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EXPRESE TRAIL A JEHO RECEPTORŮ DR5 A DcR2 V DĚLOŽNÍ TKÁNI POTKANA V PRŮBĚHU BŘEZOSTI

Diplomová práce

ve spolupráci s
UNIVERSIDADE DO PORTO
FACULDADE DE FARMÁCIA
Laboratório de Bioquímica

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PATTERN OF EXPRESSION OF TRAIL AND ITS RECEPTORS DR5 AND DcR2 IN RAT UTERINE TISSUES DURING PREGNANCY

Diploma thesis

in cooperation with
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FACULDADE DE FARMÁCIA
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ABSTRACT

Apoptosis plays a crucial role in rat uterine tissues remodelling, but the exact mechanisms involved in this process are not yet fully understood. Tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) exerts its function as apoptotic factor by binding to its death receptors DR4 and DR5. TRAIL binds also additional receptors – the decoy receptors DcR1 and DcR2 and the soluble receptor osteoprotegerin. Binding of TRAIL to these decoy receptors prevents apoptosis.

In order to clarify if TRAIL apoptotic pathway has any importance in regression of rat uterine tissues, we investigated the pattern of expression of TRAIL and its death receptor DR5 and its decoy receptor DcR2 by immunohistochemistry during gestation.

On day 8 the expression for TRAIL and receptors was observed in antimesometrial decidua, circular muscle layer, some cells from mesometrial decidua and in blood vessels. On day 10 expression of DcR2 in antimesometrial decidua became stronger. Positivity in circular muscle layer and blood vessels decreased but TRAIL and DR5 were at a similar level. On day 12 signalling for ligand and receptors was observed in mesometrial decidua and in metrial gland. However the death receptor expression was very intense in the regions around the maternal arteries invaded by trophoblast.

We observed different localization for ligand and receptors inside the cell. TRAIL was localized in cytosolic vesicles, DR5 was present in cytoplasm and DcR2 had nuclear and cytoplasmic expression.

TRAIL system was expressed from the beginning till the end of pregnancy and this expression was coincident with that of of active caspase-3, an apoptotic marker and with regions presenting cells with apoptotic morphology. These results could suggest an involvement of this system in decidual tissue regression. However on day 10 it was observed a high expression of the decoy receptor DcR2 which competes for TRAIL having an anti-apoptotic effect. Our results suggest that TRAIL has an important function in the beginning of pregnancy and as pregnancy progresses the cells could have protection from apoptosis by higher amounts of DcR2 expressed in some particular regions as decoy receptors compete for TRAIL and can be anti-apoptotic. The role of other TRAIL receptors like DR4 and DcR1 in uterine tissues regression needs to be studied.

ABBREVIATIONS

AIF	apoptosis inducing factor
AMD	antimesometrial decidua
APAF-1	apoptotic protease activating factor-1
BH	Bcl Homologous
BV	blood vessel
CML	circular muscle layer
c-FLIP	cellular FLICE-inhibitory proteins
DcR	decoy receptor
DD	death domain
DED	death effector domain
DISC	death-inducing signalling complex
DR	death receptor
EC	ectoplacental cone
FADD	Fas associated death domain
IAP	inhibitor of apoptosis proteins
LD	lateral decidua
MD	mesometrial decidua
MG	metrial gland
NF-κB	nuclear factor kappaB
NK	natural killer
OPG	osteoprotegerin
PARP	poly (ADP-ribose) polymerase
PBS	phosphate saline buffer
RANK	receptor activator of nuclear factor- κB
RIP1	receptor interacting protein 1
tBid	truncated Bid
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRADD	TNF-receptor associated death domain
TRAIL	TNF-related apoptosis inducing ligand
TRAIL-R	TNF-related apoptosis inducing ligand receptor

1. INTRODUCTION

1.1 CELL DEATH

Cell death is an important process for maintaining cellular homeostasis in organisms. There are two morphologically distinct forms of cell death: apoptosis and necrosis.

Necrosis (“accidental” cell death) is the pathological process which occurs when cells are exposed to extreme variance from physiological conditions (hypothermia, hypoxia), which may result in damage to the plasma membrane. Under physiological conditions direct damage to the plasma membrane is evoked by agents like complement and lytic viruses.

Necrosis begins with an impairment of the cells ability to maintain homeostasis, leading to an influx of water and extracellular ions. Intracellular organelles, most notably the mitochondria, and the entire cell swell and rupture (cell lysis). Due to the ultimate breakdown of the plasma membrane, the cytoplasmic contents including lysosomal enzymes are released into the extracellular fluid. Therefore necrotic cell death is often associated with extensive tissue damage resulting in an intensive inflammatory response (Van Furth and Van Zwet, 1988).

Apoptosis (“programmed” cell death, “cellular suicide”) is the physiological process by which unwanted or useless cells are eliminated during development and other normal biological processes. It is often found during normal cell turnover and tissue homeostasis, embryogenesis, induction and maintenance of immune tolerance, development of the nervous system and endocrine-dependent tissue atrophy. Inappropriate apoptosis, leading to either increased or reduced cell death, has been implicated in many human diseases, including neurodegenerative diseases, such as Alzheimer´s and Huntington´s diseases, autoimmune disorders and some forms of cancer.

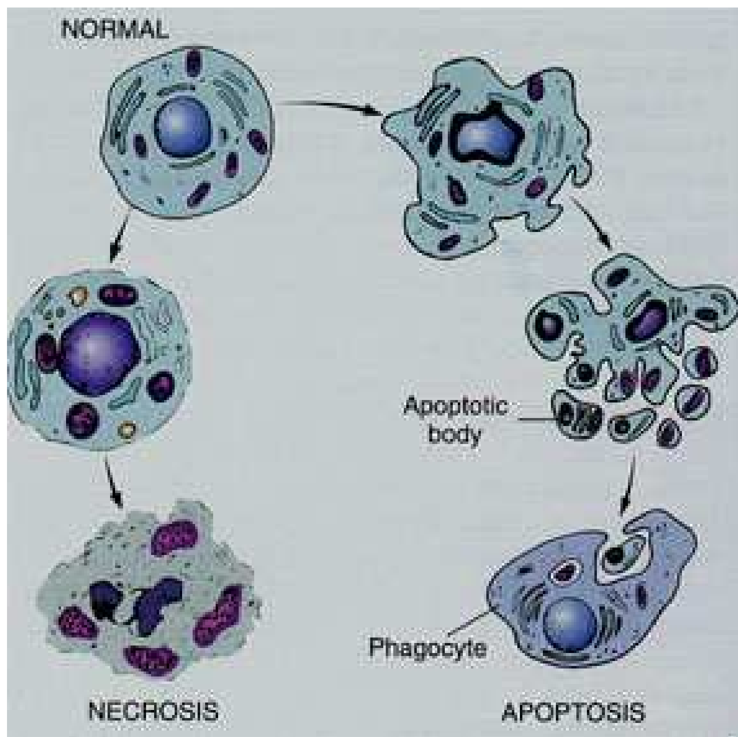

Fig 1. Morphological differences between apoptosis and necrosis (taken from www.cesc.cl).

Apoptosis is also a normal feature in reproductive organs, including the placenta, and play an important role in the normal development, remodelling and aging of the placenta. Decreased levels of the placental apoptosis are associated with pathologies such as pre-eclampsia and intrauterine growth retardation. Cells undergoing apoptosis show characteristic morphological and biochemical features (Cohen, 1993). These features include chromatin aggregation, nuclear and cytoplasmic condensation, partition of cytoplasm and nucleus into membrane bound-vesicles (apoptotic bodies) which contain ribosomes, morphologically intact mitochondria and nuclear material. *In vivo*, these apoptotic bodies are rapidly recognized and phagocytozed by macrophages or adjacent epithelial cells (Savill et al.1989). Due to this efficient mechanism for the removal of apoptotic cells *in vivo* no inflammatory response is elicited.

