Cell survival, cell death and cell cycle pathways are interconnected: Implications for cancer therapy

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Abstract

The partial cross-utilization of molecules and pathways involved in opposing processes like cell survival, proliferation and cell death, assures that mutations within one signaling cascade will also affect the other opposite process at least to some extent, thus contributing to homeostatic regulatory circuits. This review highlights some of the connections between opposite-acting pathways. Thus, we discuss the role of cyclins in the apoptotic process, and in the regulation of cell proliferation. CDKs and their inhibitors like the INK4-family (p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c}, p19^{Ink4d}), and the Cip1/Waf1/Kip1-2-family (p21^{Cip1/Waf1}, p27^{Kip1}, p57^{Kip2}) are shown both in the context of proliferation regulators and as contributors to the apoptotic machinery. Bcl2-family members (i.e. Bcl2, Bcl-X_L, Mcl-1_L; Bax, Bok/Mtd, Bak, and Bcl-X_S; Bad, Bid, Bim_{EL}, Bmf, Mcl-1_S) are highlighted both for their apoptosis-regulating capacity and also for their effect on the cell cycle progression. The PI3-K/Akt cell survival pathway is shown as regulator of cell metabolism and cell survival, but examples are also provided where aberrant activity of the pathway may contribute to the induction of apoptosis. Myc/Mad/Max proteins are shown both as a powerful S-phase driving complex and as apoptosis-sensitizers. We also discuss multifunctional proteins like p53 and Rb (RBL1/p107, RBL2/p130) both in the context of G₁-S transition and as apoptotic triggers. Finally, we reflect on novel therapeutic approaches that would involve redirecting over-active survival and proliferation pathways towards induction of apoptosis in cancer cells.

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Abbreviations: AIF, apoptosis inducing factor; ANT, adenine nucleotide translocator; Apaf1, apoptosis activating factor 1; BH3, bcl2-homology domain 3; MDM2, mouse double minute 2; MYT1, membrane-associated and tyrosine/threonine-specific-1; VDAC, voltage-dependent anion channel

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1. General introduction: linking cell cycle, cell survival and cell death

Molecular linkages between cell death, cell survival, and cell cycle have become an object of intense research in recent years (Table 1). The standard eukaryotic cell cycle is divided into four non-overlapping phases, with DNA synthesis and mitosis occurring during S phase and M phase, respectively. These landmark events are separated by G₁ and G₂ gap phases during which mRNAs and proteins accumulate continuously.

Table 1

Examples of molecules with dual or triple functions (cell cycle/apoptosis/cell survival and/or proliferation)

Molecule	Cell cycle function	Apoptotic function	Cell survival/proliferation
Cyclins			
Cyclin A	Positive regulator of G1/S transition (Girard et al., 1991)	Promotes apoptosis (Hoang et al., 1994; Meikrantz et al., 1994)	Enhances the cell survival (Hunter and Pines, 1991)
Cyclin E	Promotes the G1 phase progression (Koff et al., 1991)	Positive role during apoptosis (Mazumder et al., 2002)	Required for cell survival and cell proliferation (Dobashi, 2005)
Cyclins D1/D2/D3	Promotes entry into G1 phase (Schwartz and Shah, 2005)	Positive and negative regulator of apoptosis (Kranenburg et al., 1996; Biliran et al., 2005)	Promotes cell survival (Hinds et al., 1994)
Cyclin B1/B2	Positive regulator of G2/M phase (Pines and Hunter, 1989)	Positively involved in apoptosis (Porter et al., 2000; Borgne et al., 2006; Huang et al., 2006)	Promotes cell survival and proliferation (Coqueret, 2003)
Cyclin G	Positively regulates G1/S phase?	Positive role in apoptosis (Okamoto and Prives, 1999)	Positively promotes cell growth (Smith et al., 1997)
CDKS			
CDK1	Positively regulates both G2/M and G1/S phases (Morgan, 1997)	Positive role in apoptosis (reviewed in Ref. Golsteyn, 2005)	Promotes cell proliferation (Wolowiec et al., 1995)
CDK2	Positively regulates G1/S phase (Morgan, 1997)	Positively involved in apoptosis (reviewed in Ref. Golsteyn, 2005)	Promotes cell proliferation but not required for cell survival (Berthet et al., 2003)
CDKIs			
INK4 family (p16 ^{Ink4a} , p15 ^{Ink4b} , p18 ^{Ink4c} , p19 ^{Ink4d})	Negatively regulates G1/S phase (Carnero and Hannon, 1998)	Negatively regulates apoptosis (Ivanchuk et al., 2001)	Inhibits cell proliferation (Sherr, 2001)
Cip/Kip family (p21 ^{Cip1/Waf1} , p27 ^{Kip1} , p57 ^{Kip2})	Negatively regulates G1/S phase (Hengst and Reed, 1998)	Positive and negative regulators of apoptosis (Gartel, 2005; Hiromura et al., 1999)	Inhibits cell proliferation (Hengst and Reed, 1998)
PI3K/Akt Pathway	Promotes cell cycle progression at G1/S and G2 phases (reviewed in Ref. Liang and Slingerland, 2003)	Mainly negative regulators of apoptosis but also shown to be positive under certain conditions (Brunet et al., 1999; Aki et al., 2003; Lee et al., 2005; Lu et al., 2006; Nimbalkar et al., 2003; Ono et al., 2004; Shack et al., 2003)	Promotes cell survival and proliferation (reviewed in Ref. Vivanco and Sawyers, 2002)
Bcl-2 family			
Bcl-2, Bcl-xL, Mcl-1, Bcl-w	Delays cell cycle entry and G1/S (Greider et al., 2002; Vairo et al., 2000)	Negatively regulates apoptosis	Enhances the cell survival (Cory and Adams, 2002)
Bax, Bad, Bid, Bok	Inhibits cell cycle progression (reviewed in Ref. Zinkel et al., 2006)	Positively regulates apoptosis (reviewed in Ref. Reed, 1997)	Inhibits the cell survival (Antonsson and Martinou, 2000)
Myc family	Promotes G1/S progression (Steiner et al., 1996; Bernard and Eilers, 2006)	Positive and negative regulator of apoptosis (Desbarats et al., 1996; Alarcon et al., 1996; Spencer and Groudine, 1991; Dong et al., 1997; Rupnow et al., 1998)	Promotes cell proliferation (Borg et al., 1992; Jenkins et al., 1997; Watson, 2006)

In G₁ phase the cell is preparing for DNA synthesis, the cell is growing and the diploid cell has 2n chromosomes (Collins and Garrett, 2005; Schafer, 1998). In the subsequent S phase, DNA duplication occurs and at the end of the phase the DNA content has reached 4n. Before cells undergo mitosis, they continue in the G₂ phase with cell growth and are thus prepared for cell division. During mitosis separation into two daughter cells occurs. Cells which are in G₀ phase (quiescence) are not actively cycling (Collins and Garrett, 2005). To avoid inappropriate cell proliferation, control mechanisms exist. The key regulator proteins which allow the transition from one cell cycle phase to another are called cyclin-dependent kinases (CDKs), a fam-

ily of serine/threonine protein kinases which are activated at specific points during cell cycle (Collins and Garrett, 2005; Vermeulen et al., 2003). Positive regulation of CDK activity occurs through association with a second subunit, the cyclin and by phosphorylation of the T-loop threonine by a CDK-activating kinase (Park and Lee, 2003). Cyclins are produced at each of these phases and form a complex with their CDK partners. The levels of activating cyclins in different stages of the cell cycle differ, whereas the CDK protein levels remain stable (Vermeulen et al., 2003). During the G₀ to S phase transition, cyclins D1, D2, D3 and C get activated. Cyclins D1, D2, and D3 bind to CDK4 and CDK6 where as cyclin C binds to CDK8. Cyclins D1, D2, D3 are the first cyclins

to be induced as the G₀ cells are stimulated to enter the cell cycle. The progression through G₁ is mediated by cyclin D isoforms and CDK2, -4, and -6 (Schwartz and Shah, 2005; Vermeulen et al., 2003). Cyclin E is associated during G₁ to S phase transition and activates CDK2. Cyclin A gets activated during the S phase transition and binds to CDK1 and CDK2. B type cyclins are present during G₂ exit and mitosis phase and are associated with CDK1 (Coqueret, 2003). G and T type cyclins are associated with CDK5 and CDK9, respectively (Johnson and Walker, 1999).

