Proliferate and survive: cell division cycle and apoptosis in human neuroblastoma

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Background and Objectives. Neuroblastoma is one of the most frequent childhood cancers and a major cause of death from neoplasias of infancy. Although a wealth of studies on its molecular bases have been carried out, little conclusive information about its origin and evolution is available.

Evidence and information sources. Some intriguing findings have correlated neuroblastoma development with aberrations of two pivotal cellular processes generally altered in human cancers, namely cell division cycle and apoptosis. Indeed, it has been reported that neuroblastoma cell lines show accumulation of Id2 protein, a factor which is able to hamper the pRb protein antiproliferative activity.

State of the art. The increased Id2 is due to *N-myc* gene amplification and overexpression, a phenomenon frequently observed in neuroblastoma and an important independent negative marker. Moreover, neuroblastoma cells are frequently characterized by increased levels of survivin, an inhibitor of the apoptotic response, and by a deficiency of procaspase 8, a key intermediate of the programmed cell death cascade. These two events, probably, make neuroblastomas more resistant to programmed cell death. These recent findings might suggest that neuroblastoma cells have acquired the capability to proliferate easily and die difficultly.

Perspectives. The mechanistic meaning of these data will be discussed in the present review. Moreover, we will suggest new therapeutic scenarios opened up by the described alterations of cell cycle and apoptosis engines.

Key words: neuroblastoma, cell division cycle, apoptosis, Id2, caspase 8, survivin.

review

haematologica 2002; 87:196-214
http://www.haematologica.it/2002_02/196.htm

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Neuroblastoma (NB) is the most frequent solid extracranial neoplasia in children, and is responsible for about 15% of all pediatric cancer deaths.[1] It is an embryonal malignancy of the postganglionic sympathetic nervous system, which generally arises in the adrenal gland. Ninety-six percent of cases occur before the age of 10 years.[2] At present, although NB is sometimes diagnosed in the perinatal period,[3] no environmental factors with an impact on disease occurrence have been identified. The presence of familial cases suggests that hereditary factors may be important in the pathogenesis, but a neuroblastoma susceptibility gene has not yet been localized. Frequent areas of chromosomal loss, including chromosome band 1p36 in 30% to 35% of primary tumors, 11q23 in 44%, and 14q23-qter in 22%, may identify the location of putative neuroblastoma suppressor genes. Amplification of the *N-Myc* protooncogene occurs in 20% to 25% of neuroblastomas and is a reliable negative prognostic marker. No other oncogene has been shown to be consistently mutated or overexpressed in neuroblastoma.[4]

The clinical hallmark of NB is marked heterogeneity, with the likelihood of tumor progression varying widely according to stage, age at diagnosis and anatomical site. Generally, infants diagnosed before 1 year of age and/or with localized disease are curable with surgery and little or no adjuvant therapy. Conversely, older children frequently have extensive hematogenous metastases at diagnosis, and most of them die from NB progression despite intensive chemotherapy.

Some neuroblastomas undergo spontaneous regression or differentiate into benign ganglioneuromas. D'Angiò *et al.*[5] first recognized a distinct subset of infant patients who show a unique pattern of extensive disseminated disease, but who have spontaneous disease regression (stage 4S). This clinical diversity correlates strictly with NB

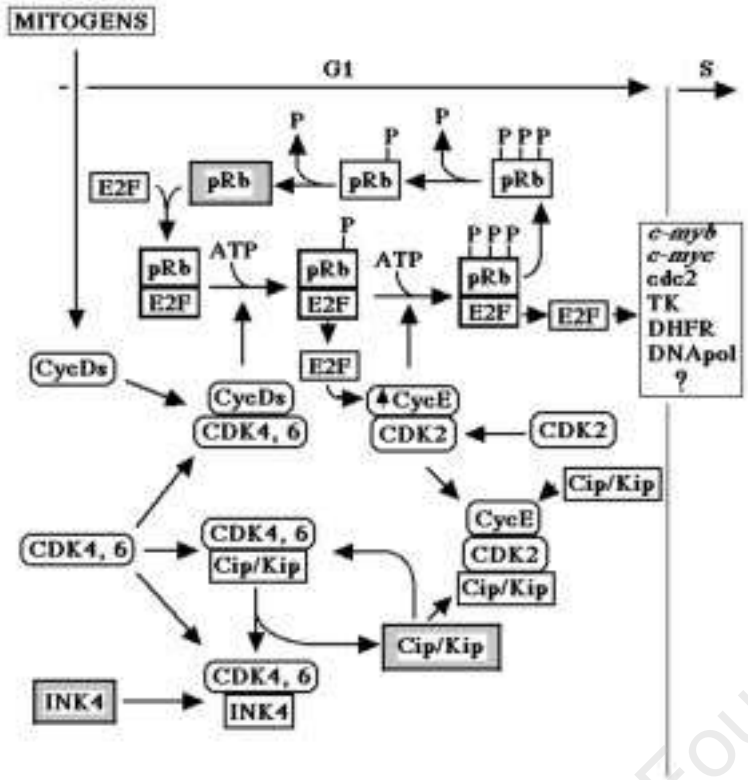

Figure 1. Major events of transition from G1 to S phase. The diagram represents some of the pivotal steps controlling S phase entry. Mitogen stimuli cause a transcriptional increase of cyclin Ds [CycDs] which bind and activate CDK4 (or CDK6) [CDK4/6]. Activated CDK4 partially phosphorylates and inactivates pRb protein [pRb-P]. The phosphorylated protein releases E2F transcriptional factor [E2F], which induces the expression of cyclin E gene and the accumulation of this cyclin [↑CycE]. Cyclin E activates CDK2 [CDK2] which completely phosphorylates pRb [pRb-PPP] and releases E2F. The transcriptional factor drives the expression of several genes required for S phase entry. CDK4 might also bind CDK inhibitors of the INK4 family [INK4], thus forming inactive binary complexes. Moreover, the CDK4-CycD complex may interact with CDK inhibitors of the Cip/Kip family [Cip/Kip]. The ternary complex is, at least in part, active. The increase of INK4 inhibitors causes the release of Cip/Kip inhibitors (and also of cycD) from the CDK4-CycD-Cip/Kip ternary complex. Finally Cip/Kip inhibitors bind and completely inhibit the activity of CDK2-CycE complex. The grey boxes show the protein whose function is involved in neuroblastoma development Additional details on the diagram are reported in the cell division cycle paragraph.

molecular features.

The possibility of undergoing differentiation is also observed *in vitro* in cell lines derived from neuroblastomas. Indeed, the treatment of these cells with different molecules (particularly, *all trans* retinoic acid, ATRA) results in the activation of an endogenous differentiation program. These findings suggest that alteration of the proliferation/differentiation interplay is particularly important in NB. In addition, several observations indicate that the physiologic apoptotic response is, at least in part, hampered in human neuroblastomas. This abnormality might play a pivotal role in the development, progression and drug-resistance of human NB.

This review will discuss the molecular biology of neuroblastoma in terms of alteration of the mechanisms controlling cell proliferation, differentiation and survival. In order to unravel the meaning and consequences of the abnormalities reported, we will first describe the main events of the cell division cycle and apoptosis.

Mammalian cell division cycle. A focus on G1 →S transition

Eukaryotic cell division cycle is composed of four phases: the time window between mitosis and a successive DNA replication (G1 phase), the phase of DNA duplication (S phase), the gap after DNA synthesis (G2 phase) and the phase which results in cell division (M phase).

The cell cycle length is directly related to the duration of the G1-phase, which might be considered as the period during which the cell makes most of its decision whether or not to proliferate. The longer the G1 phase is, the longer the cell cycle. Since knowledge of the biochemical mechanisms which allow the cell to proceed along the G1 phase is essential in order to understand the rate of cell proliferation, our description of the cell cycle will focus mainly on this phase (Figure 1).

