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The Apoptin® gene of chicken anemia virus in the induction of apoptosis in human tumorigenic cells and in gene therapy of cancer*.

Mathieu H.M. Noteborn[1,2], Astrid A.A.M. Danen-van Oorschot[2] and Alex J. van der Eb[2]

[1]Leadd BV, and [2]Laboratory for Molecular Carcinogenesis, Department of Medical Biochemistry, Leiden University, P.O. Box 9503, 2300 RA, Leiden, The Netherlands.

Correspondence: Mathieu Noteborn, Tel: 31-71-527-87 36, Fax: 31-71-527-17 36, E-mail:: leadd@leadd.nl

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Summary

Apoptosis is an active physiological process for the elimination of superfluous or altered cells from a developing or adult organism. Aberrations in the apoptotic process cause various diseases, e.g. tumor formation. Chemotherapy for the treatment of cancer often exerts its cytotoxic effect via the induction of apoptosis and its success depends mainly on the presence of functional p53 and the absence of an overexpressed Bcl-2 proto-oncogene. Unfortunately, in more than 50% of the tumors functional p53 is lacking, and/or Bcl-2 is overexpressed, which often results in resistance to anti-cancer therapy. Apoptin®,[1], a protein derived from chicken anemia virus (CAV), can induce apoptosis in cultured (human) cells derived from various tumors, e.g., osteosarcoma, lymphoma, leukemia, hepatoma, melanoma, and from tumors of breast, colon, and lung. Tumor cells lacking p53 can undergo Apoptin-induced apoptosis, and over-expression of Bcl-2 even stimulates Apoptin-induced apoptosis. Downstream inhibitors of the p53-pathway, like Bcl-2, BAG-1 and CrmA, do not inhibit Apoptin-induced apoptosis, which indicates that Apoptin induces cell death via a different pathway or that Apoptin acts at a step far downstream the apoptotic cascade. Interestingly, Apoptin fails to induce apoptosis in human primary lymphoid, dermal, epidermal, endothelial and smooth muscle cells. Co-expression of a transforming agent with Apoptin in normal diploid cells results in apoptosis. The fact that Apoptin induces a p53-independent, and Bcl-2-stimulated type of apoptosis in human tumor cells, but not in normal diploid cells, renders Apoptin a potential anti-tumor agent. Gene-therapy strategies based on viral vectors expressing Apoptin are currently under development.

I. Introduction

Apoptosis is an active and programmed physiological process for eliminating superfluous, altered or even malignant cells (Earnshaw, 1995; Duke et al., 1996). Apoptosis is characterized by shrinkage of cells, segmentation of the nucleus, condensation and cleavage of DNA into domain-sized fragments, in most cells followed by internucleosomal degradation. The apoptotic cells fragment into membrane-enclosed apoptotic bodies. Finally, neighboring cells and/or macrophages will rapidly phagocytose these dying cells (**Figure 1**; Wyllie et al., 1980; White, 1996). The apoptotic process can be initiated by a variety of regulatory stimuli (Wyllie, 1995; White, 1996; Levine, 1997). Changes in the rate of cell survival play an important role in, e.g., oncogenesis, which is caused by enhanced proliferation but also by decreased cell death (Kerr et al., 1994; Paulovich, 1997).

Several oncogenes and tumor-suppressor genes have been found to play a role in either induction or inhibition of apoptosis. The tumor suppressor p53, for example, can

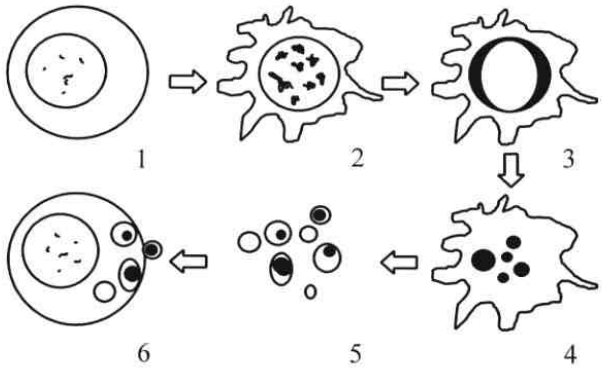

Figure 1. Schematic illustration of the subsequent morphological events, which occur during the apoptotic process. 1. Scheme of a normal cell before undergoing apoptosis. 2. The cytoplasmic membrane starts to form blebbing structures and the DNA begins to condensate. 3. The DNA condensates adjacent to the still intact nuclear membrane. 4. The nucleus becomes segregated, which is (5) subsequently followed by separation of the apoptotic cell. The complete cell content is surrounded by membrane structures, which are called apoptotic bodies. 6. These apoptotic bodies are phagocytosed by neighbouring cells and/or macrophages.

induce either cell-cycle arrest or apoptosis upon DNA damage. In many tumors, however, the p53 function is lost as a result of mutation or deletion. The proto-oncogene Bcl-2, which inhibits apoptosis, has been found to be activated in several lymphoma's due to a translocation.

