Charles University in Prague

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DIPLOMA THESIS

Expression of selected proteins of apoptotic cascade in human endometrium
Stanovení exprese vybraných proteinů apoptotické kaskády v lidském endometriu

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Abstract

Apoptosis is a process of the programmed cell death in response to severe mutations in DNA or cell stress. Apoptosis plays a key role in tissue maintenance by eliminating senescent and damaged cells. Various molecules take part in apoptosis, main participants are Bcl-2 protein family and caspases. The latter one are responsible for apoptosis execution, while Bcl-2 protein family regulates apoptotic pathway. Failure of this regulation may cause several pathologies, including development of neoplastic tissue. Human endometrium is a specific tissue, in which apoptosis is present in cycling pattern. Present study shows expression level of Bcl-2, Bax, Bad, Bid, pro-caspase-3, caspase-3 and PARP in normal, atrophic, hyperplastic and cancerous (Grade I and II) human endometrium. Bad and Bid proteins can be possible breakpoints in neoplastic transfer due to opposit expression in cancerous and hyperplastic endometrium.

Key words: endometrial apoptosis, pro-apototic proteins, anti-apoptotic proteins, caspases, PARP

Abstrakt

Apoptosa je programovaná buněčná smrt, která je vyvolána např. závažnými mutacemi v DNA nebo buněčným stresem. Hraje důležitou roli v udržování tkáňové homeostázy pomocí eliminace starých, nepotřebných nebo poškozených buněk. Účastní se jí velké množství molekul, z nichž nejdůležitější jsou zástupci proteinů Bcl-2 rodiny a výkonné proteiny apoptosy, kaspasy, zodpovědné za vlastní provedení apoptosy. Selhání regulace apoptosy může způsobit řadu malignit, včetně nádorové transformace. Lidské endometrium je specifická tkáň, ve které se apoptosa vyskytuje cyklicky. Předložená práce se zabývá studiem exprese proteinů Bcl-2, Bax, Bad. Bid, pro-kaspasy-3, kaspasy-3 a PARP v normálním, atrofickém, hyperplastickém a kancerozním (stadia I a II dle FIGO) endometriu. Výsledky této studie naznačují, že proteiny Bid a Bad mohou být jedněmi z potenciálních zlomových bodů v procesu nádorové transformace buňky, na což poukazuje i opačný vzorec jejich exprese v hyperplastickém a kancerozním endometriu.

Klíčová slova: apoptosa, lidské endometrium, pro-apoptotické proteiny, anti-apoptotické proteiny, kaspasy, PARP

Contents

Abbreviations **1**

1 Introduction **5**

1.1 History of Apoptosis 5

1.2 Overview of Apoptosis 6

1.3 Extrinsic Pathway 6

1.3.1 Fas Pathway 8

1.3.2 TNF Pathway 9

1.3.3 TRAIL Pathway 10

1.4 Intrinsic Pathway 11

1.4.1 Intrinsic pathway in Endoplasmic Reticulum 13

1.5 Caspases - a Hallmark of Apoptosis 14

1.5.1 General Features and Classification of Caspases 14

1.5.2 Caspase Targets 16

1.5.3 Regulation of Caspases 18

1.5.4 Caspase-3 20

1.6 Bcl-2 Protein Family 21

1.6.1 Bcl-2 24

1.6.2 Bax 26

1.6.3 Bid 27

1.6.4 Bad 28

1.7 PARP 30

1.8 Apoptosis in Human Endometrium 31

1.9 Apotosis in Endometrial Carcinoma and Hyperplasia 32

1.10 Targeting Apoptotic Proteins in Cancer Therapy 34

2 Main aims **37**

3 Materials **38**

4 Methods **42**

4.1 Patients . . . 42

4.2 Samples . . . 43

4.3 Preparation of Samples . . . 43

4.4 Determination of Sample Protein Concentration . . . 43

4.5 Coomassie Staining . . . 44

4.6 Electrophoresis . . . 45

4.6.1 Preparation of Gels . . . 45

4.6.2 Running Electrophoresis . . . 46

4.7 Western Blotting . . . 46

4.7.1 Blotting . . . 46

4.7.2 Detection with antibodies . . . 47

4.7.3 Chemiluminescent detection . . . 48

4.8 Statistical Analysis . . . 49

5 Results **51**

5.1 Pro-Caspase-3 and Caspase-3 Level . . . 51

5.2 Bcl-2 and Bax level . . . 54

5.3 Bid and Bad level . . . 56

5.4 PARP level . . . 59

6 Discussion **60**

7 Conclusions **65**

8 Summary **66**

Abbreviations

ADP Adenosine-5'-diphosphate

ATP Adenosine-5'-triphosphate

AE Atrophic endometrium

AIF Apoptosis inducing factor

Apaf Apoptotic protease activating factor

Bad Bcl-2-antagonist of cell death

Bak Bcl-2 homologous antagonist/killer

Bax Bcl-2–associated X protein

Bcl-2 B-cell lymphoma 2

Bcl-xL B-cell lymphoma-extra large

BH domain Bcl-2 homology domain

Bid BH3-interacting death domain

Bim Bcl-2-interacting mediator of cell death

BIR Baculovirus IAP repeat

BMI Body mass index

BSA Bovine serum albumin

CARD Caspase recruitment domains

Cdc2 Cyclin-dependent kinase 2

CLAC Cytochrome c Liberation Associated Conformation

CLIC Cytosolic Locked In Conformation

CE Cancerous endometrium

CLL Chronic lymphocytic leukemia

DED Death effector domain

DD Death domain

DISC Death inducing signaling somplex

DFF/CAD DNA fragmentation factor (DFF) / Caspase-activated DNAse

DNA Deoxyribonucleic acid

DNA-PK DNA dependent protein pinase

ECL Enhanced chemiluminescence

EDTA Ethylenediaminetetraacetic acid

ER Endoplasmic reticulum

FADD Fas-associated death domain

FLICE FADD-like interleukin-1 beta-converting enzyme

FLIP FLICE-like inhibitor protein

FSH Follicle stimulating hormone

HE Hyperplastic endometrium

HRP Horseradish peroxidase

IAP Inhibitor of apoptosis protein

IHC Immunohistochemistry

JAK/STAT Janus kinase/Signal transducer and activator of transcription

JNK c-Jun N-terminal kinase

LH Luteinizing hormone

Mcl-1 Myeloid leukemia cell differentiation protein

MLH MutL homolog

MOMP Mitochondrial outer membrane permeabilization

MSI Microsatellite instability

NAD Nicotinamide adenine dinucleotide

NE Normal endometrium

NLS Nuclear localization signal

NK Natural killer cells

PAK p^{21} activated kinase

PARP Poly-(ADP-ribose) polymerase

PBS Phosphate buffered saline

PCD Programmed Cell Death

PIDD p53-induced protein with a death domain

PKA Protein kinase A

PP1 Protein phosphatase 1

PTEN Phosphatase and tensin homolog

p90RSK p^{90} ribosomal S6 kinase

RAIDD RIP associated Ich-1/CED homologous protein with death domain

RIP Receptor-interacting protein

ROCK Rho-associated kinase

SDS Sodium dodecyl sulfate

SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis

S.E.M. Standard error of the mean

Smac/DIABLO Second Mitochondria-derived Activator of Caspases/Direct IAP Binding Protein with Low PI)

SODD Silencer of death domain

TNFR Tumor necrosis factor receptor

TRADD TNFR-associated death domain protein

TRAF TNFR-associated factors

TRAIL TNF-related apoptosis inducing ligand

XIAP X-linked Inhibitor of Apoptosis Protein

1 Introduction

1.1 History of Apoptosis

Greek word "apoptosis" means leafs falling or petals dropping off from the flower. First person, who used this word to describe tissue death was Hippocrates of Cos. [7] However, first report of the cell death attributes to Karl Vogt, who referred in 1842 about cell death in notochord and adjacent cartilage of metamorphic toads. [198] About 20 years later Rudolph Virchow described two types of the cell death, necrosis and necrobiosis. Virchow named necrobiosis a process, which is called apoptosis nowadays. [197] After that, in the second half of the 18th century Flemming came with a suggestion, that cell death is not only a result of mechanical forses, it is also evoked by some chemical changes. Moreover, he first used the word "chromatolysis" to describe chromatin disintegration in dying cells. [63]

In 20^{th} century the main work in the research of apoptosis was done by John Kerr. He observed formation of the apoptotic bodies in rat liver after obstruction of its portal blood supply. He suggested to use term "apoptosis" to describe programmed cell death . [89] Earlier, in 1965, Lokshin and Williams proposed term "Programmed Cell Death" (PCD) to describe fact that some cells are destined to die. After that, Williams had noticed, that apoptotic cells have similar pattern of the DNA fragments - "DNA ladder". [207] That was first marker to identify apoptotic cells, second marker was a biochemical feature - exposition of phosphatidylserine at the surface of apoptotic cells. [65], [60] In 1997, after huge progress in molecular methods, DFF/CAD (DNA Fragmentation Factor/caspase-activated DNase) was identified as a key endonucleas in the apoptotic DNA fragmentation. [110]

In order to detect genes responsible for PCD, *Caenorabdis elegans* was used as an animal model. It has basic apoptotic regulation, which is also found in mammals, but in more complex form. Horvitz, working with C.elegans, identified first cell death gene - ced-3. [79] In 1993 Yuan found, that ced-3 is cysteine protease and has similar properties to the mammalian interleukin-1-beta converting enzyme (now known as caspase-1). [219] Nowadays a lot of genes were identified to play role in cell death. In 1988, Vaux identified gene controlling cell death - *Bcl-2*. [194] This gene was initially found participating in B-cell lymphoma, where it was inhibiting

cell death. Thus, *bcl-2* was characterized as anti-apoptotic gene. Since its discovery, many genes were identified as *bcl-2* homologies, such as *bax*, *bcl-x*, *bcl-xl*, *bcl-xs*, *bak*, *bid*, *bad*, etc. After *bcl-2*, *p53* gene was identified as apoptotic gene, that could induce apoptosis in myeloid leukemic cells. Third identified gene was *c-myc*. Gerard Evan showed, that *c-myc* promotes cell death in the culture of rat fibroblasts. [59] Nowadays apoptosis is considered as complex process using the big amount of balancing anti- and pro-apoptotic proteins.

1.2 Overview of Apoptosis

Apoptosis is a programmed cell death, which plays the key role in tissue development and maintenance. This process enables elimination excess or defective cells. Apoptosis can be characterized by some typical changes: membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation. In contrast to the necrotic cells, apoptotic cells do not cause tissue inflammation, because they are detected and consequently engulfed by phagocytes. [167] Apoptosis is strictly controlled by the network of proteins, including balancing pro-survival signals. Failure of this regulation may lead to pathological disorders, including cancer and developmental diseases.

There are two main apoptotic pathways in mammalian cells:

- Extrinsic pathway, which is activated by interaction of ligand with cell surface death receptor.
- Intrinsic pathway, which is initiated by the release of apoptogenic factors such as cytochrome c or Smac/DIABLO (second mitochondria-derived activator of caspase) from mitochondria to cytosol. [87]

1.3 Extrinsic Pathway

Extrinsic pathway, or also called receptor pathway, is mediated by death receptors, which belongs to TNFR (tumor necrosis factor receptor) superfamily. Members of this family such as Fas(or CD95), TNFR-1, TRAIL (TNF-realted apoptosis-inducing ligand/Apo-2L), receptors DR4 and DR5 activates apoptotic pathway. Another

Table 1: Comparison of apoptosis and necrosis

Apoptosis	**Necrosis**
Morphological features	
membrane blebbing	loss of membrane integrity
shrinking of cytoplasm	swelling of cytoplasm
pore formation in mitochondria involving Bcl-2 proteins	disintegration of organelles
formation of apoptotic bodies	no vesicle formation
condensation of nucleus, aggregation of chromatin	nuclei lost
fragmentation of the cell into small bodies	total cell lysis
Biochemical features	
energy (ATP)- dependent active process	no energy required, passive process
non-random DNA prelytic fragmentation	random DNA postlytic fragmentation
activation of caspase cascade	no special protein activation
Physiological impact	
destroying individual cells	destroying group of cells
no inflammatory response	pathological process

members of TNFR superfamily, decoy receptors DcR1, DcR2 and DcR3 are not able to promote apoptosis. Decoy receptors protects cell from apoptosis by competing with death receptors. Members of TNFR superfamily, which activate apoptosis, have cysteine-rich extracellular domains to bind appropriate ligand. They contain 80 amino acids cytoplasmic domain which is called Death Domain (DD). Activation of death receptors results in their oligomerization and subsequent formation of DISC (Death Inducing Signaling Complex). DISC contains death domain of death receptors, adaptor proteins and pro-caspase dimers. This complex drives to activation of caspase-8 and caspase-10 via self-cleavage. Once activated, caspases cleave and thus activate downstream effector caspases, making apoptotic pathway irreversible. [87]

There are three major extrinsic apoptotic pathways:

- Fas pathway
- TNF pathway
- TRAIL pathway

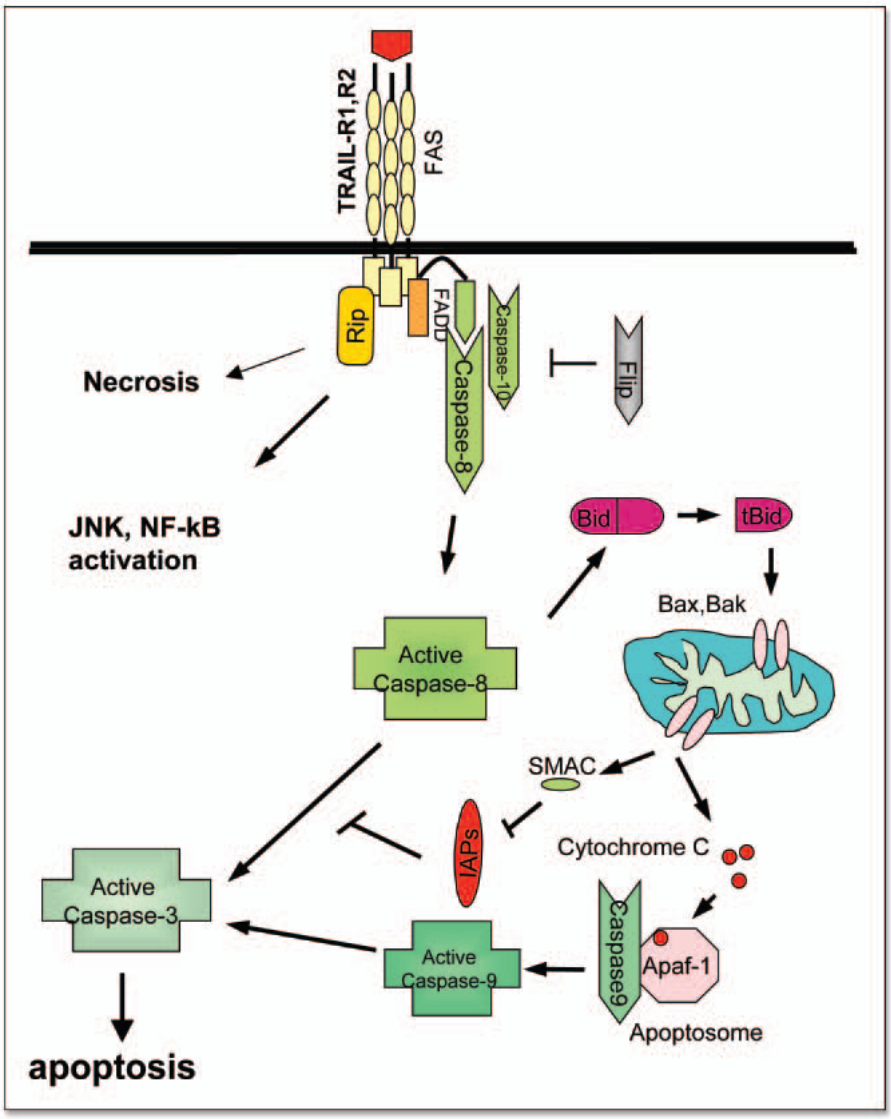

Figure 1: Fas and TRAIL signaling pathway (extrinsic pathway). See text for details.

