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CHAPTER SEVEN

Apoptosis and cancer

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Abstract: Impaired molecular regulation of active (programmed) cell death is one of the most important hallmark of cancer. Apoptosis is the best characterized biochemical and morphological form of cell death, although other forms also exist. Positive regulators of apoptosis are often lost or inactivated, while inhibitor proteins of apoptosis are often upregulated in cancer. Individual variabilities in these molecular strategies contribute to the differences in sensitivity to current anti-cancer therapies and also provide new existing molecular targets for future therapeutic approaches.

Keywords: Apoptosis, programmed cell death, necrosis, survival pathways, targetted therapy

Cell birth – via proliferation – and cell death are the two endpoints in the control of tissue homeostasis in multicellular organisms. Disturbances of either process have pathological consequences and can lead, e.g. to malformations, neurodegenerative diseases, autoimmune diseases or cancer. Till now cell death was considered as a dichotomy of apoptosis and necrosis. Whereas apoptosis is an inherent, controlled cell death programme, the counterpart, necrosis, is a more chaotic and accidental way of dying (Danial and Korsmeyer 2004). However, in the past few years different forms of non-apoptotic cell death also appeared and these should be considered when explaining cell killing process (Okada and Mak 2004).

The regulated (programmed) cell death (PCD) has an important function: to save the organism against unwanted or potentially harmful cells. According to the current paradigm the inactivation of PCD is central both in the development of cancer and its response to therapy (Okada and Mak 2004; Brown and Attardi 2005). The resistance to cell death – particularly apoptotic cell death – will lead to one of the most critical events in carcinogenesis: the accumulation of genetic errors in proliferating cells due to the quantitative or qualitative defects of DNA repair leading to genomic instability. Cancers with relative resistance to apoptosis can withstand significant DNA damage, unfavourable environments (as encountered by metastasizing cells) and the action of cytotoxic therapy. Research on new cancer therapies has therefore focused to reverse this resistance and trigger apoptosis in tumor cells.

This short review outlines the main features of different types of cell death in normal circumstances and in certain aspects of tumor growth and therapy, including certain attempts to induce cell death by modulating the activity of those components that are considered as key elements in the cell death programs.

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Programs for cell death

It is difficult to give an exclusive definition for PCD, because the different death programs have many overlaps in their signaling pathways. In addition, a cell may switch back and forth between different death pathways. It is suggested, that the dominant cells death phenotype is decided by the relative speed of the available programs, and the fastest and most effective pathway is usually the prominent.

Apoptotic pathways (caspase-dependent cell death)

Apoptosis is characterized by disintegration of the cell into small fragments ("apoptotic bodies") and that can be removed by phagocytosis without inflammatory reaction. The apoptotic cascade is initiated by two main pathways, involving either the activation of cell death receptors to respond to death ligands, or the release of cytochrome c from mitochondria. Both pathways will trigger a specific family of cystein proteases, the caspases, to execute the self-killing process (Hengartner 2000; Degterev et al. 2003).

Cell death receptor (extrinsic) pathway

Cell death receptors appear at the highest level of evolution, in mammalian cells. Death ligands are cytokines and able to bind to the death receptors and start the apoptosis program. Death receptors, -ligands and their signal transmitting pathway have the potential to initiate "self-killing" or to order another cell to kill itself ("instructive" apoptosis).

This pathway is regulated by the "death receptors" of the TNF-receptor family: FAS (CD95/APO-1), DR4 (TNF-related apoptosis-inducing ligand receptor 1, TRAIL-R1) and DR5 (TRAIL-R2). Death receptors are transmembrane glycoproteins, with a death domain in the intracytoplasmic part, to transmit the signal. Death ligands are also transmembrane proteins liberated from the surface mainly by matrix metalloproteases. The distribution of the receptors and ligands are different: Receptors are present in most tissues, while their ligand are expressed only in certain cell types or upon a stress stimulus and have systemic toxicity. On the contrary, only few normal tissue is sensitive to TRAIL.

When FAS is activated by FASL, caspase-8 will be recruited via an adapter molecule FADD (FAS-associated death domain) to make a death-inducing signalling complex (DISC). Here, caspase-8 is activated and can subsequently activate caspase cascade leading to apoptosis. In certain cells the DISC-mediated apoptotic signal, i.e. caspase-8 activity is sufficient to fully fulfill the cascade (type I cells), while in others the contribution of mitochondrial pathway by caspase-8 cleaved BID is required (type II cells) to amplify the apoptotic process. FADD has a unique domain (death effector domain, DAD, belonging to the caspase recruiting protein family, CARD). The signal complex can contain other proteins as well, supporting or inhibiting death receptors (e.g. silencer of death domain, SODD; c-FLIP (c: cellular, FADD-like interleukin-1-β-converting enzyme-inhibitory protein).