Apoptosis plays a role not only in the development of tumors but also in the treatment of cancer. Conventional therapies using cytotoxic agents often act via induction of apoptosis. In many instances, the induction of apoptosis requires functional p53 and, consequently, loss of p53 function, correlates with resistance to these therapies. For example, melanomas, lung, prostate and colon cancers often have mutated p53, resulting in a poor response to chemotherapy (Lowe et al., 1994; Levine, 1997). Over-expression of Bcl-2 in lymphomas due to a t(14;18) translocation or in chronic myelogenous leukemia due to formation of the Bcr-abl fusion product by t(9;22) translocation leads to enhanced protection against apoptosis, and sometimes to resistance against (chemo)therapy (McDonell et al., 1995).

This has led to a search for new therapies, leading for example to restoration of the function of p53. Adenovirus- or retrovirus-mediated transfer of p53 inhibits the growth of certain tumor cells in tissue culture and in mouse models (Sandig et al., 1997; Gomez-Manzano et al., 1996; Yang et al., 1995). However, restoring p53 function may not be effective in tumors which have a block in the apoptosis pathway, e.g. those which over-express Bcl-2.

The apoptosis-inducing protein Apoptin is the basis for an alternative candidate strategy, since it induces apoptosis in various human tumor cell lines, including those lacking p53 and/or overexpressing Bcl-2. In this review we discuss the potential of Apoptin as an anti-tumor agent, its tumor-specific activity and the first gene-therapy experiments.

II. Apoptin®, a CAV-derived protein induces apoptosis

Chicken anemia virus (CAV) is an avian pathogen of worldwide importance. The clinical signs of CAV in young chickens are increased mortality, loss of body weight and hemorrhages. Severe depletion of cortical thymocytes and erythroblastoid cells results in transient immunodeficiency and anemia (Coombes and Crawford, 1996).

The depletion of thymocytes observed after CAV infection is based on apoptosis (Jeurissen et al., 1992; Noteborn, 1993). DNA isolated from the thymus of infected chickens shows the apoptosis-specific laddering pattern, which is not observed in DNA isolated from the thymus of non-infected chickens. Electron-microscopic analysis of the cortex ten days after infection shows cells containing condensed chromatin adherent to the nuclear membrane, and apoptotic bodies in the cytoplasm of epithelial cells.

CAV is a small non-enveloped virus containing a single-stranded DNA genome of 2.3 kb (Noteborn and Koch, 1995). From the CAV genome, a single-polycistronic mRNA is transcribed, with three open reading frames which partially or completely overlap. These three reading frames are indeed used, and the CAV mRNA encodes three completely different proteins VP1 (51.6 kDa), VP2 (24.0 kDa) and VP3 (13.6). The products show no homology to each other or to any other known proteins. VP1 is the only capsid protein, whereas VP2 seems to be required for capsid formation (Noteborn and Koch, 1995).

To establish which CAV protein is responsible for the induction of apoptosis, plasmids encoding either VP1, VP2 or VP3 were transfected into cultured chicken mononuclear cells. The cells were analysed by indirect immunofluorescence with specific antibodies, and its DNA was stained with propidium iodide (PI), which stains DNA strongly when it is intact, but weakly and/or irregularly when it is apoptotic. Expression of VP3 alone was sufficient for the induction of apoptosis as observed in CAV infection. Therefore we renamed VP3 as Apoptin. Soon after transfection, Apoptin is dispersed throughout the nucleus. Somewhat later, when the cells become apoptotic, Apoptin becomes aggregated in the nucleus or its remnants. At this point in time, nucleosomal laddering can be seen in the DNA of Apoptin-expressing cells, whereas DNA from cells transfected with a control plasmid remains intact (Noteborn, 1994). 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 did not result in apoptosis (Noteborn, unpublished data).