From *Jin and El-Deiry, 2005*

1.3.1 Fas Pathway

Fas pathway plays central role in physiological regulation of apoptosis. [125] Fas pathway is involved in cytotoxic killing of the cells mediated by T-cells, destroying of immune cells in immune-privileged sites. The pathway is mediated by FasR (Fas Receptor). FasR is type I transmembrane receptor with molecular mass of 48 kDa. Interaction of FasL (Fas Ligand) with FasR results in receptor trimerization. This allows adaptor protein FADD (Fas-associated death domain) to interact with the cluster of receptors. FADD associates through its death domain to FasR

and by DED (death effector domain) with pro-caspase-8 or pro-caspase-10. As it was written before, DISC promotes autoproteolytic cleavage of pro-caspase by its dimerization. caspase becomes active and therefore activates downstream members of caspase cascade. According to sufficiency of caspase-8, cells can be divided into two types. In type I cells, cleaved caspase-8 is able to activate other members of caspase family and execute apoptosis. In the type II cells, activity of caspase is insufficient and mitochondrial amplification loop is required for full activation of effector caspases. Amplification loop is made by direct Bid (BH3-interacting-domain death antagonist) cleavage by caspase-8 and consecutive release of cytochrome c and Smac/DIABLO to cytosol and later formation of so-called apoptosome. This activates caspase-9, which cleaves pro-caspase-3. caspase-3 in turn activates caspase-8 and thereby completes a positive feedback loop. [96]

1.3.2 TNF Pathway

TNF is pro-inflammatory cytokine, which is mainly produced by macrophages. TNF can evoke a wide range of biological responses, including cell survival, cytoskeleton changes, inflammatory response and apoptosis. There are two major TNF receptors: TNF-R1and TNF-R2. Both of them are type I receptors. After association with ligand and oligomerization, TNFRs forms complex, which consists of adaptor protein TRADD (TNF-receptor-associated death-domain protein), kinase RIP (receptor-interacting protein) and protein TRAF2 (TNF receptor-associated factor 2). TNF-R1 complexes are preassembled and silenced by SODD (silencer of death domain). Once TNF binds to receptor, SODD is released and complex is activated. TRADD serves as a platform for binding TRAF2 and RIP. TRAF2 C-terminal domain interacts with N-terminal death domain of TRADD. RIP also interacts with TRADD via DD domain. RIP can also associate with TRAF2 via its N-terminal kinase domain or central domain. [117]

TNF-R1 activates neurotrophic factor NF-κB by ubiquitin-mediated degradation of its inhibitor I κB. This process promotes strong pro-survival signaling. NF-κB pathway activates anti-apoptotic proteins, such as cFLIP, XIAP, Bcl-xL and others. Moreover, if NF-κB activation is blocked, TNF elicit cytotoxic effect. [117]

TNFR1-induced apoptosis is based on two signaling complexes. First complex

involves TNF-R1, TRADD, RIP, TRAF2 and NF-κB. In a second complex, TRADD and RIP interacts with FADD and caspase-8. If the first complex activates NF-κB, caspase-8 becomes inhibited by FLIP and cell survives. In opposite case, cell undergoes apoptosis. Thus, TNF-R1 signalling has a checkpoint, resulting in cell death (if NF-κB activation is blocked) or cell survival (active NF-κ blocks caspase-8 cleavage). [117]

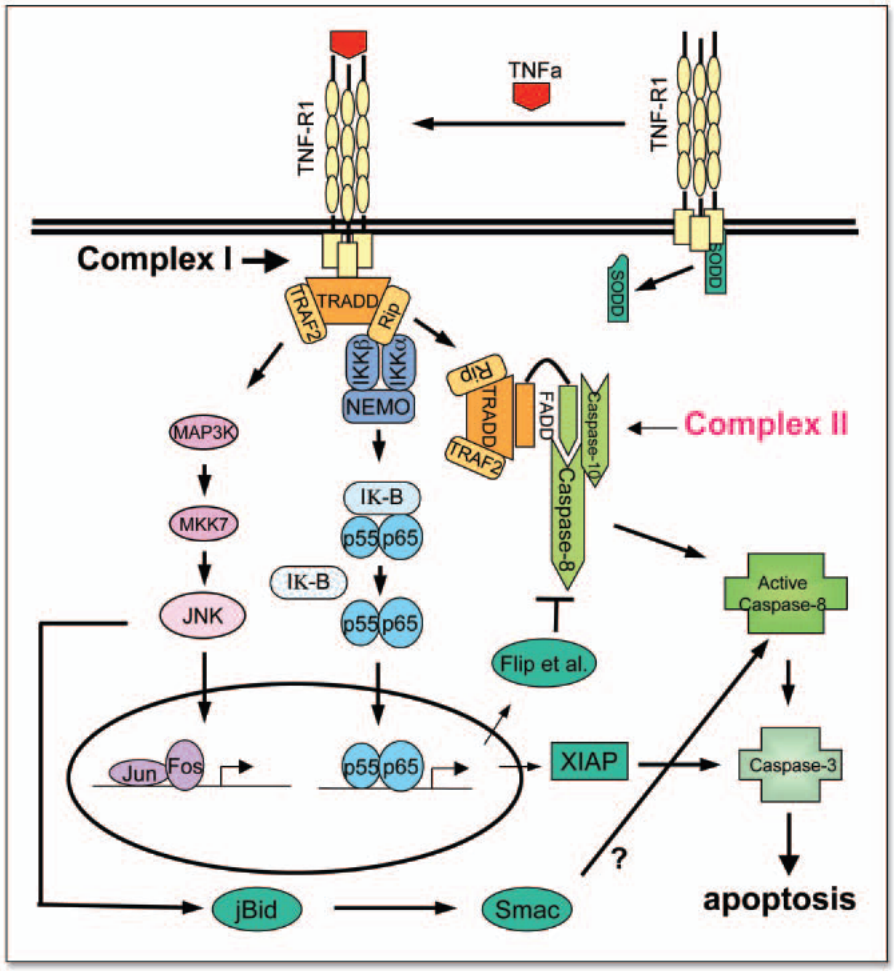

Figure 2: TNF signaling pathway (extrinsic pathway). See text for details.

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1.3.3 TRAIL Pathway

TRAIL pathway is not fully examined yet. As Fas pathway, TRAIL pathway may also play role in immune response, such as killing by cytotoxic T-cells and tumor cells killing by NK (natural killer) cells. TRAIL is type II transmembrane recep-

tor. In contrast to other TNF family members, TRAIL mRNA is constitutively expressed in almost all tissue types. Signaling through TRAIL receptors is similar to that through FAS receptors. TRAIL homotrimers bind three molecules of that ligand. After DISC complex is formed together with FADD, caspase-8 or caspase-10. TRAIL induced apoptosis also include mitochondrial amplification loop as in Fas induced apoptosis in type II cells. [211]

1.4 Intrinsic Pathway

Intrinsic pathway is also called mitochondrial, because these organelles play one of the key roles in intrinsic pathway. Mitochondrial pathway can be initiated by a various range of factors, including irradiation, oxidative stress and treatment with cytotoxic drugs. This process is controlled by the family of Bcl-2 proteins, which contains both pro- and anti-apoptotic members. Hallmark of intrinsic pathway is mitochondrial outer membrane permeabilization (MOMP) and subsequent release of cytochrome c from the mitochondrial intermembrane space to cytosol. [69]

The mechanism of MOMP still remains controversial. Nowadays there are two main possible mechanisms of MOMP. First mechanism consists of permeability transition pore formation in outer mitochondrial membrane. Water and molecules up to 1.5 kDa pass through the inner mitochondrial membrane. This results in loss of mitochondrial membrane potential and swelling of the mitochondria. In this mechanism participates adenine nucleotide transporter and voltage dependent anion channel. In the second mechanism main roles in regulation of MOMP plays members of Bcl-2 family proteins. Anti-apoptotic membres, such as Bcl-2 and Bcl-xL blocks MOMP. Proapoptotic Bcl-2 family members have two general subfamilies, which differs in amount of BH (Bcl-2 homology) domains. BH123 (also called multidomain) proteins, share three homology domains with Bcl-2, whether BH3-only proteins, as the name implies, share only one BH3 domain. BH123 proteins, Bax (Bcl-2 associated X protein) and Bak (Bcl-2 antagonist/killer-1) permeabilize outer mitochondrial membrane. BH3-only proteins, such as Bid can either directly activate Bax and Bak or interact with anti-apoptotic members of Bcl-2. [69]

MOMP results in release of intramembrane proteins from mitochondria, like cytochrome c, Smac/DIABLO, EndoG, etc. Cytochrome c directly forms apoptosome,

together with Apaf-1 and caspase-9 in presence of ATP/dATP. Formation of apoptosome results in activation of initiator caspase-9 and subsequent activation of effector caspase-3, -6 and -7. [226], [107]

Proteins Smac/DIABLO, HtrA2/Omi are so-called IAP (inhibitor of apoptosis protein) antagonists. In cytosol Smac/DIABLO inactivates IAPs by interacting of its first four amino acids at the N-terminal part with BIR3 (Baculovirus IAP Repeat) domain of IAPs. [212] BIR domain blocks activity of caspases. [217] Therefore Smac/DIABLO activates caspases by inhibition of IAPs. [57] HtrA2/Omi is a mitochondrial serine protease. This protein is more efficient suppressor of IAPs than Smac/DIABLO, because it irreversibly inactivates IAPs by cleavage. [216]

EndoG is endonuclease, which does not need caspases for its activation. After release from mitochondria to cytosol, EndoG translocates to nucleus and cleaves DNA. [106]

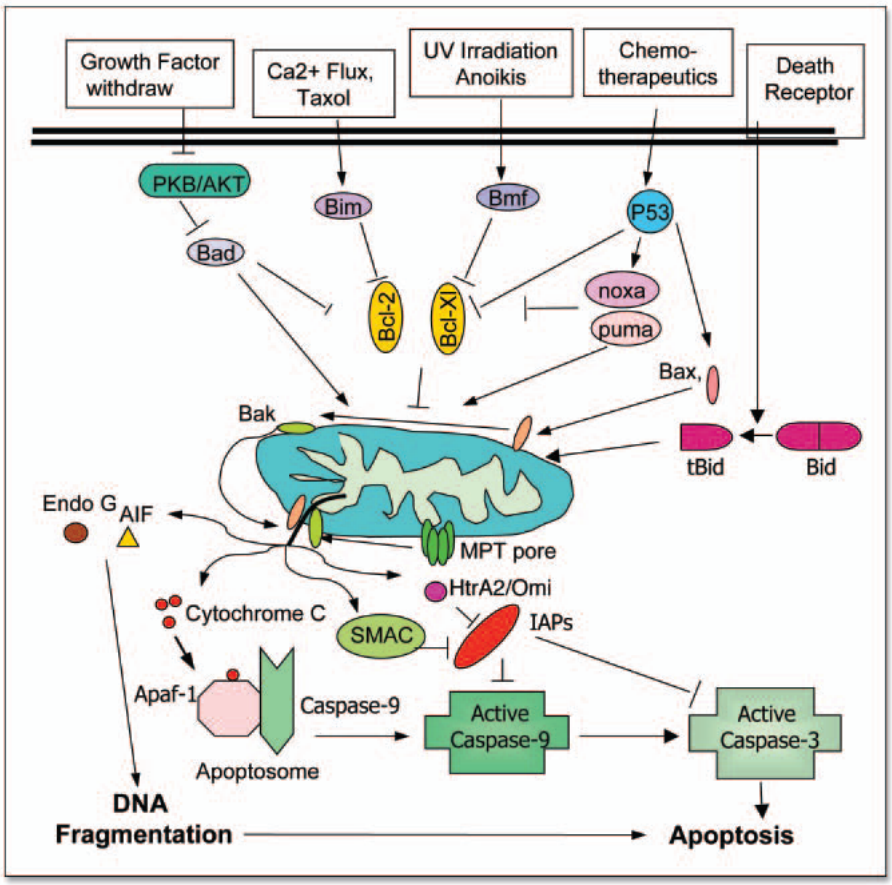

Figure 3: Intrinsic pathway mediated by mitochondria. See text for details.

From *Jin and El-Deiry, 2005*

1.4.1 Intrinsic pathway in Endoplasmic Reticulum

ER (Endoplasmic reticulum) is participating in protein maturation and secretory pathway. ER provides protein quality control to ensure their proper folding. Accumulation of unfolded proteins due to treatment with the inhibitors of glycosylation or Ca^{2+} ionophores, oxidative stress can initiate UPR (Unfolded Protein Response). UPR reduces protein synthesis, inducts chaperones, catalyzes folding and eliminates aggregates, thus enabling cell to resolve stress. However, if the damage is too strong, UPR starts apoptosis. [152]

ER has a high content of calcium, because it serves as a cofactor to proteins forming chaperons. Calcium level is regulated by two main types of channels: the inositol 1,4,5-triphosphate (IP_3R) receptor and the ryanodine receptor. It has been observed, that release of calcium from ER to cytosol is mediated by IP_3R receptor. Mechanism of its activation still remains unclear. In cytosol, calcium activates Bad, which translocates to mitochondria and facilitates cytochrome c release. Calcium also activates calpains, which are calcium-dependent cysteine proteases. Calpains can activate caspases. In addition, calpain cleaves and so activates Bax, which permeabilize mitochondrial membrane. [17]

A specific caspase is present in the ER. Caspase-12 is activated by the same stimuli as ER stress is initiated. After released to cytosol, caspase-12 directly binds and activates caspase-9 in cytochrome c independent way. [122] Caspase-12 deficient mice fail to initiate ER stress induced apoptosis. However, all experiments were performed on mice and rats, existence of human homologue of caspase-12 is still not confirmed. [128]

Bcl-2 protein has a wide range of activity, including anti-apoptotic function in the ER. For example, complex of Bcl-2 and cytochrome b5 prevents cell from ER-mediated apoptosis, induced by ceramide, Myc, ionizing radiation, or Bax overexpression. Another members of Bcl-2 family also participate in ER stress-induced apoptosis. For this type of apoptosis both Bak and Bax are required. These proteins facilitate calcium release from ER to cytosol and increase mitochondrial calcium concentration. Bcl-xL inhibits this mobilization of calcium. [17]

Prion protein diseases and neurodegenerative disorders like Alzheimer's disease, Huntington's disease, Parkinson's disease, Amyotrophic lateral sclerosis are result of missfolded protein accumulation. This implies that ER stress plays important role

in cell pathogenesis and needs further research. [70]

1.5 Caspases - a Hallmark of Apoptosis

1.5.1 General Features and Classification of Caspases

Caspases are one of the center molecules in the apoptotic process. Their activation is essential for the apoptosis. Caspases are cysteine aspartate proteases. They cleave their substrates at Asp-Xxx sites. Another common feature is presence of the catalytic triad residues, containing the active site Cys285, His237 and a carbonyl group at residue 177. Caspases are almost in all healthy cells in form of inactive zymogens (pro-caspases). Domain of pro-caspases contains large (p20) and small subunits (p10). [177] It is known, that both subunits are essential for activation of pro-caspase -1, -3. Prodomain have N-terminal peptide with variable length. It is considered, that caspases with short N-terminal repeat do not have ability of self-activation, hence are downstream. In contrast to that caspases, caspases with a long N-terminal peptide can self-activate and contain protein-protein interaction sites, which are critical for caspase activation. One of this site is called CARD (caspase recruitment domain) and serves to mediate interaction between pro-caspases and adaptor proteins, which results in caspases self-activation. Another domain is DED, which enables inactive pro-caspases to form complexes with the members of TNF superfamily molecules. Caspases DED domain interacts with the second DED in the adaptor molecules. [50]

Caspases can be divided in three groups according to their function: 1) Inflammatory caspases. Members of this group are caspase -1, -4, -5, -11, -12, -13, and -14. These caspases can be self-activating, due to presence of CARD domain. Active caspases paticipate in inflammation instead of apoptosis. 2) Apoptotic initiator caspases. Initiator caspases have on their N-terminal peptide either CARD (caspase-2, -9) or death effector domain (caspase-8, -10), therefore can also self-activate. 3) Effector (executioner) caspases. This group includes caspase-3, -6, -7. Their main features are short N-terminal peptide and insufficiency of self-activation. [50]

Almost all caspases are activated by proteolytic cleavage at two sites in the zymogens. However, mechanisms of initiator and effector caspases activation are

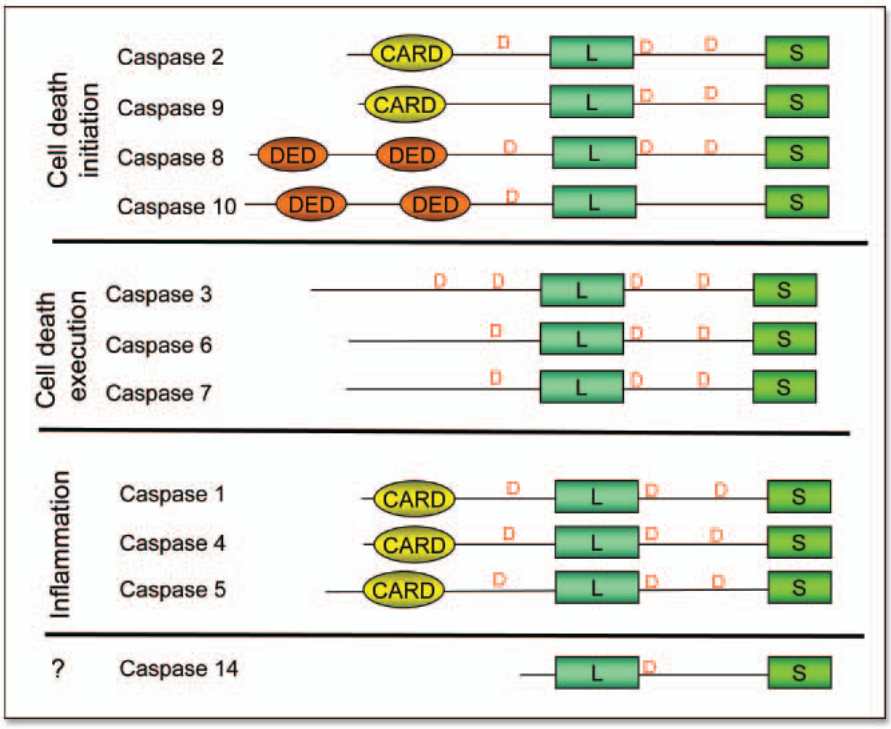

Figure 4: Classification of mammalian apoptotic caspases.

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different. It is concidered, that initiator caspases are self-activating. The model of their self-activation is called proximity induced dimerization model. Caspases are activated by cooperation with adaptor complexes for activation, for example PIDDsome complex for caspase-2, DISC for caspase-8 and apoptosome for caspase-9. PIDDsome is a large (670 kDa) complex needed for caspase-2 activation that contains PIDD (p53-induced protein with a death domain), adaptor protein RAIDD (RIP associated Ich-1/CED homologous protein with death domain) and, of course, pro-caspase-2. [153], [186] Caspase-2 CARD domain interacts with RAIDDs CARD domain and RAIDDs death domain interacts with PIDD DD. [37], [141] As written before, pro-caspase-8 forms DISC complex together with three main components: specific death ligand - FasL, death receptors from the TNF family - FasR and adaptor protein FADD (Fas-associated death domain). [145] FADD interaction with pro-caspase-8 is realized via DED domain and with FasR via its death domain. Thus, FADD binds to Fas and to pro-caspases-8, which in high local concentration induces self-activation. [164]

Term "apoptosome" is used to describe about 1.4 MDa multimeric protein com-

plex containing caspase-9, Apaf-1 (Apoptotic peptidase activating factor 1), cytochrom c and dATP/ATP. Apaf-1 is a key molecule in apoptosome, because it has CARD domain to interact with caspase-9, WD40 domain to bind cytochrome c and nucleotide binding domain, which is responsible for oligomerizaton of Apaf-1 in the presence of dATP and cytochrome c. In vitro studies shows, that presence of Apaf-1, cytochrome c and dATP result in formation of apoptosome. [163] Effector, or downstream caspases are activated by initial caspases, such as caspase-9 or -8, making "caspase cascade". [172], [189]

1.5.2 Caspase Targets

Caspases have many targets, which can be divided into several groups: 1) Proteins involved in regulation of apoptosis. 2) Mediators of apoptosis. 3) Structural proteins. 4) Proteins required for DNA repair. 5) Cell cycle proteins.

