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Prof. Dr. András Jeney

Apoptotic and proliferative activity in Neuroblastoma and PNET, tumors of the Thyroid gland and Parathyroid gland

Dr. Parvaneh Farid

Supervisor: Prof. Dr. Béla Szende

Semmelweis University
1st Department of Pathology and Experimental Cancer Research

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Introduction

A noble man of Persia by the name Baha’u’llah (1817-1892) once explained: ”the stages that mark the wayfarer’s journey from the abode of dust to the heavenly homeland are said to be seven, the first is the Valley of Search, the steed of this valley is patience”. This is a traveller’s tale. It has been hoped to tread the path of a real traveller, passing through the valleys of love, knowledge, unity, contentment, and wonderment, arriving to the seventh valley, which would lead us to a result in our research.

Over the past two decades there has been an increase in the findings on apoptosis. More than 30 new molecules have been discovered, whose known functions have exclusively to do with the initiation or regulation of apoptosis. A further twenty molecules at least, although already associated with important roles in signalling or DNA replication, transcription or repair, have been recognised as affecting the regulation of apoptosis. This remarkable process is responsible for cell death in development, normal tissue turnover, atrophy induced by endocrine and other stimuli, negative selection in the immune system, and a substantial proportion of T-cell killing. It also accounts for many cell deaths following exposure to cytotoxic compounds, hypoxia or viral infection. It is a major factor in the cell kinetics of tumors, both growing and regressing (1). Many cancer therapeutic agents exert their effects through initiation of apoptosis, and even the process of carcinogenesis itself seems sometimes to depend upon a selective, critical failure of apoptosis that permits the survival of cells after mutagenic DNA damage. It has been recognized that most if not all physiologic cell death occurs by apoptosis and that alteration of apoptosis may result in a variety of malignant disorders (2). Consequently in the last years interest in apoptosis has increased greatly. Great progress has been made in the understanding of the basic mechanisms of apoptosis and the gene products involved (3,4).

The aim of our study has been to learn more about the apoptotic and proliferative activities in certain endocrine cells, namely Thyroid, Parathyroid tumor cells, Neuroblastoma and some Primary Neuroectodermal Tumors (PNET) as they crossed our path with their rarity. The value of these parameters was studied regarding the prognostic and predictive values and their role in differential diagnosis. Having examined P53, Bcl-2 and Bax and RAR in different tumors of the tissues mentioned above, new dimension has been observed. Although few apoptotic tumor cells could be detected in our study in any of the benign or malignant tumors, this may be relevant to
the high expression of Bcl-2, since Bcl-2 is known to secure cell survival (5). The expression of Bcl-2, the qualitative and quantitative differences in different tumors of thyroid glands, be it in the adenomas or follicular adenocarcinomas, compared to its expression in papillary carcinomas had been observed to vary greatly, worth noticing while doing differential diagnosis in practice. The level of Bcl-2 expression in tumors was related to the apoptotic index as measured by morphologic assessments and terminal deoxynucleotidal transferase-mediated dUTP-biotin nick end labelling (TUNEL) assays of DNA fragmentation (6). The high expression of Bcl-2 in adenomas of the thyroid gland suggests the susceptibility to transformation to malignancy. With the knowledge that Bcl-2 inhibits apoptosis in a number of different cells, it becomes clear that prolonged survival of cells overexpressing Bcl-2 is a factor in predisposition to malignancy (5). From the practical point of view, determination of P53, Bcl-2 and Bax ratio in thyroid tumors may contribute to the differentiation between adenomas and specially follicular carcinomas (7). In parathyroid glands on the other hand the qualitative or quantitative difference between apoptotic and mitotic activity as well as P53, Bcl-2 and Bax expression has been examined when parathyroid adenomas and hyperplasias were compared. Nuclear positivity observed in some of the adenomas may be the sign of tendency to malignant transformation. Co-expression of P53, Bax and Bcl-2 has been examined to find a relation between mitotic and apoptotic activity hoping to find a good reason for the very low rate of malignancy in the parathyroid glands.

Neuroblastomas are heterogenous in terms of genetic features and clinical behaviour. A remarkable feature of these tumors is the propensity to undergo spontaneous differentiation. Furthermore some tumors are very responsive to chemotherapy, but others are quite resistant. Therefore the spontaneous apoptosis has been examined. Certain pharmacological agents with variable mechanisms of action can induce apoptosis in neuroblastoma calls. Of this group the most thorough understanding has emerged from studies on the retinoids. All-trans retinoic acid (ATRA) can induce neuroblastoma cells to differentiate or undergo apoptosis depending on the cell phenotype (8). Several studies have been dedicated to the effects of retinoids on neuroblastomas. It has been observed that the effects of the retinoids are agent-specific and dose-dependent (9). 13-cis retinoic acid is capable of lowering growth and inducing differentiation, at least in vitro. However its effects on inducing programmed cell death in neuroblastomas are less clear (8). 9-cis retinoic acid is more effective than ATRA at
inducing apoptosis in neuroblastoma cells (10). Successful induction of apoptosis by 9-cis retinoic acid requires that it be cleared from the cells by a washout technique in culture, which may have implications for in vivo dosing with this agent. In neuroblastomas the incidence of spontaneous apoptosis as well as its relation to retinoic acid receptor positivity was examined (11). In cases of PNET, however, the apoptotic index as well as retinoic acid receptor positivity was quite different from that of neuroblastoma. The level of Bcl-2 expression in neuroblastomas is inversely related to the apoptotic index. There have been conflicting reports about the relationship between Bcl-2 expression and prognostic features associated with this type of tumor when it comes to the association between Bcl-2 expression and MYCN amplification. The findings of one group were an association between Bcl-2 expression, Myc N amplification, and unfavourable histology (12). Other researchers, however, failed to confirm these correlations (13). In neuroblastoma cell lines, both Bcl-2 and Bcl-Xl (but not Bcl-Xs) are expressed (14). Bcl-2 is primarily expressed in lines of chromaffin lineage, Bcl-Xl is expressed in both chromaffin and nonchromaffin lineage neuroblastoma cell lines (6). Generally neuroblastoma cell lines that express high levels of one protein express lower level of the other (14).

**Apoptosis in general**

Like people, cells die in different ways: accident, murder, old age, even suicide. Cells that are chosen to die either because they are superfluous, diseased, or have served their useful purpose don’t just fall apart and expire, they go through a predictable, well choreographed series of events. The cells round up, their outer membranes form bulges called blebs, nuclear membranes and some internal structures break down, the nuclear DNA is fragmented by enzymes, and finally the cell breaks into pieces that are devoured by still vital neighboring cells. This mode of cell death, in which single cells are deleted in the midst of living tissues, is called ‘apoptosis’. Apoptosis is a well defined morphological phenomenon. Eventually, the cell breaks into small membrane-surrounded fragments (apoptotic bodies), which are cleared by phagocytosis without inciting an inflammatory response. The release of apoptotic bodies is what inspired the term “apoptosis” from the Greek, meaning “to fall away from” and conjuring notions of the falling of leaves in the autumn from deciduous trees (1).
Cell death can occur by either of two distinct mechanisms, necrosis or apoptosis (15,16). In addition, certain chemical compounds and cells are said to be cytotoxic to the cell, that is, to cause its death. The question here can arise of what’s the difference between these terms? To clear up these two terminologies, some basic definition will follow. Necrosis or accidental cell death is the pathological process that occurs when cells are exposed to a serious physical or chemical insult. Apoptosis (normal or programmed cell death) is the physiological process by which unwanted or useless cells are eliminated during development and other normal biological processes. Cytotoxicity is the cell-killing property of a chemical compound such as food, cosmetic or pharmaceutical or a mediator cell (cytotoxic T-cell). In contrast to necrosis and apoptosis, the term cytotoxicity does not indicate a specific cellular death mechanism. For example, cell mediated cytotoxicity (that is cell death mediated by either cytotoxic T lymphocytes (CTL) or natural killer (NK) cells combines some aspects of both necrosis and apoptosis (17,18). There are many observable morphological and biochemical differences between necrosis and apoptosis (16). Necrosis occurs when cells are exposed to extreme variance from physiological conditions e.g. hypothermia and hypoxia which may result in damage to the plasma membrane. Under physiological condition direct damage to the plasma membrane is evoked by agents like complement and lytic viruses. Necrosis begins with an impairment of the cell ability to maintain homeostasis, leading to an influx of water and extracellular ions. Intracellular organelles, most notably the mitochondria and the entire cell, swell and rupture (cell lysis). Due to the ultimate breakdown of the plasma membrane, the cytoplasmic content including lysosomal enzymes are released into the extracellular fluid. Therefore in vivo necrotic cell death is often associated with extensive tissue damage resulting in an intense inflammatory response (17). Apoptosis in contrast, is a mode of cell death that occurs under normal physiological conditions and the cell is an active participant in its own suicide. It is most often found during normal cell turnover and tissue homeostasis, embryogenesis, induction and maintenance of immune tolerance, development of the nervous system and endocrine dependent tissue atrophy. Cells underlying apoptosis show characteristic morphological and biochemical features (18). These features include chromatin aggregation, nuclear and cytoplasmic condensation, partition of cytoplasm and nucleus into membrane bound vesicles (apoptotic body) that contain ribosomes, morphologically intact mitochondria and nuclear material. In vivo, these apoptotic bodies are rapidly recognized and phagocytized by either macrophages or adjacent
epithelial cells (19). Due to this efficient mechanism for the removal of apoptotic cells in vivo no inflammatory response is elicited. In vitro, the apoptotic bodies as well as the remaining cell fragments ultimately swell and finally lyse. This terminal phase of in vitro cell death has been termed secondary necrosis.

Apoptosis or programmed cell death (PCD) is a genetically controlled process whereby cells die in response to environmental or developmental cues. The morphological characteristics of apoptosis include cytoplasmic blebbing, chromatin condensation and nucleosomal fragmentation (2). Apoptosis is relevant to a range of biological processes, including differentiation, development, cell maturation and immunologic function (3,20). Dead cells are rapidly phagocytized to prevent damage to neighboring cells. Too much cell death may produce neurodegenerative diseases and impaired development, while insufficient cell death can lead to increased susceptibility to cancer and sustained viral infection. Progress has been made in the past decade to identify many of the basic components that contribute to apoptosis, including transcriptional mediators, membrane bound receptors, Bcl-2 family members, kinases/phosphatase, and cysteine proteases. Bcl-2 was one of the first genes shown to regulate apoptosis (21) and can inhibit apoptosis in a wide variety of systems. Bcl-2 belongs to a growing family of genes that can either positively or negatively regulate apoptosis. One of these gene products, Bax, binds to Bcl-2 and antagonizes its ability to block apoptosis (22). Another critical element of the apoptotic process is the activation of cystein protease, which is currently referred to as caspases. In general, the caspases act downstream of Bcl-2-like proteins to induce apoptosis. Thus the regulation of apoptosis appears to be a precarious balance between factors that promote survival and those responsible for initiating and executing cellular suicide or maybe sacrifice.

What causes these morphological changes that we recognize as apoptosis and the biochemical changes often associated with this phenomenon? The answer is proteases, especially activation of a family of intercellular cystein proteases which cleave their substrates at aspartic acid residue, known as caspases for Cysteine Aspartyl-specific proteases. The observation that caspases cleave their substrates at Asp residues and are also activated by proteolytic processing at Asp residue makes evident that these proteases collaborate in proteolytic cascades, whereby caspases activate themselves and each other (23). Mammalian caspases appear to constitute an autolytic cascade, some members (notably caspase 8 or FLICE) being “apical” and more susceptible to modification by endogenous regulatory proteins, while others (notably capase 3 also
called CPP32, Yama and apopain) enact the final, irreversible commitment to death. Study of caspase substrates is providing interesting insights into the ways in which cells dismantle their structure and function. Such substrates include cytoskeleton proteins such as actin and fodrin and the nuclear lamins, but also an array of regulatory and chaperone-like proteins whose function is altered by cleavage in subtle and suggestive ways (24). Caspases appear to be in most if not all cells in inactive proenzyme form, awaiting activation by cleavage. One of the killing mechanisms of cytotoxic T cells is a protease, granzyme B, that is delivered to the target cell by the T cell granules and triggers these latent pro-enzyme. There are endogenous triggers also, and the first to be discovered, the C. elegans CED4 protein and its mammalian homologue, is particularly intriguing because of its mitochondrial origin. The CED4 could be the signal that initiates apoptosis under conditions of shut down of cellular energy metabolism, or when there is a critical level of cell injury affecting mitochondrial respiration. In this way CED4 may act as the link between agents long known to be associated with mitochondrial injury, such as calcium and reactive oxygen species, and the initiation of apoptosis. A second mitochondrial protein of great significance in apoptosis is BCL-2, a mammalian homologue of nematode CED9 protein. Bcl-2 has the tertiary structure of a bacterial pro-forming protein, and inserts into the outer membrane of mitochondria. It abrogates apoptosis, probably through binding CED4 and another protein Bax, with which it forms heterodimers and which, like CED4, is also a “killer” protein (22). Both Bcl-2 and Bax have several structurally and functionally similar homologues and some of this family at least also tap into other cell membranes such as the outer nuclear membrane and the endoplasmic reticulum.