The progression through the cycle is due to a biochemical process in which cyclins sequentially bind and activate distinct cyclin-dependent kinases (Cdks). Then, the activated cyclin-Cdk complexes regulate the activity of their target molecules by phosphorylation. Finally, these downstream effectors carry out the steps which ultimately allow the ordered development of the cell division cycle. Thus, regulation of Cdk activity is the key event in cell cycle progression.

Due to their pivotal regulatory roles in cell growth, Cdks are subject to several modes and lev-

els of regulation in response to both intracellular and extracellular signals.[6-8] Since Cdks form a binary complex made of an inactive catalytic subunit (Cdk) and a specific positive regulatory subunit (cyclin), the easiest control strategy is cyclin availability. Other levels of regulation are inhibitory/activating phosphorylations of the Cdk protein and binding of inhibitor proteins (CKIs; Cdk inhibitors), both of which have broad regulatory significance. However, the dominant mode of modulation for a particular Cdk is strongly context-dependent.

In mammals, two consecutive waves of Cdk activities are required for cell cycle entry and progression along G1 phase and during G1→S phase transition.[5,9,10] Cdk4 and its close relative Cdk6 function early in G1 and are activated by D-type cyclins: (i.e. cyclin D1, D2 and D3) (Figure 1). The primary target of these activities is pRb and the related *pocket proteins* (p107 and p130).[11] Cyclin E increases near to the G1→S phase transition and specifically activates Cdk2. Although cyclin E-Cdk2 complex certainly has a critical role in phosphorylating pR[12,13] (Figure 1), after cyclin D–Cdk4/6, its main target(s) in controlling S phase progression is (are) not yet identified.

Cyclin content varies along the cell cycle as a result of a strict regulation of synthesis and degradation, thus allowing a limited time window of Cdk activation. Cell cycle entry and progression along G1 phase is related to the expression of D-type cyclins, which, in turn, depends on a continuous mitogenic stimulation. Thereby, D-type cyclins provide a link between extracellular environment and the cell cycle engine.

Cyclin E starts to accumulate late in G1, peaks at the G1→S transition and decreases during S phase.[9,14] The expression of *cyclin E* gene is stimulated when pRb is hyperphosphorylated and does not exert the repression of E2F/DP1 complex, the transcription complex that probably mediates most of pRb-gene expression control. The other side of the coin is that *cyclin E* gene promoter contains a number of putative binding sites for E2F.

Beside regulation of their synthesis, cyclin levels are also regulated by a very efficacious and modifiable mechanism of degradation, the ubiquitin-proteasome pathway.[15] Often, the initial signal for protein ubiquitination is a specific phosphorylation.

A number of experiments suggest that the assembly of the cyclin-Cdk complex requires (or is facilitated by) an assembly factor. Although the identity of this key protein (if it exists) has not been defined, a chaperonin complex (Cdc37–Hsp90) specifically interacts with some Cdks (Cdk4 and Cdk6) and facilitates the formation of cyclin D1–Cdk4 complexes *in vitro*.[16,17] Moreover, it has been proposed that some specific CKIs play a function in cyclin D–Cdk4/6 assembly.[18,19]

A further mode of Cdk regulation involves phosphorylation at specific residues. Inhibitory phosphorylation has been definitely characterized for Cdk1 (Cdc2) in the context of the G2→M phase transition. However, Cdk4 and Cdk2 are also phosphorylated on tyrosine 15 and 17, respectively. Mammalian cells contain three specialized phosphatases that eliminate inhibitory Cdk phophorylation, namely CDC25A, CDC25B and CDC25C. On the basis of biochemical and cellular studies, CDC25A is considered to be responsible for Cdk2 activation.[20] Control of CDC25A is critical for the G1 response to DNA damage. Mammalian cells respond to some stressing condition (UV or ionizing radiation) by rapid ubiquitin/proteasome-mediated degradation of CDC25A.[21] This response occurs before the p53-dependent accumulation of p21Cip1 (see below) and is essential for maximal DNA repair and survival. Cdk4 is also a target of CDC25A regulation. For example, in quiescent cells, tyrosine 17 of Cdk4 is phosphorylated and UV irradiation prevents dephosphorylation during cell cycle re-entry,[22] probably by downregulating CDC25A. TGFβ and α-interferon signaling also lead to CDC25A inhibition, but the mechanism remains to be clarified.[23,24]

A primary role in the control of cell cycle progression is played by the Cdk inhibitors. Several studies have identified two classes of CKIs: the Cip/Kip and the INK4 families. The Cip/Kip family is formed by three members: $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$. The INK4 family is composed of four members: $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$.[18,25] This classification is based on the protein sequence, as well as on the affinity for the Cdk target. The Cip/Kip CKIs have conserved amino-terminal Cdk-inhibitory domains and carboxy-terminal domains which are structurally divergent. The three-dimensional structure of a ternary complex composed of the Cdk-inhibitory domain of $p27^{Kip1}$, Cdk2, and the amino-terminal (Cdk-activating) sequence of cyclin A has been reported.[26]

Cip/Kip CKIs are considered broad spectrum inhibitors, since they act on cyclin D–Cdk4/6 kinases and cyclin E/A–Cdk2. However, it has been reported that the efficiency of Cdk4/6 inhibition may vary for the different Cip/Kip proteins.[27] In addition, as anticipated above, Cip/Kip CKIs probably have a role in activating these kinases.[18]

The INK4 proteins share a common structural motif and mechanism of inhibition. They consist of either four (p15^{INK4B} and p16^{INK4A}) or five (p18^{INK4C} and p19^{INK4D}) ankyrin repeats. Importantly, INK4-bound Cdks cannot bind to cyclin.[28,29] Finally, INK4 inhibitors are narrow-spectrum CKIs; they only bind to and inhibit Cdk4 and Cdk6. These findings are extremely important in cell cycle control.

Initially, CKIs were identified as proteins which increase when cells need to cease dividing. However, this cannot explain the molecular basis of CKI regulation. Indeed, although all cells contain, more or less, identical CKIs, they regulate them in a remarkably different way, in a cell-phenotype and stimulus-specific manner. This has so far hampered any attempts at generalization.

The archetypal CKI is p21^{Cip1}. This was first isolated, independently, on the basis of its p53-dependent expression[30] and by means of its binding and inhibition of Cdk2.[31] p21^{Cip1} is largely responsible for the G1 arrest due to p53 functional upregulation during the response to stress.[32] This role has been confirmed in experiments on cells derived from p21^{Cip1} nullizygous mice, in which most of the G1 arrest, as response to DNA damage, is lost.[33,34] However, the role of p53 seems to be complicated, since the *genome guardian* regulates p21^{Cip1} at the level of gene expression and mRNA stability.[35] It is clear that p21^{Cip1} plays several functions in cell-cycle control in addition to its role as an effector of p53 responses.[36,37] Conversely, no clear function has been established for INK4 inhibitors. The only exception is the aging process of normal fibroblasts, in which p16^{INK4A} accumulation has been demonstrated to have a causative role.[38] Moreover, and most interestingly for the purpose of this review, it has been recently reported that in a neuroblastoma cell line, the induction of growth arrest and of a senescent phenotype is associated with p16 build-up.[39]

The best investigated transcriptional response of an INK4 inhibitor is the upregulation of p15^{INK4B} after addition of TGFβ to epithelial cells. However, cells lacking p15^{INK4B} gene are still inhibited by TGFβ through the downregulation of the Cdk phosphatase CDC25A.[23] This observation suggests a high degree of redundancy in cell-cycle control.