There are many observable morphological and biochemical differences (Fig.1; Table 1) between necrosis and apoptosis (Vermes and Haanan, 1994).

Table 1: Morphological and biochemical differences between necrosis and apoptosis

NECROSIS	APOPTOSIS
Morphological features	
- loss of membrane integrity - begins with swelling of cytoplasm and mitochondria - ends with total cell lysis - no vesicle formation, complete lysis - disintegration (swelling) of organelles	- membrane blebbing, but no loss of integrity - aggregation of chromatin at the nuclear membrane - begins with shrinking of cytoplasm and condensation of nucleus - ends with fragmentation of cell into smaller bodies - formation of apoptotic bodies - mitochondria become leaky due to pore formation involving proteins of the bcl-2 family
Biochemical features	
- loss of regulation of ion homeostasis - no energy requirement (passive process, also occurs at 4ºC) - random digestion of DNA (smear of DNA after agarose gel electrophoresis) - postlytic DNA fragmentation (= late event of death)	- tightly regulated process involving activation and enzymatic steps - energy (ATP)-dependent (active process, does not occur at 4ºC) - non-random mono- and oligonucleosomal length fragmentation of DNA (Ladder pattern after agarose gel electrophoresis) - prelytic DNA fragmentation - release of various factors into cytoplasm by mitochondria - activation of caspase cascade - alterations in membrane asymmetry (i.e., translocation of phosphatidylserine from the cytoplasmic to the extracellular side of the membrane)
Physiological significance	
- affect groups of contiguous cells - evoked by non-physiological disturbances (complement attack, lytic viruses, hypothermia, hypoxia, ischemia, metabolic poisons) - phagocytosis by macrophages - significant inflammatory response	- affect individual cells - induced by physiological stimuli (lack of growth factors, changes in hormonal environment) - phagocytosis by adjacent cells or macrophages - no inflammatory response

1.2 APOPTOSIS AND APOPTOTIC MECHANISMS

Apoptosis represents a model of genetically programmed cell death and a major mechanism by which tissues removes unwanted, aged or damaged cells (Rakesh and Agrawal, 2005).

The evolutionary conservation of the biochemical and genetic regulation of apoptosis across species has allowed the genetic pathways of programmed cell death determined in lower species, such as the nematode *Caenorhabditis elegans* and the fruitfly *Drosophila melanogaster* to act as models to delineate the genetics and regulation of cell death in mammalian cells. These studies have identified cell autonomous and non-autonomous mechanisms that regulate cell death and reveal that developmental cell death can either be a pre-determined cell fate or the consequence of insufficient cell interactions that normally promote cell survival (Twomey and McCarthy, 2005).

Although cells of mammalian tissues consist of a wide diversity of phenotypes and genotypes, during the development of apoptosis, all cell types undergo similar morphological changes that include chromatin condensation, nuclear fragmentation and cell body shrinkage. Characteristic apoptotic morphology reflects a drastic self-destruction of the cytoskeleton and a catabolism of intracellular macromolecules. Complex interactions between extracellular microenvironment factors and internal gene expression occur prior to the initiation of apoptosis (Rakesh and Agrawal, 2005).

The biochemical events of apoptosis have been divided into two distinct phases: an initial commitment phase, where cells receive a signal that results in commitment to cell death, followed by an execution phase, when all the characteristic morphological and biochemical features of apoptosis occur.

The execution of programmed cell death involves a family of specific apoptotic cystein proteases called caspases (**c**ysteinyl **a**spartate-**s**pecific **p**rote**ases)**. Caspases are synthetized as zymogens (inactive enzyme precursors, which need a biochemical modification to become an active enzyme) with an N-terminal prodomain of different length followed by a large subunit and a small subunit. Caspases play a critical role in the execution phase of apoptosis. “Initiator” caspases, with long prodomains, such as caspases-8, -9 and -10, either directly or indirectly activate “effector” caspases, such as caspases-3, -6 and -7 (Cohen, 1998).

These effector caspases then cleave intracellular substrates, including structural and regulatory proteins such as lamins, cytokeratins and poly (ADP-ribose) polymerase (PARP) and are directly responsible for many of the dramatic morphological features of apoptosis.

Caspase activation can be started by an intrinsic pathway including mitochondrial activation or by an extrinsic pathway through the binding of specific protein ligands to transmembrane receptors (Thonel and Eriksson, 2005).

Procaspase-8 and -10 are initiator caspases in apoptotic pathways initialized by the involvement of death receptors, whereas procaspase-9 is an “initiator” caspase in the mitochondrial pathway (Lavrik et al, 2005).

The activity of caspases is regulated at several levels, including blockage of caspase activation and inhibition of enzymatic caspase activity. Cellular FLICE-inhibitory protein (c-FLIP) proteins are inhibitors of death receptor-induced apoptosis. c-FLIP has three isoforms and under conditions of overexpression, all isoforms inhibit activation of procaspase-8. The IAP (inhibitor of apoptosis proteins) family includes 8 members, which inhibit the initiator caspase-9 and the effector caspase-3 and -7. IAPs add another level of regulation to the intrinsic apoptotic pathway to make sure that unnecessary caspase activation does not occur. On the other hand the activity of IAPs is regulated by Smac/DIABLO and Omi/HtrA2 proteins which are released from mitochondria and inhibits IAPs and thus facilitates caspase activation during apoptosis (Lavrik et al, 2005).

1.2.1 The intrinsic or mitochondrial pathway

The intrinsic cell death pathway is activated by various apoptotic stimuli, such as genomic toxicity, physical signals, such as UV irradiation or oxidative stress. These death signals result in a loss of mitochondrial membrane integrity, release of cytochrome *c* and the subsequent activation of downstream apoptotic pathways. Bcl-2 family members control the integrity of mitochondria, release of many pro-apoptotic molecules from the mitochondria and are main mediators in the intrinsic pathway. Members of Bcl-2 protein family can be either pro- or anti-apoptotic molecules. They are divided into three subfamilies based on function and BH (Bcl Homologous) domain structure. They contain at least one of four conserved BH domains. These play a role in the ability of various family members to interact with each other.

The BH1-4 subfamily contains all four homology domains and all members are anti-apoptotic. The members of this subfamily include Bcl-2, Bcl-x_L and others.

BH1-3 subfamily contains three BH domains. These proteins are pro-apoptotic. The members include Bax and Bak, which are the major cell death executioners in

the intrinsic pathway. These proteins contain three BH domains. Their function is performed by forming pores in the outer membranes of mitochondria, which leads to the loss of mitochondrial integrity and the release of pro-apoptotic factors like cytochrome *c*, SMAC/Diablo, Omi/HtrA2, DNase endonuclease G and apoptosis inducing factor (AIF). These pro-apoptotic molecules activate downstream apoptotic machinery. When cytochrome *c* reaches the cytoplasm, it binds to apoptotic protease activating factor-1 (Apaf-1) and pro-caspase-9, giving rise to the apoptosome with the help of dATP. The formation of the apoptosome leads to the cleavage of pro-caspase-9 and the activation of caspase-9. Active caspase-9 then activates downstream caspases-3, -6 and -7, which in turn cleave several cellular components and result in irreversible cell death.

DNase endonuclease G and AIF activate caspase independent pathways leading to apoptosis. Once released from the mitochondria, these proteins translocate into the nucleus to initiate chromatin degradation and apoptosis.