Apoptin consists of 121 amino acids and is rich in proline, serine and threonine residues. It contains a hydrophobic region, resembling a nuclear export signal (Wen et al., 1995; Fischer et al., 1995) and two positively-charged regions representing nuclear localization signals (Noteborn et al., 1987). A mutant Apoptin protein in which one of the two nuclear-localization signals was deleted, showed significantly reduced apoptotic activity, and was localized partially in the cytoplasm (Zhuang et al., 1995, 1995a).

It seems to be essential for induction of apoptosis by Apoptin that it co-localizes with the chromatin. The basic regions of Apoptin may allow interaction with nucleic acids, explaining the nuclear localization. The presence of Apoptin in the chromatin structure, and its high proline content, may cause disturbance of the supercoil organization, which could then result in apoptosis. Another possibility is that Apoptin acts as a transcriptional activator of genes, which directly or indirectly mediate apoptosis.

III. Apoptin-induced apoptosis is p53-independent

Apoptosis is a physiological process which is as tightly and extensively regulated as cell proliferation. Apoptosis can be divided into three phases (Vaux and Strasser, 1996). It starts with some stimulus, for example cytotoxic drugs, growth-factor deprivation, or binding of a ligand to a cell-surface receptor (e.g. Fas). In the second phase, the stimulus is detected and a transduction signal is produced. In this phase, the cell decides whether or not to undergo apoptosis, and the process can still be reversed. Many independent signal-transduction pathways can be used, but they all converge in the third phase: the execution of apoptosis. When the cell-death effectors are activated, the cell crosses the point of no return, and undergoes apoptosis rapidly.

An important regulator of apoptosis is the tumor-suppressor protein p53, which, among others, is involved in the response to excessive DNA damage caused by cytotoxic agents. On the other hand, it does not seem to be important in the regulation of apoptosis during embryonal development, since p53-knockout mice develop normally (Levine, 1997).

To determine whether p53 is involved in Apoptin-induced cell death, Apoptin was transiently expressed in human tumor cells either lacking p53, expressing a mutant form, or synthesizing wild-type p53. In all three cell lines, Apoptin was able to induce apoptosis to the same extent, indicating that it does not need functional p53 (Zhuang et al., 1995a). In consistence with this, the adenovirus E1B-55K protein, an inhibitor of p53, did not decrease the apoptotic activity of Apoptin (Zhuang et al., unpublished data).

IV. Bcl-2 inhibits p53-induced, but not Apoptin-induced apoptosis.

The proto-oncogene Bcl-2 acts downstream of p53 and is shown to inhibit p53-mediated, but also p53-independent apoptosis (White et al., 1996). Zhuang and colleagues studied the effect of several apoptosis inhibitors on Apoptin-induced apoptosis (Zhuang et al., 1995, 1995a).

Apoptin could still induce apoptosis in human hematologic malignant cells expressing high levels of Bcl-2 (DoHH-2) or BCR-ABL (K562), both inhibitors of p53-mediated cell death. In DoHH-2 cells, Apoptin induced cell death even faster than in K562 cells.

Another inhibitor of apoptosis is BAG-1, a Bcl-2-binding protein that is functionally but not structurally homologous to Bcl-2. In some cell types, Bcl-2 and BAG-1 cooperate to inhibit apoptosis, whereas Bcl-2 alone has only a minor effect. We therefore tested the effect of BAG-1, alone or in combination with Bcl-2, on Apoptin-induced apoptosis (Danen-van Oorschot, 1997a). Like Bcl-2, BAG-1 did not inhibit cell death induced by Apoptin, and neither did the combination of Bcl-2 and BAG-1. Surprisingly, Apoptin-induced apoptosis is enhanced by Bcl-2 and, to a lesser extent, by BAG-1. In parallel experiments, BAG-1 and/or Bcl-2 did inhibit p53-induced apoptosis.

In conclusion, Bcl-2 overexpression stimulates Apoptin-induced cell death. From a therapeutic point of view this feature is important, because lymphomas carry

an overexpressed Bcl-2 gene which results in their derailment and resistance to chemotherapeutic agents.

V. Caspases are differently involved in Apoptin- versus p53-induced apoptosis

In general, a step further down in the apoptotic decision cascade are the caspases, which are specific cysteine-proteases (also called ICE-like proteases). Caspases can cleave themselves, other caspases or target proteins, such as PARP, lamins and the 70-kDa U1 small ribonucleoprotein (Rao and White, 1997; Nicholson, 1996). Thus far, one assumes that all apoptotic pathways converge in the activation of the cascade of caspases, which seems to be part of the execution phase.