In addition, unique among the INK4 inhibitors, p16^{INK4A} (or *CDKN2A*, the formal name of the gene) is a tumor suppressor gene associated with an enormous number of cancers.[40] It is not yet clear why p16^{INK4A} is particularly important in counteracting the development of malignancy. However, an explanation might take into account that the gene is able to encode two different proteins, using two alternative reading frames. The second protein codified by the gene *CDKN2A*, namely p14^{ARF}, is able to increase p53 level by inactivating hMDM2, the human homolog of mouse MDM2 protein (see below).

Transcriptional control is not the unique mode for regulating CKI level. In fact, p^{27Kip1} is controlled primarily by translational and post-translational control.[41,42] Generally, transcription of the p27^{Kip1} gene is constitutive, and analysis of the p27^{Kip1} promoter demonstrated that it is controlled by the ubiquitous transcription factor Sp1.[43] Although p27^{Kip1} protein level and the rate of its synthesis increase remarkably when fibroblasts exit the cell cycle, p27^{Kip1} mRNA levels remain constant.[41] However, induction of p27^{Kip1} mRNA has also been reported in some model systems of differentiation[44,45]

For p27^{Kip1}, proteolysis is one of the key modes of regulation. p27^{Kip1} is targeted for degradation via the ubiquitin/proteasome pathway. In particular, the protein-ubiquitin ligase known generically as SCF specifically ubiquitinates p27^{Kip1} previously phosphorylated by cyclin-E–Cdk2.[46-50] In addition, the factor of specificity, or F-box protein, that recognizes p27^{Kip1}, Skp2, is synthesized periodically at the G1/S phase boundary.[50]

Thus, both cyclin E increase (and concomitant Cdk2 activation), as well as the accumulation of Skp2, are necessary to p27^{Kip1} proteolysis required for committing cells to enter the S phase.

Paradoxically, Cip/Kip inhibitors have been associated with Cdk activation as well as inhibition. Cip/Kip inhibition of cyclin-D–Cdk4 complexes is relatively poor[27] and the assembly of cyclin-D–Cdk4 complexes *in vitro* leads largely to inactive dimers.[51] Thereby, possibly on the basis of their ability to act as a bridge between cyclin and Cdk, Cip/Kip inhibitors can also serve as chaperonins, facilitating the achievement of an active conformation. Such *in vitro* findings have been recently confirmed *in vivo* by studying fibroblasts from p21$^{Cip1-/-}$ and p27$^{Kip1-/-}$ mice. Indeed, in the absence of both the CKIs, it was impossible to detect any cyclin-D–Cdk4 complexes. However, and most surprisingly, the absence of cyclin D complexes did not have any effect on the cell cycle, thus suggesting that the absence of CKIs compensated for the absence of cyclin-D–Cdk4 complexes.[52]

Finally, in the context of mechanisms regulating G1-S transition, it must be underlined that cyclin D–Cdk4/6 complexes have two roles: i) they promote cell-cycle progression by phosphorylating pRb and its homologs, and ii) they act as a reser-

voir for Cip/Kip inhibitors. In fact, an increase of cyclin D induces S phase entry by sequestering Cip/Kip inhibitors away from cyclin E–Cdk2, and, in turn, activating the enzyme. On the other hand, the dissociation of cyclin D and Cdk (caused, for example, by the accumulation of $p16^{INK4A}$) releases Cip/Kip inhibitors for binding and inhibiting cyclin-E–Cdk2. This effect appears to be important for tight cell-cycle regulation. The redistribution of Cip/Kip inhibitors to cyclin E–Cdk2 complexes (due to the increase of INK4 inhibitors) is clearly exemplified by the response of epithelial cells to the cytokine TGFβ. TGFβ stimulates the transcriptional induction of $p15^{INK4B}$, which then binds to Cdk4 and Cdk6. This results in a release of Cip/Kip inhibitors (particularly p^{27Kip1}), which associate with and inhibit cyclin E–Cdk2.[53,54]

Apoptosis: a "quiet" programmed cell death

Cells die by two major processes: i) necrosis, in which the release of intracellular lysozymes and proteases causes an inflammatory response, and ii) apoptosis (or programmed cell death), in which the cell remnants disappear, being phagocytosed by the surrounding cells.[55–59]

From a physiologic point of view, apoptosis is a general tissue phenomenon necessary for development and homeostasis, including i) elimination of redundant cells during embryogenesis; ii) tissue remodelling and repair; iii) cell atrophy upon loss of essential growth factors and/or cytokines; and, iv) removal of cells that have sustained genotoxic damage, such as cancer cells.[60–62] Interestingly, the mechanism of action of several (if not all) antineoplastic chemotherapeutic agents implies tumor cell killing by apoptosis.

Morphologically, cells undergoing apoptosis show ruffling, blebbing, and condensation of the plasma and nuclear membranes, and, subsequently, nuclear chromatin condensation. Conversely, ribosomes and mitochondria retain their structure and, at least partially, their function. The cytoskeletal architecture is disrupted; the cell shrinks and then fragments into membrane-enclosed *apoptotic bodies*, which are quickly phagocytosed by adjacent macrophages or other cells. The apoptotic bodies do not induce remarkable cytokine release. This explains why programmed cell death does not cause a concomitant inflammatory response.[55–59]

The end-stage of apoptosis is characterized by DNA endonuclease cleavage of the linker regions between nucleosomes, yielding 180 base-pair fragments. Thus, agarose gel electrophoresis analysis of apoptotic DNA reveals a characteristic ladder pattern; this pattern contrasts with the untidy DNA pattern seen in cells that have undergone necrosis. In addition, programmed cell death is an active energy-dependent process requiring RNA and protein synthesis.

The cell death process might be activated by different events, including: a) genes responding to internal stimuli, for example DNA damage; b) death signals received at the cell membrane (like Fas ligand), or c) proteolytic enzymes entering directly into the cell (granzymes). The final events, evidenced by cell structure changes and disassembly, are the activation of specific cysteine proteases, the caspases. Apoptosis regulation is strongly conserved, with a large number of the same genes regulating the process present from nematodes to humans. Although the death signal may be controlled by gene expression, programmed cell death can be activated, as described above, by diverse stimuli. Several of them (including particularly genotoxic damages *e.g.* chemotherapy, radiation) cause DNA single- or double-strand breaks or nucleotide deprivation and activate a cascade beginning with the activation of the transcription factor p53, whose targets induce either growth arrest or entry of the cell into the apoptotic pathway.

The p53 protein is a pivotal regulator of DNA transcription and metabolism; it binds directly to DNA, recognizes DNA damage (single- or double-strand breaks), and mediates at least two important cellular responses, namely the induction of G1 cell cycle arrest and the promotion of apoptosis. If the cellular damage is *reparable*, p53-dependent cell cycle block allows a time-period for DNA repair. Conversely, in the presence of a more extensive damage (to avoid proliferation of cells with altered DNA and the possible development of malignant clones), p53 activates the apoptotic pathway.