One of the most well known substrates of initiator caspases are downstream effector caspases. Another target is BH3-only protein Bid, which is cleaved by caspase-8. [105] Cleaved Bid then translocates to mitochondria to promote pore formation to release cytochrome c. [112], [94] Another caspase target is DNA fragmentation factor, which contains two subunits: caspase-3 activated 40 kDa nuclease (DFF40/CAD) and its inhibitor - DFF45/ICAD. Caspase-3 cleaves DFF45/ICAD, thus resulting in nuclease activation. [84] One of key caspase substrate in mitochodria is 75 kDa subunit (NDUFS1/NUAM) of respiratory complex I. Cleavage of this protein results in production of reactive oxygen species, loss of ATP production and mitochondrial damage. [156]

Caspases also cleave some anti-apoptotic kinases. First example can be AKT kinase, which promotes cell survival. AKT phosphorylates pro-apoptotic protein Bad that dissociates from Bcl-2/Bcl xL complex an loses its pro-apoptotic function. [144], [215] AKT also can activate NF-κB, which also promotes cell survival by activating anti-apoptotic signal pathways. [61] AKT cleavage by caspase-3 involves detachment of the epithelial cell from the matrix. [10] RIP is another kinase, that can activate NF-κB and is likewise substrate for caspases. RIP is cleaved by caspase-8 and consequently C-terminal part of the cleaved RIP inhibits NF-κB activation. [116] Next anti-apoptotic kinase is FAK (focal adhesion kinase), that participates in

Table 2: Basic Characteristics of caspases

caspase	**EC**	**Molecular weight, kDa**	**Function**	**Examples of substrates**
caspase-1	3.4.22.36	45	Inflammatory mediator	Pro-IL-1β, IL-18, IL-1F7b, PARP1, lamins, pyrin proteins, pro-caspases-1, -3, -4
caspase-2	3.4.22.55	49	Apoptotic activator	Golgin-160, Bid
caspase-3	3.4.22.56	32	Apoptotic executioner	PARP, SREBs, gelsolin, procaspases-6, -7, -9, DNA-PK, β-catenin, lamins, Topoisomerase I, ICAD, Rb
caspase-4	3.4.22.57	43	Inflammatory mediator	Similar to caspase-1
caspase-5	3.4.22.58	48	Inflammatory mediator	
caspase-6	3.4.22.59	34	Apoptotic executioner	Lamin A, PARP, pro-caspase-3, Keratin-18,
caspase-7	3.4.22.60	34	Apoptotic executioner	PARP, pro-caspase-6, steroid response element binding protein, FAK, calpastatin
caspase-8	3.4.22.61	55	Apoptotic activator	pro-caspases-3, -4, -6, -7, -9, -10, -13, PARP, Bid, c-FLIP, PAK-2, α-tubulin, vinculin, hnRNP-H, hnRNP-C1/C2
caspase-9	3.4.22.62	50	Apoptotic activator	pro-caspases-3, -7, -9, PARP
caspase-10	3.4.22.63	55	Apoptotic activator	pro-caspases-3, -4, -6, -7, -8, -9
caspase-11	3.4.22.64	45	Inflammatory mediator	
caspase-12		50	Inflammatory mediator	
caspase-13		43	Inflammatory mediator	
caspase-14		30	Inflammatory mediator	Profilaggrin, keratin intermediate filaments

process leading cell to bound to extracellular matrix. FAK cleavage occurs during apoptosis and leads to loss of pro-survival signals and matrix detachment. [206]

Pro-apoptotic kinases are also substrates for caspases. ROCK I (Rho-associated kinase) is activated by caspase-mediated cleavage of C-terminal region. [40] This kinase plays role in phosphorylation of myosin light-chains and coupling actin-myosin filaments to plasma membrane. This results in increased contractility and mem-

brane blebbing. [118] PAK (p21-Activated Kinase) is cleaved by caspase-3. [159] After cleavage, C-terminal part of PAK exhibits increased activity, which is needed for activation of JNK (c-Jun N-terminal kinase) pathway by Fas receptor. [160]

Cleavage of structural proteins is very important in apoptosis, because it leads to cell shrinkage, loss of cell shape and matrix detachment. For example, gelsolin is cleaved by caspase-3. It makes cell round, detached from matrix and promote nuclear fragmentation. [95] Substrates for caspases are also adherent junction proteins: β-catenin and γ-catenin. [169] Their cleavage leads to disruption of cell-cell interactions. [16] Cleavage of lamin results to one of the key apoptosis features: nuclear budding. After lamin breakdown follows nuclear collapse: shrinkage and fragmentation of nucleus. [151]

Process of DNA repair can compete for energy molecules, such as ATP, with the apoptotic process. Abortion of DNA repair system can accelerate apoptosis. First identified protein, that is cleaved during apoptosis, was PARP (Poly-(ADP-ribose) polymerase). PARP catalyzes ADP-ribosylation of nuclear proteins in order to accelerate DNA repair. PARP is cleaved by caspase-3 in order to store energy, that consumes PARP during DNA repair. [224] DNA-PK (DNA-dependent protein kinase) is enzyme, that repairs double-stranded breaks. It is also cleaved by caspases during apoptosis. [103], [28] However, DNA-PK in cooperation with p53 can evoke cell death in response to DNA damage. [210] Another cell repair protein ATM (ataxia telangiectasia mutated) is participating in DNA recombination repair. This protein is the key substrate of caspases-3 and -7. [80], [187]

Cell cycle proteins also affect apoptotic process. For instance, loss of Rb protein (retinoblastoma protein) leads to accumulation of pro-caspases in cell. [126] Cdc2 (Cyclin-dependent kinase 2) phosphorylates Bad and therefore activates its pro-apoptotic functions. [92]

1.5.3 Regulation of Caspases

Main regulation of caspase activity is proteolytic cleavage. However, this process is irreversible and that is why caspase activity is strictly regulated by a wide spectrum of proteins. These proteins include some viral protein, like IAPs (mammalian IAPs-like proteins) and another proteins exploited by virus to escape from host de-

fense through inhibiting apoptosis, such as p35 and v-FLIP(mammalian homologue cFLIP). These proteins inhibit caspases and therefore protect cell from apoptosis. Granzyme B, calpain and Smac/DIABLO activates pro-caspases. AKT kinase inhibits caspase-9.

IAP was identified firstly as a baculovirus protein, which was protecting cells from death and enhancing virus replication. Then were discovered some eukaryotic IAPs, such as c-IAP1, c-IAP2 and XIAP. IAPs contains BIR domain, that blocks catalyzing grooves of caspase-3, -7 and -9. IAPs also have RING domain, which transfer ubiquitin from E2 to substrate. This facilitate caspase degradation. [217] P35 is anti-apoptotic protein from baculovirus, which inhibits human caspase-1, -3, -6, -7, -8, and -10 by direct binding into active site of caspases. [225] v-FLIPs (viral FLICE-Inhibitory Proteins) are encoded by γ herpesvirus. There also exist cellular FLIPs in short and long (have caspase-like domain) forms. All FLIPs contain two DED domain, one of which binds to FADD, the second one interacts with DED caspase-8 domain, therefore inhibiting caspase-8 activation. [185]

Granzyme B is located in lytic granules in cytotoxic T-lymphocytes. It is serine protease, which cleaves and thus activates caspases-3, -7, -8 and 10. Granzyme B also cleaves Bid, creating its active truncated form. [3] Calpains are also cystein proteases, which are activated by calcium and do not cleave protein in specific sequence of amino acids. Activated cytosolic calpain moves to the ER membrane and activates caspase-12 by its cleavage. Calpain cleaves Bcl-xL as well, abolishing its anti-apoptotic activity. [127] Smac is small mitochondrial protein, which migrates to cytosol together with cytochrome-c and activates caspases. Smac interacts with IAPs and therefore block their inhibitory activity. Overexpression of Smac make cells more sensitive to apoptotic stimuli. [57] AKT kinase, as written before, participates in pathways, which suppress apoptosis. It phosphorylates caspase-9 at RxRxxS/T site. In turn, caspase can not form a tetramer and therefore be activated. [25]

Now I would like to focus on caspase-3, because its expression was examined in my diploma Thesis.

1.5.4 Caspase-3

As it was written before, caspase-3 (EC 3.4.22.56) is a key downstream caspase in both extrinsic and intrinsic pathways. CPP32 (32kDa putative cystein protease) or caspase-3 was first described by Fernandes-Alnemri et al. in 1994. [62] This enzyme is a member of CED-3 caspase subfamily and its molecular mass is 32 kDa. [62] Activated caspase-3 contains two subunits with a molecular mass of 17 kDa and 12 kDa. [119] Caspase-3 cleaves majority of cellular substrates, such as ICAD/DFF45, gelsolin, PARP, PAK and others. All caspase-3 substrates share a common motive: Asp-Xaa-Xaa-Asp (DXXD). [102] Morphological changes in apoptotic cells, like DNA fragmentation, membrane blebbing, chromatin condensation and finally formation of apoptotic bodies are also results of caspase-3 action. [209], [86] Caspase-3 deficient mice born in less amount than expected, are smaller and die at 1-3 weeks of life. These mice have failure of PCD and a variety of hyperplaisias in brain, but normal apoptosis in other tissues. Possible explanation could be that caspase-3 plays critical role in neural apoptosis program. [97] Therefrom can be made a conclusion, that caspase-3 is essential for normal development of mammalian brain.

Failure of apoptosis can lead to variety of pathologies, including cancer. Soung et al. found caspase-3 gene mutations in various human tumors, like stomach carcinoma, colon carcinoma, acute leukemia, Wilm's tumor, non-Hodgkin lymphoma, etc. These data indicates that mutation of caspase-3 together with mutations of other caspases are features of human tumors. [175] In another research, prostate tissue was studied. It turned out that prostatic intraepithelial neoplasia and cancer also has decreased level of caspase-3 expression. [6] Despite that caspase-3 has a complex role in cell and in some tissues is not essential for PCD. It can be regulation point for cancer therapy. For example, MCF7 breast cancer cell line completely lack caspase-3 expression. These cells were also resistant to chemotherapy. However, after transfection with a plasmid containing caspase-3 cDNA, the cells were more sensitive to drug therapy. [53] So that there is a hope, that further research will make possible to treat cancer via regulating caspase-3 activity.

1.6 Bcl-2 Protein Family

To date, over 25 Bcl-2 proteins have been identified. Bcl-2 protein (B cell lymphoma-2) was discovered in B-cell follicular lymphoma at the t(14,18)(q32;q21) translocation, where expression of Bcl-2 protein was enhanced by promotor of immunoglobuline heavy chain. [188], [11] Bcl-2 was proposed to be an oncogene, although subsequently turned out that Bcl-2 provides pro-survival signal, not proliferative, as it is in c-myc signalization [194] Viral Bcl-like proteins mimic pro-survival Bcl-2 proteins, therefore inhibit apoptosis to break host defense. [98]

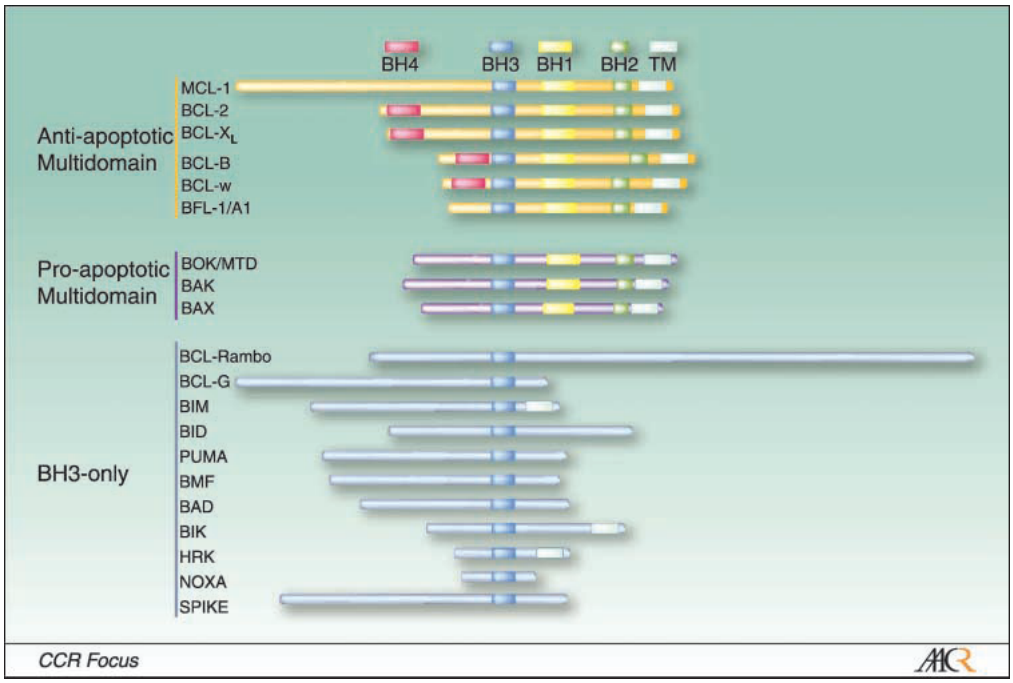


Figure 5: Classification of Bcl-2 family according to conserved domains.

From *Danial, 2007*

All proteins from Bcl-2 protein family share at least one Bcl-2 homology (BH) region. These proteins can be divided into three groups. First group contains anti-apoptotic proteins Bcl-xL and Bcl-2, which have all four (BH1-BH4) domains. To the second group belong pro-apoptotic proteins Bak and Bax with three BH domains (BH1, BH2, BH3). Members of the third group are also pro-apoptotic, but possesses only one BH3 domain, therefore they are called BH3-only proteins. To this group belong proteins, such as Bad, Bid, Bim, Puma or Noxa.

Anti-apoptotic Bcl-2 multidomain proteins contain a hydrophobic cleft, the BH3

binding groove which is formed by BH1-3 domains. It was proved, that BH4 domain makes proteins Bcl-2 and Bcl-xL anti-apoptotic, whereas caspase-mediated cleavage of this domain results in loss of pro-survival function. [34], [39] BH3 domain has four amphipathic α helixes, which possess the sequence motif: Hy-X-X-X-Hy-X-X-X-Sm-D/E-X-Hy, where Hy is hydrophobic residue and Sm is small residue, typically glycine. Because key residues of the BH3 domain and BH3 binding groove are not so conversed, there is high specifity of interactions, depending on affinity. For example, whilst proteins Bim, Puma and tBid interact with all pro-survival proteins (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1), Bad binds selectively to Bcl-2, Bcl-xL and Bcl-w. [32]

Several members of Bcl-2 family have also transmembrane domains. For example, Bak is already inserted in outer mitochondrial membrane, even in inactive form. In contrast to Bak, Bax translocates to outer mitochondrial membrane after conformational changes at both N-terminal (transmembrane domain) and C-terminal (ART domain) parts. [67] Pro-apoptotic BH3-only protein Bid is myristoylated after cleavage by caspase-8 because of exposure of glycine at its N-terminal part. [222]

Hallmark of the intrinsic pathway is cytochrome c release together with release of Smac and engoG. MOMP could mot be performed without proteins Bax and Bak. It was proven by cloning of cells lacking Bax and Bak, which failed to undergo MOMP induced by UV irradiation, growth factor deprivation, ER stress and DNA damage. [205] There are two models of Bax and Bak activation: indirect and direct. According to indirect model, Bak and Bax are activated without direct interactions with activator BH3-only proteins (Bid and Bim). Sensitizer BH3-only proteins (Bad, Noxa, Puma, Bik) compete with pro-apoptotic Bax and Bak proteins for binding to anti-apoptotic proteins Bcl-2 and Bcl-xL. Main function of Bcl-2 and Bcl-xL in this model is to bind and inhibit Bax and Bak proteins. In direct model activation of Bax and Bak requires direct interaction between these proteins and activator BH3-only proteins. Interaction leads to conformational changes and subsequent activation of Bax and Bak. Direct interaction of Bak and Bax with activator BH3-only proteins is also provided by sensitizer BH3-only proteins, which inhibit anti-apoptotic activity of Bcl-2 and Bcl-xL. [45]

Activity of Bcl-2 proteins can be regulated by various mechanisms, such as transcriptional control or post-transcriptional protein modification. For example, BH3-only proteins Puma and Noxa are activated by p53 in responce to DNA damage.