All death receptors triggers apoptosis with cytosolic death domain-containing adapter protein FADD and the FADD interacting caspase-8. The death receptor-FADD-caspase-8 pathway is called extrinsic pathway. FAS and TRAIL receptors form a membrane-associated complex also with FADD and caspase-8 named death inducing signaling complex (DISC). TNFR1 makes a complex with RIP and TRAF2 at the plasma membrane and send signals via the anti-apoptotic NF-kB pathway, whereas TNFR1 should be internalized to form pro-apoptotic complex containing TRADD, FADD and caspase-8. In these signaling complexes FADD mediates the assembly of procaspase-8 dimers, which are maturated by autoproteolysis. Mature active caspase-8 can cleave and activate effector caspases, as caspase-3 and -7. Furthermore, caspase-8 can cleave proapoptotic BCL-2 family member BID producing a truncated form (tBID). tBID is translocated to mitochondria and activate the intrinsic apoptotic pathway through the conformational change of BAX and BAK. In the TNFR1 initiated complex TRADD can also mediate recruitment of TRAF2, which interferes with TNFR1 induced apoptosis partly by inhibiting caspase-8, partly by the activation of NF-kB. TNFR2, a non-death domain containing member of the family, has a proapoptotic function, recruiting TRAF2/IAP complex, triggering its proteasomal degradation, and so liberating TNFR1 from the inhibitory action of this complex.

Death receptor-mediated apoptosis can be inhibited at different level. Proteolitic cleavage or alternative mRNA can produce soluble forms of death receptors, which can bind to the death ligands outside the cell as a protection against membrane-bound receptor

activation by consuming the available specific ligands. Furthermore FASL and TRAIL can bind to decoy receptors (DcR3 for FASL and DcR1 and 2 for TRAIL), however, these receptors have no intracellular domains necessary for DISC formation, therefore they can bind the ligand, but are unable to transmit the signal. Another, remarkable inhibitor is c-FLIP, which binds to the DISC and inhibits caspase-8 activation.

Mitochondrial (intrinsic) pathway

The mitochondrial pathway can be induced, e.g. by extra- or intracellular stress (hypoxia, DNA-damage, insufficient amount of growth factors, etc.). Inducing factor could be the p53 activated by DNA damage, ceramid, reactive oxygen species, increased intracellular Ca^{++} concentration, caused by the damage of different cell membranes due to metabolic failures. Some agents, as nitrogen-monoxid (NO), may have pro- and anti-apoptotic effect as well.

In most cases of PCD the "point of no return" is permeabilization of the outer mitochondrial membrane leading to the release of toxic proteins. The membrane permeability is controlled by pro-apoptotic (e.g. BAX, BAK, BAD, BID, BIM, BMF, NOXA) and anti-apoptotic (e.g. BCL-2, BCL-XL) members of the BCL-2 family, performing or preventing heterodimerization of proapoptotic members. BCL-2 family members are located into membranes of different cell organelles (mitochondrium, endoplasmic reticulum, even in the nucleus). Some of them (e.g. BID, BAX, BAD) sit in the cytoplasm and signal is required to translocate them into a membrane, usually into the outer membrane of the mitochondrium. All family members contain at least one of the BCL-2-homologous domains (BH1-BH4). BH3 is responsible for the anti- or proapoptotic behaviour, and certain pro-apoptotic members contain only BH3 domain. Functionally VDAC (voltage dependent anionic channel) and ANT (adeninnucleotide translocator) are active participants of the permeability control.

Permeabilization can also be resulted by the opening of a permeability transition pore in the inner mitochondrial membrane, which allows the accumulation of water and small molecules (up to 1.5 kDa), leading to the swelling of the intermembrane space and the rupture of the outer membrane. Among the released proteins cytochrome c, an important component of the respiratory chain, form a complex ("apoptosome") with ATP, APAF-1 (apoptotic protease activating factor 1) and caspase-9. Two other mitochondrial proteins, Smac/DIABLO and OMI/HtrA2 can inhibit the catalytic function of the complex. OMI/HtrA2 in this way takes part in caspase-dependent apoptosis, but using its protease activity can act as an effector in the necrosis-like PCD. Another released protein, endonuclease G, can also contribute to both caspase-dependent and -independent PCD. Apoptosis inducing factor (AIF) is normally performs an oxidoreductase function in the intermembrane space, but becomes an active cell killer when it is released to the cytosol. It is translocated to the nucleus, and helps, probably with endonuclease G, chromatin condensation and high molecular weight (50 kb) DNA fragmentation. This activity is regulated by the antiapoptotic heat shock protein 70 (HSP70). Several observation support that AIF activity can be triggered [e.g. with overactivation of poly(ADP-ribose)polymerase I by excessive calcium influx, or in pneumococcus induced apoptosis] independently from caspases. It is thought that AIF could be a safeguard death promoter in cancer cells with faulty caspase activation (Joseph et al. 2002).