Defects in cell cycle regulation can result in cancerous growth and developmental abnormalities. Therefore, insight into mechanisms of dysregulation in cell division can provide strategies for designing novel anti-cancer agents. Cell-cycle progression is a highly organized and tightly regulated process that controls cell growth, cell proliferation, and ontogenesis and is well connected to the regulation of DNA damage repair (Panigrahi and Mai, 2005; Schwartz and Shah, 2005). CDKs, the key regulator proteins, which in connection with their positive regulators, cyclins, allow the transition from one cell cycle phase to another, are also tightly regulated by inhibitory phosphorylation and by inhibitory molecules. The inhibitory phosphorylation is mediated by the kinase Wee1 and MYT1 (Park and Lee, 2003). CDKs can also be inhibited by two classes of CDK inhibitors, the INK4 group such as p16^{Ink4a} or p15^{Ink4b} and the CIP/KIP class such as p21^{waf1} or p27^{kip1} (Park and Lee, 2003; Grant and Roberts, 2003; Schwartz and Shah, 2005).

Each phase of the cell cycle contains checkpoints that allow the arrest of the cell cycle progression and activation of repair mechanisms. After passing these checkpoints cells are irreversibly committed to the next phase (Park and Lee, 2003). DNA damage and/or malfunction of other critical organelles or structures (e.g. faulty mitotic spindle) can activate the cell cycle arrest and even the apoptotic cascade, leading to cell death (Rowinsky, 2005; Siegel, 2006). Thus, the apoptotic machinery is an essential element of cell cycle checkpoints, protecting the integrity of multicellular organisms and allowing for selective removal of unwanted or damaged cells (Khosravi-Far and Esposti, 2004; Los and Gibson, 2005; Los et al., 1999). Apoptosis also plays an important role in the embryonic development, during the regulation of the immune response and in tissue homeostasis (Booy et al., 2005; Khosravi-Far and Esposti, 2004; Okada and Mak, 2004; Oliver and Vallette, 2005). The induction of programmed cell death occurs via two major pathways: the death receptor-dependent (extrinsic) pathway through TNF-family ligands, or via the mitochondrial (intrinsic) pathway induced by different factors such as UV radiation, chemotherapeutics, free radicals or DNA damage (Brouckaert et al., 2005; Los et al., 1995; Maddika et al., 2006). The induction of apoptosis leads to cell blebbing, exposure of phosphatidylserine at the cell surface, reduction of cell size, shrinkage of the cell core, DNA condensation and the formation of apoptotic bodies (Darzynkiewicz et al., 1997; Khosravi-Far and Esposti, 2004).

The death-receptor pathway is activated through the binding of cytokine ligands to receptors of the TNF-superfamily such as CD95/Apo-1/Fas, or TRAIL (Blagosklonny and Darzynkiewicz, 2005; Okada and Mak, 2004; Wesselborg and Lauber, 2005). The receptors contain different death effector domains (DEDs). Upon ligand binding, the receptors aggregate and form membrane-bound signaling complexes (Brouckaert et al., 2005). These are responsible for the recruitment of several molecules of the initiator pro-caspase-8 through adaptor molecules such as Fas-associated death domain (FADD). This event results in the formation of the death-induced signaling complex (DISC), activation of caspase-8 (cysteine-specific protease) and triggering the proteolytic caspase cascade (Brouckaert et al., 2005). Alternatively active caspase-8 can cleave the Bcl2-homology domain 3 (BH3) of the protein BID (BH3-interacting-domain death agonist) into the active form tBid (truncated BID) which provides a cross-talk between the extrinsic (death receptor-activated) and intrinsic (mitochondrial) pathway (Khosravi-Far and Esposti, 2004; Siegel, 2006). Cleaved Bid acquires a strong propensity to mitochondria and promotes the mitochondrial permeability transition (Hengartner, 2000). The mitochondrial pathway can also be triggered by various intracellular and extracellular stress signals which result in activation of pro-apoptotic proteins such as Bcl2-associated X protein (Bax) and Bcl2 antagonist/killer (Bak), or inactivation of anti-apoptotic Bcl2 family members such as Bcl2 or Bcl-x_L (Brouckaert et al., 2005; Finkel, 2001). Also, members of the Bcl2 family such as Bcl2, Bax and Bak may localize to the ER. Through the mitochondria-ER connection a further important control mechanism of apoptosis exists that either rely on caspase-2 as the apical caspase or connect to the mitochondrial death pathway over cellular-stress-signaling mechanisms (Kim et al., 2006). Anti-apoptotic proteins can be blocked by binding with BH3-only proteins such as Bad and Bim (Huang, 2000; Mendoza et al., 2005). Further proapoptotic members of the third class of Bcl2 family proteins are Bid, Noxa and Puma, which require Bax and Bak to mediate cell death. Bad and Bik cannot directly activate Bak and Bax, but act as sensitizers through binding to Bcl2 and Bcl-x_L (Kim et al., 2006). Bad-like BH3 domains are able to dissociate Bax and Bak from Bcl2 and Bcl-x_L. Activation of the dissociated pro-apoptotic molecules occurs by Bid-like activators (Kim et al., 2006). As a result of activation/inactivation of Bcl2 family proteins, changes in the mitochondrial membrane lead to the dissipation of inner membrane potential and the permeabilization of the outer mitochondrial membrane which in turn induce the release of various proapoptotic proteins such as cytochrome c, Smac, AIF (apoptosis inducing factor), HtrA2, and Endo-G (Endonuclease G) (Barczyk et al., 2005; Brouckaert et al., 2005; Hengartner, 2000).

p53 has tumor-suppressor activity and promotes the expression of several genes involved in apoptosis (Kim et al., 2006; Rowinsky, 2005). Induction of p53-dependent apopto-

sis proceeds through the downstream release of cytochrome c from mitochondria. There are various models for the opening of the permeability transition pore which allows the release of the apoptogenic proteins (Rowinsky, 2005). One possibility is that oligomerized Bak or Bax generate pores sufficient for release of the factors (Antonsson and Martinou, 2000). The proapoptotic members might be able to form complexes with mitochondrial membrane proteins such as VDAC or ANT to build pores (Loeffler and Kroemer, 2000). Further explanations are that pores are regulated by misfolded proteins and chaperones or that matrix swelling causes distortion of cristae structure and rupture of outer mitochondrial membrane (Kim et al., 2006). Once released from mitochondria, cytochrome c is bound by Apaf-1 (apoptotic protease activating factor-1) via the WD40 domain and Apaf-1 becomes competent to recruit pro-caspase-9 in the presence of ATP/dATP. Through a conformational change, the apoptosome is formed and activation of caspase-9 is mediated (Brouckaert et al., 2005; Philchenkov et al., 2004). Caspase-9 leads to activation of effector pro-caspases, such as caspase-3 and caspase-7 (Rowinsky, 2005). Furthermore, released apoptogenic factors are Smac/Diablo and Omi/Htr2A which can promote caspase activation by counteracting inhibitor of apoptosis (IAP)-mediated caspase inhibition (Cassens et al., 2003; Philchenkov et al., 2004). Endo-G and AIF might induce caspase-independent cell death and produce DNA fragmentation (Kim et al., 2006). The elimination of damaged and unwanted cells in organisms is a result of several apoptogenic factors and cascades. The mechanisms by which apoptosis occur, how it is counteracted, and how the process is linked to cell cycle is discussed below.