So are there other sources of death transducers, activating the caspase cascade because of injury to or signals arising in other parts of the cell than mitochondria? There are already examples that show that the answer is yes. Thus, the onco-suppressor protein P53 is activated following some types of DNA damage and can trigger apoptosis. One way whereby this can happen is through transcriptional activation of Bax7. The second messenger ceramide, a product of membrane linked acid sphingomyelinase activation, may act as a signal for plasma membrane damage (25). And a powerful caspase activating system is mediated by cytokine receptors of the tumor necrosis factor family, notably fas/apo-1/CD95, TNF receptor I, and others. These mechanisms for coupling cell injury to apoptosis have mostly depended on activation of pre-formed proteins. Apoptosis can also be initiated by transcriptional
mechanisms, although rather little is known about most of them. An outstanding example is Drosophila gene reaper, transcriptionally activated around two hours prior to developmental and injury-induced deaths in this organism. Drosophila apoptosis can occur without reaper transactivation but requires very substantially enhanced stimuli, suggesting that reaper adjusts a threshold for apoptosis initiation (26). Another gene whose transcription can initiate death is the familiar immediate early gene c-myc (27). Transcriptional activation of c-myc initiates entry into DNA synthesis and is required for sustained re-entry in repeated cell cycles, but c-myc activation in the absence of concurrent cytokine support triggers apoptosis. This can also be interpreted as a “threshold regulatory” effect; c-myc expression increases the cellular requirement for survival factors such as IGF-1. Impressive confirmation of the significance of these pathways to apoptosis is available from study of transforming viruses. These are hardened survivors in the labyrinth of cell regulation, and have found keys to allow escape from cell death in a variety of ways. Thus the transforming papovavirus SV40, adenovirus type 12, Human Papilloma Virus type 16 and Epstein-Barr Virus all have proteins that inactivate apoptosis through inactivation of P53 or binding of Bax (28).

There are transcriptional and non-transcriptional pathways for activation of apoptosis, and they play through common effector events mediated by caspases and regulated by members of the Bcl-2 family. Underlying this simple scheme, however, is an extraordinary complexity. Thus inactivation of Fas signalling appears to neutralize the ability of both c-myc and P53 to initiate apoptosis (29). Many of the proteins mentioned above have alternative splice variants that have apposite effect. And we still have little idea of the relevance of intracellular location or of the cell lineage to the activity of most of the apoptosis proteins. Many other gene products, including oncoproteins such as RAS and ABL, can influence susceptibility to apoptosis but in some cases a single oncoprotein may either increase or decrease susceptibility depending on the context. Perhaps it is not surprising that a cellular function as important and irreversible as death should be subject to a huge range of course and fine controls.

The TUNEL enzymatic labelling assay

One of the methods for studying apoptosis in individual cells
There are different methods by which apoptosis could be detected in individual cells, enzymatic labelling and staining with fluorochrome. In our study we have used
enzymatic labelling, the so-called TUNEL enzymatic labelling assay. Extensive DNA degradation is a characteristic event, which often occurs, in the early stage of apoptosis. Cleavage of the DNA may yield double-stranded, LMW DNA fragments (mono- and oligonucleosomes) as well as single strand breaks (nicks) in HMW-DNA. Those DNA strand breaks can be detected by enzymatic labelling of the free 3’-OH termini with modified nucleotides (X-dUTP, X= biotin, DIG or fluorescein). Suitable labelling enzymes include DNA polymerase (nick translation) and terminal deoxynucleotidyl transferase (end labelling). DNA polymerase I catalyze the template dependent addition of nucleotides when one strand of a double-stranded DNA molecule is nicked. Theoretically, this reaction (In Situ Nick Translation, ISNT) should detect not only apoptotic DNA, but also the random fragmentation of DNA by multiple endonucleases occurring in cellular necrosis. Terminal deoxynucleotidyl transferase (TdT) is able to label blunt ends of double-stranded DNA break independent of a template. The end-labeling method has also been termed TUNEL (TdT-mediated X-dUTP nick end labelling). The TUNEL method is more sensitive and faster than the ISNT method. In addition, in early stages cells undergoing apoptosis were preferentially labelled by the TUNEL reaction, whereas necrotic cells were also identified by in situ nick translation (ISNT). The in situ nick translation (ISNT) is template dependent and TUNEL is template independent; experiments suggest the TUNEL reaction is more specific for apoptosis and the combined use of the TUNEL and ISNT is helpful to differentiate cellular apoptosis and necrosis (30).

**Morphology of apoptosis**

Apoptosis may be divided into three distinct stages: Commitment, execution and clearance. It is becoming increasingly apparent that a variety of factors influence a cell’s commitment to the death program in response to an apoptotic stimulus. These include the cell’s metabolic state, the extent and type of damage induced by the agent, the cell’s genotype, and the relative expression of growth-, survival-, and death-promoting factors. Disturbances in any one of these factors may affect a cell’s susceptibility to apoptotic stimuli, resulting in an inappropriate cellular fate, possibly having significant consequences on tissue development or disease progression. Altered susceptibility to apoptosis is particularly relevant to tumorigenesis and tumor progression and for the development of effective cancer treatment strategies. In the stage of commitment the
cell having received a potentially lethal apoptotic stimulus, becomes irreversibly committed to death. It is in the execution stage where major structural changes occur. Clearance is when cellular remnants are removed by phagocytosis. The structural changes that occur during the execution phase were described by Kerr et al (1) and are now becoming better understood mechanistically. During the execution phase, coordinated morphological and biochemical changes occur within the nucleus, cytoplasm, organelles, and plasma membrane. The most easily recognizable features of these events are changes that occur within the nucleus. Chromatin condenses and aggregates along the nuclear periphery in a crescent shaped pattern. Molecular characterisation of the chromatin reveals an ordered degradation of the DNA by a cation-dependent endogenous nuclease, first into large fragments and finally into nucleosomal fragments. However, cleavage of chromatin to nucleosomal fragments does not occur in all cell types and can be inhibited without blocking the other changes of apoptosis (31,32). Parallel with chromatin condensation, the nuclear ultrastructure is changed. The nuclear lamina, an intermediate filament network that maintains nuclear envelope integrity and nuclear pore distribution, is proteolytically cleaved (33,34). Disruption of the nuclear structural framework may then allow nuclear pore clustering (35) and fragmentation of the nucleus into chromatin-containing fragments, many of which retain a trace of the nuclear membrane. Other cytoplasmic organelles remain structurally intact, although mitochondrial dysfunction is associated with apoptosis (36). Reduction in the mitochondrial transmembrane potential, uncoupling of electron transport from ATP synthesis, and increased generation of reactive oxygen species precede the nuclear changes. In the cytoplasm, protein cross-linking occurs through the action of transglutaminase (37), cytoskeletal filaments aggregate in parallel arrays, and the endoplasmic reticulum dilates and fuses with the plasma membrane, creating pock-like craters at the point of fusion. The structural integrity of the plasma membrane is further compromised by the loss of membrane phospholipid asymmetry, microvilli, and cell-cell junctions. The cell rounds up, dissociates itself from its neighbours, and shrinks dramatically, throwing out protuberances that separate into membrane bound ‘apoptotic bodies’. Within tissues, apoptotic cells and apoptotic bodies are recognized and rapidly phagocytosed by neighboring cells or macrophages. Once the execution phase of apoptosis is activated, the entire process proceeds rapidly, and is completed within a few hours (38). The duration of this phase is relatively invariant with respect to cell type and apoptotic stimulus, suggesting that the final stage of apoptosis proceed through a
common pathway. However, the commitment phase, the time from reception of the apoptotic stimulus until irreversible initiation of the execution phase is extremely variable, being dependent on cell type, apoptotic stimulus position within the cell cycle, and expression of various death modulating factors. Many of the factors that influence commitment or cellular susceptibility to apoptosis, are involved directly in the reception and transduction of the apoptotic signal. Strikingly most of the genes involved in the regulation of apoptosis that have been identified during the past few years have turned out to be also of importance in carcinogenesis.

Cell suicide or cell sacrifice

Apoptosis, or programmed cell death, is a critical process in many areas of biology such as morphological modelling of tissues during development, removal of autoreactive immune cells, hormonal or age-related tissue atrophy and normal cell turnover. During apoptosis, cells, by virtue of their own genetic makeup, die and are deleted for the overall good of the organism (39). It is also said in literature that cells in multicellular organisms can kill themselves by activating a suicidal genetic program in response to a wide variety of signals, including hormones, cytokines, ionising radiation, and chemotherapeutic agents and that this process of cell suicide is called apoptosis which usually occurs under physiological conditions (40). The phenomenon of apoptosis has long been known as programmed cell death, which is fundamental for embryonic development, such as in metamorphosis, morphogenesis and synaptogenesis (41). While apoptosis is energy requiring process, functional mitochondria are necessary to provide adequate energy for the process to reach completion, so it might not be quite fair to call the process a suicidal process, with a negative connotation of lacking energy and will power. If cells die because of a signal, be it cytokines or chemotherapy, then they have been killed and murdered. On the other hand if there is a process where cells die by virtue of their own genetic make up for the overall good of the organism then this is a sacrifice a cell makes, since sacrifice involves renouncing that which is lower for that which is higher. This happens during the embryological development, when a cell which can not serve the purpose anymore dies and is deleted from the environment. A tree which bears no fruit is good for fire. It is worthwhile reviewing the terminologies that have been given to certain processes in the life of a cell.
Apoptosis in endocrine cells

Because of the fine balance that is maintained between cell proliferation and death, there is little change in the overall number of cells during the adult life of multicellular organisms. Cell generation occurs via mitosis, a process that is relatively well understood compared to the process by which cell die. With regard to cell death, there are two distinct mechanisms by which this process can occur: necrosis and apoptosis. Necrotic cell death is characterized by cell swelling and lysis, with the cell playing no active part in the process. It is also associated with a characteristic inflammatory response. Apoptosis, in contrast, is a genetically controlled process and is probably the counterbalancing force to mitosis under normal physiological conditions. In contrast to mitosis, very little is known about how apoptosis is regulated, but it is known that the cell plays an active part in its own demise. A variety of genes that play regulatory role in apoptosis have been identified. These fall into two distinct groups, those that drive the process and those that inhibit it. In the former category are genes such as myc and P53 and in the latter genes such as Bcl-2 and Bcr-abl. What is striking about this observation is that many of them are oncogenes and have been implicated in the development of numerous cancers. Bcl-2, Bax and P53 expression has been examined by us (42) in different endocrine cells as factors influencing apoptosis by either suppressing, inhibiting or causing apoptosis. Our study has been aiming to help the differential diagnosis in the case of thyroid and parathyroid tumors and in giving weigh to predictive values. It is important to ponder upon the role and function of these proteins, finding out how and in what way they act in the process.

Role of P53 in apoptosis

The tumor suppressor gene P53 is the most commonly mutant gene in human cancers (43). P53 protein levels rise in response to genotoxic damage. This rise in p53, which results from increased transcription and protein stabilization, is responsible for initiating G1/S-phase cell cycle arrest, DNA repair and /or apoptosis. Because p53 is a principal mediator of the cellular response to genotoxic damage, its altered expression and function may allow the propagation of potentially harmful genetic lesions leading to oncogenesis. The first clear demonstration that p53 expression could induce apoptosis
was based on a cultured cell line transfected with a temperature-sensitive p53 mutant (44). P53 can induce apoptosis in different ways 1) by activating bax, a proapoptotic member of bcl-2 family, 2) by enhancing the expression of killer receptor, 3) by preventing such genes transcription which their products inhibit p53-dependent apoptosis, 4) by protein-protein mutual effect e.g. it binds to transcription/repair complex (45). In 1993 concurrent studies by Lowe et al (46) and Clarcke et al (47) demonstrated that p53 was involved in the cellular apoptotic response to genotoxic damage. In these studies, thymocytes from mice rendered homozygous null for p53 by germline targeting were found to be completely resistant to the apoptosis seen in wild type cells shortly after exposure to ionising radiation or chemical induced DNA double-strand breaks (46,47). However, p53 genotype had no effect on glucocorticoid-induced apoptosis in these cells. These findings established the existence of distinct p53-dependent and p53 independent mechanisms of apoptosis. Moreover, because p53-null mice showed a high incidence of thymic lymphomas, the results gave some support to the hypothesis that p53 mutations may facilitate tumor initiation and progression by producing defects in apoptosis. This hypothesis has been further supported by elegant in vivo experiments using transgenic mice engineered to selectively express SV40 large T antigen product, which can selectively inactivate Rb or p53, in choroid plexus cells. In the animals expressing the full length SV40 large T transgene, which inactivates both Rb and p53, aggressive choroid plexus tumor formation was observed. In contrast, tumor growth occurred at a considerably slower rate in animals expressing a truncated SV40 large T transgene capable of inactivating Rb but not p53 (48). Analysis of these tumors revealed that p53 genotype had no appreciable effect on rates of proliferation. Instead, rates of apoptosis were low in those tumors in which p53 was inactivated, suggesting that p53 associated tumor suppression due to enhanced rate of apoptosis rather than decreased rate of proliferation. Similar results have been reported in additional tissue-specific transgenic system that addressed the role of p53 and Rb in tumor development (49,50).