Caspases

Apoptosis in mammalian cells is mediated by a family of cystein proteases, called caspases. As part of the apoptosis control, caspases are initially expressed as inactive precursors, procaspases. When initiator caspases, as caspase-8 and caspase-9, are activated by oligomerization, they cleave the precursor forms of effector caspases, such as caspase-3, caspase-6, caspase-7. Activated effector caspases further cleave various cellular substrates (e.g. DNase from the ICAD/CAD complex, or even more procaspases), resulting in the well-known biochemical and morphological changes (e.g. chromatin condensation, nuclear fragmentation, DNA laddering) that are associated with apoptosis.

Family members of IAP (inhibitors of apoptosis) (c-IAP1, c-IAP2, X-IAP, survivin) can bind directly to caspases (Degterev et al. 2003; Wang et al. 2004; Leist and Jaattela 2001) and inhibit their activity. Anti-apoptotic action requires at least one BIR (baculovirus IAP domain). IAPs are negatively regulated by proteins from the mitochondrium, Smac/DIABLO and

OMI/HtrA2 using ubiquitinization and proteasome degradation. Heat-shock proteins can also interfere with apoptosis: HSP27 blocks DAXX or cytochrome c, HSP 70 the binding of APAF-1 and procaspase-9, HSP90 inhibits the oligomerization of APAF-1, and the latter as a chaperon protects the phosphorylated form of anti-apoptotic AKT.

It should be mentioned that granzym B, a powerful protease, the product of the cytotoxic cells and injected into the target cell through a pore-forming unit (porin), also can switch on the apoptotic machinery.

Non-apoptotic pathways (caspase-independent cell death)

In recent years, it has become evident that although caspases are key players in the apoptotic process, the caspase activation is not the only determinant of decisions in programmed cell death. These alternative caspase-independent models include autophagy, paraptosis, mitotic catastrophe, and apoptosis-like or necrosis-like PCD, as well as senesence (Bröker et al. 2005). These potentials protect the organism against unwanted cells when caspase-mediated pathways fail, but can also be triggered by various stimuli.

In a descriptive model cells are divided into four subclasses, based on their nuclear morphology (Leist and Jaattela 2001). In apoptosis the chromatin is condensated in often globular or crescent shaped compact figures (stage II chromatin condensation), while in apoptosis-like cell death the condensation is less compact (stage I). In necrosis-like cell death there is no chromatin condensation, but at best, clustering to loose speckles, whereas necrosis shows cell membrane rupture with cytoplasmic swelling.

Autophagy (also called type II cell death to distinguish it from apoptosis, the type I cell death) eliminate long-lived proteins and organelles by sequestering them into multimembrane autophagic vesicles to a subsequent degradation by the lysosomal system. There is evidence that lysosomal degradation of organelles is required for cellular remodelling due to differentiation, stress or damage by cytotoxins. In experimental systems the breakdown of the autophagy process, together with the heterogenous disruption of the autophagy gene (Beclin 1) may support carcinogenesis (Qu et al. 2003). AKT can also inhibit the autophagy induced proteolysis. On the contrary, tumor cells may need autophagy to survive hostile microenvironment and cytotoxic therapies. Today, the exact role of autophagy in mammalian cell death is still only partially understood.

Paraptosis is best described by cytoplasmic vacuolation due to the swelling of mitochondria and the endoplasmic reticulum. It is mediated by mitogen-activated protein kinases and switched on by TAJ/TROY (member of TNF receptor family) and insulin-like growth factor I receptor. Paraptosis but not apoptosis can be inhibited by AIP1/ALIX, which can interact with ALG-2 (calcium-binding death-related protein) (Wang et al. 2004; Sperandio et al. 2004). This also suggests that paraptosis is different from apoptosis. It is still unanswered how far autophagy and paraptosis represent independent types of programmed cell death.

Mitotic catastrophe is caused by mitotic failure due to the faulty checkpoints with the threatening possibility of the appearance aneuploid cells. Morphologically mitotic catastrophe is associated with the formation of multinucleate, giant cells that contain uncondensed chromosomes, and is distinct from apoptosis, necrosis or autophagy. It can be mainly triggered by DNA damage as well as agents stabilizing or destabilizing the microtubules (Castedo et al. 2004). However, it is still debated whether mitotic catastrophe is a fully caspase-independent type of programmed cell death. Many proteins take part in the regulation of G2 and mitotic checkpoints, e.g. CDK1 (inhibited by WEE1, MYT, and stimulated by CDC25C). If the checkpoint is not working properly, the cell can start a premature mitosis (aberrant mitosis), before DNA-synthesis is completed or DNA-damage is repaired. The same is the consequence of the damage of microtubules or mitotic spindle, caused, e.g. by cytotoxic agents (paclitaxel induces an abnormal metaphase in which the sister chromatids fail to segregate properly). All mitotic regulators, e.g. PLK (polo-like kinase), NIMA (never in mitosis, gene A9, Aurora family members, BUB1, can participate in mitotic catastrophe. It seems that survivin maintains the spindle checkpoint and prevent of accumulation of stressed cells that would otherwise undergo aberrant mitosis (Carvalho et al. 2003).