In the BH3-only subfamily all members are pro-apoptotic by either promoting the function of Bax and Bak or inhibiting the function of anti-apoptotic Bcl-2 family members. They contain one BH3 domain. BH3-only proteins include Bad, Bid, Bik and Bim. They function as guard instead of direct executors of cell death. Bid functions as a linker between the extrinsic and intrinsic cell death pathways (Zhang and Hartig, 2005).

It has been also proposed that the anti-apoptotic members bind to the pro-apoptotic type through their BH domain to block their action. The balance between the anti-apoptotic and pro-apoptotic Bcl-2 family members seems to be critical to determine cell death or cell survival.

1.2.2 The extrinsic or death receptor mediated pathway

The tumor necrosis factor (TNF) superfamily and TNF receptor superfamily (TNFR) are protein families involved in various biological processes. About nineteenth members of the TNF superfamily and thirty two members of TNFR superfamily have been identified in the human genome. These members are widely expressed in the immune system and actively involved in inflammation, adaptive immunity, lymphoid organogenesis and immune homeostasis. Many components of the TNF and TNFR superfamilies have been chosen as therapeutic targets or potential targets for the treatment of a variety of different human diseases, such as autoimmunity, osteoporosis and cancer (Zhang et al, 2005).

There have been identified six main broadly expressed death receptors (DRs): tumor necrosis factor receptor (TNFR), Fas receptor (Fas), DR3, TNF-related apoptosis-inducing ligand receptor (TRAILR1 or DR4, and TRAILR2 or DR5), and DR6. All of these DRs are characterized by a conserved death domain (DD), which works as a protein-protein binding unit that is essential to transport the apoptotic signal. The ligands for these receptors are from the family of related cytokines, the TNF family, including TNFα, lymphotoxin (LTα), Fas-ligand (FasL), Apo-3 ligand (Apo-3L), and TRAIL. They act in an autocrine or paracrine way and after binding, trigger the oligomerization of their respective receptors, an event required to start the apoptotic signalling (Thonel and Eriksson, 2005).

After ligand binding to death receptors, the DD mediates interaction with other DD-containing adaptor proteins with high specifity. There are two main DD-containing adaptor proteins involved in death receptor signalling: Fas associated death domain (FADD), which binds to Fas, TRAIL-R1 and TRAIL-R2 and TNF receptor associated death domain (TRADD), which preferentially binds to TNFR1, DR3 and DR6 (Zhang et al, 2005).

Triggering of death receptors by their ligands result in the formation of the death-inducing signalling complex (DISC). DISC consists of oligomerized receptors, the DD-containing adaptor molecule (FADD or TRADD), 2 isoforms of procaspase-8, procaspase-10 and the cellular FLICE-inhibitory proteins (c-FLIP). The DISC formation makes an assembly of procaspase-8 and -10 molecules in close nearness to each. It is believed that proximity, high local concentration and orientation of procaspase-8 and -10 molecules at the DISC lead to their autoproteolytic activation. (Procaspase-9 is activated by similar way on apoptosome in the intrinsic pathway.)

There are two types of signalling pathways in death receptor-mediated apoptosis. Type I cells are characterized by high levels of DISC formation and enhanced amounts of active caspase-8. Activated caspase-8 leads to the activation of downstream effector caspase-3 and -7. In type II cells there are lower levels of DISC and caspase-8 and the signalling needs an additional amplification loop and the engagement of the intrinsic pathway which involves the cleavage of the Bcl-2 family protein Bid by caspase-8 to generate tBid and a subsequent tBid-mediated release of cytochrome *c* from mitochondria. The release of cytochrome *c* results in the formation of apoptosome, followed by the activation of procaspase-9, which in turn cleaves downstream effector caspase-3 and -7 (Lavrik et al, 2005).

1.2.2.1 Fas mediated cell death

Fas (CD95) and FasL (CD95L) have been extensively studied in the immune system. It is well-known that Fas-FasL interactions play an essential role in maintaining homeostasis in this system. The Fas receptor activates apoptosis by recruiting a number of adaptor, signalling, and effector proteins. Together, they form a protein complex called the death-inducing signalling complex (DISC). The congregation of the DISC is triggered by Fas-receptor oligomerization that allows binding of adaptor protein FADD, which contains one death domain (DD) and one death effector domain (DED). FADD in turn recruits and allows the self-activation of procaspase-8. Active caspase-8 activates a downstream caspase cascade and leads to cell death (Fig.2) (Zhang et al, 2005; Thonel and Eriksson, 2005).

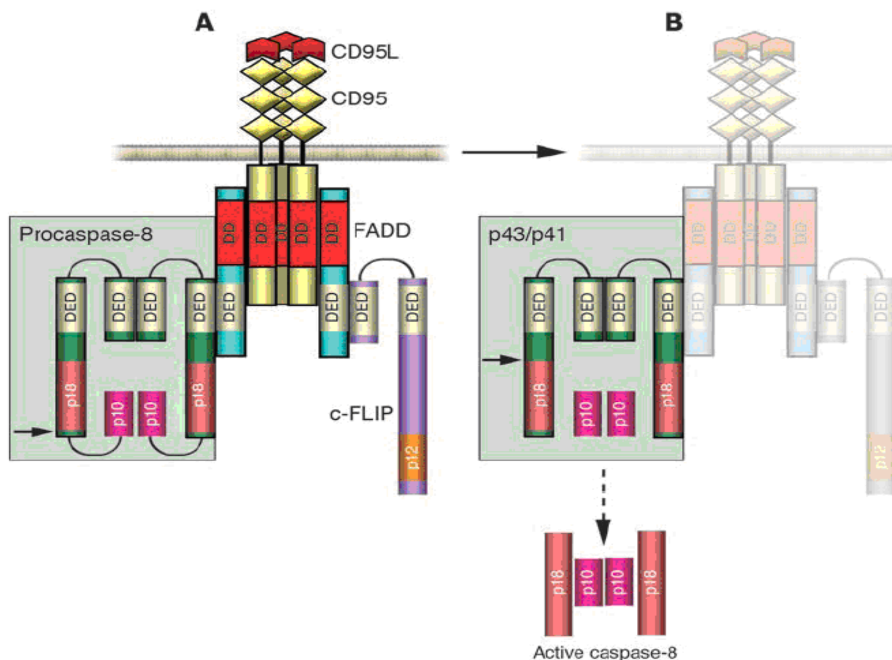

Fig 2. Scheme of procaspase-8 activation at the CD95 (Fas) DISC. CD95 DISC formation is triggered by extracellular cross-linking with FasL, which is followed by oligomerization of the receptor. FADD is recruited to the DISC by DD interactions, while procaspase-8 and c-FLIP proteins are recruited to the DISC by death effector domain (DED) interactions. After recruitment to the DISC, procaspase-8 undergoes activation by forming dimers (taken from Lavrik et al., 2005).

1.2.2.2 TNFR1 mediated cell death

Upon TNF binding, TNFR1 is capable of activating two different pathways, one that leads to the induction of apoptosis and one that leads to the activation of the nuclear factor kappa B (NF-κB) and subsequent cell survival. In several cells under normal conditions,

TNFR1 signalling induces an inflammatory response through NF-κ B. NF-κB target genes are very diverse. This transcription factor regulates expression of the genes which are involved in immunity and inflammation, cell proliferation and apoptosis, as well as genes that encode negative regulators of NF-κB. . NF-κB activates the transcription of c-FLIP, anti-apoptotic Bcl-2 family members and IAPs (Zhang et al, 2005). NF-κB is activated in response to several external stimuli, including interleukins, growth factors, viral and bacterial infections, and physical factors (e.g. UV light). Constitutively activated NF-κB has been associated with many aspects of tumour development, including promoting cancer cell proliferation, preventing apoptosis, and increasing a tumour's angiogenic and metastatic potential. Dysregulation of this transcription factor can lead to inflammatory and autoimmune diseases (Moynagh, 2005). NF-κB has an antioxidant activity and many cancer cells use it to achieve resistance to anticancer drugs, radiation and death cytokines (Luo et al, 2005).