Gene expression has been shown to be required for both apoptosis and survival depending on the cell type and stimulus. Indeed, inhibition of RNA and protein synthesis can block apoptosis induced by a number of circumstances. Others have shown that in some cases RNA and protein synthesis inhibitors either do not block or actually promote cellular demise (51). The basic machinery for apoptosis is constitutively expressed but can be modulated by changes in Gene expression in order
to trigger the death program. p53 was first detected in rodent cells transformed by simian virus SV40 in a complex with the transforming protein SV40 T antigen, suggesting that it plays a role in growth control (52). Subsequently, it was noted that p53 mutations occur in a wide variety of tumors including lung, breast, colon, esophagus, liver, bladder, ovary, brain and haemopoetic tissues (43). In fact, p53 loss-of-function mutations are the most common genetic alteration found in human cancer. Furthermore, overexpression of wild-type p53 can suppress tumor formation in culture (53,54,55). These data suggest that p53 function as a tumor suppressor gene and has sparked extensive research into understanding its mechanism of action. p53-mediated transrepression, on the other hand, occurs on genes lacking the p53 DNA binding site and is probably dependent on interactions with components of the basal transcriptional machinery (56,57). Mutational analysis of p53 has revealed distinct domains that contribute to its gene regulatory activity. Consistent with its role as a tumor suppressor gene, expression of p53 can induce either cell cycle arrest in G1 or apoptosis (58,44). Functional p53 is required for apoptosis induced by ionizing radiation and chemotherapeutic drugs (59,47) as well as transforming oncogenes like c-myc (60,61). The function of p53 is likely to depend on the cell type and/or physiological circumstances. Indeed, cytokines (44) and growth factors can affect the ability of p53 to induce apoptosis or growth arrest. These results have led to the hypothesis that genotoxic induction of p53 acts as a checkpoint control to stop further progression in the cell cycle and thereby maintain genomic integrity (62). The mechanism by which p53 induces apoptosis, however, is somewhat unclear. Generally, p53 mediated apoptosis is dependent on its gene regularity capability. Another critical issue that remains is whether p53-dependent cell death involves activation or repression of cellular genes. Studies have indicated that at least in some cases p53 induced apoptosis is not affected by the presence of RNA and protein synthesis inhibitors, suggesting that repression rather than activation is the primary mechanism (63). Several p53 responsible genes have been identified that could affect cell survival, including bcl-2 (64). Concerning thyroid neoplasms there exists various reports on the overexpression of p53 especially in poorly and undifferentiated carcinomas (65,66).
Bcl-2, an inhibitor of apoptosis

One of the first mammalian genes discovered to regulate cell death was the anti-apoptotic gene bcl-2 (67). The bcl-2 proto-oncogene was originally discovered as a common translocation in non-Hodgkins B-cell lymphoma (68,69). This chromosomal translocation event places the bcl-2 gene under the transcriptional control of the powerful enhancer elements of the immunoglobulin heavy chain resulting in high levels of bcl-2 expression and the abrogation of normal programmed cell death and promotion of malignant potential. Disruption of bcl-2 in “knock out” mice leads to impaired kidney function manifested by polycystic disease and postnatal immune failure due to dramatic cell loss through apoptosis. Thus, gain of bcl-2 function is associated with tumor development (68,69,70,71,72), while loss of bcl-2 has only restricted consequences to normal development. This suggests that there may be some redundancy in the bcl-2 family or that other members have a more critical role. Several bcl-2 like genes have been identified that can promote, rather than inhibit, apoptosis. The best-characterized and prototypic gene of this class is bax (22). While overexpression of bax can induce apoptosis, disruption of this gene in “knock out” mice leads to lymphoid hyperplasia (73). Recent data also demonstrates that expression of bax can suppress tumorigenesis in vivo (74). These data suggest that bax plays a critical role during apoptosis. Biochemical studies have indicated that bax directly interacts with itself as well as several anti-apoptotic proteins such as bcl-2 (22). Interaction between death promoting and suppressing bcl-2-like proteins has lead to a model for explaining how these proteins function. According to this model, the ratio between survival factors, such as bcl-2, and death promoters, such as bax, controls the fate of the cell. However, the biochemical mechanism of these proteins is still unclear. What is also unclear is which set of bcl-2 proteins act as effector molecules. For example, do the death promoting genes simply inhibit a required survival factor (e.g. bcl-2, bcl-xl ) or do they actually trigger apoptosis?

Bcl-2 and its family members bind to a number of unrelated proteins that has provided insight into their apoptotic activity. Indeed, bcl-2 related proteins are generally found associated with membrane structures, particularly the mitochondria, endoplasmic reticulum, and the nuclear envelope. Furthermore, *in vitro* experiments demonstrate that bcl-xl can facilitate ion transport across a lipid bilayer. It will be of interest to determine if the bcl-2 family members actually function as channels *in vivo* and if this activity is
responsible for apoptosis regulation. These and other studies have lead to several hypotheses regarding the biochemical mechanism of this family of proteins. One possibility that has emerged is that they form a channel capable of regulating ion flux, such as calcium. Calcium is a critical component of signaling pathways and high level of intracellular calcium is often associated with cell death, bcl-2 can suppress calcium release from the endoplasmic reticulum, although it is possible that this is simply a downstream consequence of its ability to inhibit apoptosis (75). Nevertheless, the experiments indicating that the bcl-2-like proteins can form ion channels add some credence to this model. Others have speculated that bcl-2 regulates the generation of reactive oxygen radicals since bcl-2 is able to attenuate cell death induced by oxidative damaging agents. (75). Free radicals can contribute to cell death by disrupting DNA, proteins, and lipids. The localization of bcl-2 at mitochondrial membranes makes it plausible that the protein is directly involved in oxidative phosphorylation (67). However further evidence demonstrates that bcl-2 can inhibit apoptosis in cells that lack mitochondrial DNA and thereby respiration (76). An alternative mechanism for bcl-2 action at the mitochondria may be the control of cytochrome C release. Release of cytochrome c from the mitochondria into the cytosol is required for caspase activation and DNA fragmentation in cell free extracts and bcl-2 can act to suppress cytochrom c efflux from mitochondria (77). It will certainly be important to determine if efflux of proteins, such as cytochrome C, from the mitochondria actually contributes to apoptosis in vivo or if this is simply a downstream phenomenon. Additionally, there is tremendous interest in elucidating the down stream events of apoptosis, most notably caspase activation.

As to the expression of bcl-2 in endocrine cells, bcl-2 has been reported to be retained in well & poorly differentiated thyroid carcinomas but absent in undifferentiated thyroid carcinomas (159,41). Several studies have found that bcl-2 is widely expressed in neuroblastoma tumors and tumor derived cell lines (13,54).

**Function of Bax**

Bax has been shown by a number of investigators to be capable of forming either homodimers with bcl-2 and bcl-Xl by immunoprecipitation and yeast two hybrid select systems (40,41). Bases on the tendency of bax to promote cell death, it has been proposed that the relative ratio of bax heterodimers to bax homodimers serves as a
switch that dictates the cell fate (78,79). According to this model, exposure of the cell to cytotoxic insults may promote the formation of bax homodimers. However this death initiation step may be blocked, if sufficient quantities of the prosurvival factors bcl-2 or bcl-Xl are present to form heterodimers with bax. Thus depending on the relative ratio of bax heterodimers to bax homodimers, the cell may proceed toward either cell survival or cell death. bax dependent cell killing appears to be linked to caspase activation (80,81). Several reports have suggested that overexpression of bax leads to the loss of inner mitochondrial potential, a phenomenon also known as mitochondrial permeability transition (82,83). Observations taken together have led to the proposal that bax may form pores on mitochondrial surface, leading to the dissipation of mitochondrial potential and to the release of cytochrome C (84). One could envision that these series of processes would first require a signalling mechanism that switches bax from its normal physiologically inactive state, possibly represented by its cytosolic form, to an active apoptotic state possibly represented by its mitochondria-bound form. Studying the expression of bax has not been given too much attention in endocrine cells. Nevertheless there have been studies that bax expression has been negative in normal thyroid tissue and weakly positive in medullary thyroid tumor (85).

**Retinoic Acid Receptor**

Retinoids have profound effects on normal vertebrate development and the maintenance of normal cellular functions in some adult tissues (86). A group of pharmacological agents with different mechanism of action can induce apoptosis in neuroblastoma cells (8). Some examples are retinoids. All-trans retinoic acid (ATRA) can induce neuroblastic cells to undergo apoptosis. It is therefore not out of place to expand on Retinoic Acid Receptor (RAR) since we have examined its expression in neuroblastomas and PNET and its relation to apoptosis (11).

The widespread cellular effects of retinoids are mediated by two classes of nuclear receptor: the retinoic acid receptors (RAR) and the retinoid X receptors (RXR) (87). Discovery of the nuclear retinoic acid receptors (RARs) and nuclear retinoid X receptors (RXRs), has significantly advanced our understanding of the biological basis of retinoic acid (RA) function. These receptors, which belong to the multigene family of steroid/thyroid hormone receptors RA-inducible transcriptional regulatory factors transduce the RA signal at the level of gene expression. There are 3 subtypes (? , ? and
Nuclear retinoid receptors are ligand-dependent transcription factors which bind specific DNA sequences, known as retinoic acid response elements (RARE) in the promoter regions of target genes. RAR-RXR heterodimers, rather than homodimers of individual receptors, bind with greatest affinity \textit{in vitro} to RAREs. Single and combination gene knockout studies in mice, for each of the nuclear retinoid receptors, have demonstrated that specific receptor subtypes have signaling roles in certain embryonal tissues, however, functional redundancy also exists between receptor subtypes in other tissues (88). A large number of tumor cell lines from different tissue origins undergo growth arrest and, in some cases, differentiation, following retinoid treatment \textit{in vitro} (88). These observations have raised the possibility that disordered retinoid signaling may contribute to some tumorigenic processes, and, moreover, that retinoids may have therapeutic or chemo-preventive roles in certain human cancers. Abnormal function or reduced expression of specific RAR, but not RXR, subtypes contribute to the malignant phenotype in several human cancers (89,90,91,92,93). Several groups have linked reduced RAR expression to the genesis of human epidermal, lung, and breast cancer (91,93,94,95).
Tumors investigated in our study

1. Neuroblastoma

The neuroblastic tumours, derived from primordial neural crest cells which ultimately populate the sympathetic ganglia, adrenal medulla and other sites, (96) are an enigmatic group of neoplasms which have the highest rate of spontaneous regression of all human malignant neoplasms yet one of the poorest outcomes when occurring as disseminated disease in children. Neuroblastoma is one of the most common childhood extracranial solid tumors. Alone, it accounts for about 15% of all childhood cancer death (97). A great deal is known about the molecular biology and genetics of this neoplasm. Studies strongly suggest that there are at least two, possibly three, clinicobiologic subsets of neuroblastoma, ranging from those that rarely kill, however hopeless the clinical situation may appear to be, to those that rapidly cause death despite all therapeutic efforts. Furthermore, some tumors are very responsive to chemotherapy but others are quite resistant. Thirty-five percent of these tumors appear during the first year of life, 20% during the second year. Altogether, 85 to 90% are found in children younger than five years of age. Most occur sporadically, but in about 20% of instances there is strong evidence of a hereditary predisposition.

In childhood, about 25 to 35% of neuroblastomas arise in the adrenal medulla. The remainder occur anywhere along the sympathetic chain, with the second most common location being the paravertebral region of the posterior mediastinum. Closely following is the paravertebral region in the lower abdomen, but tumors may arise in numerous other sites, including the pelvis and neck and within the brain. By contrast, rare neuroblastomas in adults are found in the head, neck, and legs. Macroscopically, neuroblastomas range in size from minute nodules (the “in situ lesions”) to large masses more than 1 kg in weight. In situ neuroblastomas are reported to be 40 times more frequent than overt tumors. The great preponderance of these lesions spontaneously regress leaving only a focus of fibrosis or calcification in the adult. Indeed, some clinically overt neuroblastomas have regressed or, alternatively, have undergone differentiation and maturation into a relatively benign ganglioneuroma. Some neuroblastomas are sharply demarcated and may appear encapsulated, but others are far more infiltrative and invade surrounding structures, such as the kidneys, renal vein, and vena cava, and envelope the aorta.
Histologically, there is a considerable range of differentiation among these neoplasms. Most are composed of small, primitive-appearing cells with dark nuclei, scant cytoplasm, and poorly defined cell borders growing in solid sheets.

**Classification of Neuroblastomas**

The International Neuroblastoma Pathology Committee (INPC) has adopted a prognostic system modeled on one proposed by Shimada et al (98). It is an age-linked classification dependent on the differentiation grade of the neuroblasts, their cellular turnover index, and the presence or absence of Schwanian stromal development. Based on morphologic criteria defined in this article, neuroblastic tumors (NTs) were classified into four categories and their subtypes:

1. *neuroblastoma* (Schwannian stroma-poor), undifferentiated, poorly differentiated, and differentiating;
2. *ganglioneuroblastoma*, intermixed (Schwannian stroma-rich);
3. *ganglieneuroma* (Schwannian stroma-dominant) maturing and mature;

**1/a. Primitive Neuroectodermal Tumors (PNET)**

Some tumors, although of neuroectodermal origin, express few, if any, of the phenotypic markers of mature cells of the nervous system and are described as poorly differentiated, or embryonal, meaning that they retain cellular features of primitive, undifferentiated cells. The most common is the medulloblastoma, which accounts for 20% of the brain tumors of childhood and occurs in the cerebellum. Other poorly differentiated tumors occur in other locations, and have been collectively termed primitive neuroectodermal tumors (PNET). Most classification systems continue to recognize the medulloblastoma as a distinct clinicopathologic category and acknowledge the presence of other poorly differentiated tumors that occur predominantly in children. Primitive neuroectodermal tumor is a highly controversial designation, which has been applied to neoplasms, primarily of childhood, that are histologically similar to medulloblastoma but occur in locations other than the
cerebellum, especially the cerebral hemispheres. Many would prefer not to gather all these poorly differentiated tumors into a single category until there is a clearer vision of their biological behavior; others find the designation useful to evaluate the efficacy of chemotherapeutic protocols. More information is needed to settle this issue.

**Retinoids and Apoptosis in Neuroblastoma**

A group of pharmacological agents with variable mechanisms of action can induce apoptosis in neuroblastoma cells in vitro. Some examples are retinoids, DNA strand breaking agents like cisplatin and doxorubicin, protein kinase inhibitors and others. Of this group the most thorough understanding has emerged from studies on the retinoids. All-trans retinoic acid (ATRA) can induce neuroblastoma cells to differentiate or undergo apoptosis, depending on the cell phenotype (8). Three phenotypic variants of neuroblastoma cells in culture have been described. N-type cells constitute the neuroblastic phenotype. These cells have neurotic processes and display biochemical characteristic of neuronal cells. S-type cells are substrate-adherent, epithelial in phenotype, and display biochemical features of immature Schwann, glial, or melanocytic cells. The third phenotypic variant is I-type cells, with intermediate morphology and bio-chemical features of both N-and S-type cells. S-type cells do not express Bcl-2 and are particularly sensitive to induction of apoptosis by ATRA. This is in contrast to N-type cells, which express Bcl-2 and are less sensitive to RA-induced apoptosis (99,100). Neuroblastoma cells that differentiate in response to RA are resistant to chemotherapy-induced apoptosis. This resistance is associated with regulation of Bcl-2(100). The effect of the retinoids are agent specific and dose-dependent. 13-cis RA is capable of slowing growth and inducing differentiation, at least in vitro. However its effect on inducing programmed cell death (PCD) in neuroblastomas are less clear (8,9). 9-cis RA is more effective than ATRA at inducing apoptosis in neuroblastoma cell (10). Successful induction of apoptosis by 9-cis RA requires that it be cleared from the cells by a washout technique in culture, which may have implications for in vivo dosing with this agent (10). Finally there is a novel retinoid called N-(4-hydroxyphenyl) retinamide (fenretinide), which appears to be particularly potent at inducing apoptosis (101,102). The mechanism through which the retinoids induce apoptosis has not been clearly defined.
Neuroblastoma is an embryonal childhood tumor of neural crest origin with a unique capacity for spontaneous regression and differentiation, in vivo (103,104,105). In some older patients the tumor may differentiate into a benign ganglioneuroma. Unfortunately in the majority of patients, neuroblastomas are metastatic at diagnosis and most of these children ultimately die of their disease, especially if they are over 1 year of age at diagnosis (96).