Premature senesence (senesence: type of "living cell death") could also be a way to dye, and in that sense inhibits tumor development. Cells loosing their

proliferative activity enter a form of permanent cell cycle arrest (replicative senesence). Senescent cells are metabolically active but non-dividing and show an increase in size. These cells express senesence-associated β-galactosidase and this process is generally p53-dependent. Other cell cycle inhibitors are also activated, as CDKNA1 gene (coding p21waf), CDKNA2 gene (p16), and retinoblastoma gene. One of the main duties of senesence program is to suppress tumorigenesis. Therefore, to turn the tumor cells from proliferative to senescent phase could be a strategy for therapy.

Necrosis is usually the result of extensive cellular trauma caused by pathological conditions, as trauma, infection, ischemia, etc. The normal physiological pathways that essential to maintain cellular homeostasis (regulation of ion transport, pH balance, energy production) are severely damaged. The rupture of the cell membrane and the release of intracellular components into the microenvironment trigger inflammatory response (which is absent in apoptotic cell death). The molecular program for necrosis is still a mystery.

Organelles in PCD

There is an attempt to classify caspase-independent cell death according to the organelles (mitochondria, lysosomes, endoplasmic reticulum, plasma membrane) involved. The signals from the different cellular organelles are linked and may effect both downstream and upstream of each other.

Mitochondria

See above as apoptotic (intrinsic) pathway.

Lysosomes

Recently, it has become evident that lysosomal proteins (e.g. cathepsin B) has an active role in cell death induced by several stimuli, including oxidative stress, TNF-α, bile salts or chemotherapeutic drugs. It seems that partial, selective permeabilization of the lysosomal membrane triggers apoptotic-like PCD, massive damage of the lysosomes results in unregulated necrosis. Several mechanisms are described to achieve this selective permeabilization or to control it (e.g. reactive oxygen species, proapoptotic members of BCL-2 family, HSP70). Cystein protease cathepsin B and L and the asparatic protease cathepsin D are the most abundant lysosomal proteases, and cathepsin B and D seem to have the most prominent role in apoptotic- and necrotic-like PCD (Guicciardi et al. 2004). In cell lines cathepsin B can act as effector protease downstream of caspases and execute cell death independently from apoptotic machinery. Another set of data showed that lysosomal proteases promote cell death indirectly by triggering mitochondrial dysfunction and release of mitochondrial proteins, moreover they directly cleave and activate caspases. It seems that lysosomal proteases induce PCD via multiple pathways that may overlap with the traditional mediators of apoptosis. These mediators may vary depending on the cell type and the death stimulus.

Endoplasmic reticulum

ER in case of cellular stress can maintain homeostasis by withholding protein synthesis and metabolism. If the damage to the ER is too severe unfolded protein response or release of calcium into the cytoplasm can initiate PCD (Breckenridge et al. 2003). This, or BCL-2 family member BiM, leads to the activation of caspase-12, which in its inactive state is localized at the cytosolic face of the ER. Activated caspase-12 triggers downstream caspases. However, ER stress can also induce permeabilization of the mitochondrial membrane and activate mitochondrial death proteins. BCL-2 family members and the shifts in cytoplasmic calcium ensure cross talks between ER and mitochondria (Annis et al. 2004). ER stress via intracellular calcium influx can activate calpains (calcium-activated neutral proteases), which act downstream of caspases. Calpains are controlled by calpastatin, what in turn inactivated by calpain- or caspase-mediated cleavage. Different experiments showed the cooperation of calpains and caspases, as well as calpains and cathepsins.

Cell death in tumor development and progression

Cancer cells are among the main enemies in a multicellular organism, therefore they must be eliminated. If the cell death programs fail to fulfill this task, the neoplastic cells will proliferate and accumulate with further geno- and phenotypic changes allowing invasion

and metastatization. Essentially, the loss of PCD provides a survival and growth advantage.

Antiapoptotic effect of virus proteins

It is trivial that viruses can contribute to tumorigenesis. Besides integration into the host genome, certain viral proteins have anti-apoptotic effect. It is the interest of the virus to keep the infected cell alive, at least for a while, and this function can support the survival of cells with DNA-damage. Here only few examples are mentioned. KSHV can inactivate p53 by LANA (latency associated nuclear antigen of KSHV), and caspase-8 by producing v-FLIP. EBV maintain the latent infection by the inhibition of a protein kinase (by EBER) and inducing BCL-2 (by LMP1). HHV8 release a BCL-2 homologue protein, HPV E6 blocks p53, HTLV Tax protein activates NF-kB and the FASL inactivating decoy receptor (DcR3).