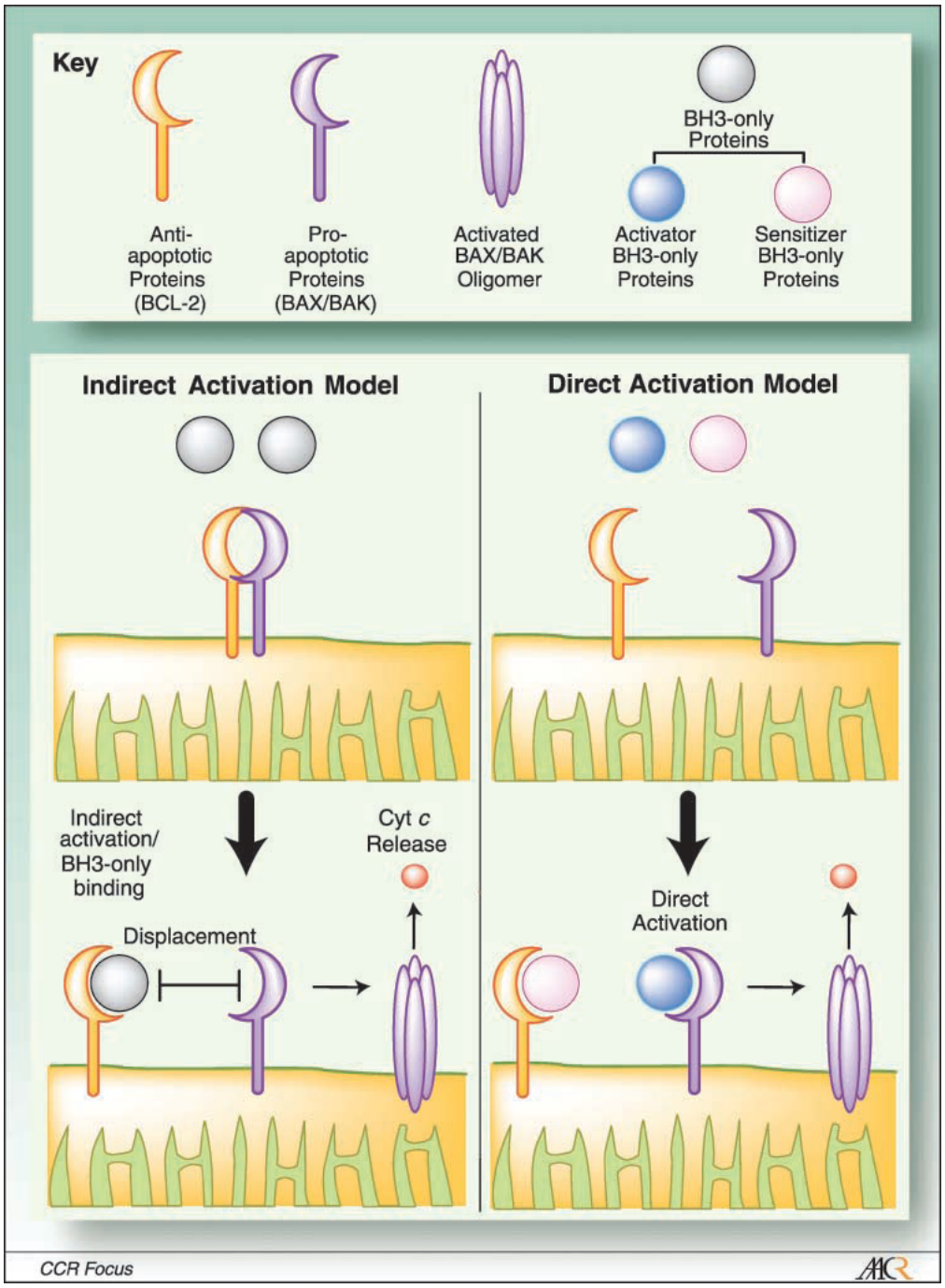


Figure 6: Models for the activation of Bax and Bak downstream of BH3-only proteins. See text for details.

From *Danial, 2007*

[129], [136] Bad is active in dephosphorylated status. Phosphorylation by pro-survival kinase AKT leads to subsequent sequestration of Bad by 14-3-3 scaffold proteins. [221] Bid protein requires cleavage provided by caspase-8 or Granzyme B for activation. [105], [179] BH3-only protein Bim can be regulated in several

ways. First, Bim is activated, when released from dynein motor complex, where is bound to dynein light chain. [149] Second, Bim activity is regulated by phosphorylation. When phosphorylation is provided by c-Jun N-terminal kinase, Bim is activated. [4] However, when Bim is phosphorylated by Erk kinase (Extracellular regulated kinase), it is degraded in proteasome. [104] Bcl-xL can be transcriptionally induced by JAK-STAT pathway to provide cell survival. [68] In cells with DNA damage, Bcl-xL is deaminated in two amino acids which are located in flexible loop region. This deamination reversibly blocks Bcl-xL activity. [54] Mcl-1 (Myeloid Cell Leukemia 1), an anti-apoptotic multi BH domain protein, is degraded in response to several cytotoxic signals. [43] Bcl-2 protein is possibly inactivated by microRNAs and phosphorylation. [38], [52] Apoptosis is precisely controlled by members of Bcl-2 protein family, which are, in turn, regulated by transriptional and post-translational mechanisms.

1.6.1 Bcl-2

Bcl-2 is an acronym for B-cell lymphoma/leukemia 2. Like its name implies, this protein was firstly described because of its participation in follicular B-cell non-Hodgkin's lymphoma due to chromosomal translocation. [188], [11] In this translocation, Bcl-2 was moved from 18q21 position into immunoglobuline heavy chain locus at 14q32. As a result Bcl-2 becomes excessively driven by gene promotor and enhancer of immunoglobuline heavy chain, thus causing Bcl-2 is overexpression. [188] Interestingly, PCR detected, that t(14;18) translocation is in 33-54 % of cases of the benign follicular hypetrophy of lymph nodes and tonsils, which occurs during infections. Although, in the majority of cases this translocation is insufficient to initiate malignancies, it still have malignant potential. [109]

Bcl-2 can be called as "generalized cell death supressor". [85] Discovery of Bcl-2 lead to creation a new class of oncogenes. Even Bcl-2 do not promotes cell growth as in case of c-Myc, it blocks apoptosis initiated by wide range of cytotoxic stimuli. [2] Because excess cells could appear not only because of increased cell proliferation, but also as a result of decreased cell death. Cancer problem could be balanced by this simple equation:

[Rate of Cell Accumulation]

=

[Rate of Cell Proliferation - Rate of Cell Death]

Since cell death and cell proliferation are in balance, excessive cells do not accumulate in tissue. Bcl-2 and other genes participating in apoptosis are at least as important as classical oncogenes, even though they do not affect cell proliferation. [154]

Bcl-2 is 26 kDa protein, which has 19 amino acids at the C-terminal, that serves as an anchor of the protein to membrane. [29], [165] Bcl-2 was found inserted into ER or outer mitochondrial membrane in such way, that the bulk of protein is oriented towards the cytosol. It was observed that C-terminal anchor is important for Bcl-2 to function as an blocker of apoptosis. [181] Bcl-2 is a multidomain protein, which has BH1-4 homology regions. The BH1, BH2 and BH3 domains of Bcl-2 forms a hydrofobic cleft, that binds BH3 domain of pro-apoptotic BH3-only proteins. Loop between BH3 and BH4 domains can be phosphorylated and thus invalidating its anti-apoptotic function. [146], [73] This protein is required for the survival of mature lymphocytes, melanocyte stem cells and cells of developing kidneys. Hence, Bcl-2 deficient mouse has abnormal death of renal epithelial progenitors, melanocyte progenitors and mature B- and T-lymphocytes. [195] Bcl-2 is also present in human endometrium, where it has cycling pattern of expression, opposite to the expression of pro-apoptotic proteins. This means, that expression of Bcl-2 is maximal at mid-proliferative phase and is minimal at the secretory phase. [85]

Several studies shown that Bcl-2 is required for maintenance of some type of tumors. It was noticed, that lymphomas and leukemias with aggressive nature have chromosomal translocations involving both c-Myc and Bcl-2. This observation results from the fact that c-Myc stimulates cell proliferation, while Bcl-2 blocks cell death. Even more, Bcl-2 inhibits cell death, which is induced by c-Myc. [14] Even if there is only expression of Bcl-2 without c-Myc, patients with several types of cancer have worse prognosis. [220], [24] That is why specific inhibition of Bcl-2 in cancer tissue can be a possible way to treat cancer.

1.6.2 Bax

Bax (Bcl-2 antagonist X) was identified as Bcl-2 interacting protein, that opposed Bcl-2 and promoted apoptotic cell death. [139] Bax is 21 kDa pro-apoptotic protein, which belongs to the Bcl-2 protein family. Bax is also a multidomain protein, but in contrast to Bcl-2 and Bcl-xL, it has three BH domains: BH1, BH2 and BH3. As it was written before, Bax together with Bak play crucial role in MOMP, which results in cytochrome c release to cytoplasm. [94] Inactive Bax is located mostly in cytosol in globular soluble form, so called CLIC (Cytosolic Locked In Conformation) form. After activation Bax is in CLAC (Cytochrome c Liberation Associated Conformation) form. [99]

Bax is composed of 9 α-helixes, two of them are the most hydrophobic and are located in the core of protein. Remaining helixes are amphipatic and expose hydrophilic residues at the surface of the protein, while hydrophobic residues are kept inside. This is the reason why Bax protein is soluble in cytosol, even if it is mostly hydrophobic. [99] C-teminal anchoring domain is tucked into the hydrophobic groove, that binds BH3-only domains. [180] This fact explains, why soluble Bax form is inactive. During initiation of apoptotic process, Bax transforms from cytosolic protein to the mitochondrial membrane-inserted. [208] Activated Bax exposes several domains, that are hidden in inactive form. In vitro studies showed that moderate basic or acidic pH changes can induce apoptotic-like conformational changes in Bax. [27] Apoptotic changes in Bax involve two steps. First step is exposure of N-terminal part, which contains mitochondrial localization signal. This process is accompanied with Bax ability to homo- or hetero-oligomerize with Bcl-xL. [26], [113] Second step is exposure of C-terminal domain, resulting into mitochondrial membrane insertion. [132] Also loosening of the Bax structure due to environmental changes enables Bax to interact with other proteins, like Bcl-xL, Bcl-2 or BH3-only proteins. [99] Bax can be transcriptionally activated by p53 and is involved in p53-regulated pathway for induction of apoptosis. [120] Bcl-2 and Bcl-xL block oligomerization of Bax and Bak in mitochondrial membrane. Anti-apoptotic Bcl-2 proteins function as chain terminating molecules of Bax/Bak oligomers. [155]

Bax-knockout mice are viable, but with hyperplastic T and B cells. Males are sterile because of defects in spermatogenesis underlying by absence of mature haploid sperm cell. [90] Cells lacking both Bax and Bak are completely resistant to

apoptosis, which is induced by tBid cytochrome c release. These cells are resistant to various apoptotic stimuli, such as UV irradiation, growth factor deprivation, tunicamycin, staurosporine and ER stress. [205]

Because mice lacking Bax have an increased risk of tumorogenesis, Bax implicates important point for regulation in anti-cancer therapy. For example, studies in ovarian cancer cell lines proved, that expression of Bax directly induced apoptosis and could increase the efficacy of chemotherapy. [214]

1.6.3 Bid

Bid (BH3-interacting death domain) was firstly described in 1996 by Wang et al. Co-immunoprecipitating studies using anti Bcl-2 antibodies in the murine T cell hybridoma line B24 identified novel Bcl-2 interacting protein. After that, cDNA library was constructed and then screened with labeled Bcl-2 protein. Interestingly, screening using Bax recombinant protein gave the same result, thus Bid protein was discovered. Bid was the first identified Bcl-2 protein, that interacts both with pro-apoptotic and anti-apoptotic proteins. [203] Bid is a member of Bcl-2 family. It is BH3-only protein. This protein is located at human chromosome 22. Although Bid does not have C-terminal transmembrane domain, it can also insert into membrane in active form. Inactive Bid is localized in cytoplasm in soluble form. Bid is expressed almost in all tissues, but the highest expression is in kidney. [203] Due to alternative splicing, Bid can have 4 protein variants. The major protein variant has 195 amino acids and 22 kDa molecular weight. [203] Bid structure is similar to Bax structure. In the core of the protein there are located two hydrophobic α-helixes surrounded by six amphipathic helixes. [36] As it was written before, Bid in contrast to Bax lacks C-terminal transmembrane domain. Instead of it, Bid helixes 6 and 7 are responsible for membrane insertion. [64] N-terminal myristoylation after cleavage also contribute to interaction with mitochondrial membrane. [222] Bid also contains N-terminal region, that negatively regulates binding to membranes. [137] Loss of this region by caspase-8 cleavage results to translocation to membranes. [105]

Due to Bid ability to interact both with anti-apoptotic proteins, as Bcl-2 and Bcl-xL, and on the other hand Bax, it plays important role in mitochondrial-dependent apoptosis. Bid-deficient mice are viable, hepatocytes are resistant to Fas-induced death. [218] In contrast, other cells, like Type II lymphoid cells are sensitive to

Fas-induced apoptosis. In hepatocytes, caspase-3 activation is suppressed by XIAP. Possible explanation could be absence of cytochrome c in cytosol, which is main inhibitor of IAPs. [108]

Key step for Bid activation is cleavage of N-teminal region. This process release the inhibition of BH3 domain. BH3 domain remains accessible even after membrane insertion of the protein. [137] Truncated Bis is about 350 times more potent than full-length Bid. [222] Native Bid is quite stable, tBid in contrast has a half-life of less than 1.5 hour. [18] Main Bid activator is caspase-8, but Bid can be also activated by Granzyme B, calpain and cathepsin. Granzyme B cleaves Bid at the loop region between helixes 2 and 3. [179] Calpain cleaves Bid and thus facilitate its translocation to mitochondria and cytochrome c release during myocardial ischemia/reperfusion injury. [33] Catepsin is a lysosomal enzyme that also cleaves Bid loop region. [178] Although, in some cases Bid could act upstream lysosomal enzymes. [72] Bid protein can be also activated by p53 pathway. Moreover, increased expression of p53 could induce upregulation of Bid mRNA. [168] Bid role could be summarized as mediator between peripheral death pathways and mitochondria, which afterwards amplificate apoptotic signalization.

Major part of anti-cancer drugs is designed to induce cellular stress and activate BH3-only proteins. P53 pathway serves as link between DNA damage caused by cytotoxic drugs and BH3-only proteins.That is why this pathway is often inactivated in cancer cells. In such case molecules mimicking BH3-only proteins can be used. [190]Nowadays the most successful are chemically created BH3 peptides, that exist in helical conformation and hydrocarbon linker, so called "stabilized alpha-helix of Bcl-2 domains". These drugs induced apoptosis *in vitro* in leukemia cells and inhibited their growth in mouse xenograft models. [200]

1.6.4 Bad

Bad (Bcl-2-associated death promoter) was the first discovered BH3-only protein. It was made by screening of an expression cDNA library with anti-Bcl-2 antibody. In human, Bad is located on the chromosome 11. Bad is 28 kDa protein, which belongs to group called sensitizer BH3-only protein. It means, that Bad interacts only with anti-apoptotic Bcl-2 proteins and inhibits their activity. Bad interacts only with

Bcl-2 and Bcl-xL. After heterodimerization with these proteins, Bad displays Bax and promotes cell death. [215]

Main regulation of Bad is provided by phosphorylation. Bad has three sites important for its activity: S112, S136 and S155. S155 lies in BH3 domain and its phosphorylation is critical for Bad activity. BH3 domain binds to the hydrophobic groove of anti-apoptotic Bcl-2 proteins. Phosphorylated serine has a negative charge which blocks interaction between negatively charged BH3 domain and hydrophobic groove. Phosphorylated S112 and S136 are docking sites for 14-3-3 proteins. Moreover, after binding of 14-3-3 to S136 and S112, there increases accesibility of S155 for phosphorylation. Phosphorylated Bad bounded to 14-3-3 translocates from mitochondria to cytosol. [221]

Phosphorylation of Bad is provided by various kinases. S136 is a preferred substrate for AKT kinase in PI3 pathway. [47] S112 and S136 are phosphorylated by both PKA (protein kinase A) and p90RSK (p90 ribosomal S6 kinase). [48], [81] Although majority of phosphorylation events results in Bad negative regulation, phosphorylation on Ser128 by cdc2 inhibits interaction with 14-3-3 proteins and enhances Bad activity. [92]

Bad phosphorylation is very important in IL-7 dependent T-cell survival. [49] Although Bad-deficient mice have no defects in thymocyte development, they showed increased death from tumors. These tumors are mostly B-cell lymphomas. [150]

Bad activity can be also regulated by caspase-dependent and Granzyme B cleavage in response to cytokine-deprivation. Cleaved Bad is poorly phosphorylated, less likely interacts with 14-3-3 proteins, but interacts more with Bcl-xL. [41]

Excluding apoptosis, Bad participates in glucose-driven mitochondrial respiration, insulin secretion and glucose homeostasis. In liver cells Bad protein forms a holoenzyme together with PKA and PP1 (Protein Phosphatase 1) catalytic units, Wiskott-Aldrich family member WAVE-1 as an A kinase anchoring protein, and glucokinase (hexokinase IV). Absence of Bad or expression of dephosphorylated Bad (Bad(3SA)) results in decreased activity of mitochondria-based glucokinase and consequently in diminished mitochondrial respiration in response to glucose. [46] Bad is also involved in cell-cycle progression. It moves cells from G_0/G_1 arrest induced by serum withdrawal to S-phase. Interestingly, this process does not depend on Bad phosphorylation or its subcellular localization, but on interaction with Bcl-xL. [31]

Bad can serve as a marker predicting tumor sensitivity to chemotherapic treatment. [51] ABT-737 that mimicking Bad BH3 peptide have promising results in lymphoma, leukemia and small cell lung cancer treatment. [138]

1.7 PARP

PARP (Poly-(ADP-ribose) polymerase) is a large family of enzymes participating in DNA repair and PCD. PARP is involved in DNA single strand and base excision repair induced by DNA nicks and breaks, leading to a 50-100 fold increase of PARP-level. These enzymes use NAD^+ directly (or ATP indirectly) as a substrate for production of ADP-ribose linear or branched polymers on different nuclear protein acceptors usually associated with chromatin, or they automodificate itself. Firstly protein-bound mono(ADP-ribose) is produced. Then polymeric chain is elongated at 2'-OH of mono(ADP-ribose) and subsequently branched at the 2'-OH of ribose moiety. [44] PARP-1 and PARP-2 are enzymes, which are directly activated by DNA strand breaks. PARP-1 contains several functional domains: 1) DNA-binding A domain, with two zinc finger structures; 2) B domain containing NLS (nuclear localization signal) and caspase-3 cleavage site; 3) automodification D domain, that interacts with various protein; 4) smallest F domain with catalytic activity. [5]

Function of PARP-1 in DNA repair was demonstrated by construction of PARP-1 deficient mice. These mice were hypersensitive to ionizing radiation and alkylating agents. They also were protected from inflammatory process caused by cerebral and cardiac ischemia, and they exhibited higher resistance to septic shock. [44]