2. PI3-Kinase/Akt pathway controls cell survival, cell cycle and apoptosis

The Phosphatidylinositol 3'-kinase (PI3-K)/Akt signaling pathway is regarded as one of the key pro-survival pathways within the cell. It is activated by many types of cellular stimuli – but also by toxic insults – and regulates fundamental cellular functions such as proliferation, growth, transcription, translation, cell cycle and also apoptosis (Cantley, 2002; Vanhaesebroeck and Alessi, 2000). Notably, it has been shown that PI3-K/Akt signaling is frequently disrupted in human cancers and plays a major role not only in tumor growth but also in the response to cancer treatment (Vivanco and Sawyers, 2002). PI3-K is a heterodimer composed of a catalytic subunit (p110) and an adapter/regulatory subunit (p85). The p85 subunit bound to p110 has both a repressor and activator function depending on its conformation. The activation of PI3-K occurs via two different mechanisms, one involving the tyrosine kinase receptor dimerization and auto-phosphorylation, the other involving intracellular non-receptor tyrosine kinases. The activated PI3-K converts the lipid PI (4, 5) P2 to PI (3, 4, 5) P3 by phosphorylating

the substrate at the C3-position of the inositol ring. The serine/threonine kinases PDK1 (3'-phosphoinositide dependent kinase 1) and Akt preferentially bind to PIP3 via their PH domains, which lead to Akt activation depending on its phosphorylation at Ser473 and Thr308 sites by PDK1 (Cuevas et al., 2001; Fruman et al., 1998). Phosphatase and tensin homolog (PTEN), also known as mutated in multiple advanced cancers (MMAC1) converts PIP3 to PIP2 and acts as a negative regulator of the PI3-K pathway (Stambolic et al., 1998). PTEN is considered to be the second most mutated gene in different cancers (Ali et al., 1999). Activated Akt (or PKB) is an important and central downstream effector of the PI3-K pathway (Marte and Downward, 1997). Akt has been estimated to have nearly 900 potential substrates in the cell and modulate their different biological functions both in the cytoplasm and the nucleus. Below, we will focus only on Akt substrates related to survival, cell cycle and apoptosis.

2.1. Inhibition of apoptosis by Akt

An important function of activated PI3-K/Akt in cells is maintaining cell survival via inhibition of apoptosis. Akt regulates the process of cell survival by phosphorylating different substrates that directly or indirectly regulate the apoptotic program. Some of the important targets for Akt during this process involve the phosphorylation of BAD (a pro-apoptotic Bcl2 family member), caspase-9, FKHRL1 (a fork head transcription factor), IKK, MDM2 and cyclic AMP response binding protein. Akt mediated phosphorylation of BAD at Ser136 promotes cell survival by inhibiting its interaction with the anti-apoptotic Bcl2 family members like Bcl-xL and further preventing the cytochrome c release (Datta et al., 1997). Akt also phosphorylates caspase-9 at Ser196, thus causing a conformational change that leads to the inhibition of its proteolytic activity (Cardone et al., 1998). Akt also indirectly regulates apoptosis by phosphorylating a forkhead transcription factor FKHRL1, and thus inhibiting the transcription of its targets FasL, Bim, IGF1BP1 and Puma (Brunet et al., 1999; Guo et al., 1999; Kops et al., 1999; You et al., 2006). Akt also promotes cell survival via activation of NF- κ B by phosphorylating IKK α , which in turn phosphorylates, and triggers the degradation of I κ B, an NF- κ B inhibitor (Kane et al., 1999; Romashkova and Makarov, 1999). This leads to further elevation in transcription of NF- κ B dependent survival genes (Bcl-xL, Bcl2, c-IAPs, c-FLIP) (Catz and Johnson, 2001; Lee et al., 1999; Wang et al., 1998). Akt-mediated phosphorylation of cyclic AMP-response-element-binding protein (CREB) also enhances survival by increasing transcription of pro-survival genes like Bcl2, Mcl-1 and Akt itself (Pugazhenthii et al., 2000; Reusch and Klemm, 2002; Wang et al., 1999). Furthermore, Akt negatively regulates apoptosis by enhancing the degradation of p53 via phosphorylation, promoting nuclear localization and p53 binding of MDM2, a negative regulator of p53 (Mayo and Donner, 2001).

2.2. PI3-K/Akt pathway and cell cycle progression

In addition to its regulatory role in cell survival, the PI3-K/Akt pathway has been suggested to have a key role in cell cycle progression. The PI3-K pathway is activated during two phases of the cell cycle, at first during early G₁ phase and the second wave of PI3-K activity is during late S phase (Jones et al., 1999). Many studies have demonstrated the functional significance of PI3-K activation during G₁/S transition, but the significance of the second wave of activation is not completely known. During G₁/S phase transition, the PI3-K/Akt pathway has multiple substrates involving – but not limited to – cyclin D, Myc, p27^{kip1} and p21^{waf1}. Akt regulates the level of cyclin D1 and Myc proteins by preventing their proteasomal degradation. GSK3 β phosphorylates cyclin D1 at Thr286 (Diehl et al., 1998) and Myc at Thr58 (Gregory et al., 2003), which promotes their degradation via ubiquitin-mediated pathway. Thus, Akt by phosphorylating and inactivating its substrate GSK3 β , prevents the degradation and cytoplasmic relocation of cyclin D1 and Myc, which then facilitates the G₁/S progression. Akt also regulates the G₁/S transition by controlling the cell cycle inhibitors p27^{kip1}, p21^{waf1} both at their transcriptional and the post-translational levels. Akt enhances the degradation of p27 by the proteasome-dependent pathway by upregulating SKP2 mRNA levels. SKP2 is a key component of the SCF/SKP2 ubiquitin ligase that mediates p27 degradation in a cyclin E/CDK2 dependent phosphorylation (Hara et al., 2001; Mamillapalli et al., 2001; Pagano et al., 1995). Previously it was shown that Akt-mediated phosphorylation of p27 at thr-157 also causes the relocation of p27 to the cytoplasm, thus relieving the nuclear substrates from p27 inhibition and enhancing the cell cycle progression (Shin et al., 2002). Recently, we have shown that p27 degradation is also regulated and dependent on its direct phosphorylation by Akt at Thr157 (Maddika and Los, submitted for publication). It has also been reported that p27 is regulated at the transcriptional level by Akt activation. Akt phosphorylates FKHRL1 and inhibits its transcriptional activity, thus leading to the downregulation of p27 at the mRNA level (Medema et al., 2000). Akt phosphorylates another cell cycle inhibitor, p21, at two different residues Thr145 and Ser146. The Thr145 residue phosphorylation by Akt results in the cytoplasmic localization of p21 and thus promotes the cell cycle (Zhou et al., 2001), whereas the Ser146 site phosphorylation enhances the stability of the protein and further increases the assembly of Cyclin D-CDK4 G₁/S transition complex (Li et al., 2002). Recently, we observed the regulation of p21 at the transcription level by Akt in a FKHRL1-dependent manner (Maddika and Los, unpublished results). Although PI3-K/Akt pathway is mostly reported to be required for G₁/S progression, there have been few studies suggesting a role for this pathway in G₂/M progression, though the mechanisms are not completely understood (Shtivelman et al., 2002). Also, it was shown that Akt activation could overcome a G₂/M cell cycle checkpoint induced by DNA damage (Kandel et al., 2002).

Though PI3-K/Akt activity might be important for G₂/M progression, it must be later transiently inactivated for mitotic exit, as the constitutive activation of this pathway leads to G₂/M cell cycle arrest in an FKHRL1/Cyclin B/PLK dependent manner (Alvarez et al., 2001).

2.3. Pro-cell death function of PI3-K/Akt pathway

Although many studies support a positive role for the PI3-K/Akt pathway in promoting cell proliferation and cell survival, there are several exceptions where the PI3-K pathway is also involved in promoting cell death pathways. The activation of PI3-K/Akt has been shown to be required for the induction of apoptosis by selected apoptotic stimuli like CD95, cisplatin, arsenite, TNF, serum withdrawal, hypoxia in some defined experimental systems (Aki et al., 2001, 2003; Lee et al., 2005; Lu et al., 2006; Nimbalkar et al., 2003; Ono et al., 2004; Shack et al., 2003).