Arrest in the cell cycle versus apoptosis

Along the carcinogenesis or after cytotoxic therapy there is a critical balance for the damaged cells between cell cycle arrest (for DNA repair and survival) and cell death. DNA damage activates kinases as ATM (ataxia-telangiectasia mutated), ATR (ATM and Rad-3 related) and DNA-PK (DNA-dependent protein kinase), which can directly or indirectly phosphorylate p53. The negative regulator of this step is MDM-2, which holds p53 for proteasome degradation. P53 has many functions as transcription factor: one of the most important is either to stop the cell is cycle (up-regulating p21waf1, GADD45) or promote its apoptosis (up-regulating, e.g. BAX, NOXA, TRAIL-R2, FAS) (Schuler and Green 2001; Petak et al. 2001). There are several models to explain how cells choose between p53-mediated cell cycle arrest and apoptosis, emphasizing the role of the extent of DNA damage, the cell types with different sets of genes available for p53, or the presence of transcriptional co-factors. The gene encoding p53, TP53, is the most frequently mutated gene in human cancers (~50%). Furthermore, the activity of wild-type p53 may be compromised by the loss of positive regulators (as p14arf), overactivation of negative regulators (as AKT), or by the mutant p53, MDM2, or viral proteins. Activity of p53 is further modulated by different regulatory proteins, e.g. ASSP, (apoptosis stimulating protein of p53). (The lack of ASSP is responsible for the inactivity of wild-type p53 in about 70% of breast cancers.) Huge amount of in vitro data support that cytotoxic agents are more effective in tumors with wild-type p53, however, the clinical relevance of p53 status in drug sensitivity (or resistance) remains controversial, therefore, need to be determined.

Besides p53, BRCA1 has also been implicated in the regulation of apoptosis. BRCA1 may function as a sensor of cell stress (DNA-damage). A recent study found that BRCA1 acts differently in breast cancer cells depending on the cytotoxic agent: increased the sensitivity to anti-microtubule agents (paclitaxel, vinorelbine), but inhibited apoptosis when etoposide or cisplatin were given (Quinn et al. 2003). This suggests BRCA1 could be a useful predictive marker to therapeutic response.

Overexpression of anti-apoptotic proteins

In many human tumors the anti-apoptotic regulators are frequently overexpressed serving the survival of tumor cells (Longley and Johnston 2005). The classical example is the constitutively active BCL-2 in follicular lymphomas as a consequence of (Sjostrom et al. 2002; Nicholson and Anderson 2002) translocation or amplification of BCL-2. BCL-2 overexpression has also ben shown to induce MYC-dependent lymphomagenesis. In this case the proliferative potential of MYC is preserved, indicating that BCL-2 counteract MYC-induced apoptosis. The clinical significance of the overexpression of decoy receptors (e.g. in colorectal cancers) is still not known, but it may influence TRAIL effectiveness. In vitro studies found that FLIP is overexpressed of in various cell lines (melanoma, or Sternberg–Reed cells in Hodgkin-lymphoma). Survivin overexpression has been found in a wide range of human tumors in vivo and was identified as being among the most common transcripts up-regulated in cancer compared with normal tissues. The finding, that clinically the low levels of survivin correlated with better response to therapy, suggested that it may be a useful clinical marker. A firm answer is still missing. In MALT lymphoma (MALT: mucosa associated lymphoid tissue) the translocation involves cIAP2 and the MLT/MALT1 genes, producing the caspase-inhibitor cIAP2 and a paracaspase (Guicciardi et al. 2004; Sjostrom et al. 2002). (It is interesting that lymphomas carrying this

translocation are resistant to anti-Helicobacter pylori therapy.) Melanomas overexpressing ML-IAP proved to be more resistant against chemotherapy-induced apoptosis, than the non-producers.

Defects in autophagic pathway of protein degradation might also be connected to cancer. For example, autophagy is partly controlled by the PI3K pathway, and constitutive activation of PI3K signalling is common in human cancers. Such activation could inhibit both apoptotic and autophagic cell death. The best evidence that impaired autophagy is connected to tumorigenesis comes from studies of BECN1 gene, encoding beclin-1. In mammalian cells beclin-1 interacts with PI3K and take part in the induction of autophagy in response to starvation. The gene (17q21) is monoallelically deleted in many ovarian, breast and prostate cancers.

Decreased production of pro-apoptotic molecules

One of the most prominent pro-apoptotic proteins is BAX. In many human tumors BAX gene has loss-of-function mutations or shifts in the reading frame. In metastatic melanoma the tumor cells can escape apoptosis by inactivate APAF-1 gene, partly by deletion or by promoter methylation. The strategy is similar in neuroblastoma, where the caspase-8 gene could be deleted or hypermethylated. In about 80% of small-cell lung cancer the procaspase-8 activity is very low. FAS gene mutation in the death domain region or deletion resulting in truncated receptor were observed in multiple myeloma and T-cell leukemia. FAS expression was decreased or absent in different tumor types (e.g. hepatocellular cc, colon cc, melanoma) compared to the relevant normal counterpart. Deletions and mutations can occur in TRAIL receptors as well. In head and neck tumors and in NSCLC the deletion in the 8p21–22 region involved TRAILR2. Mutations changed the death domain (similarly to FAS). In certain tumors the decreased production of a pro-apoptotic protein (XAF1, XIAP-associated factor 1) led to the failure antagonizing anti-apoptotic XIAP.