From a mechanistic point of view, DNA aberrations induce a series of complex processes (including the upregulation of specific kinases), whose description is behind the goal of this review.[63] In turn, such molecular events result in increased synthesis, phosphorylation and acetylation of p53. The post-synthesis modifications (*i.e.*, phosphorylation and acetylation) have profound consequences on p53 stability. They render the protein more active and reduce its binding and inactivation by MDM2 (a protein which commits p53 to destruction), thereby doubling the half-life of p53. As a result, the protein activity may increase a hundred-fold. Subsequently, p53 promotes cell cycle arrest in late G1 through upregulation of $p21^{Cip1}$ protein and the

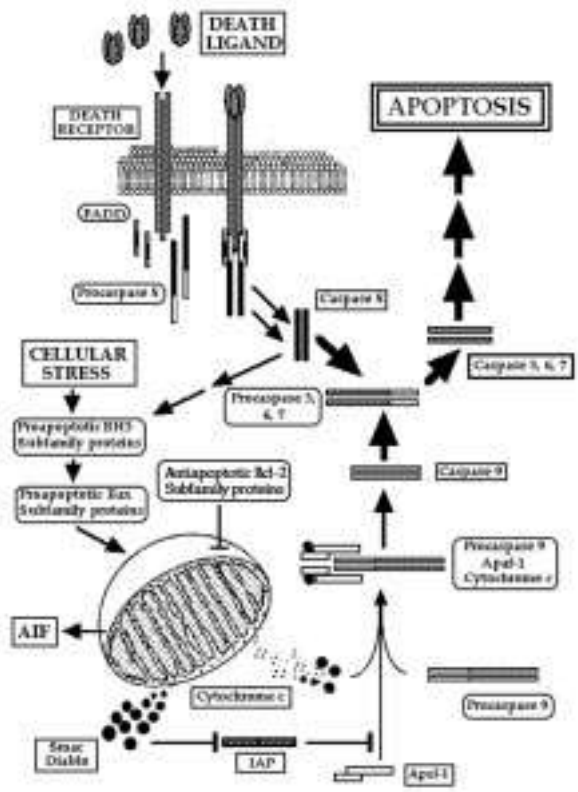

Figure 2. Schematic representation of apoptosis. The diagram shows the two major pathways causing activation of apoptosis. Extracellular stimuli, by means of specific membrane receptors (death receptors) and intracellular adaptor molecules (FADD), induce the activation of caspase 8 and, subsequently, of caspase 3 (and caspases 6 and 7). Activated caspase 3 causes the final aptoptotic events. Intracellular stimuli (cellular stress) induce the activation of specific pathways, involving proteins of the Bcl-2 family, which result in alterations of mitochondrial structure and function. The organelle releases proteins [AIF] which migrate into the nuclei and activate DNA digestion. Moreover, cytochrome c migrates from the intermembrane space into the cytosol and interacts with the Apaf-1 [Apaf-1] protein. The resulting complex activates caspase 9 and, in turn, caspase 3 (and caspases 6 and 7). Apaf-1 activation is prevented by specific inhibitor proteins [IAP]. A further factor, also released from mitochondria, Smac / Diablo, is able to hamper IAP function. Additional details on programmed cell death are reported in the text.

effects described in the previous paragraph.

In the case that DNA damage is severe and *irreparable*, p53 performs the alternative function of carrying the cell into apoptosis. Although the precise steps which allow this event are not fully clarified, evidence suggests that it occurs via the transcriptional activation of several genes (including *APO1/Fas*, *DR5*, *IGF-BP3*, *PIGs*, *PAG608*, *PERP*, *Noxa*, *PIDD*, *DRAL* and *p53AIP1*) and, particularly, through the modulation of the Bcl-2/Bax pathway (namely upregulation of Bax and downregulation of Bcl-2). Moreover, it must be underlined that recently a transcriptionally-independent p53-related apoptosis was described which involves the translocation of activated p53 from the nucleus to mitochondria.[64]

A pivotal downstream regulation of the apoptotic signals is carried out by the Bcl-2/Bax gene family. Sixteen members of this protein group have so far been recognized. Some of them, particularly, Bcl-2 and Bcl-xL, are anti-apoptotic proteins; others, including Bax, Bad, and Bid, are apoptotic activators. An upregulation of the Bax family promotes programmed cell death, while an increased expression of the Bcl-2 group inhibits apoptosis. As anticipated above, some of the pro-apoptotic effects of p53 protein might be due to modulation of the ratio of Bcl-2/Bax groups.[65-67] (Figure 2). Once the apoptotic process is activated, Bax proteins move from the cytoplasm to the *permeability transition pore* localized on the mitochondrial membrane, causing a series of events which finally allow the release into the cytosol of intermembrane space proteins (including cytochrome c, apoptosis-inducing factor *AIF*, DIABLO/Smac and others)[65–70] (Figure 2). AIF moves directly to the nucleus, where it induces chromatin condensation and nuclear fragmentation. Cytochrome c binds and activates the cytosolic protein Apaf-1 (apoptotic protease activating factor-1), which in turn is required for the subsequent activation of procaspase-9 (Figure 2). Caspase-9 directs the activation of downstream caspases, including procaspase-3, responsible for the cytological changes characteristic of apoptosis (Figure 2).

Caspases are endoproteases with a cysteine in their active site, which cleave peptide bonds having an aspartate at the N-terminus, (thus the acronym cysteine-*asp*-*ase*). They are expressed as constitutive inactive precursors and are mostly present in

the cytosol (although some reports describe the occurrence of mitochondrial caspases). Thirteen caspases have been identified. They are classified either as *initiators* (caspase 2, 8, 9, and 10), or as *effectors* (caspase 3, 6, and 7) of proteolysis. The effector caspases are localized downstream of the initiator caspases in the apoptotic pathway. Proteolysis by caspases is specific and limited: peptide cleavage occurs only after a particular aspartate residue, causing modification of particular proteins and not a general proteolysis[71–74] (Figure 2).

The temporal activation of caspases results in several important effects: i) lysis of cytoskeletal proteins; ii) nuclear membrane disruption; iii) loss of cell-cell contact, and, iv) activation of the DNA nuclease (CAD, caspase-activated deoxyribonuclease) to allow DNA fragmentation. Moreover, the limited proteolysis does not result in cellular lysis, but in the formation of apoptotic bodies. These events are responsible for the morphologic features of apoptotic cells, as well as for the DNA agarose gel ladder pattern that is highly apoptosis-specific.

A family of protein caspase inhibitors, IAPs (inhibitors of apoptosis), selectively inhibits effector caspases, blocking the apoptotic process; these proteins are overexpressed in many malignant cells.[75] The IAP family also inhibits apoptosis through non-caspase-dependent mechanisms, such as the modulation of transcription factors and the control of cell cycle progression.[75]

In addition to being activated by genotoxic events which cause DNA damage, apoptosis might be due to other causes. One is engagement of the so-called *death receptors* (see below) and the other is activation of intracellular pathways which might directly induce the release of cytochrome c. For example, exposure of cells to H_2O_2 causes upregulation of JNK activity, binding of cytochrome c to Apaf-1 and, finally, activation of caspase 9. Accordingly, embryonal fibroblasts from double JNK null mice (i.e. mice lacking functional JNK1 and JNK2 genes) are resistant to H_2O_2-induced apoptosis.[76]

As anticipated before, an alternative mechanism of programmed cell death is through the activation of *death receptors* expressed on the cell membrane (Figure 2). The archetype of this molecular system is formed by the cell-surface receptor Fas (APO1, CD95), a member of the tumor necrosis factor receptor (TNF-R) family, and its ligand.[77–79] Fas is a transducer of the apoptotic signal and is expressed on a wide range of cell types: lymphoid cells, hepatocytes and some tumor cells, as well as in lung and myocardium. Fas ligand (FasL) belongs to the TNF family; its expression is more restricted, since it is found in cytotoxic T-cells and natural killer cells. FasL, by binding to Fas, sets the apoptotic process in motion (Figure 2). This mechanism plays a pivotal role in deletion of virus-infected target cells, immune response, killing of cells in numerous pathologic conditions, and, mostly important, destruction of tumor cells.[77–79] Fas ligand, TNF (tumor necrosis factor) and TRAIL (TNF-related, apoptosis-inducing ligand) activate programmed cell death by an analogous mechanism, namely the engagement of specific receptors. Upon binding of their respective ligand, the receptors assemble into trimeric complexes. The cytoplasmic tail of each receptor contains a so-called death domain (DD) that interacts with a specific cytoplasmic protein. Such a protein, also containing DD, is able to initiate a direct pathway to apoptosis through recruitment and activation of procaspas 8 (Figure 2). Caspase 8 subsequently activates downstream effector caspases, including caspase 3, 6 and 7.