3. Cyclins and cyclin-dependent kinases

We have delineated in the introduction the role of cyclins, CDKs and their inhibitors for cell cycle progression. We will now discuss in greater detail the interactions of cyclins, CDKs and their natural and pharmacologic inhibitors, focusing on their role in cell cycle progression, apoptosis induction and cancerogenesis.

3.1. Pro- and anti-apoptotic effects of cyclins

In several kinds of malignant tumors, cyclins are overexpressed and hence rated as proto-oncogenes. For example, cyclin D was identified as an overexpressed gene in lung, breast, gastric and esophageal carcinomas at a relatively high frequency (Dobashi, 2005). Cyclin E, which has a vital role in G₁/S phase transition, has been shown to be deregulated in some human cancers such as breast, gastrointestinal cancers and ovarian cancer (Dobashi, 2005). Cyclin A has been overexpressed in several cultured cells including A549 adenocarcinoma cells of the lung. Cyclin G overexpression promotes cell growth in RKO colon carcinoma cells. However, it has also been shown that retroviral expression of cyclin G potentiates the TNF-induced apoptotic process in mouse fibroblast cells (Okamoto and Prives, 1999).

There are two mammalian A-type cyclins, namely cyclin A1 and A2. cyclin A1 is limited to male germ cells (Sweeney et al., 1996). Cyclin A1 null mice show cell cycle arrest and apoptosis of spermatocytes (Salazar et al., 2005). Apparently, Bax expression is elevated in cyclin A1 null mice's spermatocytes as compared to the normal mice leading to their apoptotic demise. This clearly shows that cyclin A1 plays a vital role in the regulation of apoptotic process by inhibiting the expression of Bax gene. Molecular mechanisms revealed that, apoptotic cell death is due to the cell cycle arrest in G₂/M transition phase by downregulation of cyclin A and

subsequent upregulation of proapoptotic Bcl2 family proteins (Palozza et al., 2002).

Recent investigations revealed that cyclin E is responsible for induction of apoptosis in hematopoietic cells (Mazumder et al., 2002). Cyclin E activity is increased substantially in the apoptotic process mediated by irradiation of RPMI 8226 cells. Overexpression of cyclin E by transient transfection did not directly induce apoptosis; however, such cells are sensitized to irradiation-induced apoptosis. Cyclin E usually exists as a 50-kDa protein (p50-cyclin E). During apoptosis it is cleaved into a 18-kDa fragment (p18-cyclin E) by caspases and it can no longer bind to the CDKs. Overexpression of Bcl2 protein downregulated the apoptotic process and at the same time reduced the expression of p18-cyclin E (Mazumder et al., 2002). These results throw some light on the roles of cyclin E in apoptosis. Unexpected evidence came from the experimental results of the same group, indicating that p18-cyclin E interacts with the Ku-70, a critical component in the non-homologous end-joining repair. It is speculated that interaction of cyclin E might inhibit the activity of Ku-70 and hence augments the apoptotic process by inhibiting the DNA repair mechanism (Mazumder et al., 2004).

Cyclin D1 has been shown to induce apoptosis in variety of cancer cells such as NIH 3T3, mouse kidney and R070B cells (Kranenburg et al., 1996). Overexpression of cyclin D1 in the above cell lines has been shown to induce apoptosis by a p53-independent pathway. There is also a report that cyclin D1 induces apoptosis in rat fibroblast cell lines under serum-depleting conditions (Sofer-Levi and Resnitzky, 1996). Ectopic expression of cyclin D in the above studies caused apoptosis by deregulation of the pRb/E2F pathway. Overexpression of cyclin D1 rendered pancreatic tumor cell lines less sensitive to cisplatin-induced apoptosis. Attenuation of cisplatin-induced apoptosis in cyclin D1 over-expressing cells was attributed to the upregulation of Bcl2 and Bcl-xL proteins. However, when the overexpressed Cyclin D1 was downregulated, using antisense RNA, the apoptotic cell death induced by cisplatin was enhanced (Biliran et al., 2005). Furthermore, cyclin D3 interacts with caspase-2, and this interaction lead to an increased caspase activity and apoptosis in HEK 293 cells (Mendelsohn et al., 2002). While over-expression of caspase-2 induced apoptosis in HEK 293 cells, co-expression of cyclin D3 and caspase-2 potentiated the apoptotic process. Accordingly, co-expression of caspase-2 mutants that does not interact with cyclin D3 does not increase apoptosis. These results clearly point out the fact that interaction of cyclin D3 and caspase-2 is required for the enhancement of apoptosis (Mendelsohn et al., 2002).

Cyclin B1 is an important regulator of apoptotic cell demise in many cell types. For example, in γ -radiation-induced apoptosis of hematopoietic cells, cyclin B1 was necessary to induce apoptosis (Porter et al., 2000). Moreover, downregulation of cyclin B1 by antisense RNA rescued cells from apoptosis (Porter et al., 2000). DNA damage-induced apoptosis was strongly dependent on the nuclear localization of cyclin B1 (Porter et al., 2003). This has been proven by

ectopic expression of either cytoplasmic or nuclear cyclin B1. Apoptotic death was seen in the nuclear localizing cyclin B1 but not in cytoplasm localized cyclin B1 (Porter et al., 2003). In HeLa cells, cyclin B1 is expressed at high levels. Downregulation of cyclin B1 expression by siRNA lead to the anti-proliferative effects of the tumor cells. Interestingly, targeting of cyclin B1 by siRNA in primary human vascular endothelial cells (HUVECS), does not decrease their proliferation (Yuan et al., 2004). Camptothecin treatment of HT29 cells induced apoptosis (Banerji and Los, 2006; Borgne et al., 2006; Huang et al., 2006). This was accompanied by an increase in the expression of cyclin B1 and cyclin E2 in these cells. siRNA-mediated inhibition of cyclin B1 prevented the cells from undergoing apoptosis, whereas siRNA-targeting of cyclin E2 had no effect (Borgne et al., 2006). Thus, cyclin B1 expression is required for apoptotic induction by camptothecin in HT29 cells. The above data clearly implicates the dual role of cyclin B1 both in apoptosis and in cell proliferation.

Cyclin L2, a recently identified human cyclin, induces apoptosis in human hepatocellular carcinoma cell lines when overexpressed (Yang et al., 2004). Apoptosis mediated by cyclin L2 is due to the upregulation of p53 and Bax proteins, and downregulation of Bcl2 protein.

4. Role of cyclin-dependent kinases in the regulation of cell cycle and apoptosis

CDKs are a family of hetero-dimeric serine/threonine kinases that are essential for the progression of the cell cycle at every phase transition of the division process. In addition, they have distinct roles in regulating transcription and neuronal function. CDKs need to be complexed with their activating partners, cyclins, to exert their role on cell proliferation. CDKs, associated cyclins and CDK inhibitors act in a coordinated manner to achieve cellular homeostasis. Dysregulation of the CDKs and/or associated cyclins have been often found in tumors and neurodegenerative disorders. Hence, to develop effective therapeutic approaches targeting CDKs, it is essential to identify and understand their complete role in regulating two major cellular processes, cell division and apoptosis.

CDK1 is the only non-redundant member of the family of CDKs. It is involved in the G₂/M transition and plays a vital role in mitosis. Inhibitors of CDK1 arrest the cell cycle at the G₂/M transition (Gray et al., 1999). Screening a budding yeast proteomic library revealed that CDK1 exerts its effect on the cell cycle by phosphorylating a number of protein substrates that are involved in cell cycle regulation. So far, there have been no reports of viable CDK1^{-/-} mice, which suggests the critical role of CDK1 in cell proliferation. The tumor suppressor protein retinoblastoma (Rb) is one of the major substrates for phosphorylation by CDK1 and CDK2.