When protein synthesis is blocked or NF-κB signalling is inhibited, TNFR1 mediated pathway becomes potently apoptotic. TNF-mediated apoptosis differs from that reduced by Fas in that TNFR1 initially recruits a different adaptor protein, TNF receptor-associated DD protein (TRADD) which is then believed to recruit FADD, thereby recruiting and activating the effector caspases.

TRADD acts as a platform for recruitment into the TNFR1 signalling complex of other signalling intermediates, such as receptor interacting protein (RIP), a DD-containing kinase, and TNF receptor-associated factor 2 (TRAF2). TNF induced signalling is believed to diverge at this point. TRAF2/RIP recruitment leads to activation of downstream kinases in the NF-κB pathway, whereas FADD recruitment leads to apoptosis (Harper et al, 2003).

1.2.2.3 TRAIL mediated cell death

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the membrane bound TNF family of cytokines, which can induce apoptosis in various tumor cells by engaging the death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2), but spare most normal cells. Two additional receptors DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4) lack functional death domains and act as decoy receptors for TRAIL (Vindrieux et al, 2005).

Preclinical studies in mice and non-human primates have shown the potential utilization of recombinant soluble TRAIL and agonistic anti-DR4 or DR5 antibodies for cancer therapy. In cancer patients, phase 1 and 2 clinical trials using agonistic mAbs that engage TRAIL receptors DR4 and DR5 have also provided encouraging results. It is now

evident that TRAIL suppresses autoimmune diseases in various experimental animal models, suggesting that the therapeutic value of the recombinant TRAIL and agonistic DR4 and DR5 antibodies might also extend to the suppression of autoimmune diseases (Cretney et al, 2005).

While the activity of TRAIL as an anti-tumour agent has been pursued, the true function of TRAIL under normal physiological conditions remains unanswered. TRAIL is highly homologous to cytotoxic Fas-ligand. Crystal structures have shown that, like other TNF ligands, it occurs like a trimer (Fig.3).

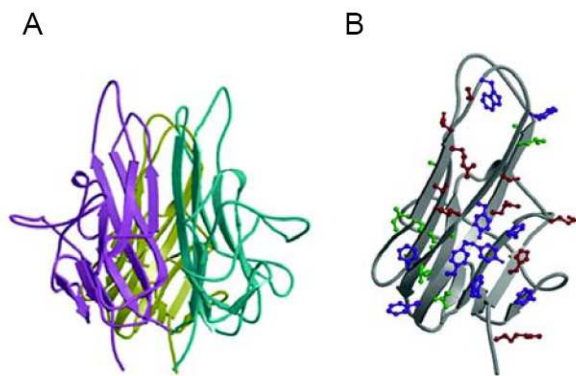

Fig 3. Crystal structure of TRAIL. (A) Structure of the TRAIL trimer. (B) Structure of TRAIL monomer showing the residues included in trimerization (taken from Kimberley and Screaton, 2004).

TRAIL can be cleaved from the membrane by cysteine proteases to create a soluble form of the ligand. Its main function is to induce apoptosis or activate the transcription factor NF-κB. Although TRAIL mRNA is expressed in a broad variety of normal tissues, the expression of functional TRAIL protein appears to be limited to immune cells, including T cells, NK cells, monocytes, dendritic cells, neutrophils, hepatocytes or cancer cells.

The cytoplasmic parts of both death receptors DR4 and DR5 contain a death domain similar to Fas and TNF-R1. TRAIL trimer cross-links three receptor molecules of DR4 or DR5 on the surface of target cells, which leads to formation of death-inducing signalling complex (DISC). The trimerization of the death domains results in recruitment of adaptor protein FADD, which in turn activates caspase-8 (Fig.4).

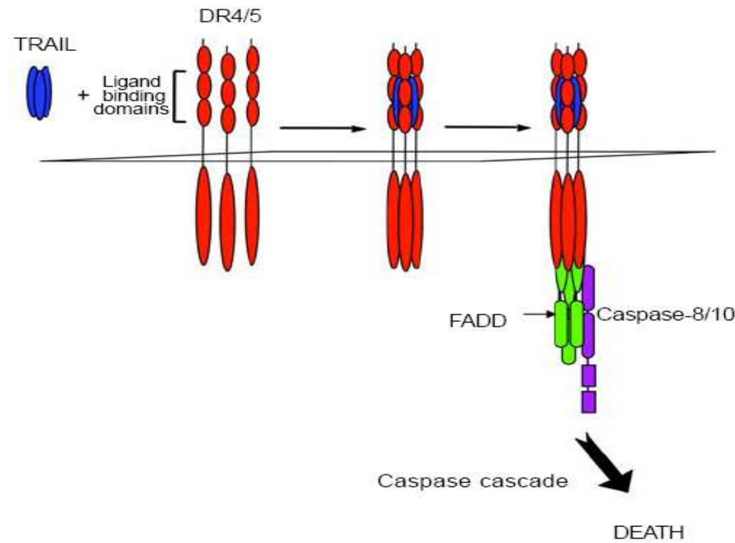

Fig 4. The TRAIL ligand induced trimerization of the receptor. The trimeric ligand puts three receptors together into a complex. Such a induced position of the intracellular domains triggers recruitment of the signalling molecules inside the cell and leads to caspase cascade and cell death (taken from Kimberley and Screaton, 2004).

In type I cells activation of caspase-8 is sufficient for subsequent activation of the effector caspase-3 to execute cellular apoptosis, while in type II cells amplification through the mitochondrial pathway is required (Fig.5).