PARP has a dual effect on cell depending on its amount and degree of DNA damage. Mild DNA damage leads to PARP activation, poly(ADP-ribosyl)ation of itself, core histones, DNA repair proteins (XRCC1, DNA ligase III, Ku70 subunit of DNA-dependent protein kinase) and subsequent repair of DNA lesions. [114], [147] In contrast, excessive DNA damage induces overactivation of PARP leading to massive PAR polymers production and release of AIF (Apoptosis-Inducing Factor) from mitochondria to nucleus in Bax oligomerization-dependent manner. [69] To explain PARP importance in cell viability, Berger with colleagues proposed "suicide hypothesis" suggesting that PARP contribute to cell death by depleting cellular NAD^+ and ATP. Total ATP depletion disables the cell to die by apoptosis and

moves cell towards necrosis. [12] That is why inactivation of PARP is critical for proceeding apoptosis. Caspase-3 cleaves PARP in DEVD sequence, which is highly conserved among different species, that indicates potential importance of PARP cleavage. [102], [42]

PARP family also participates in mitosis. It was proven by creation of PARP-deficient mice, which have misalignments of chromosomes and disruption of bipolar spindle assembly. Tankyrase-1, member of PARP family, crosslinks microtubules to spindle poles. [30]

Due to PARP involvement both in cell survival and cell death, it becomes an important point of regulation. Nowadays, PARP chemical inhibitors, that enhances chemotherapy of human cancers and cause a complete regression of xenograft tumors during preclinical tests are already developed. [23]

1.8 Apoptosis in Human Endometrium

Normal human endometrium undergoes three distinct life phases: proliferative, secretory and menstrual. Apoptosis is also present in endometrium in a cyclic manner. Hopwood and Levison first described loss of gland epithelial cells with formation of apoptotic bodies in the late secretory, premenstrual and menstrual phases. Apoptosis helps to discard senescent cells from functional layer of the uterine endometrium. [78] While apoptosis is detected in the glandular epithelium of late secretory and menstrual phases, there are almost no apoptotic cells during proliferative phase. [192] Proliferation of cells is positively correlated with estrogens. In contrast, apoptosis pattern is negatively correlated with estradiol. Progesterone has a negative effect on cell proliferation, because it promotes growth arrest. [192]

Bcl-2 protein, main inhibitor of apoptosis is also present in human endometrium. Its expression is cyclical and is highest during proliferative phase. It is considered that Bcl-2 blocks cell death during this phase. Interestingly, transcriptional factors c-Jun and Sp-3 have the same expression pattern as Bcl-2, suggesting that they can regulate Bcl-2. [140] Bcl-2 has higher expression in basal layer of endometrium, while Fas and caspase-3 are mostly present in functional layer. [157]

Other members of Bcl-2 family also participates n endometrial apoptosis. For example, levels of Bax protein were modest in proliferative endometrium and in-

creased dramatically in the secretory phase when apoptosis is most prevalent. [182] Bid cleavage and cytochrome c release were observed also in late secretory phase. [1]

Various caspases are also expressed in human endometrium. Caspase-3, -8, -9 are expressed more highly in late secretory phase of menstual cycle. [140]

As was written before, Fas and FasL are also expressed in endometrium. During proliferative phase these proteins are in vesicles in Golgi apparatus and are unable to interact. During secretory phase Fas translocates to cellular membrane. [174] Fas has the highest expression in secretory phase. Withdrawal of estrogen correlates with increased Fas and FasL expression. [204] These data indicates, that Fas-mediated apoptosis is very important for menstrual cycling. [174]

1.9 Apotosis in Endometrial Carcinoma and Hyperplasia

Endometrial carcinoma is one of the most common malignancies of female genital tract. In North America and Europe, endometrial cancer is the fourth commonest after breast, lung and colorectal cancer. [142] Risk factors for this disease are obesity, hypertension, late menopause, diabetes mellitus and exogenous estrogen usage. Majority of endometrial cancers are sporadic, but about 5% of patiens have a family history of this cancer. [71] Endometrial cancer can be divided into two types. Type I is estrogen-related cancer, which arises in the background of endometrial hyperplasia. [170] Type II cancer is estrogen-unrelated and arises in the background of atrophic endometrium. [101] This classification was made by Bokhman in 1983. [15] He postulated a hypothesis, that two types of endometriar cancer develop due to different metabolic and endocrine disturbances.

The majority of sporadic endometrial cancers (approximately 70-80%) are type I cancers. [15] In type I cancer several cheracteristic features are observed. Most frequently altered gene is PTEN (phosphatase and tensin homolog). It has tyrosine kinase function and acts as a tumour suppressor gene. [123] PTEN is inactivated in 50-80% of type I endometrial cancers. [124] Active PTEN upregulates apoptotic signalisation, including caspase-mediated Bid cleavage and downregulation of anti-apoptotic Bcl-2 proteins. [213] Next type I cancer feature is MSI (Microsatelite Instability), which is characterized by minor genetic alteration, frame-shift muta-

tions in repetitive DNA sequences. [20] Microsatelite instability is caused by defects in DNA repair process, in endometrial cancers most frequent reason of MSI is MLH1 (mutL homologue 1) repair gene mutation. [58] K-ras protooncogene is related to tumor growth and is found in 10-30% of endometrial type I cancers. [22] β-catenin is also present in endometrial cancers (about 20% of cases), although it function in tumorigenesis is still unknown. [161]

Type II cancers are more aggressive then type I endometrial cancers and are related with poor prognosis. [101] They are accounting for 10-20 % of endometrial cancers. [170] Most frequent mutation in type II cancers is p53 alteration, which is present in 90% of type II cancers. *P53* is tumor suppressor gene, which causes cell cycle arrest in response to DNA damage. [183] Another alterations are p16 inactivation, reduced E-cadherin expression and overexpression of Her2/neu. These mutations lead to loss of cell-cell interactions and uncontrolled cell growth. [158], [74], [121]

Table 3: Genetic Alterations in Endometrial Cancer: Percentage Frequency of Genetic Mutations Identified in Type I and II Endometrial Cancers

Genetic Alteration	**Type I Carcinoma (%)**	**Type II Carcinoma (%)**
PTEN inactivation	50-80	10
K-ras mutation	15-30	0-5
β-catenin mutation	20-40	0-3
Microsatellite instability	20-40	0-5
p53 mutation	10-20	80-90
HER-2/neu	10-30	40-80
p16 inactivation	10	40
E-cadherin	10-20	60-90

Bansal et al., 2009

Deregulation of apoptosis, failure of homeostasis between tissue proliferation and apoptosis play important role in cancer development. Bcl-2 was first identificated apoptosis-related gene, that participates in tumorigenesis. High Bcl-2 level was observed in various tumors, including endometrial cancer. [100], [162] It is considered, that Bcl-2 protects cells from apoptosis in various malignancies. [154] Interest-

ingly, that expression in hyperplastic endometrium was lower than in cancerous endometrium. Moreover, Bcl-2 expression in hyperplastic was even lower than in normal endometrium. Possible explanation could be that increasing of Bcl-2 expression is one of the factors leading to apoptosis resistance. [196] Another anti-apoptotic Bcl-2 proteins are also playing role in cancer development. For example, there was discovered correlation between Bcl-xL and Bcl-2 genes. Thus, overexpression of Bcl-xL downregulates Bcl-2 and cells with persisting expression of Bcl-2 have also decreased level of Bcl-xL. [148] Increased amount of NF-κB was observed in cancerous and hyperplastic endometrium. NF-κB is associated with metallo-proteinases, which can promote tumor invasiveness and formation of metastasis. Interestingly, NF-κB level is decreased in endometrial carcinoma grade II, suggesting that NF-κB has a role in protecting cells from apoptosis in hyperplasia and early carcinoma. [193] There is also evidence of receptor proteins TNF-α and Fas, participating in extrinsic apoptotic pathway. TNF-α is present in normal endometrium in secretory phase, where is up-regulated by estrogen withdrawal. TNF-α was also found in hyperplastic endometrium, but was down-regulated in endometrial carcinoma. TNF-α is supposed to regress tumors and is suppressed by NF-κB. [193] In contrast to that, Fas expression is strong both in endometrial hyperplasia and in grade II carcinomas. Fas level was lower in grade III carcinomas in comparison to grade II carcinomas. In hyperplasia and low-grade carcinomas, increased level of Fas receptors could be possible mechanism to defense host tissue against neoplastic process. [8]

Research on neoplastic tissues proves that regulation of apoptotic proteins is a part of process of tumorigenesis and suggests that apoptotic proteins could be targets for anti-cancer therapy.

1.10 Targeting Apoptotic Proteins in Cancer Therapy

Bcl-2 anti-apoptotic proteins protect cells from apoptosis and are involved in tumorigenesis. These proteins also interfere therapeutic activity of many chemotherapeutic drugs. That is why molecules that inhibits anti-apoptotic activities of Bcl-2 proteins become attractive target for basic and clinical research. Generally, small molecules that inhibits anti-apoptotic Bcl-2 proteins can be divided into three groups: 1) synthetic BH3 peptides and their modified analogs with cell membrane crossing ac-

tivities. 2) natural products discovered by random screening, 3) organic compounds made by computer-aided design. [82]

BH3-only molecules are known to block pro-survival function of Bcl-2 proteins by binding to their hydrophobic pocket. This pocket is essential for anti-apoptotic activity of Bcl-2 and Bcl-xL, mutations at this site abrogate their function. Three-dimensional structure of Bcl-2 and Bid provided structural basis for designing molecules inhibiting anti-apoptotic Bcl-2 proteins. Several BH3 peptides were synthesized. Sattler et al. synthesized peptides, containing BH3 domain from different members of Bcl-2 protein family like Bax, Bak, Bik, Bcl-2 and Bcl-xL. Peptides derived from Bak, Bik and Bax were able to inhibit activity of Bcl-xL and Bcl-2 by blocking formation of heterodimers between anti-apoptotic and pro-apoptotic Bcl-2 proteins. These peptides were competing with pro-apoptotic Bcl-2 proteins for binding hydrophobic pocket of Bcl-2 and Bcl-xL. [166] Kelekar et al. constructed 16 amino acids long peptide containing Bad BH3 domain. This peptide showed high ability to bind Bcl-xL and abrogate dimerization of Bad with Bcl-xL. [88] Cytochrome c release was observed in cells transfected by 20-amino acid Bax BH3 peptide and 15-amino acid Bak BH3 peptide. [131] After successful results with Bak and Bad BH3 peptides, there was efforts to create peptides able to permeabilize membrane. Therefore, Bak BH3 peptide containing Antennapedia internalization sequence caused activation of caspases and triggered apoptosis. Bak BH3 peptides also acted as inhibitors of Bcl-xL in cells, antagonizing its ability to suppress apoptosis induced by Fas ligation. [77] Another protein containing Bad BH3 domain was designed to carry decanoic acid as the cell permeable moiety. In mice transfected with human leukemia cells, this protein caused reduction of the tumor burden and thereby slowed the progression of tumor growth. [202]

Tetrocarcin A is a product of Actinomycete and was originally discovered as an antibiotic active against Gramm-positive bacteria and having anti-tumor activity in murine experimental tumor models. Tetrocarcin A was identificated by screening of a library of natural products using cells overexpressing Bcl-2 and Bcl-xL. Tetrocarcine A inhibits Bcl-2 mitochondrial functions, its increased concentration causes release of cytochrome c to cytoplasm and subsequent cleavage of PARP. [130]

Due to progress in drug discovery computer-aided design of specific ligands could be used. This method is working with three-dimensional structure of target protein.

[82] Using that strategy, Wang et al. created small molecule HA14-1, which binds to surface functional pocket of Bcl-2 protein and induces apoptosis in tumor cells. [201] Obatoclax, also BH3 mimetic molecule, binds and inhibits activity of Bcl-2, Bcl-xL, Mcl-1, A1 and Bcl-w. Obatoclax induced Bak and Bax pore formation in a phase I trial in patients with chronic lymphocytic leukemia (CLL). [93]

Another strategy is usage of antisense-based inhibitors or RNA interference. Oblimersen is an anti-sense oligonucleotide that downregulates Bcl-2 mRNA and protein. Research on patients with CLL discovered, that oblimersen together with fludarabine/cyclophosphamide can induce partial remission or stabilize disease. [135] Small hairpin Bcl-2 siRNA has effect on drug sensitization, it specifically inhibits Bcl-2. [83] Usage of XIAP siRNA efficiently decreases XIAP expression and together with chemotherapy treatment induce cell apoptosis in patients with esophageal squamous cell carcinoma. [223]

Promising results shows therapy targeting extrinsic apoptotic pathways. This therapy is attractive because tumor cells are more sensitive to TRAIL-dependent apoptosis than normal cells. [21] Apo2L/TRAIL stimulates cancer cell death trough DR4 and DR5 death receptors and subsequent caspase-8 activation. [199]

Key processes in apoptosis are important points for targeted anti-cancer therapy. Further research in apoptosis involvement in tumorigenesis can bring new points for targeted therapy.

2 Main aims

The aim of the Thesis was to analyze and characterize expression levels of some anti- and pro-apoptotic proteins, which are involved in endometrial apoptosis. This was performed by total protein isolation, SDS-PAGE and subsequent immunoblotting. The data were analyzed using Image Quant and Sigma Stat 3.5. programs.

Main aims of the Thesis were following:

- to analyze level of some proteins participating in apoptosis (Bcl-2, Bax, pro-caspase-3, caspase-3, PARP, Bid, Bad) in different types of human endometruim (normal, atrophic, hyperlastic, cancerous)
- to describe proliferative/apoptotic stage of selected endometrial specimens, e.g. to determine so-called apoptotic rheostat in each tissue specimen/type
- to identify some of possible turnpoints of hyperplasia to cancer switch, to uncover possible differences in levels of selected regulation proteins of the Bcl-2 family

3 Materials

Solutions for protein isolation

Homogenisation buffer, pH 7.4

20.0 mM Tris-HCl

2.5 mM EDTA

50.0 mM NaF

10.0 mM $Na_4P_2O_7$

1% Triton X-100

all the chemicals from Sigma, USA

Complete protease inhibitor cocktail for mammalian tissues

4-(2-aminoethyl)benzenesulfonyl uoride

aprotinin

leupeptin

bestatin

pepstatin A

E-64

all the chemicals from Sigma, USA

Solutions for the Lowry method

BSA (bovine serum albumin), 1mg/mL

A solution

2 % Na_2CO_3

0.1 M NaOH

B solution

2 % NaK-tartrate

C solution

1 % $CuSO_4$. 5 H_2O

Folin reagents, Sigma, USA

Solutions for Coomassie staining

Gel-fxing solution

50% (v/v) ethanol

10% (v/v) acetic acid

Gel-washing solution

50% (v/v) methanol

10% (v/v) acetic acid

Stain solution

0.1% (w/v) Brilliant Blue R350

20% (v/v) methanol

10% (v/v) acetic acid

Solutions for electrophoresis and immunodetection

Buffer 1, pH 8.8

1.5 M Tris-HCl, Sigma, USA

Buffer 2, pH 6.8

0.5 M Tris-HCl, Sigma, USA

30.0% Acrylamide - 0.8% Bis-acrylamide, ratio: 37.5:1

SDS (Sodium Dodecyl Sulphate), 10%

APS (Ammonium persulfate), 10%

TEMED (*N, N, N′, N*'-tetramethylethylenediamine)

Butanol/H_2O, 1:4 (v/v)

Running buffer, pH 8.3
25 mM Tris
192 mM Glycine
0.1% SDS

Laemmli buffer, pH 8.0
50 mM Tris/HCl
6% (w/v) dithiothreitol
5% (w/v) SDS
0.005% (w/v) Bromphenol Blue

Blotting buffeer
20 mM Tris-base
150 mM glycine
20% methanol
0.1% SDS

PBS (Phosphate buffered saline), pH 7.4
137 mM NaCl
2.7 mM KCl
10 mM Na_2HPO_4
2 mM KH_2PO_4

PBS-T buffer
PBS
0.05% (v/v) Tween 20

Blocking buffer

5% non-fat dry milk

0.1% Tween 20

Diluting buffer

1% non-fat dry milk

0.1% Tween 20

4 Methods

4.1 Patients

Endometrial samples were obtained from informed 72 patients (mean age of 58 years) admitted to Faculty Hospital Bulovka. All women underwent explorative curettage, hysteroresection or hysterectomy. Obtained endometrial samples were examined histologically at the Department of Pathology, Faculty Hospital Bulovka. Samples were classified into four groups according to histological findings: normal endometrium obtained in proliferative phase of the menstrual cycle (NE; n=21), atrophic endometrium (AE; n=9), endometrium from patients with endometrial hyperplasia (HE; n=23), and endometrium from patients with endometrial carcinoma (CE; grades I and/or II, n=19). After dissection, tissue was immediately frozen on a dry ice. Samples of venous blood for assessment of hormonal levels were collected from the same patients before operation. Clinical characteristics of the groups are shown in Table 4. Women age, weight and height data were collected by physicians of Faculty Hospital Bulovka. Hormone levels were estimated by Clinical Laboratory Unit of Faculty Hospital Bulovka. Written consent approved by local ethical committee was obtained from all subjects of the study.

Table 4: Clinical characteristics of healthy women (NE), women with atrophic endometrium (AE), women with hyperplasia (HE) and women with endomterial carcinoma (CE). Abbreviations: follicle-stimulating hormone (FSH), luteinizing hormone (LH), body mass index (BMI=*body weight[kg]/height*$[m]^2$*)*

	NE (n=21)	AE (n=9)	HE (n=23)	CE (n=19)
Age	42.75 ± 1.93	68.6 ± 3.29	53 ± 3.90	67 ± 1.94
BMI	27.8 ± 3.12	26.7 ± 0.87	28.6 ± 1.46	30.5 ± 1.81
Testosterone (nmol/L)	0.98 ± 0.11	1.48 ± 0.66	3.1 ± 1.81	1.35 ± 0.43
Progesterone (nmol/L)	1.41 ± 0.42	1.2 ± 0.34	1.17 ± 0.44	0.93 ± 0.09
Estradiol (nmol/L)	0.21 ± 0.06	0.15 ± 0.03	0.19 ± 0.03	0.23 ± 0.05
Prolactin (μg/L)	16.34 ± 4.13	11.62 ± 2.47	11.72 ± 2.11	60.78 ± 14.15
FSH (IU/L)	32.18 ± 9.95	32.34 ± 13.34	28.6 ± 8.04	32.7 ± 5.62
LH (IU/L)	18.66 ± 6.46	17.38 ± 7.33	21.65 ± 7.11	26.65 ± 9.28

4.2 Samples

In the process of curretage or hysterosection, a small amount of endometrium was dissected (0.5 - 6 cm^3; in the case of solid carcinoma whole the carcinoma was removed). About one half of obtained tissue was immediately frozen on dry ice and transported on dry ice to our laboratory. There it was stored at -80°C until use.