Several studies have found that Bcl-2 is widely expressed in neuroblastoma tumors and tumor derived cell lines (13). Moreover the level of Bcl-2 expression in tumors is inversely related to the apoptotic index, as measured by morphologic assessments and terminal deoxynucleotidal transferase-mediated dUTP-biotin nick end-labelling (TUNEL) assays of DNA fragmentation (6). There have been conflicting reports about the relationship between Bcl-2 expression and prognostic features associated with this disease. One group reported an association between Bcl-2 expression, MycN amplification, and unfavourable histology (84). Other investigations however have failed to confirm these correlations (13). In neuroblastoma cell lines, both Bcl-2 and Bcl-Xl (but not Bcl-Xs) are expressed. Bcl-2 is primarily expressed in lines of chromaffin lineage, Bcl-Xl is expressed in both chromaffin and nonchromaffin lineage neuroblastoma cell lines. Generally neuroblastoma cell lines that express high levels of one protein express lower levels of the other (6).

Retinoid treatment of most neuroblastoma tumor cell lines induces growth arrest and neuritic differentiation in vitro (106) and, thus provides an excellent model for studying whether abnormal retinoid signaling is a component of the neuroblastoma tumorigenic process. Untreated neuroblastoma cells express high levels of RAR-?, low level of RAR-?, and barely detectable levels of RAR ?, while retinoid treatment induces RAR-? expression some 40-fold and RAR ? expression by four-fold (107,108,109). Comparative studies of neuroblastoma cells transfected with each of the 3 RAR subtype have shown that only RAR-? transfectants demonstrates enhanced retinoid sensitivity and growth inhibition in the absence of added retinoid (110).

Genetic heterogeneity of neuroblastoma

Several genetic features have been found to be characteristic of subsets of neuroblastomas, including hyperdiploidy with whole chromosome gains, amplification of the MYCN oncogene, and deletions of the short arm of chromosome 1 (1p)(111).
Hyperdiploidity is characteristic of tumors in infants, particularly those who are prone to undergo spontaneous regression or who respond well to chemotherapy. However, the most aggressive tumors particularly those who are metastatic in older patients frequently have MycN amplification and the majority of these also have 1p deletion (111). Partial trisomy for 17q is also a common genetic change in neuroblastomas and its presence in the karyotype is generally associated with a worse outcome. Other deletion and rearrangements are also found such as deletion of 11q or 14q, but so far these features have not been consistently associated with clinical behaviour.

**Apoptosis and response of neuroblastoma to chemotherapy**

A variety of DNA damaging agents have been shown to induce apoptosis in neuroblastoma cells in vitro (112,113). Several studies have indicated that neuroblastoma cells undergo apoptosis in response to DNA damaging agent after arrest in G2/M of the cell cycle (114). Neuroblastoma cells treated with these agents show classic morphologic features of apoptosis and evidence of DNA damage. The CD95 system has recently been shown to mediate apoptosis induced by chemotherapy in neuroblastoma (115). CD95, a member of the TNFR superfamily, triggers cell death in a variety of cell types. In neuroblastoma cells, doxorubicin, cisplatin, and etoposide induce expression of CD95, as well as the ligand, CD95L (115). Chemotherapy induced apoptosis mediated by CD95 in neuroblastoma involves activation of ced-3-like proteases, resulting in cleavage of PARP an enzyme involved in DNA repair (115). These results suggest that autocrine or paracrine activation of CD95/CD95L leads to a death response in neuroblastoma cells after treatment with these specific chemotherapeutic drugs.

Both Bcl-2 and Bcl-XI can modulate chemotherapy induced apoptosis in neuroblastoma cells. Single-gene transfection studies have shown that deregulated expression of Bcl-2 or Bcl-XI in a dose dependent manner can confer resistance to apoptosis induced by cisplatin or 4-hydroxycyclophosphamide, and delay apoptosis induced by etoposide (116,112,113).

Not all cytotoxic agents induce apoptosis by engaging CD95. Betulinic acid (BA), a pentacyclic triterpene, induces apoptosis in a number of cell types, including neuroblastoma, and this appears to be independent of the Cd95 and P53 pathways (117). BA-induced apoptosis is associated with increased caspase activity, enzymatic
processing of the upstream caspase component, FLICE (caspase-8) and PARP cleavage. BA-induced apoptosis, however, is independent of CD95/CD95L activation. BA-induced apoptosis is associated with induction of the apoptosis-facilitating proteins, Bax and Bcl-Xs. Both Bcl-2 and Bcl-Xl can inhibit apoptosis induced by BA in a manner similar to its protection from DNA-damaging agents (115). Caspase activation appears to be the final common pathway for induction of apoptosis by most agents. Staurosporin, an alkaloid inhibitor of protein kinases, is also able to induce apoptosis in neuroblastoma cells (118). Staurosporin-induced apoptosis is associated with proteolytic processing of CPP32 (caspase-3), PARP activation, and PARP cleavage. In some systems, however, PARP activation is not always associated with proteolytic processing of PARP. Peroxynitrite (PN), which forms through a reaction of superoxide with nitrous oxide has recently been shown to induce apoptosis in the NSC34 neuroblastoma spinal cord cell line (119). In this system, PN-induced apoptosis was associated with morphologic features of apoptosis and increased PARP immunoreactivity, but there was no evidence of PARP cleavage. It has been suggested that PN induce DNA damage that stimulates PARP, which depletes intracellular energy stores, resulting in cell death (119). This possibility will require further investigation, however, these results suggest that additional pathways may function to mediate specific drug-induced apoptosis in neuroblastoma.

Therapeutic strategies based on in vitro studies of neuroblastoma cells have led to several current clinical trials in patients with neuroblastoma. Most notable are the protocols utilizing retinoic acid (RA) in the treatment of advanced stage disease, which are based on in vitro assays indicating RA can inhibit neuroblastoma proliferation. A phase 1 trial utilizing 13 cis-RA following bone marrow transplantation for stage 4 neuroblastoma has suggested that the drug is well tolerated, with minimal toxicity (120), and its therapeutic efficacy is currently under investigation. An improved understanding of the molecular pathways controlling the survival/death response of neuroblastoma cells should provide insight into mechanism that could ultimately be targeted for novel therapeutic intervention. In support of this possibility are studies indicating that neuroblastoma cells, regardless of their Bcl-2 or Bcl-Xl expression, are sensitive to induction of apoptosis by adenoviral-mediated expression of the death-facilitating protein Bcl-Xs (116). The possibility of therapeutically engaging the death pathways directly is intriguing, but will require the development of targeted gene therapy approaches. Finally, therapeutic interventions aimed at either blocking the
Trk ligand-receptor interaction, or selectively inhibiting the Trk family of receptors, may provide a therapeutic approach that is more effective and less toxic than currently used chemotherapeutic agents. The blue letters are my own work.

Our studies on apoptosis in Neuroblastoma & PNET

Aim: RAR-β and apoptosis: therapy with RA

The specimen has been received from the 2nd Department of Pediatrics, Semmelweis University of Medicine, Budapest, Hungary. The great help and assistance of Prof. Dr. Dezso Schuler and the co-operation of Dr. Peter Hauser and Dr. Maria Babosa is deeply appreciated.

Little is known about the spontaneous apoptotic activity in neuroblastomas and PNET. These tumors have a poor prognosis even when treated with irradiation and/or cytotoxic drugs (121,122). Recently retinoic acid has been reported to be effective in the therapy of NB, promoting the differentiation of the tumor cells (123). In vitro studies of NB cells showed that retinoic acid administration induces apoptosis in cell cultures and this effect was mediated by a retinoic acid receptor (124). The possibility of inducing apoptosis by retinoic acid treatment has been studied in vitro (124). Nuclear localization of the receptor by Western blot has been reported, we investigated the possibility to localize the receptor by immunohistochemical method within the tumor cells of NB and PNET.

Materials and methods

Twenty-two cases of NB and four cases of PNET were investigated. In a retrospective study basic clinical data, such as age, sex, location of the tumor, chemotherapy and/or surgical removal irradiation and survival were registered (Table 1).

Among the children suffering from NB 14 were males (average age 3.40+0.80) and 8 were female (average age 3.40+1.5 years). Three boys (2.5 and 10 years old) and one girl (4 years old) had PNET. Seven neuroblastomas and two PNETS were in Evan’s stage IV, four neuroblastomas were in Evan’s stage II, one neuroblastoma and one PNET were in Evan’s stage I, and one PNET was in Evan’s stage II at diagnosis (Table
2). The tumor tissues were obtained by surgical excision before treatment from the primary tumor, and after formalin fixation and paraffin embedding 8um thin sections were cut.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (Year)</th>
<th>Chemotherapy</th>
<th>Radio-therapy</th>
<th>Location</th>
<th>Operation</th>
<th>Stage/Evans</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.NB</td>
<td>F</td>
<td>0.8</td>
<td>Endoxan + ADR + steroid + OPEC</td>
<td>None</td>
<td>Mediastinum</td>
<td>Part.res.</td>
<td>St.IV.</td>
</tr>
<tr>
<td>2.NB</td>
<td>F</td>
<td>1.2</td>
<td>NB-90 RG-C Vac+OPEC+OJEC(REITIN OID)+ Autotransplantation</td>
<td>None</td>
<td>Mediastinum</td>
<td>Part.res.</td>
<td>St.III.</td>
</tr>
<tr>
<td>3.NB</td>
<td>F</td>
<td>8</td>
<td>None</td>
<td>None</td>
<td>Retro-peritoneum</td>
<td>Part.res.</td>
<td>St.III.</td>
</tr>
<tr>
<td>4.NB</td>
<td>F</td>
<td>1</td>
<td>None</td>
<td>None</td>
<td>R. adrenal gland</td>
<td>Total resection</td>
<td>St.I.</td>
</tr>
<tr>
<td>5.NB</td>
<td>F</td>
<td>6</td>
<td>OPEC/OJEC</td>
<td>None</td>
<td>Retro-peritoneum</td>
<td>None</td>
<td>St.III.</td>
</tr>
<tr>
<td>6.NB</td>
<td>M</td>
<td>4</td>
<td>NB-90-RG-C OPEC+Newcastle+Autotranspl.</td>
<td>32 Gy</td>
<td>Inoperable</td>
<td>Retro-peritoneum</td>
<td>St.IV.</td>
</tr>
<tr>
<td>7.NB</td>
<td>M</td>
<td>1</td>
<td>No clinical data / foreign person</td>
<td>None</td>
<td>Inoperable</td>
<td>Retro-peritoneum</td>
<td>St.III.</td>
</tr>
<tr>
<td>8.NB</td>
<td>M</td>
<td>4</td>
<td>None</td>
<td>None</td>
<td>Retro-peritoneum</td>
<td>None</td>
<td>St.IV.(?)</td>
</tr>
<tr>
<td>9.NB</td>
<td>M</td>
<td>4</td>
<td>ALL-BFM-88</td>
<td>None</td>
<td>Retro-peritoneum</td>
<td>None</td>
<td>St.IV.</td>
</tr>
<tr>
<td>10.NB</td>
<td>M</td>
<td>6</td>
<td>OPEC</td>
<td>30 Gy</td>
<td>Pelvic lymph node</td>
<td>Part.res.</td>
<td>St.IV.</td>
</tr>
<tr>
<td>11.NB</td>
<td>M</td>
<td>3</td>
<td>OPEC</td>
<td>None</td>
<td>Retro-peritoneum</td>
<td>Part res.</td>
<td>St.IV.</td>
</tr>
<tr>
<td>12.NB</td>
<td>M</td>
<td>5</td>
<td>OPEC</td>
<td>None</td>
<td>Inoperable</td>
<td>Auto.bone. transplant</td>
<td>St.IV.</td>
</tr>
<tr>
<td>13.NB</td>
<td>F</td>
<td>6</td>
<td>VP-16+Carboplatin</td>
<td>None</td>
<td>Retro-peritoneum</td>
<td>Bone m. transplant</td>
<td>St.IV.</td>
</tr>
<tr>
<td>14.NB</td>
<td>F</td>
<td>2</td>
<td>n.d.</td>
<td>None</td>
<td>Bone marrow</td>
<td>Bone marrow transplant</td>
<td>St.IV.</td>
</tr>
<tr>
<td>15.NB</td>
<td>M</td>
<td>8</td>
<td>OPEC/OJEC</td>
<td>None</td>
<td>Bone marrow</td>
<td>Bone marrow transplant</td>
<td>St.IV.</td>
</tr>
<tr>
<td>16.NB</td>
<td>M</td>
<td>3</td>
<td>n.d.</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>NA</td>
</tr>
<tr>
<td>17.NB</td>
<td>M</td>
<td>0.3</td>
<td>n.d.</td>
<td>None</td>
<td>Adrenal gland</td>
<td>None</td>
<td>NA</td>
</tr>
<tr>
<td>18.NB</td>
<td>F</td>
<td>0.5</td>
<td>HR-NBL-1/ESIOP</td>
<td>None</td>
<td>Retro-peritoneum</td>
<td>None</td>
<td>St.IV</td>
</tr>
<tr>
<td>19.NB</td>
<td>M</td>
<td>2</td>
<td>n.d.</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>St.I.</td>
</tr>
<tr>
<td>20.NB</td>
<td>M</td>
<td>3</td>
<td>n.d.</td>
<td>None</td>
<td>Adrenal gland</td>
<td>None</td>
<td>St.I.</td>
</tr>
<tr>
<td>21.NB</td>
<td>M</td>
<td>3</td>
<td>n.d.</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>St.IV</td>
</tr>
<tr>
<td>22.NB</td>
<td>M</td>
<td>1</td>
<td>n.d.</td>
<td>None</td>
<td>Mediastinum</td>
<td>None</td>
<td>NA</td>
</tr>
<tr>
<td>23.PNET</td>
<td>F</td>
<td>4</td>
<td>OPEC/OJEC</td>
<td>None</td>
<td>Retro-peritoneum</td>
<td>Part.res</td>
<td>St.IV.</td>
</tr>
<tr>
<td>24.PNET</td>
<td>M</td>
<td>2</td>
<td>NB-A-89</td>
<td>None</td>
<td>Retro-peritoneum</td>
<td>Part.res</td>
<td>St.I.</td>
</tr>
<tr>
<td>25.PNET</td>
<td>M</td>
<td>5</td>
<td>VAIA</td>
<td>90 Gy</td>
<td>Right. forearm</td>
<td>Part.res.</td>
<td>St.IV.</td>
</tr>
<tr>
<td>26.PNET</td>
<td>M</td>
<td>10</td>
<td>NHL-BFM-95</td>
<td>None</td>
<td>Mediastinum</td>
<td>Part.res.</td>
<td>St.II</td>
</tr>
</tbody>
</table>