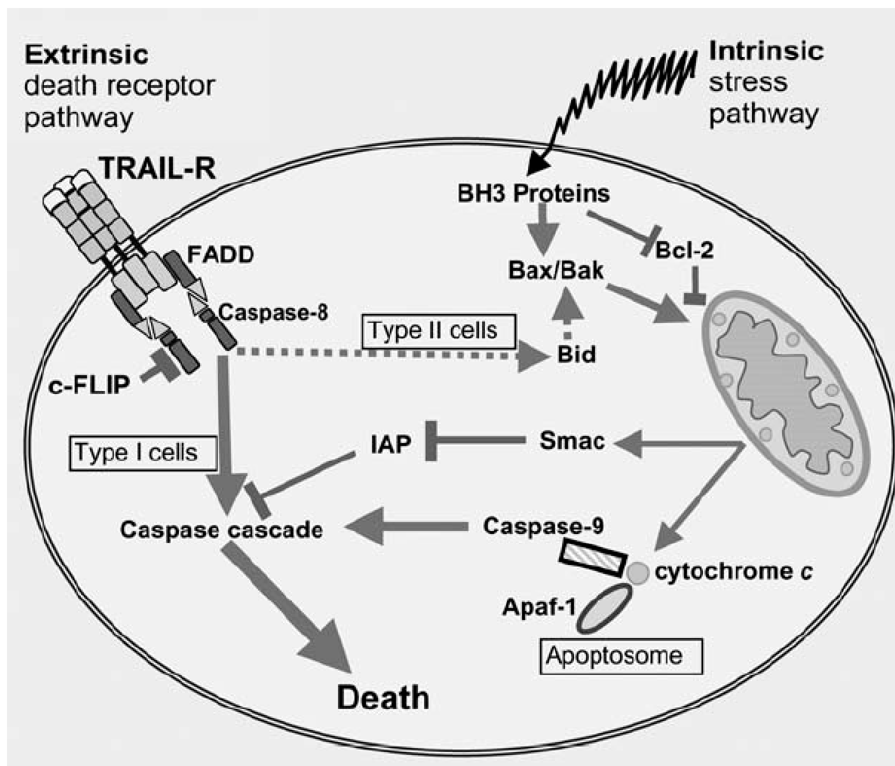

Fig 5. TRAIL-induced apoptosis signalling pathways. Trimerization of DR4 or DR5 by a TRAIL trimer leads to recruitment of an adaptor protein FADD, which in turn activates caspase-8. In certain cell types (type I), activation of caspase-8 is sufficient for activation of caspase-3, which executes apoptosis (extrinsic pathway). In other cell types (type II), amplification through the mitochondrial pathway, which is initiated by cleavage of Bid by caspase-8 and translocation of truncated Bid to mitochondria, leading to Bax/Bak mediated release of cytochrome-*c* and thereby caspase-9 activation by Apaf-1, is required for the caspase-3-mediated cellular apoptosis (intrinsic pathway). As potential resistance mechanism c-FLIP can prevent the recruitment of caspase-8. Bcl-2 can suppress the Bax/Bak mediated release of cytochrome-*c* and Smac/DIABLO from mitochondria. IAPs can attenuate the activities of caspase-9 and -3, although Smac/DIABLO can counteract IAPs (taken from Cretney et al, 2006).

The decoy receptor DcR1 is a glycosylphosphatidylinositol (GPI)-anchored membrane protein, which does not contain the DD and does not signal apoptosis. The decoy receptor DcR2 contains a truncated death domain, which also does not signal apoptosis (Fig. 6).

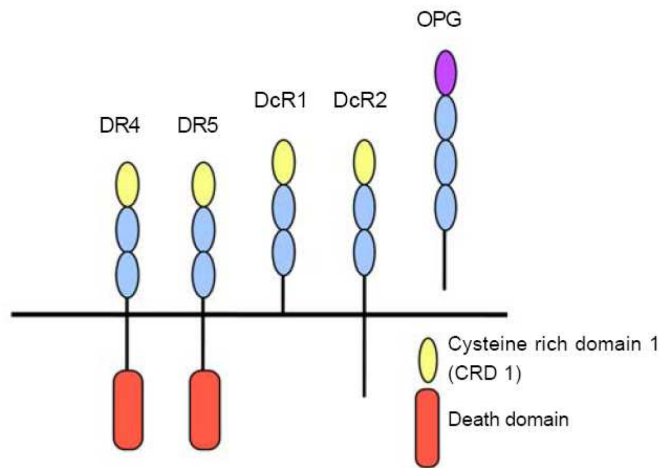

Fig 6. Scheme of the five TRAIL receptors. The extracellular cysteine-rich domains are displayed by yellow and blue ovals, the death domain is represented in red (taken from Kimberley and Screaton, 2004).

It is believed that they can act as decoy receptors competing for TRAIL with death-inducing DR4 and DR5 receptors. It is also important that the cytoplasmic regions of DR5 and DcR2 contain potential TRAF-binding motifs, which could be responsible for NF-κB activation. Although DR4 and DR5 transcripts and TRAIL mRNA are expressed in many tissues, most normal cells are resistant to apoptosis induction by this ligand. It has been therefore suggested that DcR1 and DcR2 receptors may contribute to the physiological resistance to TRAIL. In contrast, several tumor cell lines express DR4 and DR5, but little DcR1 and DcR2 proteins, suggesting that cancer cells could be more sensitive to the TRAIL apoptotic signal.

In addition to TRAIL-specific receptors, osteoprotegerin (OPG), which is a soluble inhibitor of receptor activator of nuclear factor kappa B (RANK) ligand and regulator of osteoclastogenesis, also bind to TRAIL in humans and mice, and may serve as a soluble decoy receptor for TRAIL when over-produced (Yagita et al, 2004; Kimberley et al, 2004; Vindrieux et al, 2005).

1.3 MORPHOLOGY OF RAT UTERUS AND CHANGES OF UTERINE TISSUES DURING PREGNANCY

1.3.1 Morphology of rat uterus

The rat uterus is a heterogeneous organ composed of three layers, the endometrium, myometrium and perimetrium or external serosa. The myometrium is the muscular part of the uterus and it consists of an inner layer of circular and an outer layer of longitudinal muscle fibers. The endometrium is composed of a luminal epithelium, which lines the uterine lumen, glandular epithelium which invaginates into the stroma and fibroblastic stromal cells which form a matrix for supporting of the glands. Both the structure and the function of the endometrium is under the influence of ovarian steroid hormones progesterone and oestrogen. In rodents and primates these stromal cells are transformed into decidual cells in the beginning of pregnancy.

1.3.2 Implantation and decidualization

Implantation is a complex sequence of events that begins with the acquirement of a fixed position of the blastocyst in the uterus as a result of its attachment to the luminal epithelium. In rodents and other mammals that develop hemochorial or endotheliochorial placentae, this attachment is followed by invasion of the endometrium by the trophoblast cells of the blastocyst. During early pregnancy in laboratory rodents the appearance of the uterine lumen changes from an irregular shape to a lumen with mucosal crypts that possesses a mesometrial-antimesometrial orientation. The blastocyst usually attaches to the uterine epithelium on days 4-6 of pregnancy on the antimesometrial side. Implantation is accompanied by morphological and biochemical changes in the endometrium. These consist mainly of modifications in the shape, organization, and metabolism of the stromal cells of the endometrial connective tissue, that undergo proliferation and differentiation leading to the formation of a distinctive form of tissue called the decidua. Decidualization can be also induced artificially in the absence of the blastocyst, resulting in the formation of a tissue (called deciduoma) which is very similar to true decidua. Decidualization may be induced by prostaglandins, leukotrienes, platelet-activating factor and a complex network of cytokines and growth factors (Salamonsen et al, 2003). Several functions for decidua have been proposed, including physical barrier to trophoblast invasion, an immunological barrier that

functions to prevent rejection of the embryo, supplying nutrients, and producing hormones and other specific products (Abrahamsohn and Zorn, 1993; Welsh and Enders, 1985).

After implantation the transformation of the stromal fibroblast cells starts first in a small region and then spreads in a crescent area around the blastocyst forming the antimesometrial decidua. On day 9 decidualization reaches the basal region, adjacent to the circular muscle layer, but by day 11 the antimesometrial decidua regresses completely by apoptosis and secondary necrosis to form decidua capsularis (Correia da Silva et al, 2004). On day 8-9 in the lateral mesometrium proliferation of endothelial cells leads to the formation of large sinusoids. Between the lateral sinusoids and the antimesometrial deciduas, a region in cells rich in glycogen appear forming the glycogenic wing area. After this period mesometrial decidual cells appear in the central region of the mesometrial pole.

On day10 of pregnancy (when decidualization reaches its maximum level of complexity and cell number) there are several types of cells in the rat uterus: the antimesometrial decidual cells, the mesometrial decidual cells, the lateral decidual cells (glycogen-rich cells) and undifferentiated stromal cells. The fully transformed antimesometrial decidual cells are large and rounded and are close together and have often two large oval nuclei with well-developed nucleoli. The blood vessels in this region are fenestrated capillaries. In the mesometrial region there are two types of cells: mesometrial decidual cells and round cells, precursors of the granulated metrial gland cells. Mesometrial decidual cells are smaller, less densely packed and have numerous processes, giving the cells a spiny appearance. The round cells are found initially in the spaces between the decidual cells and from there they migrate toward the mesometrium, where they will form the metrial gland presenting, after maturation, the characteristic granules in the cytosol. They belong to the natural killer (NK) cell lineage and their granules contain perforin and granzyme B. It has been suggested that they may kill aberrant trophoblast cells migrating through the mesometrial pole and have a role in the maternal tolerance of foetal tissues. The middle of the implantation site, between the antimesometrial and mesometrial decidua, the lateral decidua, is occupied by the glycogen-rich cells. These cells contain many organelles and are closely packed. This region has a rich labyrinth of large capillaries.