Similarly to Fas ligand, TRAIL (Apo2L) has been shown to be a potent inducer of apoptosis in various cells and particularly in those established from cancers.[80] However, differently from TNF and Fas ligand, which are both lethal when injected into mice,[81] TRAIL exerts potent antitumor activity without showing systemic toxicity.[82,83] Four TRAIL receptors have so far been identified; two agonistic (TRAIL-R1/DR4[84] and RAIL-R2/DR5/KILLER[85] and two antagonistic TRAIL-R3/DcR1/TRID[84, 85] and TRAIL-4/DcR2/TRUNDD.[86] Although TRAIL and TRAIL-R1 and -R2 are expressed in a large number of human tissues, normal cells are not responsive to TRAIL-mediated killing.[87] This might be due to the expression of the antagonistic receptors in normal tissues which protect normal tissues from TRAIL-induced apoptosis by competing with the activating receptors for limited amounts of TRAIL.[84-86]

A similar complex behavior is shown by the nerve growth factor (NGF) and its receptors: the high affinity receptor tyrosine kinase TrkA, and the low affinity receptor p75. Since p75 is also a member of the TNF family of death receptors, its engagement by NGF stimulates sphingomyelin hydrolysis and ceramide synthesis, and, finally, apoptosis. As a low affinity receptor, on the other hand, p75 modulates ligand binding and some signaling pathways activated by TrkA.[88] Indeed, TrkA and the other neurotrophin receptors play important roles in the survival and differentiation of neuronal progenitors in the central and peripheral nervous systems.

Neuroblastoma and cell division cycle engine alterations

Prelude

Among the numerous genes designed (probably too simplisticly) as potential tumor suppressor genes (TSGs), very few of them seem to be involved truly in the origin and/or progression of human cancers. In particular, a large body of evidence suggests that only *RB1* and *p53* genes are significantly altered in a wealth of different tumors. It is intriguing that both these genes encode proteins which directly (pRb) or indirectly (via $p21^{Cip1}$ protein) participate in the control of the cell cycle. However, as will be shown below, several studies suggest that these genes are scarcely modified in human neuroblastoma.

An additional TSG has been discovered more recently, the *CDKN2A* (or $p16^{INK4A}$) gene, which encodes two different proteins able to regulate G1/S phase transition and p53 function. This gene, like *RB1* and *p53*, appears inactivated in a tremendous number of cancers through different mechanisms.

A further component of the cell cycle engine which seems strictly correlated to cancer development is the CKI $p27^{Kip1}$ protein. Numerous reports have demonstrated a strong correlation between the levels of $p27^{Kip1}$ protein and human cancer outcome.[89-96]

In the following sections, we will report a brief description of the major genetic and functional alterations of the cell cycle engine observed in human neuroblastoma. Other changes which are frequently detected in human cancers, namely amplification of cyclin D1, cyclin E and CDK4 genes and, in a minor way, altered expression of $p21^{Cip1}$ and CDK6, are not described in this manuscript, since very few and confusing reports correlate them to NB origin and development.

CDKN2A inactivation

CDKN2A gene (the gene which encodes $p16^{INK4A}$ and $p14^{ARF}$ proteins) is inactivated in a very large number of human cancers.[97-100] Three different mechanisms might account for this event: homozygous deletion, point mutation and expression silencing (by CpG islets promoter hypermethylation). Importantly, the inactivation mechanism is highly tumor-specific, although homozygous deletion (HD) is the most frequently observed.[101,102]

On this basis, several studies have investigated the intriguing hypothesis that *CDKN2A* gene might be inactivated in human neuroblastoma. Several loss of heterozygosity studies cumulatively demonstrated 9p21 (the region where *CDKN2A* maps) allelic loss in about 22% of primary neuroblastomas.[103-107] Conversely, *CDKN2A* HD has not been found in primary tumors[103-10] and has been observed in a small percentage (9%) of neuroblastoma cell lines.[111]

Furthermore, only a single *CDKN2A* mutation, a missense mutation at residue 52 of $p16^{INK4A}$ exon 2, was identified out of a large number of primary tumors and cell lines (178 and 28, respectively) screened.[103-105,107,109,110] Although exon 1beta (encoding $p14^{ARF}$) was not included in the screening of mutations, these results together suggest that a purely genetic disruption of the *CDKN2A* locus in neuroblastomas is uncommon. Finally, the possibility that the *CDKN2A* gene is silenced by epigenetic processes, like hypermethylation of the promoter region, has been investigated in a few instances,[103,104,107] and the results obtained indicate the absence of this event in NB.

It is also conceivable that the gene codifying $p16^{INK4A}$ and $p14^{ARF}$ proteins is inactivated by alternative mechanisms. In this case, expression studies (both at mRNA and protein level) might furnish intriguing information. However, the literature data give conflicting findings[104,107,112] and, in general, do not allow a clear conclusion or any obvious correlation with other parameters.

N-myc/pRb, the Id2 connection

So far, structural and functional alterations of *RB1* gene have not been reported as a major mechanism of neuroblastoma origin. Conversely, it is well established that *N-myc* gene amplification represents one of the most peculiar genetic features of human NB, as well as an important independent prognostic marker.[113-115] Moreover, targeted expression of *N-myc* in transgenic mice causes development of neuroblastoma.[116] However, the precise functional correlation between the high level of *N-myc* protein and neuroblastoma development and progression is, at present, almost obscure.

Recently, an important experimental breakthrough was made in this field. Indeed, it has been possible to correlate the increase of *N-myc* protein and the loss of pRb function. Id2, a member of the Id family, is a dominant negative protein able to sequester pRb (and the other *pocket proteins*, p107 and p130, see the previous paragraph on the cell cycle) and, in turn, to abolish the antiproliferative effect of this tumor suppressor protein.[117–119] *N-myc* amplification (and the consequent accumula-

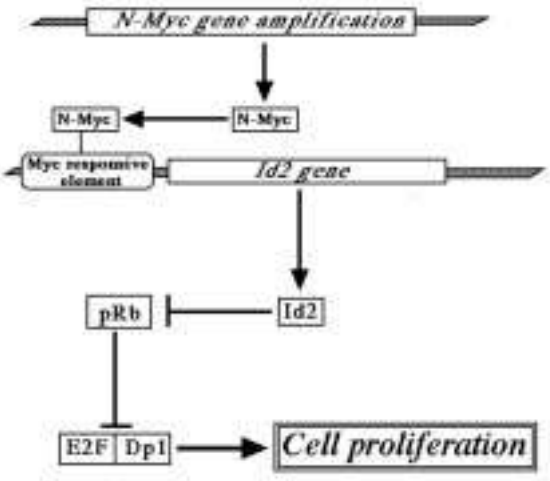

Figure 3. Diagram of the effects of *N-myc* gene amplification on pRb antiproliferative function. *N-myc* gene amplification induces *N-myc* protein accumulation. The transcriptional factor, interacting with a DNA-specific responsive element, causes activation of the Id2 gene and upregulation of the relative protein. Id2 binds and inactivates the pRb protein, thus causing loss of the antiproliferative function of this protein.

tion of *N-myc* protein) induces a remarkable increase of *Id2* gene expression and of *Id2* protein level[120] (Figure 3). This is due to the occurrence on the *Id2* gene regulatory region of two high affinity myc–binding regions[120] (Figure 3). Moreover, analysis of *Id2* protein content clearly demonstrated that neuroblastoma cell lines having an amplified *N-myc* gene accumulate the protein.[120]

In conclusion, it is possible to suggest that in neuroblastoma, although *RB1* gene is not altered, its function is abolished by *N-myc*-dependent *Id2* accumulation (Figure 3). This important and intriguing hypothesis again points to *RB1* inactivation as a central event in the development of human cancers, including neuroblastoma.

p27^{Kip1} and neuroblastoma

It has been established that low levels of p27^{Kip1} correspond to poorer outcomes in several human cancers.[89-92] Aggressive cancers appear to contain a more active p27^{Kip1}-specific degradation system when compared to neoplasias with a favorable outcome.[93] Therefore, the content of cellular p27^{Kip1}, as estimated by immunologic methods, represents an independent prognostic factor for several cancers, including tumors of colon,[91] rectum,[93] stomach,[92] breast,[90] prostate,[95] liver[96] and several other tissues.