To determine whether caspases are also involved in Apoptin-induced cell death, the cowpox-virus protein CrmA was co-expressed with Apoptin. CrmA mainly inhibits the activity of caspase 1, but also of other caspases, and thereby prevents apoptosis. Co-expression of CrmA with Apoptin, however, could not prevent apoptosis, whereas in-parallel experiments it was revealed that p53-induced cell death was suppressed by CrmA (Danen-van Oorschot, unpublished results). Similar results were obtained with modified peptides, known to specifically inhibit caspases, also those more downstream in the cascade.

These data indicate that Apoptin-induced apoptosis does not involve caspases, or at least not those interfering in the p53-apoptotic pathway. Alternatively, Apoptin may act downstream of the caspases. As Apoptin was found to co-localize with chromatin, it may bind to DNA and disturb its structure, or even act as an endonuclease. However, no evidence of binding to DNA has been found so far (Noteborn, unpublished results). Another possible explanation is that Apoptin associates with DNA-binding proteins, and indirectly influences the chromatin structure. We are currently trying to identify proteins that associate with Apoptin via the yeast-two-hybrid system.

A schematic overview of the pathways via which Apoptin and p53 induce apoptosis, is shown in **Figure 2**.

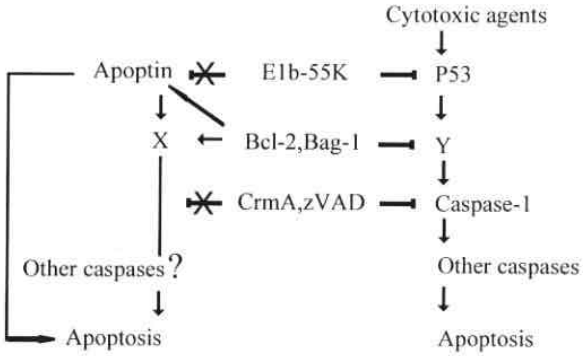

Figure 2. Schematic representation of the Apoptin-pathway versus the p53 pathway of apoptosis induction. The adenovirus E1B 55K protein inhibits p53 activity but not apoptin-induced apoptosis. The Apoptin-pathway is strongly stimulated by Bcl-2 and to some extent by BAG-1, whereas in contrast, the p53-pathway is inhibited by both Bcl-2 and BAG-1. The caspase-inhibitors CrmA and peptide zVAD inhibit p53, but not Apoptin-induced apoptosis.

VI. Apoptin induces apoptosis in human tumor cells, but not in normal cells

The fact that Apoptin does not need the tumor suppressor p53 to induce apoptosis, and is even stimulated by the proto-oncogene Bcl-2 makes Apoptin an interesting candidate anti-tumor agent.

To explore this potential, the apoptotic effect of Apoptin has been examined in many human and other mammalian tumor cell lines of different origins. In all examined tumorigenic/transformed cell lines Apoptin was proven to induce apoptosis. Some of these cell lines are ones derived from breast and lung tumors, from neuroblastoma, hepatoma, melanoma, lymphoma, leukemia, or colon carcinoma (see **Table 1** and **Table 2**). In some tumor cell lines, programmed cell death was induced faster than in others, but apoptosis had always reached 90-100% at 5-6 days after transfection.

Interestingly, Apoptin did not induce apoptosis in normal, diploid (human) cells. We have examined human keratinocytes, vascular endothelial cells, smooth muscle cells, primary T cells and two types of human diploid fibroblasts under tissue-culture conditions, and murine and rat embryonal fibroblasts: in none of these cells Apoptin was able to induce apoptosis (**Table 3**). Among the above cell lines, several ones do not express (functional)

Table 1. Human tumorigenic and transformed cell lines in which Apoptin induces apoptosis.