By day 12 the mesometrial decidua reaches its maximum of development, the ectoplacental cone of the embryo invades the central mesometrial decidua and differentiation of its inner core results in the formation of the definitive placenta. After this period mesometrial decidua regresses by apoptosis and persists till the end of pregnancy as a thin layer of tissue to form the decidua basalis, a part of the definitive placenta (Bell, 1983).

After middle of pregnancy the region between the circular and longitudinal mucle coats give rise to the metrial gland which reaches its maximum development on day 14 of gestation. The most prominent morphological characteristic of this typical rodent tissue is the presence of the NK cells, the granulated metrial gland (GMG) cells. Their number increases rapidly during decidual differentiation reaching a peak of development on days 14-15 of pregnancy. Later they degranulate and display apoptotic features suffering cell death (Delgado et al, 1996). The development and regression of uterine tissues (decidua and metrial gland) is shown in Fig.7 and 8.

The regression of the decidua in rodents takes place in an organized manner. The first cells to die very early in pregnancy, are the antimesometrial decidual cells. These cells are sloughed into the lumen followed by the deeper decidual cells, causing the enlargement of the implantation chamber to accommodate the growing embryo. The dead cells are phagocytosed by the trophoblasts and only a thin layer of antimesometrial decidual cells remains, while the mesometrial cells stay until middle of pregnancy showing degenerative features after day 12 of gestation. It has been showed that apoptosis plays a major role in the decidual cells degeneration (Welsh and Enders, 1985; Correia da Silva et al, 2004). However the exact mechanisms and type of pathways involved remain unknown.

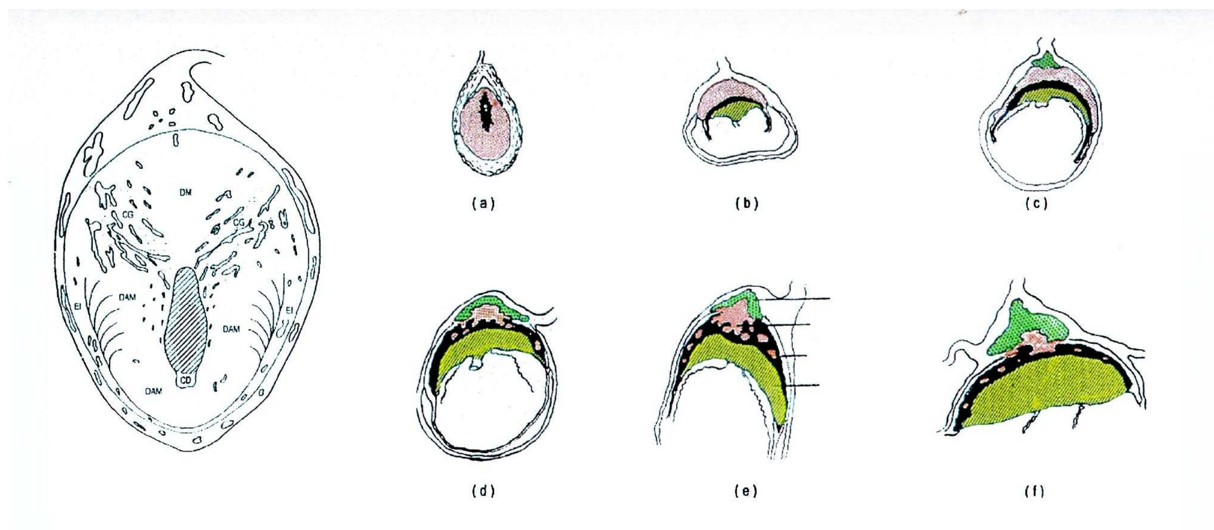

Fig 7. Schematic representation of the alterations that occur in the rat uterus during pregnancy (taken from Welsh and Enders, 1985 and Glasser and Davies, 1968).
AMD – antimesometrial decidua, LW – lateral wings, MD – mesometrial decidua, MT – metrial triangle.

2. AIM OF STUDY

Apoptosis is a significant biological process which plays a key role in the rat uterine tissue development and decidual regression during pregnancy. In response to the implanting embryo the uterine stromal cells undergo proliferation and differentiation forming a new tissue, the decidua which is fundamental for placentation and normal pregnancy outcome. The decidua suffers regression in an organized manner to allow placental development. This process is regulated by many protein molecules, but the whole mechanism is not yet fully understood. One of the proteins that could be involved in apoptotic regression of decidua is TRAIL, a widespread cytokine invoved in apoptosis in various types of tissues. TRAIL performs its function by binding to its two death receptors and in addition it has two decoy receptors. To understand its role in decidua remodelling it was investigated the pattern of expression of TRAIL as well as its receptors DR5 and DcR2 in the maternal rat uterine tissues during pregnancy. For this study an immunohistochemistry technique was carried out. Localization of these proteins may help to assess the function of TRAIL, present in the uterine environment during pregnancy, in reorganization of uterine tissues and may also contribute to a better understanding of the remodelling process that occurs during gestation.

3. MATERIALS AND METHODS

3.1 ANIMALS

Adult Wistar rats (200-250g) were bred under controlled conditions of light (12 hours of dark, 12 hours of light) with free access to water and standard food. Rat females were mated with fertile males and in the morning after mating the presence of spermatozoons in the vaginal smear was marked as the first day of pregnancy.

3.2 MATERIALS

Goat polyclonal antibody TRAIL (K-18): sc-6079, Goat polyclonal antibody DR5 (M-20): sc-19529 and Goat polyclonal antibody DcR2 (D-15): sc-11638 were bought from Santa Cruz Biotechnology, INC, CA, USA. Vectastain ABC-AP kit was purchased from Vector Laboratories, CA, USA. Sigma Fast TM Fast Red TR/Naphtol AS-MX Tablet set (F-4648), 3-amino propyltriethoxysilan (A3648) and Meyer´s Hematoxylin solution were from Sigma-Aldrich, CA, St.Louis, USA. Aquamount Gurr® and DePeX mounting medium were purchased from BHD Laboratory supplies, England.
All the other reagents were from Merck, Germany.

3.3 METHODS

3.3.1 Preparation of tissues

Rats were anesthezized with ether and killed by cervical dislocation. The foetal-placental units from days 8, 10, 12, 14, 16 and 19 of pregnancy were fixed for 24 or 48 hours in 10% buffered formal saline at room temperature. After fixation they were dehydrated and included into paraffin wax (Appendix 3). Serial sections (4µm) were cut on the microtome, through each implantation site in the area containing the embryo and mounted on glass slides coated with 3-aminopropyltriethoxysilan. The slides were deparaffinized and hydrated through a graded alcohols till PBS (Appendix 5).

For the study of the general morphology was used Hematoxylin and Eosin staining (appendix 6).