In the case of NB, an increase of p27^{Kip1} protein is associated with the retinoic-dependent differentiation of several neuroblastoma cell lines.[121, 122] This event precedes the accumulation of cells in G1 phase and, thus the blockage of cell growth,[123] indicating that the CKI build-up is a cause, not a consequence, of the proliferation arrest. Additional data indicate that the mechanism of p27^{Kip1} increase is related to a not completely identified mechanism which hampers CKI degradation.

The data on retinoic-treated neuroblastoma cell lines and the findings in other cancers collectively suggest that the analysis of p27^{Kip1} content in neuroblastoma could be of interest.

At present, few data are available on this topic. A genetic study on 25 neuroblastoma samples[124] demonstrated the complete absence of p27^{Kip1} gene abnormalities, including deletions and point mutations. A different pattern was observed by analyzing the CKI protein content by immunohistochemistry. The analysis of 30 childhood cancers showed that p27^{Kip1} was expressed in all cases, although the staining intensity and intracellular localization varied in association with tumor differentiation. Primitive, small, round neuroblasts presented negative or only weak nuclear staining, while differentiating tumor cells displayed, besides the nuclear staining, a novel, intense cytoplasmic positivity.[125] This finding leads to the conclusion that, not only *in vitro* but also *in vivo*, the differentiation process is associated with accumulation of p27^{Kip1}.

Finally, a very recent immunohistochemistry analysis, performed on 100 primary tumor biopsy samples from neuroblastoma patients with a documented follow-up, suggests that the level of the CKI is an independent prognostic factor.[126] This study, in addition, ruled out a possible interplay between p27^{Kip1} cellular content and the degree of *N-myc* gene amplification.[126]

Thus, although a definite conclusion on the importance of p27^{Kip1} levels in neuroblastoma cannot be reached, several findings suggest the occurrence of an inverse correlation between the protein content and NB progression.

A brief conclusion

It is widely believed that the p16^{INK4A}/Cdk4/pRb pathway is altered in almost all human tumors. Apparently, neuroblastoma escapes this widely accepted view. Indeed, *RB1* alterations are rare in this cancer, as are *CDKN2A* abnormalities (although 9p21 LOHs are frequently observed). In addition, no definite data on p27^{Kip1} level have been reported.

A possible explanation for these findings is that the pRb pocket protein is functionally inactivated

as a consequence of a different mechanism, *i.e.N-myc*-dependent Id2 protein upregulation. This mechanism might link the increased level of *N-myc* protein (a marker of a negative prognosis) and the loss of pRb activity. Future studies should confirm this pivotal observation in primary samples. However, some questions still remain open. Is the Rb pathway in cell lines with a physiologic *N-myc* dosage normal? And, if not, what is the mechanism which inactivates the function of the pocket proteins or of the other gatekeeper genes of cell division cycle? The answers must await future investigations on cell lines, primary NB samples and modified animal models.

Moreover, and most importantly, inactivation of the $p16^{INK4A}$/Cdk4/pRb perhaps should not be considered as a mechanism of *malignant transformation*. Possibly, it could simply accelerate the rate of proliferation leading to the loss of some cell cycle checkpoints. Such phenomena might increase the possibility of cancer development but should not be considered as the initial cause. Thus, the isolation of a possible *neuroblastoma gene* still remains elusive, as does, in part, the role of the *N-myc* gene.

Neuroblastoma and resistance to programmed cell death: molecular bases and clinical meaning

Prelude

An inappropriate increase of cell number is one of the hallmarks of malignant cell transformation. It can be a result of either a faster growth rate or reduced apoptosis. Moreover, since many chemotherapeutic approaches are based on the induction of apoptosis, the development of drug-resistance might depend on the development of genetic changes which result in inefficient activation of programmed cell death.

Defective apoptosis in human cancers may result from a large number of different phenomena, which include: i) inactivation of genes involved in cell death induction, or ii) constitutive activation/overexpression of genes which counteract the apoptotic process. The next paragraphs will briefly describe the major alterations of apoptosis so far observed in neuroblastoma.

p53 gene family abnormalities

Mutations of the p53 gene are very rare in primary neuroblastomas, and deletions or LOH at 17p (where *p53* maps) are uncommon.[127] There is, however, evidence that inactivation of the p53-dependent pathway to apoptosis is important in some cases of neuroblastoma . Amplification of the p53-inhibitory *MDM2* locus has been identified in several neuroblastoma cell lines and in a single tumor.[128–130] Furthermore, immunohistochemistry studies suggest that p53 protein is sequestered in the neuroblastoma cytosol, a phenomenon which has been hypothesized to be a post-translational mechanism for functional repression of the tumor suppressor protein.[131,132]

Recently, it has been demonstrated that *p53* gene is a member of a large family which includes at least two other genes, *p63* and *p73*, both sharing a remarkable sequence homology.[133,134] *p73* gene in particular has attracted the attention of neuroblastoma researchers, since it was initially thought to be the long sought tumor suppressor gene localized on 1p36 and, perhaps, responsible for the development of this cancer.[135]

However, more detailed studies dampened the enthusiasm and demonstrated that, at best, this gene is inactivated in some cases of neuroblastoma, possibly by hypermethylation of its regulatory regions.[136]

Alterations of Fas/Fas ligand and Bcl-2/Bcl-xL pathways in neuroblastoma development and drug resistance

Neuroblastoma has the highest rate of spontaneous regression observed in human cancers. Children with 4S NB often have initial progression of multifocal disease followed by rapid tumor involution. Delayed implementation of normal apoptotic pathways has been proposed as an explanation for this phenomenon.[137]

As discussed before, entrance into a programmed cell death pathway can be originated by exogenous (presence or absence of ligands) or endogenous (DNA damage) signals. Members of the tumor necrosis factor receptor family, such as p75 (the low affinity NGF receptor cited earlier) and Fas (which binds Fas ligand) can mediate the induction of apoptosis in some neuroblastoma cell lines.[138, 139] In addition, increased Fas expression seems an essential component of chemotherapy-induced apoptosis of neuroblastomas.[139]

Intracellular molecules responsible for relaying the apoptotic signal include proteins of the *Bcl-2* family. Apoptosis-suppressing genes, such as *Bcl-2* and *Bcl-xL* are highly expressed early in neuronal ontogeny. *Bcl-2* is strongly expressed in most neuroblastoma cell lines[140-142] and primary tumors,[143-147] and the level of expression is inversely related to the proportion of cells undergoing apoptosis and to the degree of cellular differentiation.[146-148]

There have been conflicting reports regarding the correlation between the level of expression of *Bcl-*

2 in primary tumors and prognostic variables,[144, 148-150] but overall, the evidence suggests that there is no significant interplay. However, the *Bcl-2* family of proteins may play an important role in acquired resistance to chemotherapy. Transfection of cDNA encoding either *Bcl-2* or *Bcl-xL* into neuroblastoma cells causes resistance to alkylating agent–induced apoptosis in a dose-dependent manner.[141,151] These observations are consistent with the hypothesis that neuroblastomas prone to undergoing apoptosis are more likely to regress spontaneously and/or to respond well to cytotoxic agents.