4.3 Preparation of Samples

To isolate cytosol fraction, tissue was brought to 0°C, cut into small pieces in homogenisation buffer containing complete protease inhibitor cocktail for mammalian tissues. Then it was homogenised in 10 volumes of the buffer on wet ice using tight 15 mL teflon-glass homogeniser. Homogenisation was run for 3 times for 10 min at 2000 rpm; Brown, Germany. The homogenate was centrifuged at low-speed at 10 000 g for 2 times for 10 min at 4 °C. Arising supernatant and the pellet were subsequently separated. Supernatant was then collected, samples was immediately frozen and stored at -80°C until use.

4.4 Determination of Sample Protein Concentration

The concentration of the proteins was determined by the method of Lowry. [111] The Lowry method was modified as follows. Standards were prepared using BSA in concentration 1mg/mL in distilled water. Standards were used to prepare the standard curve as described in Table 5. In the sample tube,10 μL of sample was added; distilled water was added to 500 μL. Both standards and samples were prepared in triplets. 1.5 mL of ABC solution was added to each standard and sample. ABC solution was prepared by mixing solution A + solution B + solution C in a ratio of 50:1:1. Tubes were incubated 10-15 minutes at room temperature. After that, 150 μL of Folin reagents was added to each tube. Tubes were mixed thoroughly and incubated 30 minutes in dark at room temperature. Absorbance was measured at 720 nm. Calibration curve was prepared and fitted to $y = ax^2 + bx + c$ (Figure 7). The sample protein concentration was then estimated as radix given by curve solution.

Table 5: BSA and distilled water concentration for standard preparation

Standard	1	2	3	4	5	6	7	8	9	10
BSA (μL)	0	10	20	30	40	50	75	100	150	200
H2O (μL)	500	490	480	470	460	450	425	400	350	300
BSA (μg)	0	10	20	30	40	50	75	100	150	200

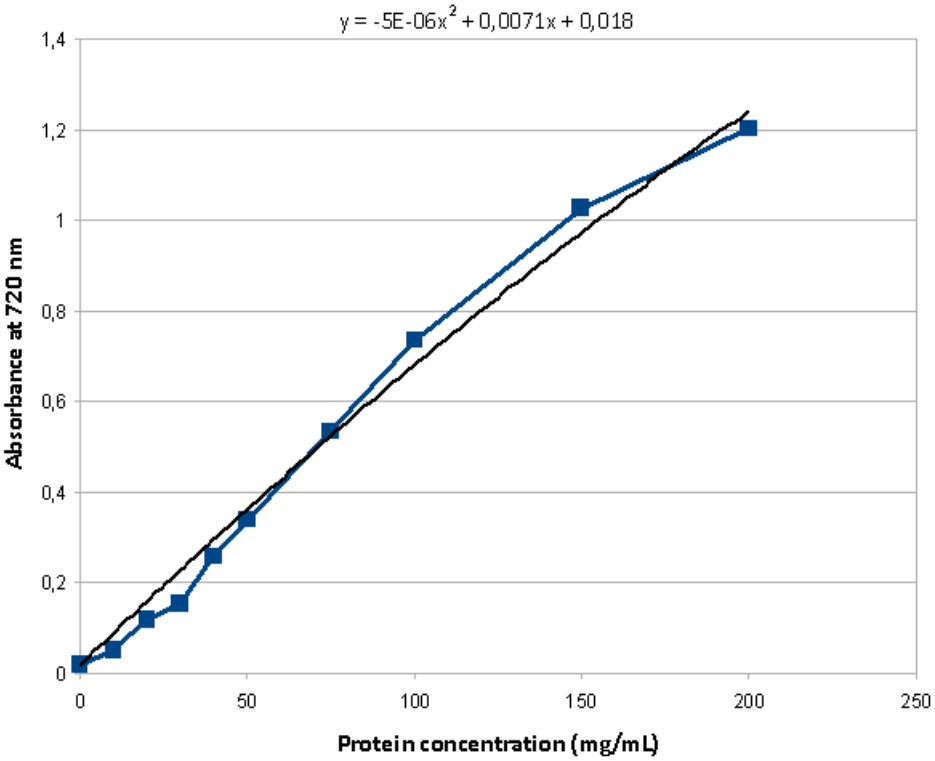


Figure 7: An example of protein concentration determination by the modified method of Lowry

4.5 Coomassie Staining

For assessment of protein sample equality added to each well Coomassie staining was performed (Figure 8). This staining detection method does not differ between specific proteins and thus is used for total protein content. In these preliminary experiments the following Coomassie protocol modification was used.

After electrophoresis, the apparatus was disassembled and the gels were washed off the glass plates with 500 mL of the gel-fixing solution and soaked in that solution

for 1 hour. After incubation, solution was removed. Then gels were covered with 500 mL of the gel-washing solution and incubated overnight at room temperature with gentle agitation. Subsequently, gels were covered with 400mL of the Coomassie stain solution and stained at room temperature for 3 to 4 hour with gentle agitation. Then, Coomassie stain solution was removed and gels were intensively washed with distilled water.

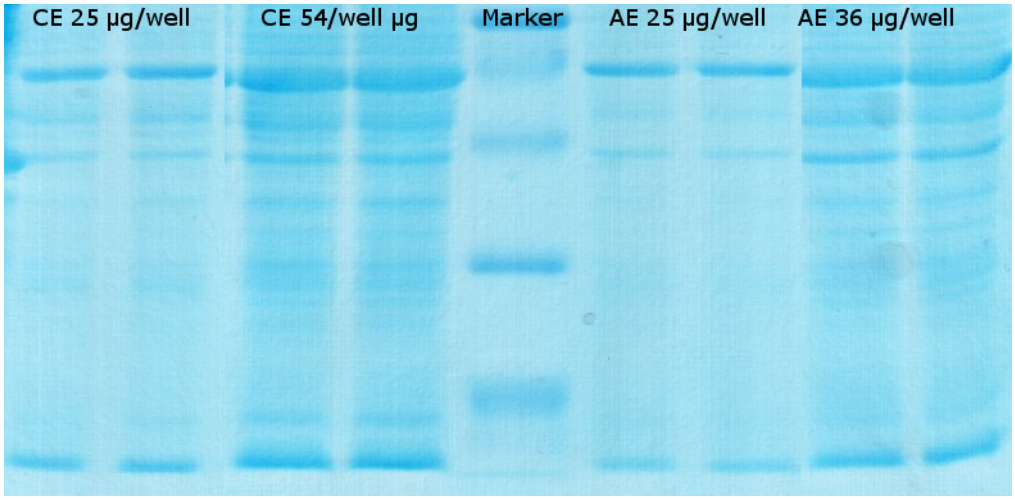


Figure 8: An example of Coomassie stained gel

4.6 Electrophoresis

4.6.1 Preparation of Gels

Proteins were resolved by standard SDS-PAGE (Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis) using Mini Protean II Gel Kit (Bio-Rad). Briefly, gels for electrophoresis are composed of so-called "running" gel and "stacking" gel. "Running gel" is the gel of selectable concentration of acrylamide-bisacrylamide monomer. The choosing of concentration depends on molecular mass of separated proteins. In current study 15 % running gel was used. Gels were prepared according Table 6.

Mixture was poured between two electrophoretic glasses and superimposed by mixture butanol/H_2O in rate of 1:4 to avoid upper part gel part drying during polymerisation process. When polymerisation was finished (20-30 minutes), butanol/H_2O was poured out and upper part of the polymerized gel was carefully washed by distilled water for several times. Then it was carefully dried using filter paper and

Table 6: Reagents for gel preparation.

	15% running gel	stacking gel
H_2O	4.7 mL	6 mL
Buffer 1	5 mL	-
Buffer 2	-	2.5 mL
30% Bis-acrylamide	10 mL	1.3 mL
10% SDS	200 μL	100 μL
10% APS	90 μL	90 μL
TEMED	8 μL	8 μL

stacking gel mixture was poured on. A plastic comb was inserted into the stacking gel mixture to make 15 wells. After polymerization of stacking gel mixture, the comb was removed and polymerised gel was intensively washed several times with distilled water. Then, gel was replaced to electrophoretic apparatus (Mini Protean) and a running buffer was poured into the tank.

4.6.2 Running Electrophoresis

The sample containing 25 μg of protein was solubilised in Laemmli buffer. The proteins were resolved by standard SDS-PAGE (15% gels). To denaturate proteins, samples were shortly boiled in sample tubes in destilled water (1 minute at 100°C). The electrophoresis was run at constant voltage 200 V for 1 h, using a Mini Protean II gel kit.

4.7 Western Blotting

4.7.1 Blotting

After the SDS-PAGE, proteins were transferred to nitrocellulose membrane using wet apparatus. Briefly, nitrocellulose membrane was placed on filter paper placed on a sponge wetted in blotting buffer. The gel with divided proteins was placed on the second filter paper and sponge and was covered with the nitrocellulose membrane part (see Figure 9). Blotting was run at 35-50 mA overnight using wet apparatus on a Mini Protean II Gel Kit.

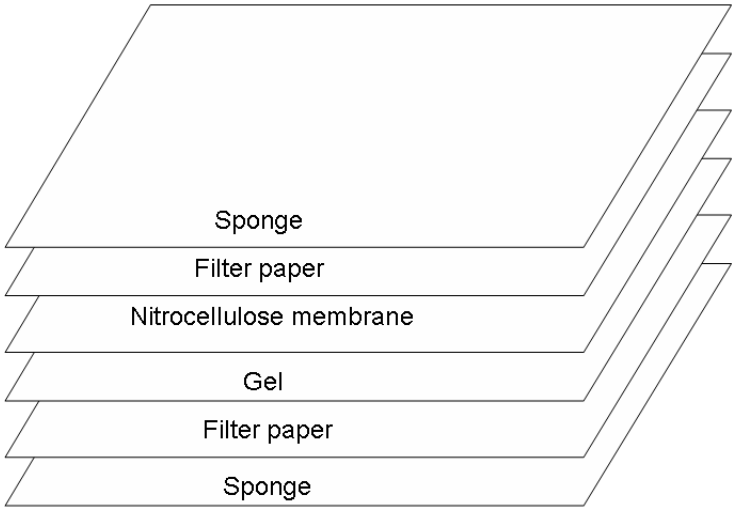


Figure 9: Schematic assembly of so-called "Western blot sandwich"

4.7.2 Detection with antibodies

Detection using antibodies contains two main steps. First step is based on primary antibodies, which precisely interact with the parts of proteins. Second step include secondary antibodies, which are conjugated e.g. with HRP (Horseradish peroxidase). These antibodies bind to primary antibodies. After that, HRP, which is linked to secondary antibodies, cleaves a chemiluminescent agent and therefore produces luminescence, which is detected by X-ray film.

The membrane was blocked for 1 h in blocking buffer in plastic container in the total volume of 20 mL at room temperature. Then the membrane was incubated for 2 h at room temperature in appropriate primary antibody in diluting buffer. The following primary antibodies were used in this study (see Table 7). After the primary antibody was removed, the blot was washed 3 times for 10 min in PBS-T buffer. Subsequently, the membrane was incubated for 1 h at room temperature with appropriate HRP-conjugated secondary antibody in diluting buffer. The following secondary antibodies were used in this study (see Table 7). After the removing of secondary antibody, the membrane was intensively washed 3 times for 10 min in PBS-T buffer.

Table 7: Primary and secondary antibodies used in current study

Name	Type	Source	Raised against	Dilution
Primary antibodies				
Actin (sc-1615)	polyclonal	goat against human	C-terminus	1:500
Bad (sc-6542)	polyclonal	goat against human	N-terminus	1:500
Bax (sc-65532)	monoclonal	mouse against human	amino acids 3-16	1:500
Bcl-2 (sc-65392)	monoclonal	mouse against human	full length	1:500
Bid (sc-56025)	monoclonal	mouse against human	amino acids 61-118	1:500
Caspase-3 (sc-22171)	polyclonal	goat against human	Ser 176 at cleaved p11 unit	1:500
Pro-caspase-3 (sc-7272)	monoclonal	mouse against human	full length	1:500
PARP (sc-8007)	monoclonal	mouse against human	C-terminus	1:500
Secondary antibodies				
Goat IgG-HRP (sc-2005)	polyclonal	goat against mouse	Bax (sc-65532), Bcl-2 (sc-65392), Pro-caspase-3 (sc-7272), PARP (sc-8007)	1:20 000
Donkey IgG-HRP (sc-2020)	polyclonal	donkey against goat	Actin (sc-1615), Bad (sc-6542), Caspase-3 (sc-22171)	1:20 000

4.7.3 Chemiluminescent detection

After an intensive washing of incubated nitrocellulose membrane, it was carefully dried between two sheets of filter paper. Then, luminescent substrate was spread on the membrane for 1 minute. ECL (Enhanced chemiluminescence) Western blotting kit (Amersham Life Science; RPN2108) was used. After 1 minute incubation, membrane was exposed to the Kodak film. The optimal time of membrane exposure to the Kodak film depend on both the protein signal intensity as well as background luminescence. The average optimal time was 30 seconds. After exposure, films were developed, scanned and analyzed.

4.8 Statistical Analysis

The levels of pro-caspase-3, caspase-3, Bcl-2, Bax, Bid, Bad, PARP, actin and the Bax/Bcl-2 ratio were analysed and quantified by scanning densitometry (Image-Quant TL v 2005, Amersham Biosciences). Protein expression was normalized to band intensities observed for F-Actin used as internal control. Thus, values of protein levels are given in relative units (R.U.) determined as ratio of mean protein band optical density to mean Actin band optical density on the same membrane.
Detection of Bcl-2 and Bax was performed separately on the same membrane to determine the protein ratios in the same sample.

Statistical analysis was performed using the SigmaStat 3.5 program (Systat Software, Inc.). For comparison of protein levels between four studied groups, One-way ANOVA test was used. When the normality (data distribution was not approximately normal) test failed, Kruskal–Wallis one-way analysis of variance by ranks was performed. Data from 7 dependent experiments (= 7 SDS-PAGE were run on material collected and pooled from all the samples of the experimental group) made in triplets for each protein and experimental group were analysed.

The null hypothesis H_0 was "population means are equal", or, in the other words, there are no differences between the population means of the studied groups. In formula:

$$H_0: \mu_1 = \mu_2 = \mu_3 = \ldots = \mu_n$$

where:

H_0 = the null hypothesis
μ_1 = the mean of population 1,
μ_2 = the mean of population 2, and
μ_n = the mean of population n.

The significance level (critical p-value) was 0.05. If a test of significance gave a p-value lower than the significance level, the null hypothesis was rejected.

Concerning statistics, data are presented with following characteristics:

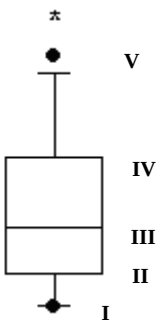
n = number of subjects in each group

p; p-value = the probability of the occurrence of a given event. Here, the given event is "no difference exists between the groups means" (null hypothesis). The p-value is used as an alternative to rejection points to provide the smallest level of significance at which the null hypothesis would be rejected. In this study, p-value 0.05 (5%) was used.

S.E.M. = standard error of the mean; the standard deviation of the sample mean estimate of all samples mean (or in other words: the standard deviation of the sample mean estimate of a population mean)

= graphical representation of positive S.E.M in column plots

* = graphical representation of statistically significant differences in data means of compared group on the significance level 0.05, e.g. critical p-value 0.05 (5%)



= **box plot** (or a box-and-whisker diagram or plot). Plot depicting groups of numerical data through their five-number summaries: I) the smallest observation (sample minimum), II) lower quartile (Q1, P_{25}), III) median (Q2), IV) upper quartile (Q3, P_{75}), and V) largest observation (sample maximum). The median is shown as a line across the box. If the mean is shown, it is usually drawn in as a dotted or a dashed line. Therefore 1/4 of the distribution is between this line and the top of the box and 1/4 of the distribution is between this line and the bottom of the box.

5 Results

5.1 Pro-Caspase-3 and Caspase-3 Level

Level of pro-caspase-3 and caspase-3 was analyzed using two different specific non cross-reactive antibodies recognizing either pro-caspase-3 or caspase-3. Bands of 35 kDa and 18 kDa representing pro-caspase-3 and caspase-3 respectively were observed in all tissue specimens (Figure 10 and Figure 11).

Pattern of the pro-caspase-3 and caspase-3 level was similair, the highest level both of pro-caspase-3 and caspase-3 was detected in patients with cancerous endometrium (375 % and 1680 %, respectively, Table 8). Hyperplastic endometrium has also higher level of pro-caspase-3 and caspase-3 (160 % and 700 %, respectively) compared to normal endometrium. Level of pro-caspase-3 and caspase-3 was significantly lower in comparison to normal, hyperplastic and cancerous endometrium.

Interestingly, increase of caspase-3 level was more dramatic in cancerous and hyperplastic endometrium with respect to normal endometrium, than increase of pro-caspase-3. Level of caspase-3 was more than four times higher than level of inactive enzyme form pro-caspase-3.