n.d. = no data;
All sections were examined with HE staining. Neuroblastomas were of the small round cell type and PNET showed the characteristics described elsewhere (125). ApopDetek (simply sensitive in situ Detection Kit, Dako, Denmark) was used to show apoptosis (126).

Table 2.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Sex</th>
<th>Age (Year)</th>
<th>Apoptosis index (%)</th>
<th>RAR-B index (%)</th>
<th>Survival (months)</th>
</tr>
</thead>
<tbody>
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<td>1.</td>
<td>NB</td>
<td>F</td>
<td>0.8</td>
<td>4.8</td>
<td>22</td>
<td>5</td>
</tr>
<tr>
<td>2.</td>
<td>NB</td>
<td>F</td>
<td>1.2</td>
<td>1.0</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>3.</td>
<td>NB</td>
<td>F</td>
<td>8.0</td>
<td>0.0</td>
<td>0</td>
<td>&gt;24</td>
</tr>
<tr>
<td>4.</td>
<td>NB</td>
<td>F</td>
<td>1.1</td>
<td>0.0</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>5.</td>
<td>NB</td>
<td>F</td>
<td>6.0</td>
<td>0.0</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>6.</td>
<td>NB</td>
<td>M</td>
<td>4.0</td>
<td>2.0</td>
<td>19</td>
<td>13</td>
</tr>
<tr>
<td>7.</td>
<td>NB</td>
<td>M</td>
<td>1.0</td>
<td>0.0</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>8.</td>
<td>NB</td>
<td>M</td>
<td>4.0</td>
<td>0.0</td>
<td>9</td>
<td>&gt;24</td>
</tr>
<tr>
<td>9.</td>
<td>NB</td>
<td>M</td>
<td>4.0</td>
<td>1.0</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>10.</td>
<td>NB</td>
<td>M</td>
<td>6.0</td>
<td>3.8</td>
<td>17</td>
<td>23</td>
</tr>
<tr>
<td>11.</td>
<td>NB</td>
<td>M</td>
<td>2.3</td>
<td>3.4</td>
<td>34</td>
<td>17</td>
</tr>
<tr>
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<td>M</td>
<td>2.5</td>
<td>0.0</td>
<td>11</td>
<td>20</td>
</tr>
<tr>
<td>13.</td>
<td>NB</td>
<td>F</td>
<td>6.0</td>
<td>0.0</td>
<td>30</td>
<td>n.d.</td>
</tr>
<tr>
<td>14.</td>
<td>NB</td>
<td>F</td>
<td>2.0</td>
<td>0.0</td>
<td>10</td>
<td>n.d.</td>
</tr>
<tr>
<td>15.</td>
<td>NB</td>
<td>M</td>
<td>8.0</td>
<td>0.0</td>
<td>90</td>
<td>n.d.</td>
</tr>
<tr>
<td>16.</td>
<td>NB</td>
<td>M</td>
<td>3.0</td>
<td>0.0</td>
<td>0</td>
<td>n.d.</td>
</tr>
<tr>
<td>17.</td>
<td>NB</td>
<td>M</td>
<td>0.3</td>
<td>0.0</td>
<td>80 nuclear 20 cytopl.</td>
<td>n.d.</td>
</tr>
<tr>
<td>18.</td>
<td>NB</td>
<td>F</td>
<td>0.5</td>
<td>0.0</td>
<td>85 cytopl.</td>
<td>n.d.</td>
</tr>
<tr>
<td>19.</td>
<td>NB</td>
<td>M</td>
<td>2.0</td>
<td>3.0</td>
<td>50 cytopl.memb.</td>
<td>n.d.</td>
</tr>
<tr>
<td>20.</td>
<td>NB</td>
<td>M</td>
<td>3.0</td>
<td>0.0</td>
<td>70</td>
<td>n.d.</td>
</tr>
<tr>
<td>21.</td>
<td>NB</td>
<td>M</td>
<td>3.0</td>
<td>0.0</td>
<td>50 nuclear</td>
<td>n.d.</td>
</tr>
<tr>
<td>22.</td>
<td>NB</td>
<td>M</td>
<td>1.0</td>
<td>0.0</td>
<td>-</td>
<td>n.d.</td>
</tr>
<tr>
<td>23.</td>
<td>PNET</td>
<td>F</td>
<td>4.0</td>
<td>5.0</td>
<td>43</td>
<td>4</td>
</tr>
<tr>
<td>24.</td>
<td>PNET</td>
<td>M</td>
<td>2.0</td>
<td>19.5</td>
<td>68</td>
<td>25</td>
</tr>
<tr>
<td>25.</td>
<td>PNET</td>
<td>M</td>
<td>5.0</td>
<td>6.8</td>
<td>41</td>
<td>42</td>
</tr>
<tr>
<td>26.</td>
<td>PNET</td>
<td>M</td>
<td>10.0</td>
<td>4.1</td>
<td>16</td>
<td>&gt;20</td>
</tr>
</tbody>
</table>

Sections were treated with proteinase K to make the tissue permeable to the reagents of the labelling and detection procedure. The apoptosis index in the tumor tissue was determined by examining 2000 tumor cells. The presence of RAR was
examined by immunoperoxidase reaction using RAR-B Ab (BIOMOL, Germany, cat.No.SA-17). This antibody has been reported to show RAR in Western blot (127). The sections were treated with proteinase K and, to inactivate endogenous peroxidase, incubated in methanol and H2O2. Undiluted anti-retinoic acid receptor-B was applied as primary antibody and was incubated overnight at –4 C. The secondary Ab-mouse immunoglobulin was used in 1:100 dilution the next day. DAB (diaminobenzidine) was used as a chromogen and methyl green as a counterstain.

The same procedure was performed with RAR-B diluted in 1:5 and 1:10 to test the sensitivity of the reaction, since according to our knowledge this is the first attempt to use immunohistochemistry to show RAR. RAR index in tumor tissue was determined by examining 2000 tumor cells.

**Results**

The clinical data of our patients are shown in table1. With the exception of two cases (case 3 and case 6), death was due to progression of the tumor 5-42 months after the diagnosis, irrespective of localization, histological types, or mode of therapy. Only one case (case 3) received RA therapy, this patient is still alive. Apoptosis was shown in 6 NB tumor samples (table-2).

The apoptotic index was zero or 1% in 8 children.

![Fig. 1. NB case scattered apoptotic cells. ApopDetec staining (x300).](image1)

![Fig. 2. NB RAR positivity on cell surface. Anti-RAR-beta immunoperoxidase (x300)](image2)
and relatively low (2-4%) in the other four cases, while it was higher (4.1-10.5%) in PNET. The RAR index determined by immunoperoxidase reaction in NB was zero to 3% in five cases and 9-34% in seven children (table-2). Scattered apoptotic cells in a section of neuroblastoma is shown in Figure 1.

RAR index in PNET was 16-68% in all four cases. As shown in table 2, apoptotic index and RAR index varied parallel to each other, i.e., when apoptosis was frequent, the RAR index was also elevated. The correlation coefficient between apoptosis and RAR index was $r = 0.47$ according to Pearson-Bravis.

RAR positivity appeared in the nuclei of tumor cells both in NB and PNET when the antibody was applied undiluted (figures 2 and 3), but if 1:5 or 1:10 dilution was also applied, cell surface and slight cytoplasmic positivity could be detected in the same cell (Figure 4, 5), along with nuclear positivity, probably due to better resolution of the cell organelle. There was no evaluable relationship between apoptotic index, age, and stage.

Fig. 3. RAR positivity in nuclei of NB. Anti-RAR-beta immunoperoxidase (x150)
Discussion

A variety of tumors have been studied for spontaneous and induced apoptosis. Inducibility of apoptosis in tumor tissue by various drugs or irradiation is considered as a possible predictive factor (121,122,128). The spontaneous apoptosis index in NB and PNET has been found to be very low (129, 130). The possibility of inducing apoptosis by retinoic acid (RA) treatment was first studied by Smith et al (124) in vitro. In their experiment RA-induced apoptosis in cultured NB cells was studied and this effect was linked to RAR. Since RA therapy has been administered in some cases of NB (131,132)
detection of RAR may predict the effectiveness of RA treatment. Although nuclear localization of the receptor by western blot (133) has been reported we demonstrated by immunoperoxidase reaction that at least a certain proportion of the receptor may be situated on cytoplasmic membranes.

As for NBs, not all were RAR positive in our study. The apoptotic index was low in general compared to other types of tumors, but it was elevated in cases with a strong RAR positivity. In all cases of PNET, however, a relatively high apoptotic index as well as strong RAR positivity was found.

To the best of our knowledge, immunohistochemical demonstration of RAR has not been reported. Our findings show that the RAR-B Ab can be used successfully also for immunohistochemical purposes. The results suggest that spontaneous apoptotic activity in NB may be caused by endogenous retinoic acid and mediated by RAR.
**Tumors in Thyroid glands**

Nodules in the thyroid have always commanded a great deal of attention because of their fear of being cancerous. The female-to-male ratio is about 3 to 4:1. Most of these solitary masses prove to be dominant nodules within a multinodular goiter, cysts, or asymmetric enlargements of various non-neoplastic conditions, such as Hashimoto’s thyroiditis. When such nodules prove to be neoplastic, in well over 90% of instances, they are *adenomas*. Virtually all adenomas of the thyroid are present as solitary, discrete masses. With rare exception, they all are derived from follicular epithelium and so might all be called follicular adenomas (134). Microscopically, a variety of patterns can be identified that recapitulate stages in the embryogenesis of the normal thyroid, and so they have been divided into fetal, embryonal, simple, and colloid subtypes or, more simply, into microfollicular and macrofollicular patterns. There is little virtue in these classifications because mixed patterns are common, and ultimately all have the same clinical and biologic significance. Numerous studies have made it clear that adenomas are not forerunners of cancer except in the exceptional instance.

**Malignant Tumors in Thyroid glands**

Thyroid carcinoma is two to three times more common in middle aged women than in men. Before puberty, there is no sex difference, and the female preponderance disappears in postmenopausal life. Possibly relevant is the fact that most well-differentiated thyroid carcinomas have estrogen receptors. The morphologic variants of thyroid carcinoma with their approximate frequencies are as follows:

- Papillary carcinoma 75-85%
- Follicular carcinoma 10-20%
- Medullary thyroid carcinoma 5%
- Anaplastic carcinoma (rare)

**Papillary Carcinoma**, is the most common type of thyroid carcinoma, comprising 45% to 70% of all cases (123,135). It is a slow-growing tumor that usually appears clinically as a solitary thyroid nodule. The primary tumor is in most cases limited to the gland, but
especially in older people it may invade the surrounding structures, such as muscles, the esophagus, or the larynx. The prognosis of papillary carcinoma is relatively good, with a 10-year survival rate in the order of 80% to 95% (135,136,137). Histologically papillary carcinoma is composed of papillae, usually accompanied by follicles and sometimes by solid sheets of cells. The papillae are often long and slender and have a fibrovascular core that is usually thin but may be thick and hyalinized. The nuclei of papillary carcinoma often overlap and have many typical features including a pale outlook, large size compared with normal follicular cells, irregular outline with deep grooves and cytoplasmic pseudoinclusions, rare mitosis, and an inconspicuous nucleolus (138,139,140).

**Follicular carcinoma** comprises about one fourth of all thyroid carcinomas. It is more common in females than in males and is a disease of middle and old age. Distant metastases are common, occurring in as many as 25% of patients at diagnosis (141). Their most common sites are the bones and lungs. Histologically the tumor is often encapsulated. The capsule usually contains large, thin-walled blood vessels. Vascular invasion -often to these vessels- is common, a finding that explains the frequent occurrence of distant bloodborne metastases in follicular carcinoma. Follicular carcinoma shows follicular differentiation but lacks the diagnostic features of papillary carcinoma (142). Like follicular adenoma, follicular carcinoma is composed of follicular, trabecular, or solid structure (143).The degree of follicular differentiation varies, but usually the follicles are smaller and less well differentiated than the follicles in papillary carcinoma. Follicular carcinoma is divided into two subtypes: minimally invasive (also called encapsulated) and widely invasive (142). In the first subtype the tumor is encapsulated, and only minimal vascular or capsular invasion is seen. In the second subtype the tumor is either nonencapsulated or encapsulated with considerable invasion. These two subtypes should be distinguished in prognosis (144) because the 10-year survival rate for the first type is 80% to 95% and for the second type 30% to 45% (135,136,145).