3.3.2 Immumohistochemical staining

Expression of TRAIL, and the receptors DR5 and DcR2 was analysed using an avidin-biotin alkaline phosphatase complex immunohistochemical technique (Vectastain ® ABC-AP kit) for paraffin sections. Deparaffinized and hydrated sections were incubated for 20 minutes in diluted normal blocking serum from the species in which the secondary antibody was made. After this, the sections were incubated with each primary antibody overnight (for 18 hours) at 4°C. Primary antibodies were diluted in PBS as follows: TRAIL (1:200), DR5 (1:150) and DcR2 (1:400). Then the sections were washed in PBS for 10 minutes and incubated with biotinylated secondary antibody solution for 30 minutes and again washed in PBS for 10 minutes. Afterwards the sections were incubated with Vectastain ABC-alkaline phosphatase reagent for 30 minutes, washed in PBS for the next 10 minutes and in water for 2-5 minutes. The substrate reaction was started with Sigma Fast Red TM tablets, monitorized in the microscope and stopped by washing in running tap water for 5 minutes. The sections were then counterstained with Meyer´s Hematoxylin solution for 3 minutes and mounted with Aquamount improved medium. For negative control was used PBS instead of primary antibody. For experiments were used samples from at least three different animals.

4. RESULTS

The general uterine morphology was studied by Hematoxylin and Eosin staining (Fig.8).

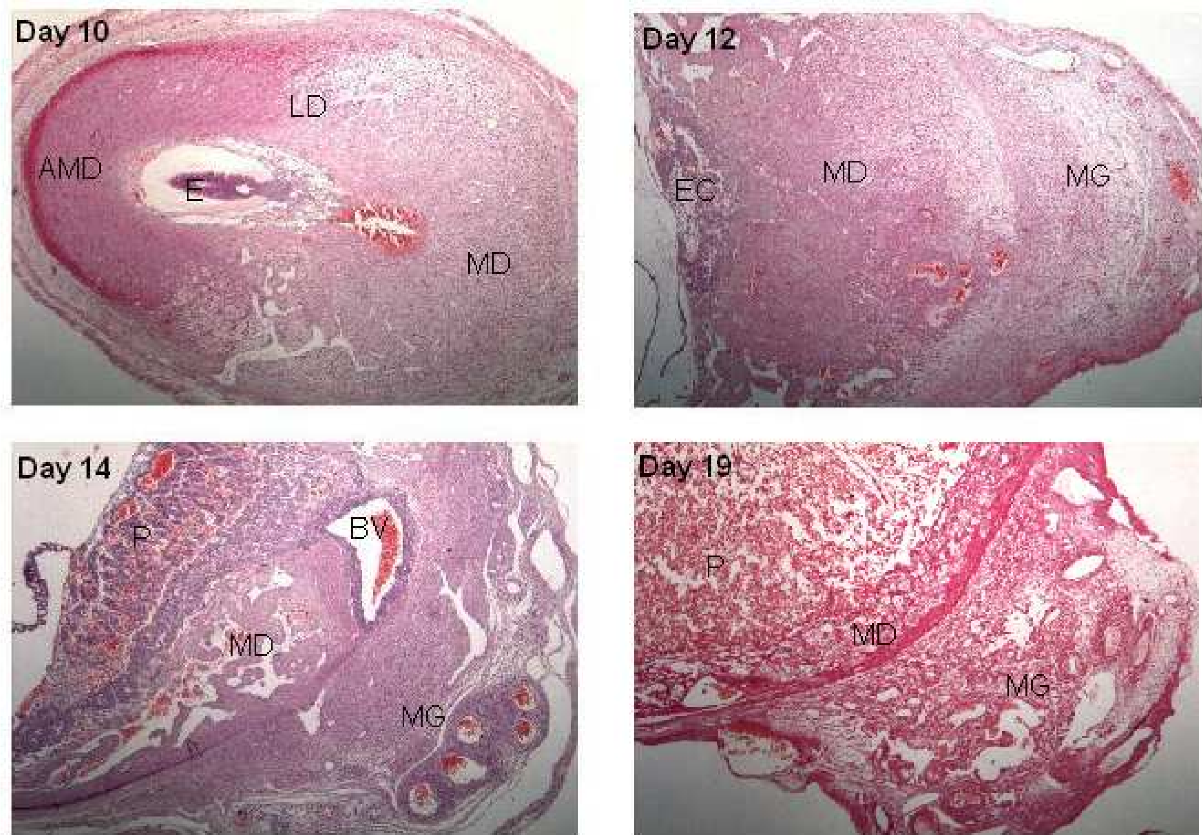

Fig 8. The morphology of rat uterus – development of decidua, metrial gland and placenta during pregnancy. The transverse sections are stained with Hematoxilin and Eosin (original magnification x20).
E – embryo, AMD – antimesometrial decidua, LD – lateral decidua, MD – mesometrial decidua, EC – ectoplacental cone, MG – metrial gland, P – placenta, BV – blood vessel.

The presence of TRAIL, DR5 and DcR2 in rat uterine tissues during pregnancy was investigated by immunohistochemistry.

Day 8 – 10 of pregnancy

From day 8 till day 10 of gestation, the antimesometrial decidua reached its maximum development and the formation of mesometrial decidua was initiated.

During this period it was observed a positive signal for TRAIL mainly in the antimesometrial region and the higher expression could be found in the cells adjacent to the uterine lumen. Also some cells from the lateral decidua were positive. On day 8 expression of TRAIL in blood vessels of certain animals was observable, but not on day 10. Some muscle cells in the circular muscle layer show staining for ligand and receptors on day 8 though the signal decreased on day 10. Expression of TRAIL seems to be situated in cytoplasm (Fig. 9 A), while the nucleus is negative and some cells mainly in antimesometrial area showed vesicular expression of the ligand (Fig. 9 B). It was observed a decrease in expression of TRAIL from day 8 to day 10.

The death receptor DR5 was expressed antimesometrial decidua on days 8 and 10. Positive staining was situated in cytoplasm and the nuclei were negative (Fig. 9 C; Fig. 10 D, E, F). Some empty cells in the glycogenic cells area had, like TRAIL, a positive cytoplasm.

On day 8 the expression for DcR2 could be observed in antimesometrial decidua. The cytoplasm and some nuclei of the decidual cells had a positive signal (Fig. 9 D), while on day 10 most of the cells of the antimesometrial and lateral decidua presented a strong nuclear expression (Fig. 11 C). On day 10 some cells of the mesometrial region, the round precursors of the granulated metrial gland cells were positive (Fig. 11 A, B).

