	Preclinical studies	Phase I and II clinical trials	Phase I and II clinical trials	Phase I and II clinical trials	Preclinical studies	Preclinical studies	Preclinical studies

Addendum

Since the first PKTC meeting, a number of other proteins exhibiting tumor-selective toxicity have been identified. These will be discussed below.

Noxa

Noxa is a BH3-only protein with proapoptotic activity that functions downstream of the p53-mediated apoptotic pathway. It was recently found to selectively induce apoptosis in tumor cells (Suzuki, et al., 2009). Infection with a recombinant adenovirus, contrived to express the Noxa gene, induced apoptosis in several human breast cancer cell lines *in vitro*, but not in normal mammary epithelial cell lines. Furthermore, intratumoral injection of the Noxa-expressing adenovirus resulted in marked shrinkage of transplanted tumors derived from breast cancer cells, without any notable adverse effect on the surrounding normal tissue. In contrast, the expression of Puma, another BH3-only protein that also functions downstream of the p53 pathway, induced apoptosis in both cancer and normal cells. Thus, the results suggest a mechanism wherein Noxa, but not Puma, selectively induces apoptosis in human tumor cells.

ORCTL3

The organic-cation transporter like-3 gene (ORCTL3) was identified in a systematic, high-throughput screen for genes specifically inducing cell death in transformed tumor cells (Irshad, et al., 2009). ORCTL3 was found to be inactive in normal rat kidney cells (NRK), but induced apoptosis in NRK cells transformed by oncogenic H-ras. ORCTL3 also induced cell death in v-src-transformed cells and in various human tumor cell lines but not in normal cells or untransformed cell lines. Accordingly, ORCTL3 was shown to be down-regulated in human kidney tumors. Though ORCTL3 is a member of the organic-cation transporter gene family, data indicate that it induces apoptosis independently of its putative transporter activity. Rather, experimental evidence suggest that ORCTL3-induced apoptosis is executed via an ER stress mediated mechanism.

Par-4

The prostate apoptosis response-4 (par-4) gene was first identified as an immediate early apoptotic gene, which was up-regulated in response to elevated intracellular Ca2+ concentration in rat prostate cancer cells treated with ionomycin. Human Par-4, which was found to share significant sequence similarity with its rat counterpart, was subsequently identified in yeast-two hybrid studies as a partner of the atypical Protein Kinase C (Diaz-Meco, et al., 1996) and tumor suppressor Wilm's tumor-1 (Johnstone, et al., 1996). Studies conducted in cell culture models show that over-expression of Par-4 is sufficient to directly induce apoptosis in many cancer cell types, and that this ability is associated with Par-4 nuclear translocation. Moreover, the apoptotic action of Par-4 can overcome cell protective mechanisms, such as the presence of Bcl-xL, Bcl-2, or absence of wild-type p53 or PTEN function (Shrestha-Bhattarai and Rangnekar, 2010). Interestingly, Par-4 is retained in the cytoplasm of normal and immortalized cells through an as yet unidentified mechanism, rendering it incapable of inducing apoptosis in these cells.

Accordingly, Par-4 was found to be down-regulated in many cancers, including renal cell carcinoma (Cook, et al., 1999), neuroblastoma (Kogel, et al., 2001), endometrial cancer (Moreno-Bueno, et al., 2007), and breast cancer (Zapata-Benavides, et al., 2009). Animal studies have demonstrated that Par-4 knockout mice are prone to spontaneous development of tumors in various tissues, e.g. lungs, liver, urinary bladder and endometrium, and Par-4 knockout mice are more susceptible to chemical- or hormone-induced lesions, exhibiting a significantly shorter life span compared to wild-type animals, due to death by spontaneous tumors (Garcia-Cao, et al., 2005). Par-4 is down-regulated by oncogenic Ras via the MEK-ERK pathway, and this is considered an important step towards Ras-induced transformation. Restoration of Par-4 levels, either by MEK inhibition or by stable expression of ectopic Par-4, abrogates cellular transformation. This tumor-suppressor action of Par-4 appears to be distinct from its apoptotic function (Barradas, et al., 1999; Pruitt, et al., 2005; Qiu, et al., 1999). In the case of prostate

cancer, Par-4 is not down-regulated, silenced or mutated, but inactivated due to phosphorylation by Akt1, which prevents nuclear translocation of Par-4, thereby retaining it in the cytoplasm and rendering it incapable of causing apoptosis (Goswami, et al., 2005).

In contrast, phosphorylation of the threonine 155 residue of Par-4 by Protein Kinase A (PKA), as well as its nuclear translocation, is essential for Par-4 apoptotic activity (Shrestha-Bhattarai and Rangnekar, 2010). The apoptotic effect of Par-4 involves either an activation of the cellular apoptotic machinery or inhibition of the cellular pro-survival mechanisms. Par-4 induces apoptosis in hormone-independent cancer cells by activation of the extrinsic apoptosis cascade through FADD. Therefore, in these cancer cells, over-expression of Par-4 is a sufficient signal for cell death (Chakraborty, et al., 2001). In parallel, Par-4 translocates to the nucleus and inhibits NF-κB-mediated cell survival mechanisms (Nalca, et al., 1999).

While most studies on Par-4 focused on the apoptotic effect mediated by intracellular Par-4, recent findings indicate it is also secreted by both normal and transformed mammalian cells (Burikhanov, et al., 2009). *In vitro*, Par-4 secretion could be induced by exogenous stimuli capable of causing stress in the endoplasmic reticulum (ER), and secreted Par-4 was shown to induce apoptosis via the cell-surface protein GRP78, which is coincidentally also found in the ER lumen. Par-4 was also found to be secreted *in vivo*, and could be detected in the serum of Par-4 transgenic mice. In addition, Par-4 activity in the serum was able to induce apoptosis specifically in cancer cells.

In view of these findings, both systemic (extracellular) and intracellular Par-4 may be applied for cancer therapy. In fact, TRAIL has been shown to cause the nuclear translocation of Par-4, and this mechanism is purportedly responsible for the ionizing radiation-induced bystander effect triggered in response to high-dose X-rays (Shareef, et al., 2007).

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