CDK2 complexes with E-type and A-type cyclins. Initially, CDK2/cyclin E was thought to exclusively promote the G₁/S transition; however, CDK2 knock-out mice are viable,

though females and males remain infertile which suggests that CDK2 has a specific role in gametogenesis and meiosis while its role in cell proliferation can be compensated (Berthet et al., 2003; Ortega et al., 2003). This view is supported by the finding that CDK1/cyclin E complexes can compensate for the absence of CDK2 activity (Aleem et al., 2005; Kaldis and Aleem, 2005).

CDK1 activity following binding to its respective cyclins has been observed in cells that were committed to enter apoptosis after treatment with death inducing compounds like granzyme B (Shi et al., 1996), perforin, fragmentin-2 and camptothecin (Borgne and Golsteyn, 2003; Shimizu et al., 1995). Moreover, exposure of tumor cells to CDK1 inhibitors for longer than 24 h can induce apoptosis (Vassilev et al., 2006). In non-cycling G₁ CD4⁺ CD8⁺ thymocytes, during exposure to apoptotic stimuli such as dexamethasone, heat-shock, γ -irradiation, or Fas/CD95 cross-linking, CDK2 activity was found to be increased indicating its role in thymocyte selection (Hakem et al., 1999).

5. Effect of CDK inhibitors during cell cycle and apoptosis

Cyclin-dependent kinases exert their cell cycle-regulatory functions by phosphorylating an array of substrates. The activity of these kinases can be limited by specific, endogenous or exogenous, cyclin dependent kinase inhibitors (CDKIs). There are two major families of endogenous CDKIs. The first family includes the INK4 (inhibitors of CDK4) proteins, which bind and inhibit CDK4 and CDK6 specifically during the G₁ phase. There are four such proteins: p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c}, p19^{Ink4d}. These proteins prevent transition from G₁ to the S phase by forming either an inactive ternary INK4-CDK4/6-cyclin D or binary INK4-CDK4/6 complex (Jeffrey et al., 2000). Unlike INK4 inhibitors, the Cip/Kip (CDK interacting protein/kinase inhibitory protein) family includes the inhibitory proteins, which bind and inactivate multiple cyclin D, E, A/CDK complexes during all phases of the cell division cycle. This family contains three inhibitory proteins: p21^{Cip1/Waf1}, p27^{Kip1}, p57^{Kip2} (Sherr, 2001).

In addition to these endogenous CDKIs, there are few, small molecule inhibitors of CDKs: roscovitine/CYC202, olomoucine, flavopiridol and SU9516. These synthetic compounds can block or induce apoptosis depending on experimental conditions. The compounds reduce proliferation of tumors by stopping the cell cycle via inhibition of CDK1 and CDK2 (Wadler, 2001; Crews and Shotwell, 2003; Golsteyn, 2005).

5.1. The INK4-family inhibitors

The INK4 group of CDKIs is characterized by a narrow substrate specificity centered on CDK4 and CDK6. A member of this group, p16^{Ink4a} is encoded by p16 gene, a

tumor suppressor, which opposes the activity of the cyclin D-dependent kinases. Its inhibitory effect is based on the ability to form complexes with CDK4/6 in order to prevent CDK4/6-cyclinD binding and S phase entry, respectively. Moreover, p16^{Ink4a}-mediated CDK4/6-cyclinD binding disruption cancels the Rb-E2F mitogenic signal transduction and cells remain in G₁ phase (Sherr, 2001). There is a tight linkage between p16^{Ink4a} and p14^{ARF} protein. The ARF protein is encoded by an alternatively spliced variant of INK4a gene and inhibits cell cycle progression by stabilizing p53. This stabilization depends on the ability of ARF to create a complex with p53-MDM2 (mouse double minute 2; a small extra-chromosomal nuclear body) in order to prevent p53 from degradation by the proteasome. In addition, ARF is positively regulated by E2F, a transcription factor induced by the Rb, that activates the promoters of genes encoding for cyclin E and A, which are crucial for S phase of the cell cycle. Furthermore, loss of p16^{Ink4a} is functionally equivalent to loss of Rb whereas loss of ARF amounts to loss of p53 (James and Peters, 2000; Sherr, 2001).

The second inhibitory protein from INK family, p15^{Ink4b}, regulates the cell cycle clock by inhibiting CDK4/6-cyclinD mediated phosphorylation of Rb. Induction of p15^{Ink4b}-triggered G₁-phase arrest occurs in response to TGF- β (Hannon and Beach, 1994; Reynisdottir et al., 1995). Loss of p15 gene is associated with lymphoproliferative disorders and tumor formation (Latres et al., 2000). The p15^{Ink4b}-mediated pathways that control G₁/S transition are frequently deregulated in human cancers such as prostate cancer, melanoma, pituitary adenoma, acute myeloid leukemia, gastric cancer.

The other members of INK4 class, p18^{Ink4c} and p19^{Ink4d} are expressed during fetal development and seem to play a key role in terminal differentiation (Phelps et al., 1998; Zindy et al., 1997). Expression of these inhibitors increases during G₁/S transition, mimicking G₁ arrest caused by pharmacologic CDKs inhibition (Hirai et al., 1995). The p18^{Ink4c} inhibitor plays a key role in growth control consistent with the wide expression of the gene encoding for this protein in variety of tissues. It has been suggested that loss of p18 function results in shortening the G₁ phase and driving the cell cycle machinery. Furthermore, the p18^{Ink4c} acts synergistically with p27^{Kip1} in mediating suppression of pituitary tumorigenesis. Mice lacking both p18 and p27 genes develop pituitary tumors almost as quickly as mice lacking the p18 gene alone (Franklin et al., 1998). Since loss of Rb gene leads to pituitary tumor (Hu et al., 1994), p18^{Ink4c} and p27^{Kip1} are suspected to control the function of Rb, which can be a common target for these two collaborating CDKIs. This suggests that two members of INK4 family are involved in growth suppression which is linked with the wild-type Rb function (Guan et al., 1994).

5.2. Kip/Cip-family of CDK-inhibitors

p21^{Cip1/Waf1} is a member of the Kip/Cip, a broad specificity class of CDKIs. This inhibitor was identified

simultaneously by two independent research groups, and named as Waf1 (wild-type p53-activated fragment 1) and CDK-interacting protein 1 (Cip1). As the name indicates, the p21^{Cip1/Waf1} is under the control of the p53 tumor suppressor, which drives faulty cells into apoptosis. The main role of p21^{Cip1/Waf1} in cell cycle regulation lies in its ability to inhibit the activity of cyclin A,E/CDK2 required for G₁/S transition and therefore G₁ arrest.

Beyond the cell cycle, p21^{Cip1/Waf1} can perform as a negative regulator of p53-dependent apoptosis. In DNA damaged cells, p21^{Cip1/Waf1} is responsible for p-53 dependent G₁ arrest (Deng et al., 1995; Finlan and Hupp, 2005). After 48h, the expression of p21^{Cip1/Waf1} in cells treated with DNA-damaging agent decreased and the presence of a cleaved fragment of p21^{Cip1/Waf1} was observed. This event points at the cleavage of the cell cycle regulator protein p21^{Cip1/Waf1} during the terminal G₁ arrest leading to apoptosis. The cleavage and inactivation of p21^{Cip1/Waf1} is mediated by caspase-3 (Los et al., 2001). But p21^{Cip1/Waf1} may also act as apoptosis inhibitor. TGF- β , TNF, IFN- γ , and interleukin-6 induce p53-independent expression of p21^{Cip1/Waf1} and cause not only cell cycle inhibition but also suppress apoptosis. The probable mechanism of this action includes: (a) interaction with pro-apoptotic molecules such as pro-caspase-3, caspase-8 or ASK-1 (apoptosis signal-regulating kinase 1); and (b) induction of G₁ arrest in response to binding to cyclin A, E/CDK2 complexes (Gartel and Tyner, 2002).