**Medullary Carcinoma** basically differs from other thyroid carcinomas in showing evidence of C-cell differentiation. Like normal C-cells, the tumor cells produce and secrete calcitonin. The tumor may also produce prostaglandins histaminase (146), and express carcinoembryonic antigen (CEA) (147), and occasionally excretes 5-
hydroxytryptamine (5-HT) or adrenocorticotrophic hormone (ACTH). Although most medullary carcinomas occur sporadically, about 10% have a genetic background (148). The sporadic carcinoma is generally unilateral but the familial tumor is usually bilateral and multicentric and often accompanied by C-cell hyperplasia, which precedes the development of medullary carcinoma (149,150). Also, the associated pheochromocytoma is usually bilateral (151). Medullary carcinoma is rather rare, comprising about 5% to 10% of all thyroid carcinomas. It is as common in men as in women and is usually detected at middle age. The most frequent finding is a solitary thyroid nodule, but sometimes enlarged cervical lymph nodes may be the first symptom. Regional lymph node metastases are common; they have been detected in about half the patients at the time of surgery (152). There are often irregular calcifications in the stroma that may resemble psammoma bodies but do not show regular laminations. It is not always possible to find amyloid in medullary carcinoma. In such cases the diagnosis can be confirmed by use of silver stains for argyrophil cells (153) or immunohistochemical methods based on the calcitonin content of the tumor cells (154). Mixed medullary-follicular carcinoma (156,157) denotes a very rare tumor with morphologic features of medullary carcinoma with immunoreactive calcitonin, accompanied with morphologic features of follicular carcinoma with immunoreactive thyroglobin.

Anaplastic carcinoma: Fortunately fewer than 5% of thyroid carcinomas fall into this category. They tend to occur in elderly individuals, particularly in endemic goitrous regions. There are basically three histologic patterns: 1) spindle cell carcinomas, 2) giant cell leions, and 3) rarest of all the small cell carcinoma (155). All grow quite rapidly and are usually large masses by the time they come to clinical attention.

II. Apoptosis in Thyroid glands

Our studies on apoptosis in thyroid gland tumors

This study has been carried out by the co-operation of the:
National Institute of Oncology, Budapest, Hungary with the full support of Dr. Ilona Peter, whom without her assistance the work would have been impossible. And the
Introduction

Thyroid nodules are found in as many as 1% to 10% of the population, but malignant tumors of the thyroid account for only about 1% of all cancer and 0.4% of cancer related death (156). Females are affected three times as often as males (106). The aim of this study was to measure spontaneous apoptosis and the expression of some oncogenes which influence this process: Bcl-2 by securing cell survival (5), mutant P53 by suppressing and bax by promoting cell death (22). With the knowledge that Bcl-2 inhibits apoptosis in a number of different cells and bax is antagonizing the protective effect of Bcl-2 it becomes clear that prolonged survival of cells overexpressing Bcl-2 is a factor in predisposition to malignancy (5). Nevertheless, investigation of these oncogenes in benign tumors—adenomas could indicate a chance of malignancy in such tumors.

Materials & Methods

Sixteen cases of thyroid carcinoma and nine cases of adenoma were investigated in a retrospective study. Basic data (age, sex, tumor type) were registered (table 3.).

<table>
<thead>
<tr>
<th>No</th>
<th>Age (year)</th>
<th>Sex</th>
<th>Tumor type</th>
<th>Apoptosis %</th>
<th>P53 %</th>
<th>Bax %</th>
<th>Bcl-2 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>24</td>
<td>F</td>
<td>F. adenoma</td>
<td>0</td>
<td>2.6</td>
<td>12.7</td>
<td>Neg.</td>
</tr>
<tr>
<td>2.</td>
<td>35</td>
<td>F</td>
<td>F. adenoma</td>
<td>0</td>
<td>4.9</td>
<td>1.9</td>
<td>Neg.</td>
</tr>
<tr>
<td>3.</td>
<td>44</td>
<td>F</td>
<td>F. adenoma</td>
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<td>0.9</td>
<td>0</td>
<td>Neg.</td>
</tr>
<tr>
<td>4.</td>
<td>39</td>
<td>F</td>
<td>F. adenoma</td>
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<td>0</td>
<td>11.9</td>
<td>Neg.</td>
</tr>
<tr>
<td>5.</td>
<td>45</td>
<td>F</td>
<td>F. adenoma</td>
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<td>33.1</td>
<td>43.5</td>
<td>Neg.</td>
</tr>
<tr>
<td>6.</td>
<td>55</td>
<td>F</td>
<td>F. adenoma</td>
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<td>5.1</td>
<td>0</td>
<td>59</td>
</tr>
<tr>
<td>7.</td>
<td>20</td>
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<td>F. adenoma</td>
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<td>0.6</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td>8.</td>
<td>50</td>
<td>F</td>
<td>F. adenoma</td>
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<td>0.3</td>
<td>9.2</td>
<td>47</td>
</tr>
<tr>
<td>9.</td>
<td>51</td>
<td>F</td>
<td>F. adenoma</td>
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<td>3.5</td>
<td>40.5</td>
<td>Neg.</td>
</tr>
<tr>
<td>Subtotal</td>
<td>40</td>
<td></td>
<td>F. adenoma</td>
<td>0</td>
<td>5.7</td>
<td>13.3</td>
<td>41.7</td>
</tr>
</tbody>
</table>

Table 3.a.1. Percentage of P53, bax, bcl2 and apoptosis in thyroid adenoma
The tumor tissues were obtained by surgical excision, and after formalin fixation and paraffin embedding 8 μm thin sections were cut. All sections were examined with HE staining. The criteria described by Wyllie (3) were used to recognize apoptotic cells. ApopDetec (simply sensitive In situ Detection System Kit, DAKO, Denmark) was also used to show apoptosis. Sections were treated with proteinase K to make the tissue

Table 3.a.2. Percentage of P53, bax, bcl2 and apoptosis in thyroid follicular adenocarcinoma

<table>
<thead>
<tr>
<th>No</th>
<th>Age (year)</th>
<th>Sex</th>
<th>Tumor type</th>
<th>Apoptosis is %</th>
<th>P53 %</th>
<th>Bax %</th>
<th>Bcl-2 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.</td>
<td>64</td>
<td>F</td>
<td>F.ad. carcinoma</td>
<td>0</td>
<td>94</td>
<td>12.3</td>
<td>Neg.</td>
</tr>
<tr>
<td>11.</td>
<td>50</td>
<td>F</td>
<td>F.ad. carcinoma</td>
<td>0</td>
<td>22.8</td>
<td>4.8</td>
<td>2</td>
</tr>
<tr>
<td>12.</td>
<td>68</td>
<td>M</td>
<td>F.ad. carcinoma</td>
<td>0</td>
<td>46.7</td>
<td>0</td>
<td>Neg.</td>
</tr>
<tr>
<td>13.</td>
<td>71</td>
<td>F</td>
<td>F.ad. carcinoma</td>
<td>0</td>
<td>53.1</td>
<td>1.6</td>
<td>Neg.</td>
</tr>
<tr>
<td>14.</td>
<td>67</td>
<td>F</td>
<td>F.ad. carcinoma</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>15.</td>
<td>55</td>
<td>M</td>
<td>F.ad. carcinoma</td>
<td>0</td>
<td>81.7</td>
<td>1.1</td>
<td>Neg.</td>
</tr>
<tr>
<td>16.</td>
<td>17</td>
<td>M</td>
<td>F.ad. carcinoma</td>
<td>0</td>
<td>100</td>
<td>4.2</td>
<td>Neg.</td>
</tr>
<tr>
<td>Subtotal</td>
<td>56</td>
<td>F.ad. carcinoma</td>
<td>0</td>
<td>71.1</td>
<td>3.4</td>
<td>2.5</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.a.3. Percentage of P53, bax, bcl2 and apoptosis in thyroid papillari adenocarcinoma

<table>
<thead>
<tr>
<th>No</th>
<th>Age (year)</th>
<th>Sex</th>
<th>Tumor type</th>
<th>Apoptosis is %</th>
<th>P53 %</th>
<th>Bax %</th>
<th>Bcl-2 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>17.</td>
<td>64</td>
<td>F</td>
<td>P.ad. carcinoma</td>
<td>0</td>
<td>0</td>
<td>3.4</td>
<td>Neg.</td>
</tr>
<tr>
<td>18.</td>
<td>44</td>
<td>F</td>
<td>P.ad. carcinoma</td>
<td>0</td>
<td>100</td>
<td>5</td>
<td>Neg.</td>
</tr>
<tr>
<td>19.</td>
<td>43</td>
<td>M</td>
<td>P.ad. carcinoma</td>
<td>0</td>
<td>0.6</td>
<td>4.6</td>
<td>Neg.</td>
</tr>
<tr>
<td>20.</td>
<td>17</td>
<td>M</td>
<td>P.ad. carcinoma</td>
<td>0</td>
<td>0.6</td>
<td>0</td>
<td>Neg.</td>
</tr>
<tr>
<td>21.</td>
<td>11</td>
<td>F</td>
<td>P.ad. carcinoma</td>
<td>0</td>
<td>26</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>22.</td>
<td>23</td>
<td>M</td>
<td>P.ad. carcinoma</td>
<td>0</td>
<td>0</td>
<td>13.1</td>
<td>Neg.</td>
</tr>
<tr>
<td>23.</td>
<td>65</td>
<td>F</td>
<td>P.ad. carcinoma</td>
<td>1%</td>
<td>53.1</td>
<td>3.2</td>
<td>Neg.</td>
</tr>
<tr>
<td>24.</td>
<td>42</td>
<td>M</td>
<td>P.ad. carcinoma</td>
<td>0</td>
<td>96.1</td>
<td>0</td>
<td>Neg.</td>
</tr>
<tr>
<td>25.</td>
<td>72</td>
<td>F</td>
<td>P.ad. carcinoma</td>
<td>0</td>
<td>0.5</td>
<td>1.2</td>
<td>4</td>
</tr>
<tr>
<td>Subtotal</td>
<td>42</td>
<td>P.ad. carcinoma</td>
<td>0</td>
<td>39.6</td>
<td>3.4</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.b. Average of P53, bax, bcl2 and apoptosis in thyroid tumors

<table>
<thead>
<tr>
<th>Average Age (year)</th>
<th>Tumor Type</th>
<th>Apoptosis %</th>
<th>P53</th>
<th>Box</th>
<th>Bcl - 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 ? 12</td>
<td>F. adenoma</td>
<td>0</td>
<td>5.7 ? 10,4</td>
<td>13.3 ? 10,0</td>
<td>41.7 ? 20,5</td>
</tr>
<tr>
<td>56 ? 18</td>
<td>F.ad.carcinoma</td>
<td>0</td>
<td>71.1 ? 30,4</td>
<td>3.4 ? 4,6</td>
<td>2.5 ? 0,7</td>
</tr>
<tr>
<td>42 ? 22</td>
<td>P.ad.carcinoma</td>
<td>0</td>
<td>39.6 ? 44,3</td>
<td>3.4 ? 4,1</td>
<td>3.0 ? 1,4</td>
</tr>
</tbody>
</table>

The tumor tissues were obtained by surgical excision, and after formalin fixation and paraffin embedding 8 μm thin sections were cut. All sections were examined with HE staining. The criteria described by Wyllie (3) were used to recognize apoptotic cells. ApopDetec (simply sensitive In situ Detection System Kit, DAKO, Denmark) was also used to show apoptosis. Sections were treated with proteinase K to make the tissue
permeable to the reagents of the labeling and detection procedure. The apoptosis index in the tumor tissue was determined by examining 2000 tumor cells. The sections were examined for mutant p53, bax and bcl-2 by immunoperoxidase reaction. All antibodies to p53, bax and bcl-2 were the products of DAKO (Denmark) and were used in dilution 1:100 overnight at 37 °C. The sections were treated with proteinase K and, to inactivate endogenous peroxidase, incubated in methanol and H₂O₂. The secondary antimouse IgG (product of DAKO) was used in dilution 1:100 the next day. DAB (diaminobenzidine) served as a chromogen and methyl green as counterstain. p53, bcl-2 and bax positivity were determined by examining 2000 cells.

**Results**

In adenomas p53 positivity was 5.7% (fig. 6.), bax positivity was 13.3% and bcl-2 positivity was 41.7%. As for the follicular adenocarcinomas p53 showed 71.1% positivity (fig. 7.), bax was positive in 3.4% and (Fig. 6.)

![Graph showing percentages of bcl2, bax, and p53 in thyroid tumors]

Fig. 6. Percentage of bcl2, bax and P53 in thyroid tumors
bcl-2 was positive in 2.5%. In papillary adenocarcinomas (Fig.7,8,9,) the value for p53 was 39.6%, for bax was 3.4% positivity and for bcl-2 was 3%. Apoptosis was almost absent in all cases.