Survivin gene expression

Survivin is a recently described member of the IAP family.[152] The gene encoding for human survivin was first identified by screening a human genomic DNA library with a complementary DNA putatively encoding the coagulation factor Xa receptor, effector cell protease receptor-1 (EPR-1).[152] The mechanism of action of the protein has not been completely understood. Survivin seems to be involved in the control of the mitotic spindle checkpoint[153] and, thereby, in cell cycle progression. Moreover, the protein interacts with caspase 9[154] suggesting that it prevents the activation of caspase 3 and 7. These effects should prevent the upregulation of the key apoptotic pathways.[154,155] However, the ability of survivin to inhibit caspase 3 activity directly has been strongly questioned.[156]

An important observation concerning survivin is that the protein occurs at a high level in embryonal tissues and in various cancers, but, unlike other IAP proteins, not in normal adult tissues. In 1998, an immunochemical analysis of 73 neuroblastoma samples demonstrated that this cancer shows a particularly elevated amount of the protein.[157] Moreover, the amount of survivin is directly correlated with the more aggressive cancer stages (stage 3 and 4).[157] More recently, a high gene dosage of survivin occurring in neuroblastoma cases with a poor prognosis has been described.[158] Although the mechanism of survivin accumulation in neuroblastoma has not been clarified, it is conceivable that it depends (at least in some cases) on gene amplification. In turn, this might suggest that the high level of survivin contributes to the drug-resistance of the most aggressive form of this childhood neoplasia.

Caspase deficiency

The cascade of activated caspases represents the heart of the apoptosis biochemical engine. Thus, structural or functional alterations of caspase genes might give remarkable advantages under specific conditions. For example, some viruses, once they have infected cells, induce the expression of an IAP protein which hampers the destruction of the host cells by apoptosis.[159,160]

Alterations of caspase genes have been little analyzed in human primary cancers and in malignant cell lines. However, recent investigations have demonstrated that caspase 8 expression is absent in a significant percentage of neuroblastoma cell lines.[161] It has also been reported that protease deficiency is mostly an epigenetic phenomenon due to DNA methylation while in a few cases it is correlated to gene deletion.[161] Furthermore, complete inactivation of caspase occurred almost exclusively in neuroblastomas showing *N-myc* amplification.[161] While the deficient cells were resistant to death receptor- and doxorubicin-mediated apoptosis, the re-expression of caspase resulted in a normal response to cell death stimuli.[161] Two subsequent papers confirmed the absence of caspase 8 expression in neuroblastoma cell lines[162,163] and associated this event with an increased resistance to TRAIL.[162,163]

The deficiency of caspase 8 appears to be extremely intriguing even if, so far, no data on primary samples are available in the literature. Preliminary studies, carried out in the laboratories of the authors, confirmed the absence of cysteine-protease expression *in vivo*, ruling out any correlation with *N-myc* gene amplification (*Iolascon et al., unpublished data*).

From a mechanistic point of view, the cancer advantage related to procaspase 8 absence is not immediately clear. However, very recently it has been demonstrated that caspase 8 is necessary for the transcription-independent cell death process activated by p53 protein.[164] Thus, the absence of caspase 8 might confer two distinct advantages to neuroblastoma cells: i) it might decrease the antitumoral activity of the immunologic system linked to the engagement of cell death receptor (by Fas ligand, TNF and, particularly, TRAIL), and ii) it might down-regulate p53-dependent apoptosis.

Neurotrophin receptors levels and neuroblastoma

A large body of evidence has demonstrated that the levels of different neurotrophin receptors (*i.e.* the above mentioned Trk) family play an important role in the biology and clinical behavior of neuroblastomas[165] In fact, neurotrophins, such as NGF, brain-derived nerve growth factor (BDNGF), and NT/3, are important growth factors for embryonal

development and remodeling of nervous systems, but they have also been associated with the survival, differentiation and apoptosis of neuroblastomas.[165] Importantly, forced overexpression of TrkA in neuroblastoma cell lines induces NGF responsiveness and differentiation.[166]

Observations from several independent studies suggest that high expression of TrkA is present in NB with favorable biological features and highly correlated with patient survival.[167] Conversely, the BDNGF receptor, TrkB, is mainly expressed on unfavorable, aggressive NB with *N-Myc* amplification.[168] TrkC, which might be activated (like TrkA) by NGF, as well as by NT/3, is generally increased in favorable prognosis NB.[169] All together these findings represent important clinical markers for the prognosis of neuroblastoma patients.

The rationale for this observation is obviously related to the different functions of the respective neurotrophins and to the pathway which is activated by the specific receptor. Less obvious is the cause of their different expressions. In other words whether this is a cause or a consequence. For example, no compelling evidence links *N-Myc* amplification and TrkB expression.

An additional intriguing recent observation is the absence of p75 protein in neuroblastoma samples as detected by immunochemistry.[170] Since engagement of this receptor (see also the discussion in a previous paragraph) is able to activate the apoptotic process, its deficiency might contribute to the general apoptotic alterations characteristic of human neoplasias. The data on p75 need further confirmation before they can be fully evaluated

Is apoptosis altered in human neuroblastoma?

A number of reports suggest that the apoptotic response is modified in neuroblastoma cells. Indeed, data from immunohistochemical investigations indicate that the level of survivin in aggressive stages of the cancer is higher than that in the forms with a positive prognosis (Figure 4). This is due perhaps to an increased gene dosage or perhaps to different mechanisms. Moreover, studies on cell lines demonstrate a deficiency of caspase 8 due to silencing of gene promoter through, possibly, a methylation mechanism (Figure 4). These observations appear to be associated with an altered response to the TRAIL molecule.

Both these findings, if further confirmed, highlight the possibility that neuroblastomas have an impaired cell death response. The advantage of malignant clones is related to their difficulty in being destroyed by physiologic mechanisms (such as TRAIL) which are devoted to removing transformed cells. Indeed, 4S neuroblastoma patients, in whom apoptosis is not altered, show spontaneous recovery. Obviously, this does not mean that survivin and caspase 8 are dominant oncogenes or tumor suppressor genes. These findings may mean that a defective apoptotic response might be of aid in causing the survival of cells with genetic alter-

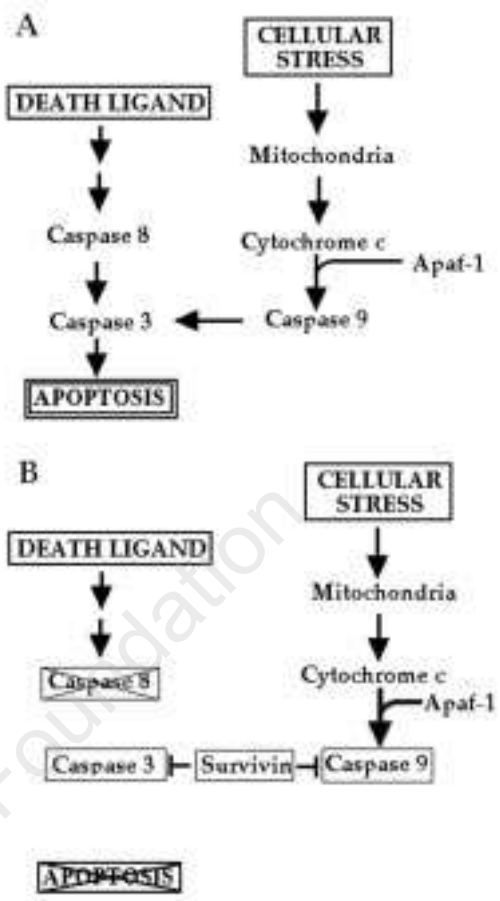

Figure 4. Alterations of apoptosis identified in human neuroblastoma. Panel A summarizes the physiologic apoptotic pathways (see also Figure 1). Panel B reports the alterations of apoptosis which have been observed in neuroblastoma. In particular, the absence of caspase 8 has been demonstrated in several specimens. This alteration should hamper the activation of caspase 3 and the following apoptosis due to extracellular stimuli. In addition, several neuroblastoma cells show accumulation of survivin, an IAP protein. Survivin downregulates the activation of caspases 9 and 3. Thereby, programmed cell death due to conditions of stress is also, at least in part, inactivated. Additional details on the mechanisms shown are reported in the relative paragraph.

ations. In other words, the absence of an efficacious cell death program is a strong basis for accumulating DNA abnormalities, but not the cause of the genomic alterations. In this scenario, it is not known whether the inability to induce normal apoptosis precedes or follows the genetic abnormalities which give a neuroblastoma phenotype.