Pro-survival signalling (apoptosis is blocked by the overproduction of pro-survival signals)

Tyrosine kinase receptors (e.g. EGFR family, including EGFR/ERBB1/HER1, HER2/ERBB2/NEU, HER3/ ERBB3, HER4/ERBB4) can influence efficacy of cytotoxic agents by regulating anti-apoptotic signalling. Receptor dimerization results in cross phosphorylation of the key tyrosine residues in the cytoplasmic domain, which offers docking sites for downstream signal transducers. Such signals are generated along the phosphatydilinositol-3-kinase (PI3K)/AKT (protein kinase B, PKB) pathway and the STAT (signal transducers and activators of transcription) pathway.

Activation of PI3K can lead to phosphatidylinositol 3,4,5-triphosphate, which translocates AKT to the plasma membrane, where it is phosphorylated by 3-phosphoinositide-dependent kinase I (PDK-1). AKT further activates and regulates the function of many cellular proteins, including key regulators of apoptosis, e.g. BAD (Longley and Johnston 2005). Phosphorylation of BAD stops its inhibition by anti-apoptotic BCL-xL, furthermore, can inhibit caspase-9 and activate transcription factor –kB (NF-kB). As a whole, activated BAD will work against apoptosis. AKT also effect p53 activity as it promotes phosphorylation and translocation of MDM-2 to the nucleus, where it down-regulates p53 expression. AKT is frequently activated in human cancers due to mutations or amplifications of upstream regulators. In vitro studies demonstrated that inhibiting the PI3K/AKT pathway increases the cytotoxic effects of different chemotherapeutic agents (Nguyen et al. 2004). Recently, in NSCLC patients the phospho-AKT-positive tumors showed better clinical response following gefitinib therapy, than the negative tumors (Cappuzzo et al. 2004).

STAT proteins carry cytoplasmic signals from growth factor and cytokine receptors to the nucleus and activate transcription of various target genes. Recruitment of STATs to the activated receptors and their phosphorylation is usually mediated by a receptor-associated tyrosine kinase of the Janus kinase (JAK) family (Yu and Jove 2004). Persistent activation of STATs, especially STAT3 and STAT5 is a frequent finding in human cancers due to the constitutive activation of upstream tyrosine kinase (similarly to AKT activation). Among others STAT3 and STAT5 can regulate the expression of different anti-apoptotic proteins (e.g. BCL-xL, Mcl-1, BCL-2, survivin).

NF-kB is a key player in oncogenesis by promoting proliferation and inhibiting apoptosis. NF-kB is not a single gene but a family of closely related transcription factor genes: NF-kB1 (p50/p105), NF-kB2 (p52/p100), RELA (p65), c-REL, RELB, which produce

seven of proteins with REL homology domain mediating their dimerization, interaction with specific inhibitors and DNA binding. NF-kB dimers are mainly cytoplasmic kept in a transcriptionally inactive form by IkBs. Upon phsphorylation by IkB kinases (IKKs), IkB undergo proteasome-dependent degradation and so the NF-kB is activated, translocated into the nucleus (Lin and Karin 2003; Dolcet et al. 2005). In most instances NF-kB has an anti-apoptotic effect by up-regulating the expression of various anti-apoptotic proteins: e.g. IAPs, TNF-receptor associated factors (TRAFs), c-FLIP, BCL-2, BFL-1 (A1), BCL-X_L or down-regulate pro-apoptotics, e.g. PTEN. (PTEN suppresses the pro-survival PI3K/AKT pathway.) Whether inducibly or constitutively activated, NF-kB seems to be a critical factor in drug resistance. In vitro studies demonstrated that inhibition of NF-kB can sensitize cancer cells to chemotherapy-induced apoptosis. NF-kB is probably a major target for proteasome inhibitors, as proteasome inhibition prevents degradation of IkB, blocking NF-kB nuclear translocation (Cusack 2003). In contrast, recent evidences support that certain dimers of NF-kB could have pro-apoptotic effect, therefore it is possible, that NF-kB exerts dual function, either activator or inhibitor of apoptotic cell death, depending on the levels of RELA and c-REL.

Therapeutic induction of tumor-cell death

Development of cancer is partly due to the failure to eliminate damaged cells by the apoptotic program, either because the program is faulty, or the overproduction of survival factors inhibit the function of otherwise existing program. Since the cancer cell's susceptibility to apoptosis is severely compromised, other forms of cell killing become more important in a response to DNA-damaging (cytotoxic) agent. Success of chemotherapy is largely dependent on the ability of cytotoxic agents to induce cell death. Majority of agents trigger mitochondria pathway, but the death receptors are also involved. Recent evidences suggest that certain forms of chemotherapy induced cell death are more apoptosis-like/necrosis-like PCD than apoptosis or necrosis. It is a question, what will determine the form of cell death induced by a particular chemotherapeutic agent or radiotherapy? Presumably it depends on the context, including cell type, the type of DNA damage to which the cell is exposed or the dose of the agent (Bröker et al. 2005; Abend 2003).