Table 8: Level of pro-caspase-3 and caspase-3 in HE, AE, CE and NE. Results are in relative units and represent mean of seven dependent experiments ± S.E.M.($p < 0.05$)

	HE (n=23)	AE (n=9)	CE (n=19)	NE (n=21)
pro-caspase-3	0.78 ± 0.15	0.03 ± 0.01	1.80 ± 0.23	0.48 ± 0.10
caspase-3	1.47 ± 0.18	0.04 ± 0.01	3.52 ± 0.27	0.21 ± 0.06

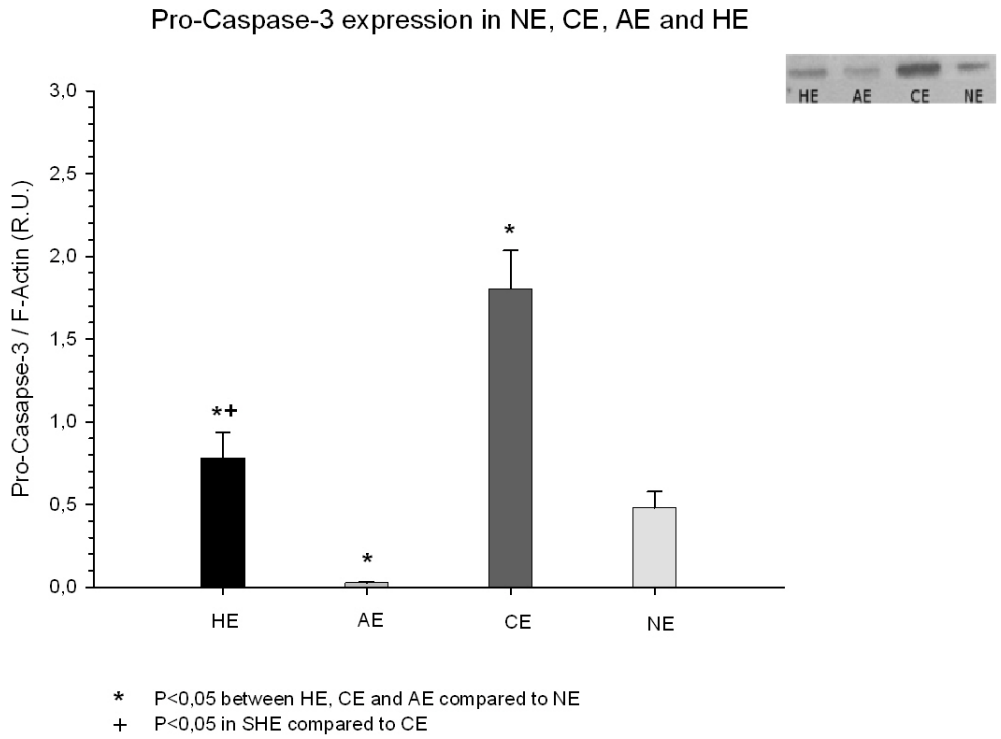


Figure 10: Western blot analysis of pro-caspase-3 levels in HE, AE, CE and NE. 25 μg of protein was loaded to each lane, pro-caspase-3 band bands was detected at 35 kDa. Protein intensities were normalized to intensities observed for F-Actin as internal control. Results are in relative units and represent mean of seven dependent experiments $\pm$ S.E.M.(standard error of the mean) ($p < 0.05$)

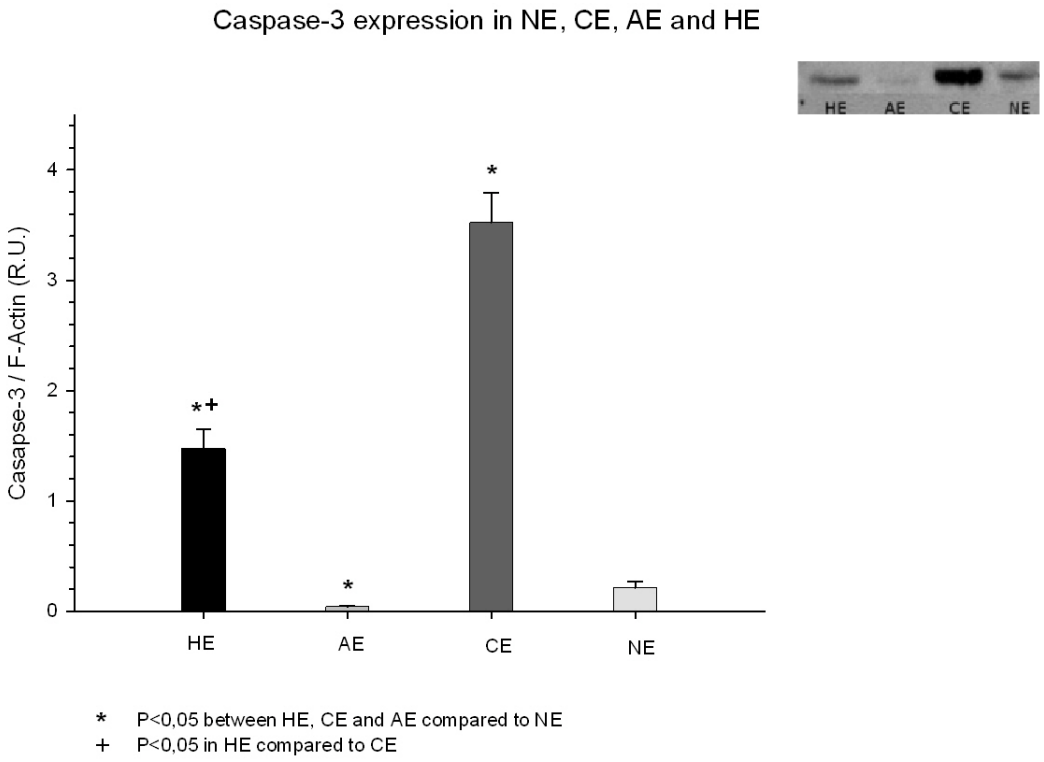


Figure 11: Western blot analysis of caspase-3 levels in HE, AE, CE and NE. 25 μg of protein was loaded to each lane, caspase-3 bands was detected at 18 kDa. Protein intensities were normalized to intensities observed for F-Actin as internal control. Results are in relative units and represent mean of seven dependent experiments $\pm$ S.E.M. ($p < 0.05$)

5.2 Bcl-2 and Bax level

In all groups of endometrium a band of 25 kDa for Bcl-2 and a band of 23 kDa for Bax were observed (Figure 12). Profile of Bax and Bcl-2 level was similar in all groups of endometria studied, although Bax reached only 60-70 % of Bcl-2 level (Table 9). In cancerous endometrium, level of Bcl-2 was higher in respect with normal endometrium (150 %), whereas changes in Bax level were non-significant. Interestingly, Bcl-2 and Bax level was diminished in hyperplastic endometrium as compared to cancerous endometrium. Bcl-2 level in hypeplastic endometria was about 60 % of Bcl-2 level in cancerous endometrium. Bax level reached in hyperplastic endometrium 55 % with respect to cancerous endometrium. Level both of Bcl-2 and Bax was significantly lower in atrophic endometrium in comparison with normal endometrium.

The analysis of Bcl-2/Bax ratio, which is one of the key factors that determine cell survival showed the highest ratio in cancerous endometrium, which was about 1.45 (Figure 13) . Bcl-2/Bax ratio in normal and atrophic endometria was also more than one (1.04 and 1.18 respectively, Table 10). Bcl-2/Bax ratio in hyperplastic endometrium was about half less than Bcl-2/Bax ratio in cancerous endometrium. Interestingly, slightly higher Bcl-2/Bax ratio was observed in atrophic endometrium as compared to normal endometrium.

Table 9: Level of Bcl-2 and Bax in NE, CE, AE and HE. Results are in relative units and represent mean of seven dependent experiments ± S.E.M. ($p < 0.05$)

	NE (n=21)	CE (n=19)	AE (n=9)	HE (n=23)
Bcl-2	0.78 ± 0.03	1.17 ± 0.04	0.06 ± 0.01	0.71 ± 0.05
Bax	0.56 ± 0.07	0.77 ± 0.08	0.04 ± 0.01	0.42 ± 0.07

Table 10: Descriptive characteristics of Bcl-2/Bax ratio in HE, AE, CE and NE. Results are in relative units.

	NE (n=21)	CE (n=19)	AE (n=9)	HE (n=23)
Mean	1.28 ± 0.16	1.68 ± 0.14	1.35 ± 0.20	0.90 ± 0.09
Median	1.04	1.45	1.18	0.86

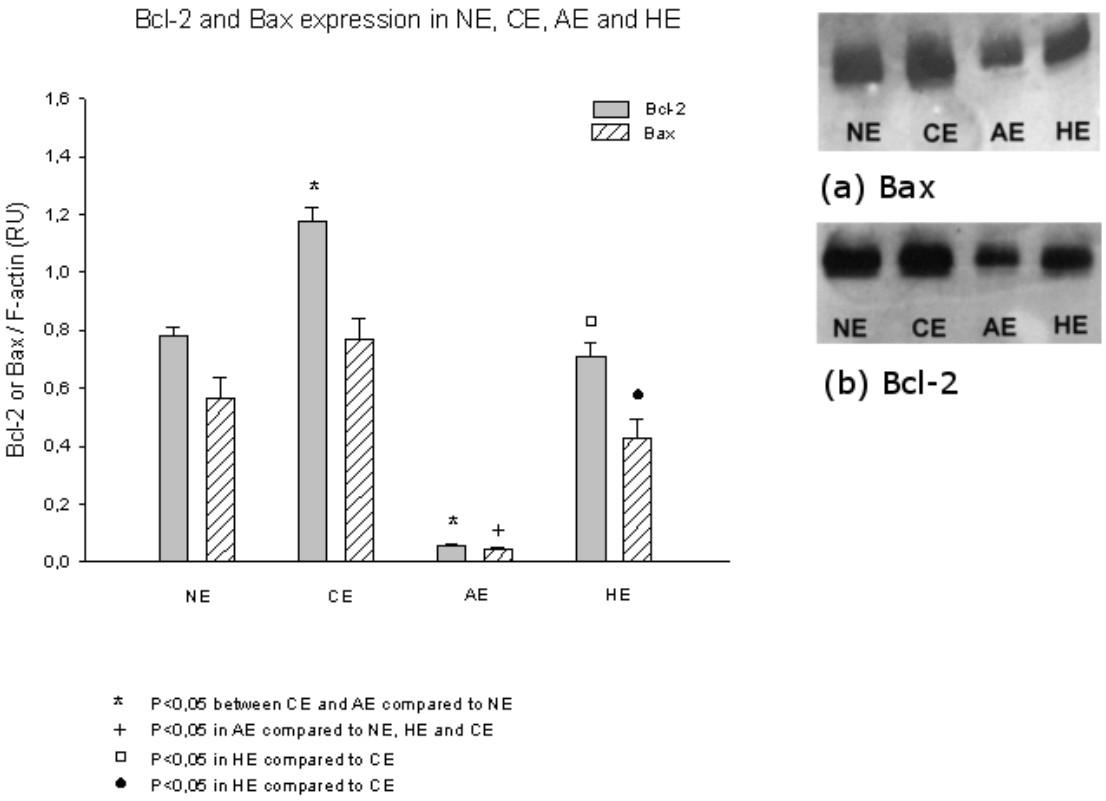


Figure 12: Western blot analysis of Bcl-2 and Bax levels in HE, AE, CE and NE. 25 μg of protein was loaded to each lane. Bcl-2 band and Bax bands were detected at 25 and 23 kDa, respectively. Protein intensities were normalized to intensities observed for F-Actin as internal control. Results are in relative units and represent mean of seven dependent experiments $\pm$ S.E.M. ($p < 0.05$)

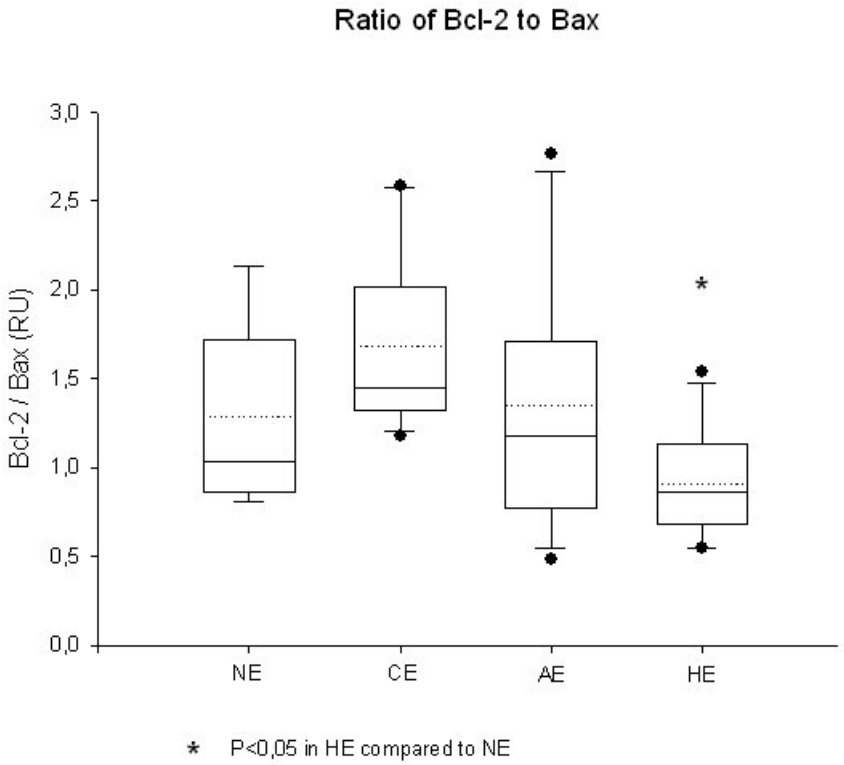


Figure 13: Bcl-2/Bax ratio in HE, AE, CE and NE. The level of protein was determined by Western blot, as described in Methods. Results are in relative units and represent mean of seven dependent experiments ± S.E.M. ($p < 0.05$)

5.3 Bid and Bad level

Bid and Bad proteins were detected in all tissue samples (Figure 14 and Figure 15). Bid and Bad level has opposite tendencies in hyperplastic and cancer endometrium in respect to normal endometrium. Bid protein level was more than 50 % lower in cancerous endometrium as compared to hyperplastic endometrium (0.25 and 0.53 R.U. respectively, Table 11). In contrast to that, there was no statistically significant difference of Bid level between hyperplastic and normal endometrium.

In case of Bad protein level highest level of Bad protein was in cancerous endometrium (0.5 R.U.). Level of Bad protein in normal endometrium was slightly lower, but there was to statistically significant difference (Table 12). Bad protein level in hyperplastic endometrium was only 40 % of Bad protein level in normal endometrium.

Table 11: Level of Bid in HE, CE and NE. Results are in relative units and represent mean of seven dependent experiments ± S.E.M. ($p < 0.05$)

	HE (n=23)	NE (n=21)	CE (n=19)
R.U.	0.53 ± 0.08	0.50 ± 0.05	0.25 ± 0.03

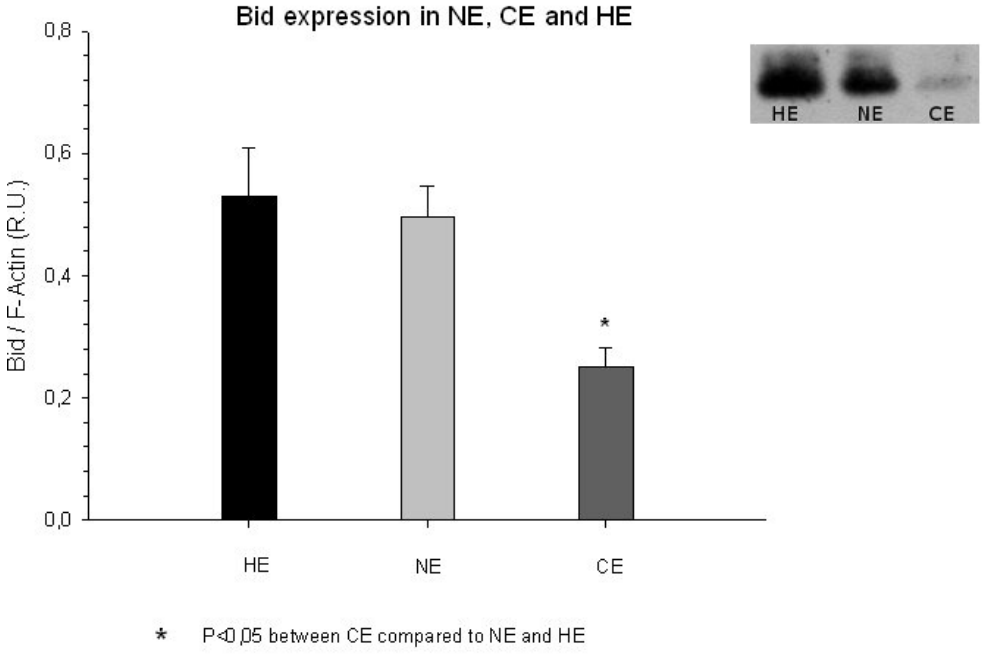


Figure 14: Western blot analysis of Bid levels in HE, NE and CE. 25 μg of protein was loaded to each lane. Bid band was detected at 23 kDa. Protein intensities were normalized to intensities observed for F-Actin as internal control. Results are in relative units and represent mean of seven dependent experiments ± S.E.M. ($p < 0.05$)

Table 12: Level of Bad in HE, CE and NE. Results are in relative units and represent mean of seven dependent experiments ± S.E.M. ($p < 0.05$)

	HE (n=23)	NE (n=21)	CE (n=19)
R.U.	0.18 ± 0.02	0.45 ± 0.08	0.50 ± 0.06

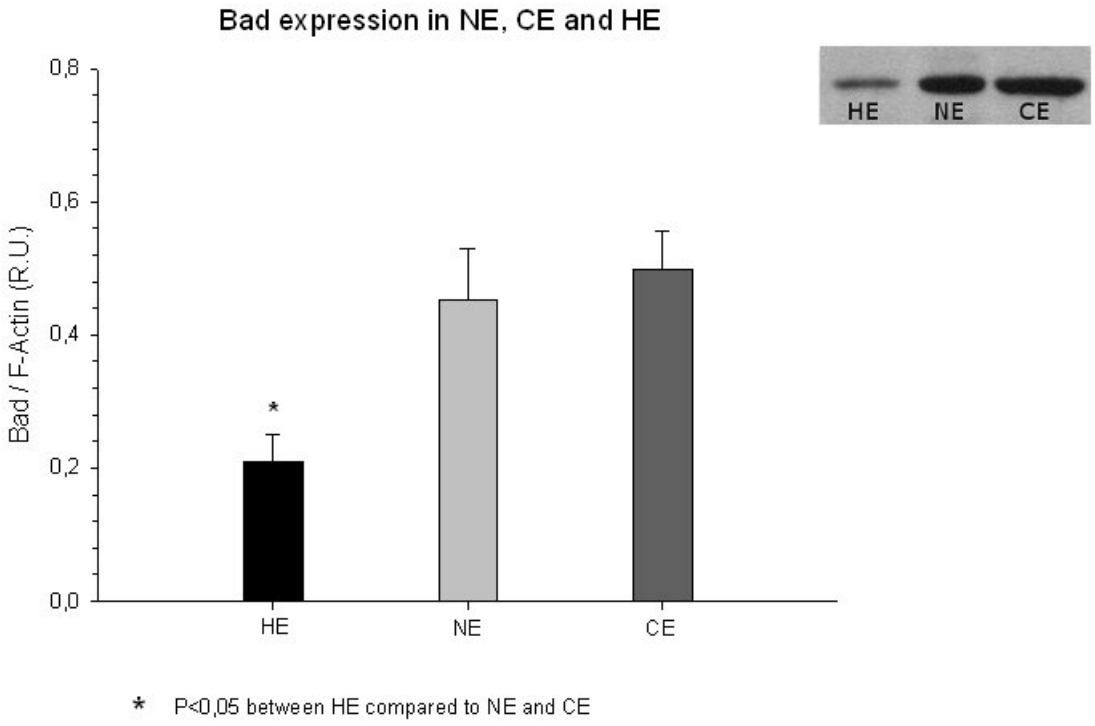


Figure 15: Western blot analysis of Bad levels in HE, NE and CE. 25 μg of protein was loaded to each lane. Bid band was detected at 25 kDa. Protein intensities were normalized to intensities observed for F-Actin as internal control. Results are in relative units and represent mean of seven dependent experiments $\pm$ S.E.M. ($p < 0.05$)

5.4 PARP level

A 110 kDa PARP fragment was observed in all tissue specimens (Figure 16). Level of PARP in cancerous endometrium reached 130 % of PARP level in normal endometrium, although the difference was considered to be not-significant (Table 13). The only significant difference was found in PARP level in atrophic endometrium when compared to all other tissue specimens. The PARP level in atrophic endometrium was only 4.5 and 3 % in comparison with normal, hyperplastic and cancerous endometrium, respectively.