The second member of Cip/Kip family of CDKIs, p27^{Kip1} inhibits cyclinE/CDK2 and regulates G₁/S transition. This inhibition occurs in cells arrested by TGF- β or cell-cell contact. As mentioned before, p27^{Kip1} plays an important role in pituitary tumor suppression by controlling the function of Rb. The activity of p27^{Kip1} is regulated by its fluctuations during the cell cycle. This means that the concentration of p27^{Kip1} decreases in response to mitogen stimulation and increases when mitogens are withdrawn (Sherr, 1996). The degradation of p27^{Kip1} mediated by interleukin-2 is required for cell cycle entry (Nourse et al., 1994). In addition, p27^{Kip1} is reported to have an effect on apoptosis, suggesting its induction by over-expressed p27^{Kip1} (Tenjo et al., 2000). In contrast, decreased expression of p27^{Kip1} in tumor cells correlates with poor prognosis (Sherr, 1996).

The p57^{Kip2}, another member of the Kip/Cip family of CDKs inhibitors, plays a major role in embryonic development and its loss leads to developmental disorders (Yan et al., 1997). Reduction in expression of this inhibitor correlates with tumorigenesis in laryngeal mucosa, which can work as a good marker for diagnosis. The implication of p57^{Kip2} in the control of cell cycle is not clear.

5.3. "Small Molecule" CDK-inhibitors

The inhibition of CDKs activity can also be achieved by small chemical compounds. The inhibitors such as: roscovitine/CYC202, flavopiridol, olomoucine, and SU9516 represent a group of very specific CDKs inhibitors which

are able to stop the cell cycle by inhibiting CDK1 and 2.

Another inhibitory compound, SU9516 exhibits a selective inhibition towards CDKs. Similarly to roscovitine and flavopiridol, SU9516 is involved in the induction of apoptosis. Furthermore, SU9516-mediated CDK2-inhibition entails a downturn in Rb protein phosphorylation required for CDK2/Cyclin complexes (Golsteyn, 2005).

The CDK inhibitors play a guarding/controlling role for the cell cycle machinery. They participate in crucial checkpoints within the cell cycle. Some of them act as tumor suppressors. All of the mentioned inhibitors aim at stopping the cell cycle by interaction with different CDKs and prevent the cell from faulty division. Disruption of individual checkpoints is a common event to all cancers. The pro-apoptotic role for CDKIs is still not well understood.

6. Role of p53, E2F and Retinoblastoma protein in the control of apoptosis and cell cycle

Many reviews discuss the role of p53, Rb and E2F in the regulation of cell proliferation and cell death; thus this paragraph aims only to highlight the most important facts about these proteins (Finlan and Hupp, 2005; Yu, 2006). It is considered that tumor suppressor genes have appeared during evolution probably to protect multicellular organisms from uncontrolled cellular division caused by arising mutations. This concept is supported by the fact that the corresponding proteins or pathways are mutated or inactivated in most human cancers.

Cell cycle control at the G₀-S phase involves a complex interaction of various proteins. There are two major pathways involved in the cellular progression from G₀-S phase: (a) Rb (Rb, cyclin D1, and p16^{INK4A}) cell cycle pathway, and (b) p53/p21^{Waf1} G₁-S checkpoint arrest pathway. Despite both pathways acting largely independently, they share complex interaction patterns (Burke et al., 2005; Hsieh et al., 2002).

6.1. p53-dependent cell cycle checkpoint machinery

In response to stress stimuli, p53 which normally has a low expression level, accumulates within cells due to an increase in the protein's stability (Finlan and Hupp, 2005; Hsieh et al., 2002). Indeed, p53 integrates signals from various pathways that become activated as a result of different stimuli such as DNA damage, hypoxia, and oncogene activation. Under these conditions, p53 initiates various cellular responses that can lead to cell-cycle arrest, senescence, differentiation, DNA repair, apoptosis, and inhibition of angiogenesis (Finlan and Hupp, 2005; Giono and Manfredi, 2006). Most of these responses are carried out via its ability to function as a transcription factor. It (a) mediates cell growth arrest by inducing the expression of p21, 14-3-3s, Cdc25C, and GADD45; (b) stimulates DNA repair by inducing the expression of p21, GADD45, and the p48 xeroderma pigmentosum protein;

Fig. 1. Different pathways resulting from activation of p53 due to cell injury (see main text for details).

and (c) induces apoptosis by upregulating the transcription of Bax, PUMA, Noxa, p53-AIP, PIG3, Fas/APO1/CD95, and KILLER/DR5 (Fig. 1). These responses are regulated through different mechanisms and are highly dependent on the type and amount of stimuli. For example, low levels of stress or DNA damage would induce levels of p53 that induces growth arrest genes. In contrast, under severe cellular stress, the higher levels of expressed and stabilized p53 activate apoptotic pathways. It has also been proposed that specific post-translational modifications of p53 are important for final outcome. Finally, it has been shown that p53 has a role in the apoptotic mitochondrial pathway which is largely independent from its transcriptional regulation (Mashima and Tsuruo, 2005; Giono and Manfredi, 2006). By inducing the expression of the CDKI p21^{Waf1} in cells with damaged DNA, p53 prevents initiation of DNA replication in the G₁/S checkpoint. p21^{Waf1} inhibits the phosphorylation of Rb (Finlan and Hupp, 2005; Giono and Manfredi, 2006). In addition, expression of p21^{Waf1} induces growth arrest at the G₁ and G₂ phases (Giono and Manfredi, 2006).

p53 may be predominantly involved in maintenance rather than initiation of either G₁ or G₂ arrests. It is shown that G₁ arrest consists of two phases; the first is independent of p53, but the second is p53-dependent and involves the p53-activating kinases ATM, ATR, and Chk2. Thus, it is proposed that p53-dependent arrest in G₁ extends the delay initiated by Cdc25A, providing the cell with sufficient time to repair the damaged DNA. During S phase, an isoform of p53 (Δ p53) induces cell cycle arrest by binding to p21^{Waf1} and 14-3-

3s growth arrest genes, but not the apoptotic PIG3 gene (Giono and Manfredi, 2006). p53 also prevents aneuploidy by blocking endoreduplication of tetraploid cells that result from mitotic failure (Giono and Manfredi, 2006). In addition to growth arrest and apoptosis, p53 is involved in regulating DNA repair after genotoxic stresses; p53 can directly interact with proteins involved in DNA repair and a variety of DNA structures. p53 binds to double-stranded and single-stranded DNA in a non-specific way, to ends of double-strand breaks, to Holliday junctions, and to DNA bulges caused by DNA mismatches (Giono and Manfredi, 2006).

6.2. The retinoblastoma tumor suppressor gene

Rb was the first tumor suppressor identified (Lee et al., 1987) and has a very significant role in controlling cell cycle. Besides tumor suppression, Rb is critical for stem cell maintenance, tissue regeneration, differentiation, and developmental programs. Rb protein is a 928 amino acid, nuclear phosphoprotein that possesses weak, nonspecific DNA binding activity. Rb protein reacts with a wide array of proteins that are mostly involved in transcription control. Among these proteins E2Fs are very important. E2F and its heterodimer partner, DP, are central regulators of cell cycle gene expression, and directly regulate the expression of genes involved in DNA replication, DNA repair, and G₂/M progression (Knudsen and Knudsen, 2006).

Rb and its homologous family members, RBL1/p107 and RBL2/p130 mediate cell cycle arrest by antagonizing transcription factor E2F/DP. This is regulated in turn by the phosphorylation status of Rb family proteins. Only hypophosphorylated forms of the Rb protein family can interact with E2Fs during the cell cycle (Hsieh et al., 2002; Jackson and Pereira-Smith, 2006). Phosphorylation of Rb is catalyzed by the activity of CDK/cyclin complexes. In general, mitogenic signals (e.g. growth factors) lead to activation of CDK/cyclin complexes, while anti-mitogenic signaling pathways (e.g. as initiated by confluence or nutrient depletion) inhibit activation of the G₁ CDK/cyclins. Sufficient phosphorylation of Rb inactivates its transcriptional repressor function, thus allowing expression of E2F target genes, whose activity is essential for entry into S phase. Rb is held inactive (e.g. hyperphosphorylated) throughout the rest of the cell cycle (S, G₂, and M phases) (Knudsen and Knudsen, 2006).