**Discussion**

Data from this study on mutant p53, bcl-2 and bax expression in benign and malignant thyroid tissue reveal considerable results. Mutant p53 is very low in
adenomas compared to its expression in papillary and follicular carcinomas. Bcl-2 expression is high in adenomas and low in carcinomas according to our data. Bax expression on the other hand, is relatively high in adenomas but still lower than bcl-2 and it is low in carcinomas. In agreement with our results, bcl-2 and bax had been reported to be co-expressed in aggressive thyroid carcinomas (159). As for the positivity of bax expression in papillary carcinomas Branet et al (5) obtained similar results as described here by us. It has been supposed that when bcl-2 is present in excess, cells are protected against apoptosis (159,160,161). However, when bax is in excess, cells are susceptible to apoptosis. As apoptosis is concerned, only very few apoptotic tumor cells could be detected in our study in any of the benign or malignant tumors. This may be relevant to the high expression of bcl-2. On the other hand, high expression of bcl-2 in adenomas suggests the susceptibility to transformation to malignancy. From a practical point of view, determination of p53, bcl-2 and bax ratio in thyroid tumors may contribute to the differentiation between adenomas and especially follicular carcinoma (7).
Fig. 11. P53 positivity in follicular adenocarcinoma. P53 immunoperoxidase (x600)

Fig. 12. Bcl-2 positivity in follicular carcinoma. Bcl2 immunoperoxidase (x300)
With the request of WHO-IARC (International Agency for Research on Cancer), after the publication of our paper on thyroid tumours (Farid P, Gomba SZ, Peter I, Szende B. Bcl-2, P53, and Bax in thyroid tumours and their relation to apoptosis. Neoplasm 2001; 48(4): 299-301) the following text was sent, along with a chart (fig.6) and a photo (fig.11) and was accepted for publication in the WHO book on Pathology and genetics of tumours of Endocrine organ.

**Thyroid follicular carcinoma, Somatic genetic, expression profiles/proteomics**

Follicular carcinoma is a much more aggressive lesion than papillary carcinoma. This cancer occurs more often in females with a peak incidence in the fifth and sixth decades. Mortality rates are 70% at 5 years.

There is no sign of increased apoptosis, or programmed cell death, as defined by morphological change in non-pathologic cell loss and relevant to a wide spectrum of biology; in follicular carcinoma, however as shown by immunohistochemical method (presumably mutant) p53, bax, and bcl-2 were expressed.

When bcl-2 is present in excess, cells are protected against apoptosis (4,5,7) since it secures cell survival (2). Mutant p53 suppresses, while bax promotes cell death (6,1).

When Bax is in excess, cells are susceptible to apoptosis. BCL-2 and Bax had been reported to be co-expressed in aggressive thyroid carcinoma (4).

From a practical point of view the determination of P53, BCL-2 and Bax ratio in thyroid tumors, contributes to the differentiation between adenomas and especially follicular carcinomas (3). In adenomas the rate of P53 positive cells was 5.7%, while it was 13.3% for Bax and 41.7% for Bcl-2 in the tumor cell. The identification of the above mentioned oncogenes, and many more, is facilitated by proteomics, that studies the proteins in general and in particular their changes resulting from various disorders or the effect of external factors (8).
References


III. Tumors of the Parathyroid glands

Normal parathyroid glands: In adults, most of the parathyroid glands are composed of *chief cells*, but these give rise to several transitional forms as will be evident subsequently. The chief contain lipofuscin pigment and secretory granules of parathyroid hormones (PTH). Sometimes these cells have a ‘*water clear*” appearance owing to lake of glycogen. *Oxyphil* cell and transitional oxyphils are found throughout the normal parathyroid either singly or in small clusters.

The metabolic function of PTH in supporting the serum calcium level can be summarized as follows, as a short review for better understanding the dysfunctions and hyperplasia of the parathyroid glands (158):

1. PTH mobilizes calcium from bone.
2. It increases renal tubular reabsorption of calcium, thereby conserving it.
3. It promote renal production of 1,25-(OH)2D3, active in intestinal absorption of calcium.
4. It lowers the serum phosphate level by enhancing phosphaturia.

**Adenoma**

Almost always solitary adenomas average 0.5 to 5.0 gm in weight but maybe much larger. Although two may be present concomitantly in the same or different glands, nodular hyperplasia must be ruled out when more than one adenoma is present. As a rule, normal parathyroid parenchyma contains evenly distributed cytoplasmic fat droplets, whereas adenomas do not contain such a fat, and hyperplastic parenchyma has reduced or absent fat (162). The adenomas are well-encapsulated, soft, tan to red lesions. Most are composed predominantly of chief cells, but often they contain foci of oxyphil and transitional cells usually present as islands within a background of the chief cells. Although oxyphil cells are traditionally thought to be non-functional, rarely a hyperfunctioning adenoma is composed of these cells. In larger lesions, there may be areas of infarct necrosis or hemorrhage.
Primary hyperplasia

Primary hyperplasia may occur sporadically or in the MEN syndromes I and II a. The hyperplasia may be either diffuse or nodular and most often is composed predominantly of chief cells but sometimes water clear cells and, in almost all instances, islands or nodules of oxyphils. Poorly developed, delicate, fibrous strands may envelope the nodules. The cells in the diffuse hyperplasia or within the nodules are arranged in a wide variety of patterns: solid sheets, nests, trabeculae, or sometimes follicular structures. One of the more distinctive features of primary hyperplasia is the dispersal of occasional fat cells throughout the hyperplastic areas, but overall the total amount of fat is reduced (163,164).

Secondary Hyperplasia

The syndrome most often appears in patients with renal failure (uremic hyperparathyroidism) but also with marked vitamin D deficiency or osteomalacia. The origin of hyperparathyroidism in renal failure is attributed to phosphate retention and hypocalcemia leading to compensatory parathyroid hyperfunction. In addition, the renal disease may contribute to the hypocalcemia by 1) a reduction in the synthesis of 1,25-(OH)2D3 with impaired intestinal absorption of calcium and 2) some poorly understood state of skeletal resistance to the calcemic action of vitamin D and PTH. Uremic hyperparathyroidism may be observed in the absence of hypocalcemia. Similarly, vitamin D deficiency may directly stimulate PTH secretion. Whatever the precise mechanism, it is clear that patients with renal failure and rarely the other conditions mentioned develop secondary parathyroid hyperfunction with hyperplasia principally of the chief cells. In many instances, the glands revert to normal if the basic clinical derangement is brought under control by, for example renal transplantation. With long standing secondary hyperplasia, however, reversion to normal may not occur, contributing to the possibility that secondary hyperplasia may in time convert into autonomous adenoma formation, sometimes referred to as tertiary hyperparathyroidism (165,166).
Parathyroid carcinoma

Fewer than 5% of cases of PHPT are caused by carcinomas. These tumors present as gray-white, irregular masses that sometimes exceed 10 gm in weight and enlarge usually one parathyroid gland. Many are small, however, and readily mistaken for an adenoma. Histologically, the neoplastic cell may be of variable size and shape but are usually remarkably uniform and not too dissimilar from normal parathyroid cells. There is general agreement that a diagnosis of carcinoma based on cytologic detail is unreliable, and local invasion and metastasis constitute the only reliable criteria of malignancy (167).

III. Apoptosis in parathyroid tumors

Our studies have been done with the co-operation of the Department of Surgery and Transplantation, Semmelweis University, Budapest-Hungary, our gratitude goes to Prof. Dr. Ferenc Perner and dr. Gyula Végso.

Hyperplastic and adenomatous lesions of the parathyroid glands are not uncommon, but malignant tumors of this gland are extremely rare (168). Hyperplasias may be primary lesions but in most cases parathyroid hyperplasia is the consequence of chronic renal failure. Adenomas may appear as a subject of both primary and secondary hyperparathyroidism (HP), but the majority of these tumours appear without anamnestic data on renal disease (169). Of course, renal damage develops regularly as an effect of elevated serum Ca level in HP.

Differential diagnostic difficulties are well known concerning hyperplasia, adenoma and carcinoma of the parathyroid gland. The most important sources refer to morphological criteria and biological behaviour of the tumours (170). The significance of DNA diploidy in indicating the benign or maliginanat character of parathyroid tumours was studied by Bocsi et al (171). They found that DNA index had no value in deciding the benign or malignant character of a given sample.

A few but important data are available on p53, bcl-2 expression in parathyroid hyperplasia, adenoma and carcinoma. bcl-2 and p53 expression was found both in hyperplasias and adenomas but carcinomas failed to express bcl-2 (172,173). The role of other gene products in the differential diagnosis of parathyroid neoplasia like P27, cyclin D1, and Ki67 was also emphasized (174,175,176).
We investigated the mitotic and apoptotic activity and also the expression of P53, Bcl-2 and Bax in hyperplasia and benign as well as malignant tumors of the parathyroid glands. The aim of this study was to look for differences in this respect between the above-mentioned proliferative alterations and further to find out whether the prevalence of enhancing or inhibitory factors of apoptotic activity may explain the rarity of parathyroid carcinomas.

**Materials & Methods**

*Clinical and laboratory parameters:*

The parathyroid lesions presented here were clinically diagnosed and surgically removed at the Clinical Department of Transplantation and Surgery of the Semmelweis University, Budapest Hungary. Altogether 107 patients were operated on because of hyperparathyroidism (HP), 55 proved to be primary (PH), 52 suffered from secondary hyperparathyroidism (SH). Clinical symptoms and laboratory parameters (serum Ca and P, creatinine and parathormone level as well as radiological examination) leading to diagnosis of HP were registered together with the period in time between the onset of clinical symptoms or the start of hemodialysis or the date of renal grafting and the diagnosis. Localisation and number of enlarged and removed parathyroid glands; postoperative serum Ca and parathormone levels were also registered.

**Histological studies:**

All removed parathyroid glands were fixed in buffered neutral formalin and embedded into paraffin. 8µm thin sections were cut and stained with hematoxylin and eosin. Immunoperoxidase reactions to detect P53, Bcl-2 and Bax as well as TUNEL reaction to show apoptosis were performed. Anti P53, anti-Bcl-2 and anti-Bax antibodies were the product of DAKO (Glostrup, Denmark). The antibodies were diluted 1:100 and applied overnight at 37ºC. Sections were treated with proteinase K, incubated in methanol and H2O2. The secondary anti-mouse IgG (DAKO, Glostrup, Denmark) was used in dilution 1:100 the next day. Diaminobenzidine (DAB) served as chromogen and methyl green as counterstain. The TUNEL reaction was performed using Apop Detec (Simply sensitive In Situ Detection System Kit, DAKO, Glostrup, Denmark) which detects DNA fragmentation along nucleosomes and stain the nuclei of apoptotic cells.
Cellular constitution of the parathyroid lesions and mitotic index (considering 1000-cells) were determined using H&E stained sections. The criteria described by Roth (168) were applied to differentiate between hyperplasia, adenoma and carcinoma. Apoptotic index was established after TUNEL reaction considering 1000 cells. Both mitotic and apoptotic indices were expressed in percent. bcl-2 and bax, as well as P53 expression was studied considering chief cells, oxyphil cells, and clear cells. Positivity was stated if 50 percent or more of a cell type showed cytoplasmic or nuclear reaction with DAB.

**Results**

The age and sex distribution and the per cent of adenomas, hyperplasias and carcinomas are shown in fig. 13.a and 13.b. The overwhelming majority of patients with primary lesions was female. SH occurred in females and males in nearly equal number. The age of patients suffering from PH was in average higher than that of patients with secondary hyperparathyroidism.

![Graph](image1)

**Fig. 13.a. Primary hyperparathyroidism age, sex distribution and percent of adenomas, hyperplasias and carcinomas**

![Graph](image2)

**Fig. 13.b. Secondary hyperparathyroidism age, sex distribution and percent of adenomas, hyperplasias and mixed lesions**

<table>
<thead>
<tr>
<th>Total</th>
<th>55</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 males</td>
<td>49 females</td>
</tr>
<tr>
<td>Age: 23-70</td>
<td>32-76</td>
</tr>
<tr>
<td>(59,2)</td>
<td>(60)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total</th>
<th>52</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 males</td>
<td>27 females</td>
</tr>
<tr>
<td>Age: 14-71</td>
<td>20-71</td>
</tr>
<tr>
<td>(49,5)</td>
<td>(43,3)</td>
</tr>
</tbody>
</table>
The majority of primary lesions were adenomas and only three carcinomas were found. In case of secondary lesions hyperplasia was the most common finding.

The clinical symptoms and events leading to the diagnosis of HP are shown in table 4.a. PH resulted in nephrolithiasis and in osteoporosis as well as other bone and joint alterations in a high number of cases. SH was predictable by the fact of pre-existing chronic renal failure treated by dialysis or renal transplantation. We have to mention that no MEN cases and no vitamin D deficiency or marked osteomalcia cases were found in our material. The most characteristic laboratory findings are listed in table 4.b.

<table>
<thead>
<tr>
<th>PH</th>
<th></th>
<th>SH</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptoms and events</td>
<td>N° of cases</td>
<td>Symptoms and events</td>
<td>N° of cases</td>
</tr>
<tr>
<td>Nephrolithiasis</td>
<td>23</td>
<td>Osteoporosis</td>
<td>14</td>
</tr>
<tr>
<td>Osteoporosis</td>
<td>25</td>
<td>Bone-joint pain</td>
<td>16</td>
</tr>
<tr>
<td>Bone fracture</td>
<td>3</td>
<td>Bone fracture</td>
<td>5</td>
</tr>
<tr>
<td>Bone-joint pain</td>
<td>13</td>
<td>Nephrolithiasis</td>
<td>1</td>
</tr>
<tr>
<td>Elevated se Ca, P</td>
<td>9</td>
<td>Soft tissue calcification</td>
<td>3</td>
</tr>
<tr>
<td>gastrointestinal</td>
<td>5</td>
<td>Gastrointestinal</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Renal graft</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dialysis</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Renal graft plus renal failure</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Renal failure</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 4.a. Clinical symptoms and events leading to the diagnosis of HP
<table>
<thead>
<tr>
<th>Laboratory findings</th>
<th>Pre-operative</th>
<th>Post-operative</th>
<th>Pre-operative</th>
<th>Post-operative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Se Ca (mmol/l)</td>
<td>3 (2.73-3.5)</td>
<td>2.23 (1.4-2.62)</td>
<td>2.74 (2.2-3.21)</td>
<td>2.14 (1.44-2.7)</td>
</tr>
<tr>
<td>Se P (mmol/l)</td>
<td>0.83 (0.59-1.24)</td>
<td></td>
<td>1.71 (0.63-3.0)</td>
<td></td>
</tr>
<tr>
<td>Se PTH (pg/ml)</td>
<td>247 (68.6-1301)</td>
<td>20.3 (18.6-49.2)</td>
<td>821.9 (77.3-2510)</td>
<td>769 (180-1194)</td>
</tr>
<tr>
<td>Se creatinine (dialysis)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mol/l)</td>
<td></td>
<td></td>
<td>183 (95-338)</td>
<td></td>
</tr>
<tr>
<td>Se creatinine (renal graft)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(? mol/l)</td>
<td></td>
<td></td>
<td></td>
<td>50.6 (1.2-251)</td>
</tr>
</tbody>
</table>

Table 4.b. Characteristic laboratory findings in HP patients

High serum Ca and P levels as well as PTH level were registered in both PH and SH cases, higher values being characteristic to SH. Preoperative serum Ca and PTH levels reflected high PTH activity. Definitive preoperative diagnosis was secured by ultrasound and/or CT imaging as well as by isotope scanning (Table 5.).