There are normal tissues (e.g. thymocytes, spermatogonia, hair-follicle cells, stem cells of the small intestine and bone marrow and tissues of the developing embryos) which are sensitive to the induction of apoptosis by DNA-damaging agents. Tumors originated from these tissues (e.g. lymphomas, some hematological tumors) are also sensitive to DNA-damaging cytotoxic agents. The induced apoptosis is p53-dependent, therefore if this pathway is inhibited (e.g. p53 mutation, BCL-2 overexpression), the sensitivity to the treatment will decrease (Gudkov and Komarova 2003). In solid tumors, however, the main reason for cell death is not the induction of apoptosis. Although it can happen, but mitotic catastrophe or senesence-like irreversible growth arrest are more frequent. It has been shown in vitro and in vivo that changing the sensitivity to apoptosis will not influence the overall sensitivity of these tumor cells to cytotoxic treatment. One can conclude that the view which made apoptosis synonymous to "cell killing" and any manipulation that altered the level of apoptosis was considered a way to reach a similar change in the overall cell killing, is probably wrong. It would be a consequence that other types of cell death should be involved and also that the resistance to therapy is not explained by inadequate apoptosis program. Nevertheless, considering the importance of this issue, more preclinical and clinical data are needed to make firm, clinically useful statements.

Induction of pro-apoptotic effect

Therapy by targeting or inducing death receptors

Targeting and activation of death receptors to induce apoptosis as a therapeutic goal has received enormous interest over the past decade, when it was proved that death receptor ligands and cytotoxic agents operate via the same mechanism, by inducing PCD, often in a cooperative manner. The problems for protein-based and virus-based gene therapy are similar: improving delivery and tumor-directed actions, preventing off-target action, and preventing or reducing immunogenicity of the used therapeutics (Wajant et al. 2005).

FAS/FASL The loss of the death-receptor expression have been reversed by chemotherapy and the FAS-negative tumor cells expressed FAS. Such induction

is usually p53-dependent, but in p53-mutated tumors interferon-γ can make the stimulation of FAS expression. This explains how interferon-γ increases the therapeutic effect of 5-fluorouracil (Schwartzberg et al. 2002).

It would be a good idea to give death-ligand, if the tumor cells have death-receptor. Recombinant death ligands and agonistic antibodies worked effectively in vitro inducing and/or supporting chemotherapy-induced apoptosis. However, activation of FAS as a therapeutic action was challenged due to the acute hepatotoxicity of FAS-agonistic antibodies. Therefore, the principal aim is to avoid the unwanted side-effects. The soluble, trimeric FASL has no bioactivity per se, but becomes activated, when immobilized by binding to the extracellular matrix. Immobilization of FASL was achieved with a trimeric fusion protein, consisting an antibody domain-recognizing tumor stroma marker fibroblast activation protein (FAP) and soluble FASL. The anti-FAP-FASL fusion protein showed no hepatotoxicity or systemic toxic effect in mice. It is a question how active is this fusion protein in the local activation of FAS. Another possibility to circumvent systemic side-effects in gene therapy is the use adenovirus vector for targeting with inducible and/or tissue-specific promoters. The usefulness of this method in vivo has not been shown yet. Limitations of the broader clinical use are the inefficient delivery to the target, strong immunogenicity and inflammatory response. Intratumoral application can reduce these obstacles (Moon et al. 2003). Further possibility is to use FAS-specific antibodies, that, for poorly understood reasons, have tissue-restricted agonistic properties. Future studies will show whether such antibodies can make clinical use.

TRAIL Nowadays TRAIL is probably is the best candidate of a death ligand for systemic administration. In normal (non-transformed) cells apoptosis induction by soluble TRAIL is prevented by an unknown mechanism what is missing or less active in tumor cells. It seems that aggregated (cross-linked) TRAIL variants are more efficient than the non-aggregated TRAIL, but serious side effects can be expected. The task is to ensure strictly tumor localized action of such reagents. Trimeric single chain antibody-TRAIL fusion proteins (immobilized on the cell surface as FASL, see above) activate TRAILR2 more efficiently target antigen expressing than non-expressing cells, although they are equally TRAIL sensitive. The potential systemic toxicity of trimeric TRAIL variants when combined with cytotoxic agents is only begun to study in preclinical models. For example, proteasome inhibitors, which are often used to sensitize tumor cells for apoptotic action of TRAIL, also makes non-transformed cells sensitive to TRAIL-induced apoptosis (Leverkus et al. 2003). To fully activate the TRAIL receptors on tumor cells may require to stimulate with transmembrane TRAIL and/or to sensitize the cells for death receptor-induced apoptosis, e.g. by cytotoxic drugs. It is important to avoid hepatotoxicity and systemic side effects. In animal model the local administration of transmembrane TRAIL encoding adenovirus produced strong anti-tumoral effect. Studies are at early phase with TRAILR-specific antibodies.