Cell line	Type
1. MCF-7	Breast tumor
2. Saos-2	Osteosarcoma, p53-
3. U2OS	Osteosarcoma, p53+
4. SLCC-1	Small lung cell carcinoma
5. SLCC-2	Small lung cell carcinoma
6. Hep3B	Hepatoma, p53-
7. HepG2	Hepatoma, p53+
8. G401	Kidney rhabdoid tumor
9. KG-1	Acute myeloid leukemia
10. K562	Acute myeloid leukemia, Bcr-abl
11. DoHH-2	Immunoblastic B-lymphoma
12. Jobo-O	Epstein-Barr virus transformed B cells
13. Pre	SV40-transformed pre-crisis fibroblasts
14. Post	SV40-transformed post-crisis fibroblasts
15. NW-18	SV40-transformed tumorigenic fibroblasts
16. SCC-15	Squamous cell carcinoma
17. SVK14	SV40-transformed keratinocytes
18. HaCaT	Spontaneously transformed keratinocytes
19. 911	Adenovirus-5-transformed embryonal retinoblasts
20. 293	Adenovirus-5-transformed embryonal retinoblasts
21. HT29	Colon carcinoma

Table 2. Non-human tumorigenic and transformed cell lines in which Apoptin can induce apoptosis.

Cell line	Type
1. N1E-115	Murine neuroblastoma
2. P19	Murine embryonal teratocarcinoma
3. BRK-Xho	Adenovirus-5-transformed baby rat kidney cells
4. CC-531	Rat colon carcinoma
5. Cos-7	Monkey SV40-transformed kidney cells
6. MDDC- MSB1	Avian lymphoma
7. HD-11	Avian myeloblastosis virus (AMV)-transformed cells.

Table 3. Normal diploid cells in which Apoptin does NOT induce apoptosis

Human cells	Type
1. T cells	Phytohemagglutinin-stimulated primary T cells
2. HUVEC	Umbilical-cord vascular endothelial cells
3. HSMC	Smooth muscle cells
4. FSK-1	Epidermal keratinocytes
5. VH10	Diploid fibroblasts
6. VH25	Diploid fibroblasts
Non-human cells	**Type**
7. REF	Rat embryo fibroblasts
8. MEF-1	Mouse embryo fibroblasts, p53+
9. MEF-2	Mouse embryo fibroblasts, p53-

p53, indicating once again that Apoptin induces apoptosis independently of p53.

It might be expected that tumorigenic and/or transformed derivatives from the above primary cell types also are resistant to Apoptin-induced apoptosis. We, therefore, examined the effect of Apoptin in transformed cells derived from normal diploid fibroblasts and keratinocytes. Apoptin was found to induce apoptosis in VH-10 fibroblasts and keratinocytes that were transformed with SV40, and also in spontaneously immortalized keratinocytes. Apparently, these cells had become susceptible to Apoptin-induced cell death upon transformation (Danen-van Oorschot et al., 1997). This result indicates that Apoptin specifically induces apoptosis in transformed and tumorigenic cells, but not in normal diploid cells. Even more fascinating is the observation by Zhang and Noteborn (unpublished data) that co-expression of the transforming agent large T antigen of DNA tumor virus SV40 and Apoptin in normal diploid human cells results in Apoptin-induced cell death. An early event during tumorigenesis seems to be the switch for Apoptin-induced apoptosis. All these experiments have been carried out under cell-culture conditions, and it is now of great interest to determine whether Apoptin shows the same specificity in vivo.

VII. Apoptin is differential located in normal diploid cells versus transformed and/or tumorigenic cells.

An explanation why Apoptin specifically induces apoptosis in transformed and tumorigenic cells came from the analysis of its localization by indirect immunofluorescence. In tumorigenic/transformed cells

Apoptin is localized in the nucleus, initially in finely dispersed form, and when the cell becomes apoptotic it starts to aggregate. In normal diploid cells, however, Apoptin is found predominantly as fine granules in the cytoplasm. As mentioned above, a truncated version of Apoptin, lacking one of the putative nuclear localization signals, is also localized mainly in the cytoplasm, but in a more diffused form. This truncated Apoptin has a strongly reduced apoptotic activity. These observations suggest that the nuclear localization of Apoptin is an important factor in its ability to induce apoptosis. It is possible, however, that nuclear localization as such is not sufficient and that, in addition, Apoptin also needs to be modified in order to be able to trigger the apoptotic event in the nucleus.

The fact, that the transforming SV40 large T antigen translocates, besides Apoptin, also the tumor-related Hepatitis B virus HBx protein into the nucleus (Doria et al., 1995), suggests that Apoptin-induced apoptosis is linked to cell transformation. Paradoxically, the nuclear location of HBx will result in carcinoma formation, whereas nuclear Apoptin induces apoptosis.

The answer to the interesting question why Apoptin is differential located in normal versus transformed cells might help to unravel the mechanism of Apoptin-induced apoptosis and also one of the (early) steps in tumorigenesis.