<table>
<thead>
<tr>
<th></th>
<th>PH</th>
<th>SH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultrasound</td>
<td>35</td>
<td>34</td>
</tr>
<tr>
<td>CT</td>
<td>39</td>
<td>41</td>
</tr>
<tr>
<td>Isotope</td>
<td>54</td>
<td>48</td>
</tr>
<tr>
<td>One of the above</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Two of the above</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Three of the above</td>
<td>24</td>
<td>24</td>
</tr>
</tbody>
</table>

Table 5. Number of cases diagnosed and localized by ultrasound, CT and isotope techniques
In 34 cases of PH postoperative Ca administration became necessary. Subcutaneous autotransplantation of parathyroid tissue was performed postoperatively in 13 cases of SH. Serum creatinine level was high in nearly all SH patients with chronic renal disease. The time gap between the onset of symptoms in PH was relatively long (5.6 years) calling the attention to the difficulties of diagnosis of HP. The period between the start of diagnosis on the renal grafting was in average 4.2 (Table 6.) and 9 years, respectively.

The number of the removed parathyroids is indicated in table 7. Primary lesions were solitary in the majority of cases and were localized in the inferior right and left parathyroids.

<table>
<thead>
<tr>
<th></th>
<th>PH</th>
<th>SH (start of dialysis)</th>
<th>SH (renal grafting)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of enlarged parathyroids</td>
<td>5-6 (0.5-30)</td>
<td>4.2 (0.25-13)</td>
<td>9 (1.5-28)</td>
</tr>
</tbody>
</table>

Table 6. Time gap between the onset of clinical signs and events and the diagnosis of HP (years)

<table>
<thead>
<tr>
<th></th>
<th>No of cases</th>
<th>No of cases</th>
<th>No of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>48</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

Table 7. Number of the parathyroid lesions

Postoperative complications such as bleeding, infection, recurrent laryngeal nerve damage occurred in altogether 6 cases. Regarding HP all operations for PH and all but two operations for SH proved to be successful.
Histological examination of the removed parathyroids showed the structural and cellular features of hyperplasia, adenoma or carcinoma (see Fig 13 a, b).

The typical histological appearance of an adenoma and the immunostaining for parathormone are shown in figure 14. a. and 14. b.

Fig. 14. a. Parathyroid adenoma HE (x300)

Fig. 14. b. Parathormone adenoma. Parathormone immunoperoxidase (x300)
The cellular constitution of adenomas and hyperplasia is shown in fig. 15. Chief and oxyphil cells were both present in the lesions and clear cell component was also found in hyperplasia. No difference could be detected between the primary and secondary lesions in this respect.

Apoptotic index was equally low in adenomas and hyperplasias and so was the mitotic ratio. The carcinomas showed slight elevation in mitotic and apoptotic activity (fig. 16).

Fig. 15. Distribution of cells types in hyperplasia and adenoma.

Fig. 16. Apoptotic and mitotic index.
The ratio of P53, Bcl-2 and Bax expression was studied considering chief cells, oxyphil cells and clear cells. **Hyperplasias** showed p53 positivity in the oxyphil cells in 10% of the cases, chief cells were positive in 5 percent. The positivity was nuclear in general, but in some cases cytoplasmic positivity was seen. The ratio of bcl-2 positivity was 60 percent in the oxyphil, 20 percent in the chief and 10 percent in the clear cells, on average.

The bax expression was ten percent higher in all types of cells, compared to bcl-2 expression (fig. 17.a., 17.b.).

Fig. 17.a.

Fig. 17.b.
Figure 18.a. shows Bcl-2 positive cells in hyperplasia. bax positive cells in hyperplasia are shown in figure 18. b. Co-expression of bcl2 and bax, as well as p53 was registered in most of the positive cases. Adenomas were positive for p53 in 15 percent of the cases, considering oxyphil cells and in 5 percent of the cases considering chief cells. Positivity appeared in the nucleus in the majority of cases but in few cases cytoplasmic positivity was observed (Fig. 19.a, 19. b).
The expression of bcl-2 and bax in the oxyphil cells of adenomas was equally high and a relatively high expression of bcl-2 and bax was present in the chief cells. Figure 20.a. and 20. b. shows bcl-2 and bax positive cells of adenomas, respectively. Co-expression of the three genes was characteristic to adenomas as well.

Fig. 20.a. Bcl2 positivity in parathyroid adenoma. Bcl2 immunoperoxidase (x300)

Fig. 20.b. Bax positivity in parathyroid adenoma. Bax immunoperoxidase (x600)
The few cases of carcinomas showed nuclear p53 positivity (Fig. 21.a.) and the cytoplasm was negative for bcl-2 and bax (Fig. 21.b.).

Fig. 21.a. P53 nuclear positivity in parathyroid cancer. P53 immunperoxidase (x 300)

Fig. 21. b. Bcl2 negativity in parathyroid cancer. Bcl2 immunperoxydase (x600)
Discussion

The results of our study on a relatively large number of parathyroid hyperplasias and adenomas clearly indicate that mitotic or apoptotic activity can not differentiate between these two pathological entities. Both mitotic and apoptotic indices were equally low in hyperplasias and adenomas. In accordance with the study of Ricci et al (172) p53 and bcl-2 expression was found in hyperplasias as well as adenomas. The percent of cases considered as positive for p53 or bcl-2 was slightly higher in adenomas, but differential diagnosis can not be based on p53 or bcl-2 immunostaining. The same may be applied to bax expression. According to our knowledge expression of bax has not been studied yet in proliferative parathyroid lesions. The most interesting finding in our study was the co-expression of bcl-2 and bax in most hyperplasias and adenomas. Oxyphil cells and chief cells were studied separately regarding p53, bcl-2 and bax expression. Oxyphil cells showed positivity for these gene products in a significantly higher proportion of cases than chief cells or modified (clear) chief cells. This fact may be due to the abundance of mitochondria in oxyphil cells. The appearance of p53 positivity in the cytoplasm of both hyperplastic and adenomatous lesions may point to the increased activity of inhibitor of P53 amino acid terminal nuclear signal (177) which means an increased defense against transport of mutant P53 protein into the nucleous. The antibody used by us shows predominantly mutant P53 by immunostaining. Nuclear positivity of some of the adenomas may be a sign of the tendency towards malignant transformation.

The very low number of carcinomas does not allow us to draw definite conclusions comparing these tumours with adenomas. In accordance with the literature (178,179) mitotic as well as apoptotic activity were slightly elevated in carcinomas. Nuclear p53 positivity and the absence of bcl-2 as well as bax expression in carcinomas are in accordance with the findings of Stojadinovic et al (173). Co-expression of p53, bcl-2 and Bax may be a factor responsible for the low mitotic and apoptotic activities well as for the very low rate of malignomas in the parathyroid glands.

Our clinical findings may contribute to the differential diagnosis of hyperplasia and adenoma of the parathyroid glands. Single lesions are more likely adenomas than multiple ones. Adenomas occurred overwhelmingly in females. The gender distribution in case of hyperplasia was practically even. The average age of patients with PH was close to 60, SH occurred in earlier years of life.
Serum Ca and PTH levels were significantly higher in hyperplasias compared to adenomas. Chronic renal failure, dialysis or renal grafting are anamnestic data pointing to SH and the likelihood of hyperplasia. The presence of bone and joint lesions in both PH and SH and the very long time between the onset of these symptoms and the surgical removal of the parathyroid lesions calls attention to the importance of considering the possibility of HP first of all in case of osteoporosis.
Conclusions

In Neuroblastomas and PNET apoptotic index and RAR index varied parallel to each other, i.e., when apoptosis was frequent, the RAR index was also elevated. As for NBs, not all were RAR positive in our study. The apoptotic index was low in general compared to other types of tumors but it was elevated in cases with a strong RAR positivity. In all cases of PNET, however, a relatively high apoptotic index as well as strong RAR positivity was found.

To the best of our knowledge, immunohistochemical demonstration of RAR has not been reported yet. Our findings show that the RAR\(\beta\) Ab can be used successfully also for immunohistochemical purposes. The results suggest that spontaneous apoptotic activity in NB may be caused by endogenous retinoic acid and mediated by RAR. From the practical point of view the treatment of patients with RA was dependent on the result of our examination and in the case of positivity for the RAR the treatment was carried out at the clinic.

Having examined p53, bcl-2 and bax in different tumors of the Thyroid, a new dimension has been observed. Mutant p53 is very low in adenomas compared to its expression in papillary and follicular carcinomas, and mutant p53 suppresses cell death. Bcl-2 expression is high in adenomas and low in carcinomas according to our data, and bcl-2 secures cell survival. bax expression on the other hand, is relatively high in adenomas (but still lower than bcl-2) and low in carcinomas, and bax promotes cell death.

The high expression of Bcl-2 explains the low ratio of apoptotic cells in the examined thyroid tumors, benign and malignant. The high expression of Bcl-2 in adenomas suggests the susceptibility to transformation to malignancy. Although few apoptotic tumor cells could be detected in our study in any of the benign or malignant tumors this may be relevant to the high expression of bcl-2, since bcl-2 is known to secure cell survival.

From the practical point of view, determination of P53, Bax and Bcl-2 ratio in thyroid tumors contributes to the differentiation between adenomas and especially follicular carcinomas.

As to the parathyroid lesions the overwhelming majority of patients with primary lesions were female. Secondary hyperparathyroidism occurred in females and males in
nearly equal numbers. The age of patients suffering from PH was on average higher than that of patients with SH. The majority of primary lesions were adenomas and only three carcinomas. In case of SH, hyperplasia was the most common finding. Apoptotic index was equally low in adenomas and hyperplasia and so was the mitotic ratio. The carcinomas showed slight elevation in mitotic and apoptotic activity. The results of our study on a relatively large number of parathyroid hyperplasias and adenomas clearly indicate that mitotic or apoptotic activity cannot differentiate between these two pathological entities. Bcl-2 and bax and in some cases p53 expression was found in hyperplasias as well as adenomas. The percent of cases considered as positive for p53 or bcl-2 was slightly higher in adenomas, but differential diagnosis can not be based on p53 or bcl-2 immunostaining. The same may be applied to bax expression (according to our knowledge expression of bax has not been studied yet in proliferative parathyroid lesions). The most interesting finding in our study was the co-expression of bcl-2 and bax in most of hyperplasias and adenomas. Spontaneous apoptosis is generally low in endocrine cells, but overall knowledge in this field reveals that hormones produced by different endocrine cells like PTH or LH induce apoptosis. This by itself probably can build up a protective mechanism in endocrine cells against apoptosis. The great emphasis in our study had been given to shed light over this subject and has called our attention to the co-expression of bax and bcl-2 and its relation to the protective mechanism against apoptosis in endocrine tumors.
**Abbreviations**

NB, Neuroblastoma

PNET, Primary neuroectodermal tumors

PCD, Programmed Cell Death

INPC, International Neuroblastoma Pathology Committee

NTs, Neuroblastic Tumors

OPEC, Oncovin+Cisplatin+Etoposid+Cyclophosphamid

OJEC, Oncovin+Cyclophosphamid+Etoposid+Carboplatin

VAIA, Vincristin+Actinomycin/Adriamycin+Ifosfamid

VAC, Vincristin+Actinomycin/Adriamycin+Cyclophosphamid

ALL-BFM-88/NHL-BFM-95,

Prednisolon+Vincristin+Daunorubicin+Cyclophosphamid+L-Asparaginase+Leupurin+Metotrextat.

NB-A-89, Dacarbazin+Adriamycin+Mustarnitrogen+Vincristin

Newcastle, Vepesid+Ifosfamid/Vincristin+Cisplatin/Vincristin+Ifosfamid+Adriamycin

RAR, Retinoic Acid Receptor

DAB, Diaminobenzidine

PTH, Parathormone

PH, Primary Hyperparathyroidism

SH, Secondary Hyperparathyroidism
Acknowledgement

It seems that the journey has ended, but as Baha’u’llah said in one of his masterpiece the seventh valley is the valley of true poverty and absolute nothingness, he added that these journeys have no visible ending in the world of time, but the severed wayfarer-if invisible confirmation descend upon him and the Gurdian of the Cause assist him-may cross these seven stages in seven step, nay rather in seven breaths.
The invisible ending is when one finding leads us to search for another, and that is the beauty of research work. The invisible confirmation was deeply felt, and the heart-warming fact that I indeed was not alone on this journey was a daily experience. I would like to express all my respect, gratitude and appreciation to beloved Professor Bela Szende without whom none of the my imagination would take the color of reality. His patience and his love to the research work has been exemplary. I greatly appreciate the possibilities given by Professor László Kopper the head of the department for fullfiling my dream in the 1st Department of Pathology and Experimental Cancer Research. My utmost thank to Professor András Jeney for his continous support. I would like to thank my dear husband Dr.Ajang Farid, for being extremely supportive and for loving me for who I am. My hearfelt appreciation goes to our assistant in the laboratory Dr.Paczolay Gyozone and dear Mrs.Erzsebet Kiss who has been a great help with the computer work. I also would like to thank Mr.Andrew Singer for proof reading my paper with the accuracy he did.
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