Rb family members also regulate gene expression by directing changes in chromatin structure. These chromatin changes can be implemented by recruiting histone deacetylases (HDACs), histone methyltransferases, SWI/SNF complex members, and, less well-characterized, DNA methyltransferases and polycomb proteins (Jackson and Pereira-Smith, 2006). It has been shown that the integrity of the Rb pathway could influence the activity of p53 and vice versa (Hsieh et al., 2002).

Rb's function may be downregulated, or completely ablated by variety of mechanisms: (a) excessive expression of

CDK4 or cyclin D which results in enhanced Rb phosphorylation, (b) loss or mutation of p16^{INK4a} CDKI, (c) sequestration by oncoproteins such as the human papilloma virus E7 protein, and finally (d) mutations of Rb gene (such as occurring in retinoblastoma) (Knudsen and Knudsen, 2006).

E2F/DP complexes are critical down-stream mediators of the p16^{INK4a} /RB pathway. Six different E2F-like proteins (E2F1, E2F2, E2F3, E2F4, E2F5, and E2F6) have been so far described. These complexes can be roughly classified into two subgroups: (a) activating E2Fs (E2F1, E2F2, and E2F3) are potent transcriptional activators and (b) repressive E2Fs (E2F4, E2F5, and E2F6) that apparently repress transcription. Recent findings suggest that E2F/DP activity is essential for cell proliferation and its reduction immediately provokes a senescence-like cell cycle arrest (Maehara et al., 2005).

Overexpression of E2F1 can induce apoptosis independent of p53. There is also increasing evidence that in tumor cells E2F1 cooperates with p53 to induce apoptosis. This is independent of the transactivation function of E2F1, and may occur upon binding of E2F1 to p53 via its cyclin A-domain. Apparently, the level of cellular cyclin A determines this interaction. However, within normal cells and in the presence of Rb, which down regulates E2F1, p53-stabilization induced by DNA damage cannot be activated to induce apoptosis by proteins like E2F. In addition, mitogen-induced E2F would transactivate genes like cyclin A, which prevents E2F binding to p53, thereby keeping cells alive. This could explain why in primary normal cells, a predominantly p53-dependent G₁ arrest and not apoptosis occurs (Hsieh et al., 2002).

7. Dual role of Myc family members in regulating cell cycle and apoptosis

Twenty-five years have passed since c-Myc was discovered as the cellular homologue of the transduced oncogene of several avian retroviruses (Sheiness et al., 1978; Vennstrom et al., 1982). The gene encodes a transcription factor of the HLH/leucine zipper family of proteins that activates transcription as part of a heteromeric complex with a protein termed Max. Altogether, Myc family members function as regulators of gene transcription by heterodimerizing with Max through a network of Myc/Mad/Max proteins at the E-box element (Blackwell et al., 1990). Some of the biological functions of Myc family proteins are accomplished by sequence-specific DNA binding that is mediated by the carboxyl-terminal region of the protein (Blackwell et al., 1990) and a negative feedback mechanism can act as a homeostatic regulator of c-Myc expression in vivo (Penn et al., 1990). In mammalian fibroblasts, Myc acts as an upstream regulator of CDKs and functionally antagonizes the action of at least one CDKI, p27 (Steiner et al., 1996). In general, Myc is one of the few proteins that can solely drive resting cells into cell cycle and promote DNA synthesis. Interestingly, overexpression of Myc in these cells

also blocks their differentiation. It is now well documented that translocations of c-Myc locus into the heavy or light chain immunoglobulin loci frequently occurs in Burkitt's lymphoma and amplification of N-Myc and L-Myc genes in neuroblastoma and small cell lung cancer, respectively (Heerema et al., 2005). Myc genes are among the most frequently affected genes in human cancers (Henriksson and Luscher, 1996).

Along with its transforming effect, Myc can also sensitize the cells towards apoptosis, that indicates Myc is a part of the intracellular Life-and-Death switching system (Desbarats et al., 1996). In fact, the expression of Myc proteins is deregulated approximately in one third of human cancers by a number of different mechanisms (Spencer and Groudine, 1991). Likewise, overexpression of Myc is common in certain advanced cancers with poor prognosis such as colorectal cancers, hormone dependent breast and prostate cancers (Borg et al., 1992; Jenkins et al., 1997; Watson, 2006).

The effects of human c-Myc on cells' susceptibility to apoptosis were investigated by introducing them into immortalized rat fibroblasts, or by introduction of Myc that could be "on demand" activated by the addition of a small molecule (i.e. estrogen) (Alexander et al., 2007; Stroh et al., 2002). The transfected cells proliferated to a similar extent upon Myc activation or over expression, but differed by up to 15-fold in the level of apoptosis, that correlated inversely with the expression of Myc. A necessary role of c-Myc was observed in apoptosis induced by T-cell receptor activation and further studies also established that c-Myc is a critical determinant of apoptosis induced by TNF (Dong et al., 1997; Shi et al., 1992). However, it now appears that c-Myc is required for the efficient response to a variety of apoptotic stimuli, including transcription and translation inhibitors, heat shock, hypoxia, glucose deprivation, chemotherapeutic agents and DNA damage (Alarcon et al., 1996; Dong et al., 1997; Rupnow et al., 1998; Shim et al., 1998). It is also quite well documented that retroviral Myc proteins (v-Myc proteins) and other Myc family members like S-Myc, N-Myc and L-Myc have the capacity to induce apoptosis similar to c-Myc (Nesbit et al., 1998). The Myc-induced apoptotic program is counteracted by antiapoptotic Bcl2-family members (Bissonnette et al., 1992). The role of Bcl2-family members in cell cycle regulation and cell death is discussed to the greater detail in the next paragraph.

So far, several studies have revealed the regulatory circuits that link death receptors to c-Myc. Members of the TNF transmembrane receptor family, expressed on mesenchymal and epithelial cells use an adaptor system to activate the caspase directly (Los et al., 1995; Philchenkov et al., 2004). c-Myc sensitizes cells to death signals triggered by TNF receptors and CD95/Fas (Ashkenazi and Dixit, 1998).

Strong support has been established for the dual intrinsic function model of c-Myc as a coordinate activator of cell proliferation and apoptosis. These effector signals are hypothesized to include 'priming' and 'triggering' mechanisms associated with separable caspase-dependent

Fig. 2. A dual signal model for c-Myc function. The model proposes that c-Myc activates proliferation and primes apoptosis through one pathway and triggers apoptosis through a mechanistically distinct second pathway. In this model, death priming and proliferation involves regulation of specific target genes by c-Myc/Max complexes. The links between TRRAP and the transcription complex SAGA suggest possible involvements with the proliferation pathway. The interaction of p107 is proposed to co-ordinate the regulation of cell cycle-relevant genes. Max and gene regulation is required for proliferation and death priming but may be dispensable for death triggering. Conversely, Bin1 is dispensable for proliferation and death priming but necessary to trigger death once it is primed. p53 may contribute to sensitizing cells to the trigger, but is mechanistically distinct from Bin1 since the latter can mediate p53-independent death. Survival signals from cytokine and adhesion receptor pathways are proposed to target the inactivation of triggering pathway (adapted with modifications from (Prendergast, 1999; Wechsler-Reya et al., 1997).

and caspase-independent process (Fig. 2) (Prendergast, 1999).