In preclinical studies TRAIL proved to be effective, e.g. in glioma and colon cc, while in breast cancer the result was dependent on the chemotherapeutic drug used in the combination: effect of doxorubicin and 5-fluorouracil was enhanced by TRAIL, but not with melphalan, methotrexat or paclitaxel. In certain tumor cell type the contribution of the mitochondrial apoptotic pathway was necessary, which predict that the TRAIL will not be effective in all tumor types.

Other attempts

A peculiar way to increase the probability of apoptosis induction is the artificial enhancement of mitochondrial membrane permeability (e.g. by lodinamine, arsenit, betulinic acid, CD437, amphipatic cationic a-helical peptide), which would promote the escape the pro-apoptotic molecules, if the conventional chemotherapy fails.

Replacement of missing or inactive pro-apoptotic molecule is still a challenge. In experimental systems the introduction of BAX gene using an Ad-DF3-BAX vector-gene complex destroyed almost all implanted tumor cells. Another option is to decrease the methylation (e.g. by 5-aza-deoxy-cytidine), when the promoter region of a pro-apoptotic gene (e.g. caspase-8) is hypermethylated.

Inhibition of antiapoptotic effect

Antisense therapy

With the revolutionary development of high-throughput genomic, transcriptomic and proteomic technologies,

hundreds of potential therapeutic targets have been identified. Many of these gene products are not easily reached by small molecules or antibodies, and so other strategies to influence gene-expression are attractive. Known nucleotide sequences of a given gene offer the possibility to rapidly design antisense oligonucleotides (ASO) or short interfering RNA (siRNA) duplexes. The better chemical modifications of ASO increase resistance to nucleases, prolong tissue half-lives and improve scheduling. Recent clinical trials support the activity of these drugs to effectively suppress target-gene expression (Gleave and Monia 2005).

Among the most promising targets for antisense therapy are anti-apoptotic regulators overexpressed during tumor development (e.g. BCL-2, BCL-X_L, survivin, XIAP, MCL-1). G3139 (oblimersen, Genasense, Genta) is a first generation 18-mer phosphorothioate ASO, complementer to the first six codons of the initiating sequence of human BCL-2 mRNA. Promising preclinical results allowed to start clinical studies and they still continue in Phase I-III trials in different tumor types, in combination with different cytotoxic agents (Rai and Moore 2004; Chanan-Khan 2005; Chi et al. 2003). BCL-X_L is another anti-apoptotic member of BCL-2 family and in certain tumors both BCL-2 and BCL-X_L are overexpressed. A bispecific ASO targeting both molecules was recently tested as a second-generation 2 -MOIE modified ASO, and found to be a powerful inhibitor of BCL-2, BCL-X_L and MCL-1. Similarly, an ASO was developed against MCL-1, which acts as an anti-apoptotic agent, heterodimerizing with proteins known to promote apoptosis. Survivin, a member of IAP family, is expressed at a high level in various human tumor types (e.g. lung colon, pancreas, breast, prostate). Survivin antisense (LY2181308) showed pro-apoptotic activity in tumor cell line, including the sensitization of tumor cells to chemotherapy-induced apoptosis (e.g. in non-Hodgkin lymphoma) (Ansell et al. 2004). Further apoptosis-related targets for ASOs are, e.g. XIAP (AEG35156/GEM640, Aegera Therapeutics), clusterin (OGX-011, OncoGeneX Technologies), STAT3 (ISIS 345794), HSP27 (OGX-427, OncoGeneX Technologies), MDM2 (GEM240, Hybridon). As is true for clinical development of all targeted therapies, important issues include optimal biological dose, relevance in the patient population being studied, and rationales for combination strategies to yield unambiguous end points.

RNAi targeting c-FLIP dramatically sensitizes colon cancer cell lines to 5-FU, oxaliplatin and CPT-11. FLIP can inhibit the perforin–granzym B pathway. Overexpression of survivin has been shown to challenge chemotherapy-induced apoptosis in vitro, which was overruled by RNAi targeting (Nakamura et al. 2004).

Inhibition of the survival pathways, e.g. inhibition of the lipid-kinase route (PI3K-AKT-TOR) is probably an efficient indirect way to help cell death induction. It is highly possible, that this mechanism is at least partly responsible for the therapeutic effectiveness of tyrosine-kinase inhibitors, as imatinib (Gleevec) targeting BCR-ABL fusion product.

Conclusion

At the advent of targeted tumor therapy we have to learn more about those mechanisms which are mainly responsible for tumor growth and progression. Cell death is a critical cellular phenomenon, and today the monopolium of apoptosis seems to be shaken. Certain technologies of a therapy based on the stimulation of death are already known, and many of these are over the preclinical stage and entered into the clinical trials.

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