The importance of apoptotic abnormalities in human cancer is strongly confirmed by the occurrence of p53 mutations in a tremendous number of neoplasias as well as by *Bcl-2* gene alteration in specific hematologic tumors. Thus, it is not surprising that neuroblastoma, like other cancers, shows less efficient programmed cell death. What is intriguing is the molecular mechanism responsible for such impairment. However, future studies will be necessary to understand the importance of the findings described and to verify that apoptosis functions poorly in NB samples.

Are neuroblastoma molecular alterations important in hematologic infant neoplasias?

The molecular mechanisms responsible for neuroblastoma development described above might be important in other neoplasias of infancy, particularly in hematologic cancers which represent a large percentage of childhood cancers. Very few (or not consistent) data are at present available regarding Id2 upregulation and caspase deficiency in acute leukemia, the major childhood blood tumor. Survivin overexpression has been consistently reported in adult hematologic cancers,[171,172] and, in our opinion, future studies should be devoted to investigating this alteration in pediatric hematologic neoplasias too.

Among cell division cycle aberrations, *CDKN2A* gene inactivation certainly plays a pivotal role in infant hematologic cancers and particularly in acute lymphoblastic leukemias (ALL).[173-176] At least two studies merit being reported in detail.

Zhou *et al.*[173] evaluated 105 pediatric ALL cases and reported *CDKN2A* gene deletions in 28% of B-ALL and in 68% of T-ALL samples. In 37 out of 38 cases with *CDKN2A* deletion, the *CDKN2B* gene was also deleted. However, isolated *CDKN2B* deletions were not detected. The study also suggested an increased prevalence of *CDKN2A/CDKN2B* deletions in T-ALL subtypes ($p<0.005$), associated with other adverse factors, such as age <1 year and white cell count (WBC) number greater than 50×10^9/L. The five-year disease-free survival was 68% for patients without *CDKN2A/CDKN2B* deletions and 35% for those with *CDKN2A/CDKN2B* deletions ($p<0.005$).

Given the relevant incidence of *CDKN2A/CDKN2B* gene deletions in pediatric T-ALL, Diccianni *et al.*[85] investigated the prognostic value of these genetic abnormalities within a subgroup of 103 patients with T-ALL. *CDKN2A* alteration was detected in over 60% of cases at first presentation. Of the primary samples with *CDKN2A* deletions, 72% also had *CDKN2B* mutations. In contrast to other reports, which included all ALL sub-types, Diccianni *et al.* found that the presence of *CDKN2A/CDKN2B* deletions did not correlate significantly with other poor prognostic factors (higher WBC or mediastinal mass), nor was it predictive of relapse rate or duration of first remission.

In conclusion, it is interesting to note that, although different from a mechanistic point of view, both neuroblastoma (via N-myc/Id2 up-regulation) and ALL (via *CDKN2A* loss of function) show functional inactivation of pRB growth restraining activity. This, once again, demonstrates that this pathway is pivotal in the development of human cancers.

The importance of a bridge between molecular studies and clinical applications: a potential basis for new neuroblastoma treatments

A frequent and major concern of clinicians is whether the enormous amount of information deriving from molecular studies (both biochemical and genetic investigations) is really important or whether it is just an interesting description without any relevance for the diagnosis, prognosis and treatment of a disease. This is particularly important in the case of childhood cancers, for which very few efficacious treatments exist.

This review describes the state-of-art of the basic investigations on two pivotal cellular processes, *i.e.* cell division cycle and apoptosis, in human neuroblastoma in the hope of proposing new working hypotheses useful for both basic researchers and clinicians.

Three major novel abnormalities have been highlighted, namely i) *N-myc* gene amplification causes Id2 protein overexpression, which in turn inactivates the pRb-dependent antiproliferative pathway; ii) the anti-apototic protein survivin accumulates in poor survival neuroblastomas, and, iii) procaspase 8 gene is frequently silenced in neuroblastoma causing impairment of the apoptotic response.

At present, it is difficult to evaluate the importance of these discoveries in the origin and/or development of neuroblastoma. *N-myc* gene amplification is certainly important in this child-

hood cancer and a direct correlation between the transcriptional factor protein level and aggressivity of neuroblastoma has been demonstrated.[115] However, there is no direct proof that *N-myc* gene dosage is directly involved in neuroblastoma onset, and in addition, a significant percentage of neuroblastomas do not have the gene amplification. It is highly probable that, like other molecular events, the loss of Rb function (due to *N-myc* amplification) results in an increased rate of proliferation, thus worsening the patient's survival. On the other hand, it is still to be demonstrated that *N-myc* acts exclusively through Id2 protein especially since myc proteins may have pleiotropic activities.

Survivin overexpression and procaspase 8 gene silencing should both inactivate, at least in part, anticancer apoptotic defenses. Intriguingly, from a mechanistic point of view, the contemporaneous presence of the two alterations is additive (Figure 4). This should result in inefficient destruction of cells carrying altered DNA and in accumulation of cancer-inducing genetic abnormalities. The origin of both the observed phenomena is unknown and might be a consequence of epigenetic processes. However, since neuroblastoma frequently develops in the first year of life, the putative epigenetic events must occur very early, perhaps during intrauterine life.

Thus, although the consequence of survivin increase and caspase 8 deficiency, *i.e.* increased survival, is conceivable (but not experimentally defined) their origin is so far obscure. Moreover, their relationship with the cause (or causes) of neuroblastoma remains an open question.

On the other hand, an interesting feature is the possibility of developing therapies which allow the modification of the events described. Firstly, *N-myc* downregulation is, at present, obtainable *in vitro* by treatment with retinoic acid, an approach which also results in $p27^{Kip1}$ upregulation.[121] Both, these effects should result in Id2 decrease and in inhibition of Cdk2 activities, and in turn, upregulation of pRb function. Interestingly, the effect of retinoic acid is potentiated by treatment with histone deacetylase inhibitors which are themselves able to induce $p21^{Cip1}$. Since caspase 8 silencing seems to be overcome by treatment with azacytidine, it could be possible to reconstitute the pool of the proteins with this treatment or discover mechanims able to potentiate the activity of caspase 10, a functional analog of caspase 8.

In conclusion, although the importance of the reported mechanisms in neuroblastoma is not clear, and, particularly, their relationship with the origin of this tumor remains elusive, their discovery opens up new paths for the development of therapies based on manipulating gene expression. Indeed, we believe that modifying gene expression by non-toxic agents might yield very intriguing new therapies. However, only detailed knowledge of the functions of target genes and of the specificity of potential drugs will provide the sound bases on which to develop new pharmacologic strategies. Thus, more knowledge on the molecular biology of neuroblastoma is still necessary.

Contributions and Acknowledgments

AB, FDR and AI analyzed the data present in the literature on neuroblastoma and the cell division cycle. RR and FDR summarized and evaluated the findings related to NB and apoptosis. AB wrote the paper. FDR and AI acted as final supervisors. All authors approved the final version of the manuscript.

Funding

This work was supported in part by grants from the Fondazione Italiana per la Ricerca sul Cancro (FIRC), Associazione Italiana per la Ricerca sul Cancro (AIRC), MURST (Progetti di Rilevante Interesse Nazionale), Cluster di Biomedicina - Progetto1 - MURST and the Associazione Italiana per la lotta al Neuroblastoma.

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