VIII. Apoptin is a potential anti-tumor agent

Treatment of tumors with cytotoxic agents has been shown to kill tumor cells, often via the induction of apoptosis. Many tumors, however, are resistant to induction of apoptosis by such chemotherapy due to the loss of functional p53 or to overexpression of Bcl-2. For these categories of tumors, the development of new, effective therapies would be very welcome.

The results obtained so far with Apoptin suggest that this viral protein is a promising candidate for the treatment of tumors, including those that lack functional p53 or overexpress Bcl-2. The apoptosis inhibitor Bcl-2 not only fails to block, but even seems to enhance Apoptin-induced apoptosis. Furthermore, Apoptin induces apoptosis in transformed/tumorigenic cells, but does not in normal diploid cells grown under tissue-culture conditions.

In conclusion, these observations imply that Apoptin is a serious candidate as an anti-tumor agent.

In order to use Apoptin as an anti-tumor agent, gene-therapy strategies can be used. A major problem that needs to be overcome is the efficient delivery of Apoptin throughout the entire tumor and to all metastases. Presently, the most commonly used gene delivery systems are viral vectors because of their high efficiency.

Retroviral vectors are used in the majority of approved gene-transfer protocols (reviewed by Roth and Cristiano, 1997). The advantage of this system is that the viral DNA can stably integrate into the host genome, which ensures long-term expression of the introduced gene. However, this may also be disadvantageous because of the risks associated with random integration. Nevertheless, retroviral vectors can infect only dividing cells and they are, therefore, potentially useful for the treatment of cancer (Vile et al., 1996).

Other commonly applied vectors are based on human adenovirus 5 (Ad5), which can infect many different cell types and does not require cell division. They are specifically targeted to the respiratory and gastrointestinal epithelia, as well as to the liver. This system is less suitable for long-term expression as adenoviral DNA does not integrate into the host genome. This feature has the advantage that no risk exists for insertional mutagenesis. These properties, and the fact that adenoviral vectors have a high transduction efficiency and can be produced at high titers, renders these vectors particularly suitable for gene therapy against cancer (Ginsberg, 1996; Kozarsky and Wilson, 1993; Roth and Cristiano, 1997).

Rodent parvoviruses like H-1 and MVM viruses (that can also grow in human cells) have been shown to exert an oncosuppressive activity, i.e. they inhibit the formation of spontaneous, as well as chemically or virally induced tumors in laboratory animals. Cancer cells provide these viruses with an environment beneficial to their multiplication, which means that they appear to be oncotropic. However, these rodent parvoviruses are not always able to prevent the cancer cells from multiplying. Therefore, Dinsart et al. (1996) have developed a system for recombinant parvoviral particles expressing cytotoxic agents. In collaboration with Dinsart and Cornelis, DKFZ, Heidelberg, Germany, we are developing a recombinant parvovirus expressing Apoptin. The first aim will be to examine whether parvovirus-expressed Apoptin will kill those tumor cell lines, that are resistant to lysis by H1 and/or MVM parvovirus.

It was unclear, initially, whether viral vectors expressing the Apoptin gene could be produced at all, since the required helper cells for growing the replication-defective viral vectors are transformed cell lines and might be susceptible to apoptin-induced apoptosis. Indeed, the murine packaging cell lines PA317 and Psi-crip expressing Apoptin were killed within four days after the onset of recombinant retrovirus production (Noteborn et al., unpublished results). Hence, retroviral vectors carrying the gene encoding Apoptin need to be produced in a batchwise fashion.

At the moment, we are investigating a recombinant replication-deficient adenovirus expressing Apoptin. The transformed helper cells (Fallaux et al., 1996) used for growing adenoviral vectors, as expected, also underwent apoptin-induced cell death. Fortunately, however, the lytic cycle of the adenovirus vector seemed to proceed faster than the induction of apoptosis by Apoptin, resulting in recombinant-Apoptin viral stocks with high titers (Pietersen et al., unpublished data).

Animal studies with the recombinant-apoptin adenovirus will reveal whether in-vivo expression of Apoptin will indeed have anti-tumor activity and no toxic side effects. Preliminary results of preclinical trials assaying the in-vivo toxicity of Apoptin, show that adenovirus-expressed Apoptin causes only very minor toxic side effects, if any. Currently, anti-tumor studies using perfusion of isolated rat livers containing colon-metastases are under way.

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