8. The dual role of Bcl2-family members in cell cycle regulation and apoptosis

Bcl2 family members are present in cells of all multicellular organisms and they play the key role in the regulation of mitochondria/apoptosome-dependent apoptosis. Proteins of the Bcl2 family can be further classified as either pro-apoptotic or anti-apoptotic and are characterized by distinct, evolutionarily conserved Bcl2 homology domains of which there are four (BH1–4). There are two types of pro-apoptotic Bcl2-family members. The majority of the pro-apoptotic Bcl2-family members are known as “BH3-only” molecules because they lack domains BH1, –2 and –4. Examples of BH3-only Bcl2 family members are Bad, Bid, Bim_{EL}, Bmf, and Mcl-1_S. These molecules appear to be expressed in a tissue restricted manner. The smaller and more ubiquitously expressed group form multi-domain pro-apoptotic Bcl2-family members. Beside the BH3-domain these proteins also contain other domains typical to anti-apoptotic Bcl2-family members. That family contains Bax, Bok/Mtd, Bak, and Bcl-X_S. Oppositely, anti-apoptotic members contain at least a BH1 and BH2 domain (i.e. Mcl-1_L) or otherwise encompass all BH1–4 domains such as Bcl2 and Bcl-X_L. Bio-

chemical and physiological regulations of these two groups of proteins determine whether a cell survives or undergoes apoptosis (Lanave et al., 2004). Accumulating evidence suggests that some Bcl2 family members have dual roles, serving as molecular mediators of both apoptosis and cell cycle progression. It has recently been proposed that the dual functions of Bcl2 family members in regulating apoptosis and cell cycle are primarily governed by the multi-domain members, whilst the role of BH3-only molecules are more variable (Zinkel et al., 2006).

The anti-apoptotic function of Bcl2 is characterized by mono- (Ser70) or multisite (Thr69, Ser70, and Ser87) phosphorylation in the flexible loop domain (FLD). Interestingly, Bcl2 phosphorylation has been shown to regulate intracellular reactive oxygen species (ROS) levels and subsequently inhibit cell cycle progression by delaying the G₁/S transition (Deng et al., 2003). Bcl2 has also been shown to delay cell cycle entry by retarding the accumulation of E2F1, a critical inducer of cell cycle entry, and acting through mechanisms independent of Rb but dependent upon p130 and p27, all of which are negative regulators of the cell cycle (Vairo et al., 2000). Both Bcl2 and Bcl-X_L delay serum- and Myc-induced cell cycle entry via a mechanism involving the CDKI, p27, and G₁ CDKs. Bcl-X_L and Bcl2 elevate p27 levels thereby impeding the activation of CDK2 and CDK4 during progression to S-phase (Greider et al., 2002). In addition, Bcl-X_L prolongs the G₀ phase and enhances its arrest and induces a reduction in cell size and total RNA content during cell cycle arrest and entry, however this process has been shown to be reversible by Bad (Janumyan et al., 2003).

Pro-apoptotic Bad inhibits both cell cycle progression, and the anti-apoptotic functions of Bcl-X_L and Bcl2 via BH3 binding. Phosphorylation of Bad at Ser128 by Cdc2 has been implicated to serve as a functional link between the role of Bad in regulating apoptosis and cell cycle. Cdc2-mediated phosphorylation at Ser136, and subsequent activation of Bad has been demonstrated to induce apoptosis in primary neuronal cells by inhibiting the interaction between Bad, and 14-3-3 (Konishi et al., 2002). Furthermore, Bad is regulated by reversible phosphorylation where dephosphorylation of Ser112, predominantly by protein phosphatase 2A (PP2A), is a prerequisite for dephosphorylation of pSer136, presumably by multiple phosphatases. Moreover, PP2A competes with 14-3-3 for Bad binding upon apoptosis induction by the withdrawal of survival factor interleukin-3 (IL-3), suggesting that PP2A is a positive regulator of Bad-mediated apoptosis (Chiang et al., 2003).

Other pro-apoptotic Bcl2 proteins including Bax and Bok/Mtd (Bcl2-related ovarian killer/Matador) have also been described to play a role in the unique coupling of apoptosis and cell cycle. The cell cycle arrest, and apoptosis-inducing tumor suppressor p53, possesses the ability to mimic BH3-only molecules by catalyzing the liberation of Bcl-X_L-sequestered pro-apoptotic Bcl2 family members. Moreover, p53 facilitates direct activation of Bax thus inducing mitochondrial membrane permeabilization and apoptosis (Chipuk

and Green, 2004). Bax has also been described to accelerate S-phase progression (Zinkel et al., 2006), although the underlying molecular processes are poorly understood. A recent study demonstrated that Bok/Mtd is cell cycle-regulated and can sensitize H1299 cells to anticancer chemotherapeutics-induced apoptosis. It was shown that Bok mRNA is low in quiescent cells although it progressively increases with serum stimulation and that this process is dependent upon E2F1 over expression as it induces upregulation of Bok mRNA (Rodriguez et al., 2006).

9. Conclusion and future directions

As our understanding of cancer biology increases, so do the chances for the development of more effective and selective, targeted cancer therapies (Broxterman and Georgopapadakou, 2005). So far, much success has been achieved in the development of therapies that target malignancies associated with the overexpression of BCR-Abl (i.e. Gleevec), or a broad array of tumors that hyperactivated the EGFR/Her/Neu pathway (Booy et al., 2006; Brown and Gibson, 2005; Johnston et al., 2006; Krzemieniecki et al., 2006).

While cancer cells inactivate elements of apoptotic pathway, they never disable the entire signaling cascade, implying that at least some molecules share function between cell proliferation and cell death machinery. Accumulating knowledge on the functional significance of Bcl2 family members in linking apoptosis and the cell cycle continues to empower the pharmacological quest for therapeutic peptides and peptidomimetics in pathophysiological states such as cancer, acute liver damage, and ischemia of the myocardium and brain (Ghavami et al., 2005; Hauff et al., 2005; Huang, 2000; Kreuter et al., 2004; Mendoza et al., 2005). Many novel small molecule approaches integrating peptides or peptidomimetics are being constructed to mimic the pro-apoptotic function of the BH3 domain, a fundamental target model whose function, in excess, can overcome the molecular roadblocks impeding the success of conventional chemotherapy (Mendoza et al., 2005). Pharmacological optimization of BH3-peptides and derived mimics using “hydrocarbon stapling” to produce stable, protease-resistant α -helices (Anderson et al., 2006; Krocak et al., 2006; Walensky et al., 2004) or conjugation to cell penetrating peptides, such as the membrane translocation domain of the Antennapedia (Ant) protein to enhance membrane permeability (Hauff et al., 2005; Mendoza et al., 2005; Vieira et al., 2002), are proving to be efficacious methods in the design of potential drug candidates both in vitro and in vivo. The sophisticated dual role of the Bcl2 family members in the intricate regulation of apoptosis and the cell cycle makes them ideal therapeutic targets in diseases characterized by deregulated apoptotic pathways.

Since cell survival, cell death, and cell cycle progression pathways are interconnected, at least theoretically it should be

possible to develop pharmacologically active substances that would “hijack” cell proliferation pathways and redirect them into apoptotic process. Several viral molecules like for example E4orf4, apoptin and VpR are able to selectively kill cancer cells (Maddika et al., 2005, 2006). Thus, they may harbor the potential, or even directly interact with key components of cell survival and proliferation pathways that are hyperactive in cancer cells, and redirect them towards the activation or amplification of the cell death machinery. Since cyclins play a dual role both as pro-apoptotic and anti-apoptotic proteins, at least theoretically it should be possible to selectively harness their pro-apoptotic potential and direct it towards selective activation of programmed cell death in cancer cells. Thus, understanding of the roles of these proteins will certainly pave a way towards more scientific advancement in the field of cancer biology and will provide solid foundations for the discovery of novel drug targets that would kill-, or at least control cancer progression.

Beside the outlined above specific approaches, researchers also try other non-conventional approaches in the battle with cancer, like for example, manipulation of the concentration of certain biologically important ions, or building blocks for DNA and/or RNA synthesis, or the utilization of naturally occurring proteins produced by the immune system (Frey and Brauer, 2006; Grote et al., 2006; Hashemi et al., 2005, 2007; Hashemi and Krocak, 2005; Li et al., 2005). Most likely, the cure of cancer will be achieved by the combination of traditional approaches with selected targeting of cell cycle regulators and simultaneous sensitization to apoptosis. Likely, the rapidly accumulating knowledge of cancer immunology will aid us in either achieving of satisfactory level of remissions, or even in eliminating most of the cancer altogether by immunizations, as this is the case with the majority of morbid viral and many bacterial diseases.

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