Table 13: Level of PARP in HE, AE, CE and NE. Results are in relative units and represent mean of seven dependent experiments ± S.E.M. ($p < 0.05$)

	HE (n=23)	AE (n=9)	CE (n=19)	NE (n=21)
R.U.	2.18 ± 0.42	0.10 ± 0.02	3.18 ± 0.30	2.49 ± 0.24

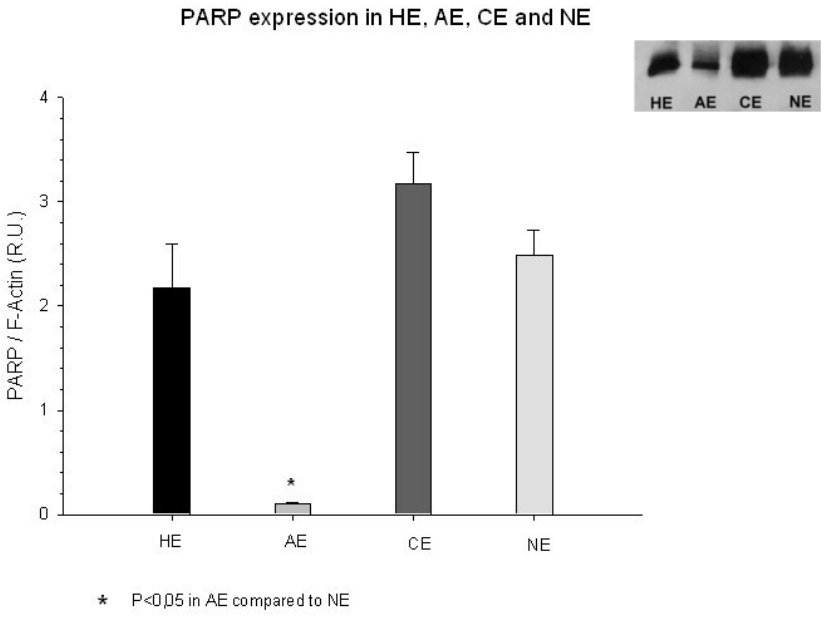


Figure 16: Western blot analysis of PARP levels in HE, AE, CE and NE. 25 μg of protein was loaded to each lane. PARP band was detected at 110 kDa. Protein intensities were normalized to intensities observed for F-Actin as internal control. Results are in relative units and represent mean of seven dependent experiments ± S.E.M. ($p < 0.05$)

6 Discussion

Balance between cellular proliferation and death is critical for preventing oncogenesis. Not only increased proliferation, but also decreased cell death can lead to the cancer development. Dysregulation of the key molecules participating in apoptosis, such as Bcl-2 protein family and caspases, are one of the start points for cancerogenesis. Plenty of researches has been made to analyze and understand a process of cancerogenesis. Despite this fact, the process of endometrial cancer development remains still not fully understood. In present study, expression level of some proteins involved in apoptotic pathway in different types of human endometrium was detected. There was an effort to find some possible turnpoints in development of cancerous endometrium in a backgroung of hyperplastic endometrium. This study was based on Western blot immunodetection. In the effort to thoroughly compare obtained data with previously published works, we run up against problem of substantial methodical differences. Most of the works in this field used immunohistochemistry (IHC) as the main detection method of apoptotic markers. Unfortunately, Western blot immunodetection and IHC data can not be compared directly (as commented below) and results outcoming from these two methods should be also compared with caution. Despite the intensive search, there is no paper directly comparing data arising from IHC and Western blot technique on the same material.

There is almost no apoptotic cells in endometrium during proliferative and early secretory phase of the menstruation cycle. Because of that, healthy endometrium in proliferative phase was chosen as a control tissue specimen. In current study levels of Bcl-2 protein family (Bcl-2, Bax, Bid, Bad), pro-caspase-3, caspase-3 and PARP expression level were analyzed in normal, atrophic, hyperplastic and cancerous human endometrium. High level both of pro-caspase-3 and caspase-3 were found in hyperplastic and cancerous endometrium. These data agreed with the results of Atasoy et al., who immunohistochemically showed higher expression of caspase-3 in cancerous endometrium in comparison with hyperplastic endometrium. [8] Peiro et al. also observed, that normal and atrophic endometrium have lower caspase-3 expression than cancerous endometrium. [143] Both authors conclude, that caspase-3 could serve as a marker, predicting patients outcome. Di Paola did not find any statistically significant difference between caspase-3 expression in normal and cancerous

endometrium. [55] On the other hand, Villavicencio did not detect caspase-3 in normal and hyperplastic endometrium, pro-caspase-3 was detectable. [196] Possible explanation could be in usage of different antibodies in Di Paola and Villavicencio group. Interestingly, detection of specific protein can also depend on the antibody supplier and type. In the study of the Villavicencio group, the used antibody was polyclonal caspase-3 antibody from Dako, USA. In the other work of this group published at the same time (fall of 2006) and using the same electrophoresis as well as Western blot protocol, caspase-3 was detectable using polyclonal caspase-3 antibody from BD Pharmigen. [9]

Bcl-2 protein is the main inhibitor of apoptosis and is considered to be oncogene when overexpressed. [154] In present study, higher level of Bcl-2 in cancerous endometrium and slightly lower Bcl-2 expression in hyperplastic endometrium with respect to normal endometrium. These findings correlate with those reported by the other researchers. For example, Villavicencio et al. observed higher Bcl-2 level in normal endometrium when compared to hyperplastic as well. [196] Marone et al. found out, that Bcl-2 expression is higher in cancerous endometrium in comparison with hyperplastic. [115] Very low expression of Bcl-2 in atrophic endometrium and a slightly lower in hyperplastic endometrum regards to normal endometrium was shown by Vaskivuo et al. [193] In contrast to findings presented in current study, Vaskivuo et al. observed decreased expression in endometrial adenocarcinoma and found, that Bcl- 2 level is decreased with the increasing tumor grade. Although other authors did not observe any correlation between tumor grade and Bcl-2 expression. [76], [143], [91] Some studies also detected decreased pattern of Bcl-2 expression in endometrial carcinomas. [184], [133], [35] These quite contrary results could be explained by usage of different methods and different protocols, as it was in case of caspase-3 expression. Whereas in present study detection of Bcl-2 expression level were perfomed using immunoblotting method, other studies, detected it immunohistochemically.

As mentioned above, there are substantial difficulties concerning direct comparison of IHC and Western blot technique on the human endometrium. Chemiluminescent systems used for Western blot are based e.g. on horseradish peroxidase (HRP, as used in this study) and offer rapid highly sensitive results with excellent signal-to-noise ratio. In many laboratories, they are the predominantly used detection

method. On the other hand, IHC methods had a relatively low sensitivity, but a high specificity. Immunohistochemistry is a key technique in both investigative and diagnostic pathology. [75] However, on the request of University Hospital Bulovka, IHC technique was not content of this study.

We think three main obstacle areas that can cause results difference with the respect to Western blot and IHC techniques. First, observed results contrary can come from different antibodies used in different studies (in the name of monoclonal vs. polyclonal antibodies, different epitopes antibodies are raised against, different antibodies suppliers etc.). Second, signal intensity is also affected by choose of chemiluminescent substrate used for Western blotting. And finally, results and published data also depend on examination practices in particular laboratory. These possibilities are shortly discussed bellow.

Western blots are detected with antibodies specific to the target protein. These antibodies are known as primary antibodies. A labeled secondary antibody is then added, which binds to the primary antibody. The principle of the IHC staining procedure is to visualize target antigens through antigen-antibody interactions that are visualized by enzymatic reactions. The IHC staining protocol consists of several key steps: 1) blocking, 2) epitope recovery and 3) detection. [75] Antibodies attach to the proteins by affinity tags.

The accessibility of the affinity tags varies with the structure of the individual protein. It also depends on the place of the attachment of the antibody. This can cause that the detection limit can vary from one detection method to another despite of the usage of the antibodies detecting the same protein, because their tags on the particular protein are raised against different sequences that can differ in the accessibility to the antibody.

The second difference underlying discrepancy in the same protein detection can be caused by usage of different chemiluminescent systems or Western blot Kits. Chemiluminescent systems based on HRP can use oxidation of different substrates. [56] Different laboratories can use either standard or enhanced systems for chemiluminescent detection. Sensitivity of the latter one can be up to 20 times greater and can vary in the range from femtograms to picograms of detected protein.

There are also papers indicating that experimental outcomes are affected by differences between standard of laboratories. [191] These differences of standards can

include different examination practices that depended on the experience of the operator. In addition, the results also depend on the use of different antibody reagents, different antibody dilutions, preparation of solution, or different enzymatic treatments of tissue. All of these may contribute to the observed variation.

Bax expression pattern was similar to that of Bcl-2. In accordance with present research, results of immunoblot analysis of Villavicencio et al. observed lower level of Bax in hyperplastic endometrium in comparison with normal endometrium. [196] Peiró et al. immunohistochemically detected decreased Bax expression in hyperplastic endometrium with respect to cancerous endometrium. [143] In contrast to that, Vaskivuo et al. observed peak of Bax expression in simplex hyperplasia and its consequent decreasing in proliferation endometrium, complex and atypical hyperplasia, atrophic endometrium and cancerous endometrium. [193] Although Kokawa et al. showed higher Bax level in endometrial carcinoma rather than in hyperplasia, which agrees with results presented in current study.

Bcl-2/Bax ration is very important factor for predicting cell fate. Whereas high Bcl-2/Bax ration makes cells resistant to apoptotic stimuli, low Bcl-2/Bax ratio predisposes cells to apoptosis. In current study, Bcl-2/Bax ration in endometrial carcinoma and normal endometrium is > 1. Interestingly, Bcl-2/Bax ratio was > 1 also in atrophic endometrium. Hyperplastic endometrium has Bcl-2/Bax ratio < 1. Similar results have Villavicencio et al. in their immunoblot study. [196] On the other hand, Vaskivuo et al. observed by immunohistochemical method Bcl-2/Bax ration > 1 only in proliferative endometrium, whereas all other endometrial tissue specimens (hyperplastic, atrophic, cancerous) have this ratio < 1. [193] Interestingly, Bcl-2/Bax ratio was higher in simplex hyperplasia, than in other types of endometrial hyperplasia, atrophic and cancerous endometrium, however its value was < 1.

Pro-apoptotic Bid protein mediates both extrinsic and intrinsic apoptotic pathways. Truncated Bid translocates to outer mitochondrial membrane and facilitates cytochrome c release to cytoplasm. [87] In present study lowest expression of Bid protein was observed in adenocarcinoma cells. Downregulation of Bid expression could be one of the features of neoplatic transformation. Hyperplastic and normal endometrium have almost similar level of Bid expression, thus there was no statistically significant difference.

Bad is also pro-apoptotic protein, which is involved in intrinsic apoptotic pathway. Bad forms heterodimers with Bcl-2 and Bcl-xL and thus inactivates their activity. [87] Bad expression pattern was quite opposite to that of Bid. Hyperplastic andometrium has lowest Bad level. Expression of Bad was only slightly lower in normal endometrium when compared with cancerous endometrium. To best author knowledge, there is no studies, which were analyzing Bid and Bad proteins expression in endometrial carcinoma or hyperplasia. Altough there are studies, that analyzed Bad expression in another tissues. For example, Smith et al. demonstrated, that Bad expression enhances tumor growth in prostate cancer cells. [173] Different expression of Bid and Bad proteins in heperplastic and cancerous endometrium could be a good marker for diagnosis determination, however it needs a further research.

Information on PARP expression in endometrial tissues is also limited. Although there are studies carried out on another tissues, which indicate change of PARP expression in neoplastic transformation and its consequent progression. For example, PARP protein lever is higher in malignant cells when compared to normal cells in breast cancer, hepatocellular carcinoma and malignant melanoma. [13], [134], [171], [176] In the human endometrial tissue, Ghabreau et al. found by immunohistochemical staining increasing level of PARP from endometrial hyperplasia to endometrial cancers Grade I and sharply decreses in advanced endometrial cancers. [66] These findings are in good agreement with results in current study, although in present study no statistically relevant changes were oserved. Similar data were also observed in study of Brustmann et al. [19] In their immunostaining study PARP expression pattern increased significantly from normal endometrium via non-atypic hyperplasia to atypic hyperplasia and decreased from atypic hyperplasia to carcinomas. Authors did not find statistically significant difference in PARP expression between atypic hyperplasia and endometrial carcinoma Grade I, and between endometrial carcinoma Grade II and III and serous carcinomas or clear cell carcinomas.

7 Conclusions

Results of this study showed presence of apoptosis in normal, atrophic, hyperplastic and cancerous endometrium. Higher expression level of the main effector caspase in intrinsic apoptotic pathway, pro-caspase-3 and its cleaved active form caspase-3 in hyperplastic and cancerous endometrium. This could be explained by the tissue effort to eliminate pre-neoplastic and neoplastic cells.

Inspite of some discrepancy in results due to usage of different methods, data presented in current study together with the results of other researchers suggest, that pre-neoplastic and neoplastic states of human endometrium are caused not only by failure of apoptotic signalization, but also by several changes in cell proliferation. Observed higher Bcl-2/Bax ratio in cancerous endometrium means inhibited apoptosis and could be one of the mechanism of cancer development. In contrast to that, hyperplastic endometrium has low Bcl-2/Bax ratio. This fact could be also explained as an effort of the cells to undergo apoptosis and eliminate malignant tissue. However, in cancerous endometrium, this effort seems to be overpowered by a strong proliferation signal. Thus, neoplastic progression could be linked with increased proliferation and failure of apoptosis.

Decreased Bid expression could point to some defects in apoptotic program. However, whether Bid expression level is regulated at the point of mRNA or protein remains unknown. It is possible, that mutations in *bid* gene contributes to oncogenesis. On the other hand, expression of Bad reaches its peak in cancerous endometrium. Bad can stimulate tumor growth, hence this could provide selective pressure to increase Bad expression in tumors. [173] Further research including analyzing of mRNA expression and mutations in DNA is needed.

Despite absence of statistically relevant differences of PARP expression in different tissue specimens, PARP level was slightly higher in cancerous endometrium. It could be explained by the function of PARP and its involvement in process of DNA reparation. This explanation is also supported by fact, that PARP level is decreased in advanced endometrial carcinomas. [19]

Current results give a basis for further research. e.g. in the field of mRNA level, possible apoptosis-related genes mutations etc. Also in vitro and in-vivo studies consider treatment of cancerous cells by BH3 mimicking molecules are necessary.

8 Summary

Present study focused on expression level analysis of several proteins involved in apoptotic pathways in human endometrium has yielded following results:

- higher level of pro-caspase-3 and caspase-3 in hyperplastic and cancerous endometrium could signalize tissue effort to eliminate pre-neoplastic and neoplastic cells
- high Bcl-2/Bax for CE can point to deregulation of endometrial tissue apoptotic program, which should eliminate malignant cells
- higher PARP expression in CE and lower in HE, with respect to NE, although we did not observe statistically relevant changes
- trend of Bid and Bad protein expression changes is opposite in HE and CE with respect to NE

Development of neoplasia is a complex process involving both cellular proliferation and cell death. Further research is needed, including different methods and different experimental approaches. It would be extremely important to disclose and define a turnpoint of hyperplasia-to-cancer switch referring to protein life cycle from its transcription to degradation. It would be also important to describe main alterations in different cell cycle programmes and define them in the terms of enhanced enhanced expression of selected protein or protein functional changes, eventually protein higher or lesser degradation rate.

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