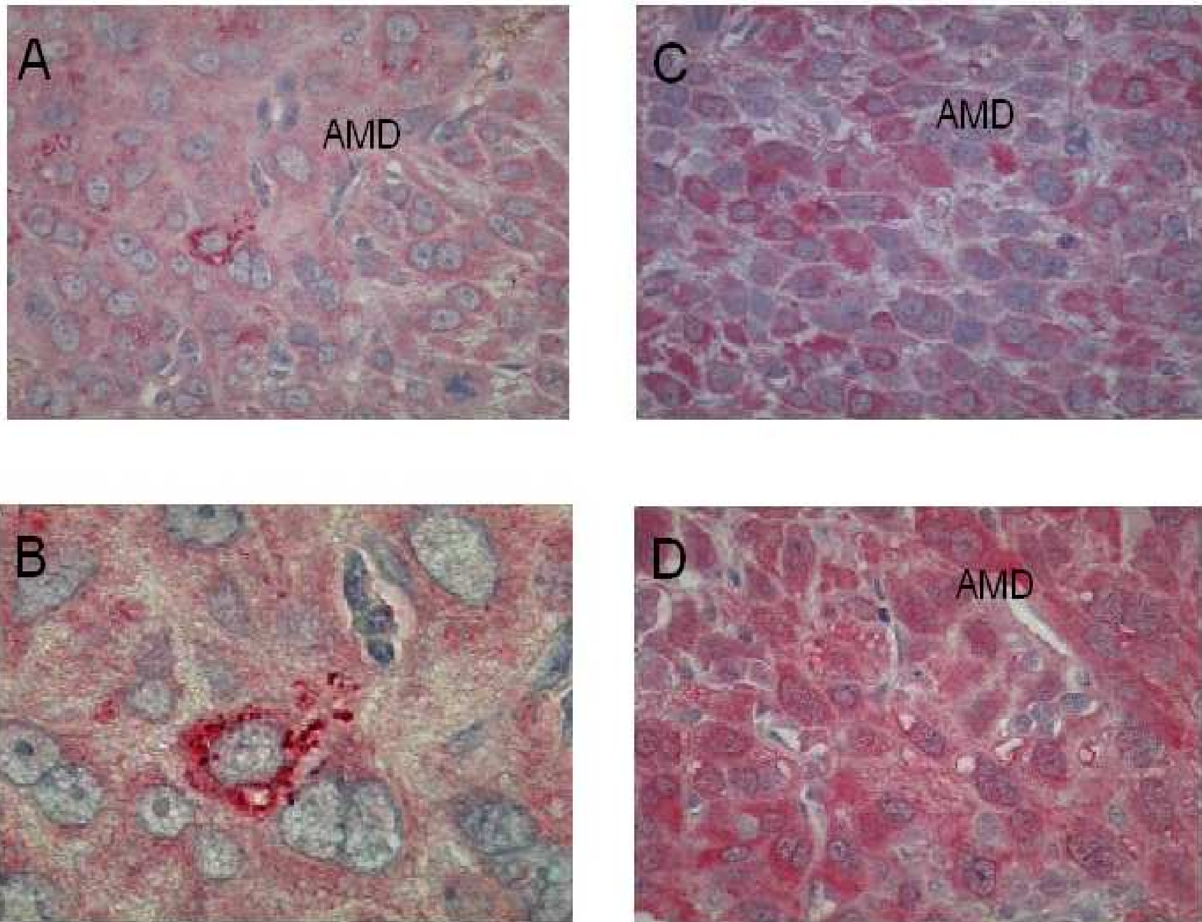

Fig 9. Expression of TRAIL, DR5 and DcR2 in antimesometrial decidua on day 8 of pregnancy. (A) The section is showing TRAIL expression in antimesometrial decidua. The positive staining is situated in the cytoplasm and the nuclei are negative (original magnification x400). (B) Higher magnification of antimesometrial decidual cells. TRAIL presents a vesicular appearance in the cytosol of the cell (original magnification x 1000). (C) DR5 has positive cytoplasmic signal in antimesometrial area, the nuclei are negative (original magnification x400). (D) Expression of DcR2 in antimesometrial decidual cells is different from TRAIL and DR5. Some nuclei start to have positive staining (original magnification x400).
AMD – antimesometrial decidua.

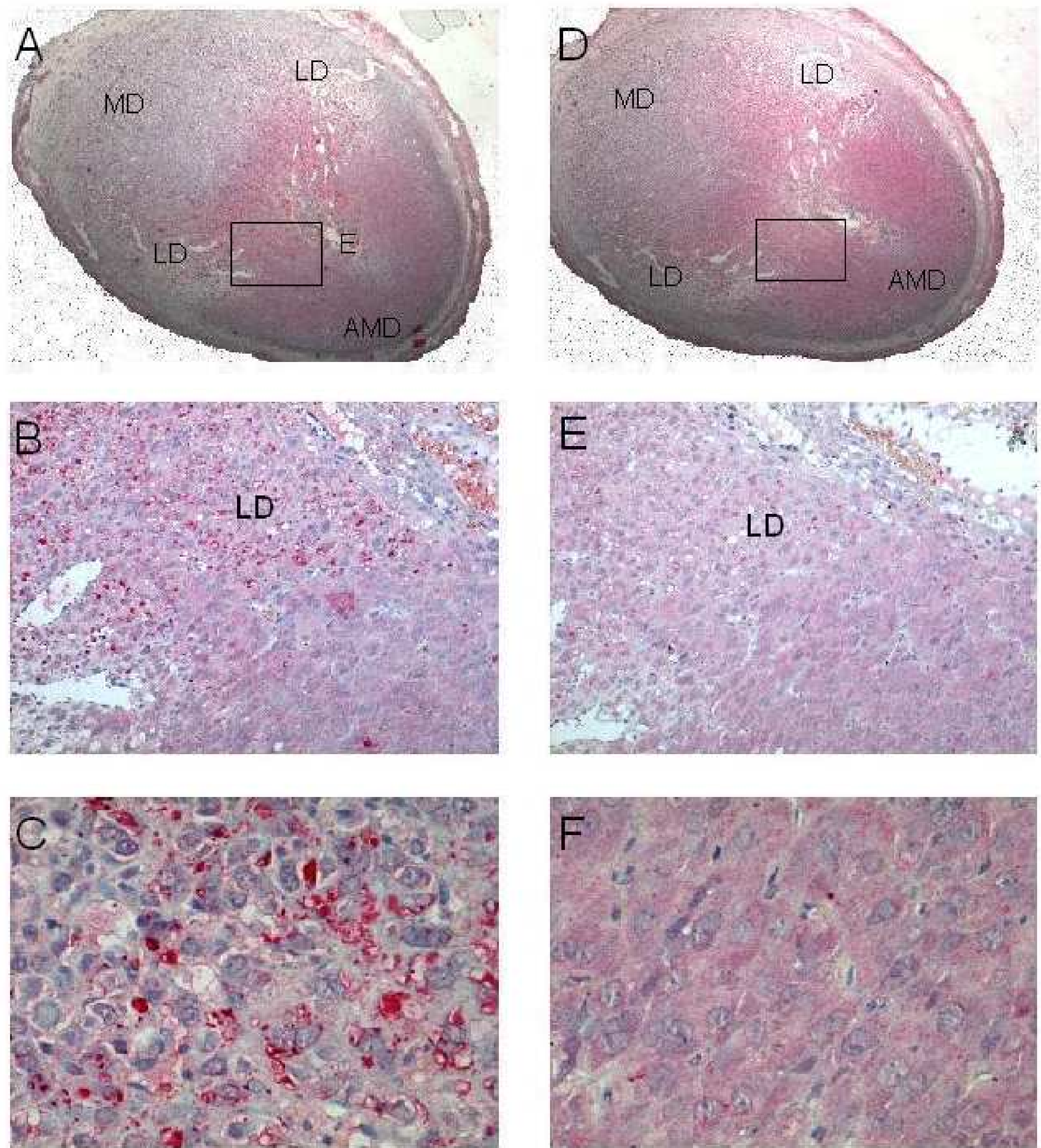

Fig 10. Expression of TRAIL and DR5 in rat uterus on day 10 of pregnancy. (A) The section shows TRAIL expression in antimesometrial decidua and lateral wing area. The staining is spread all over these tissues while the mesometrial decidua is weakly positive. TRAIL expression is more restricted to the region close to the embryo than DR5 and DcR2 (original magnification x 20). (B) Higher magnification of lateral decidua from (A). The glycogenic cells have a positive signal (original magnification x100). (C) Antimesometrial decidual cells present TRAIL expression in cytosolic vesicles (original magnification x400). (D) DR5 expression is localized in antimesometrial, lateral and mesometrial decidua (original magnification x 20). (E) Higher magnification of lateral decidual area from (D). The expression is weaker than TRAIL in the same regions (original magnification x100). (F) Higher magnification of antimesometrial decidua showing the staining in cytoplasm (original magnification x400).

AMD – antimesometrial decidua, E – embryo, MD – mesometrial decidua, LD – lateral decidua.

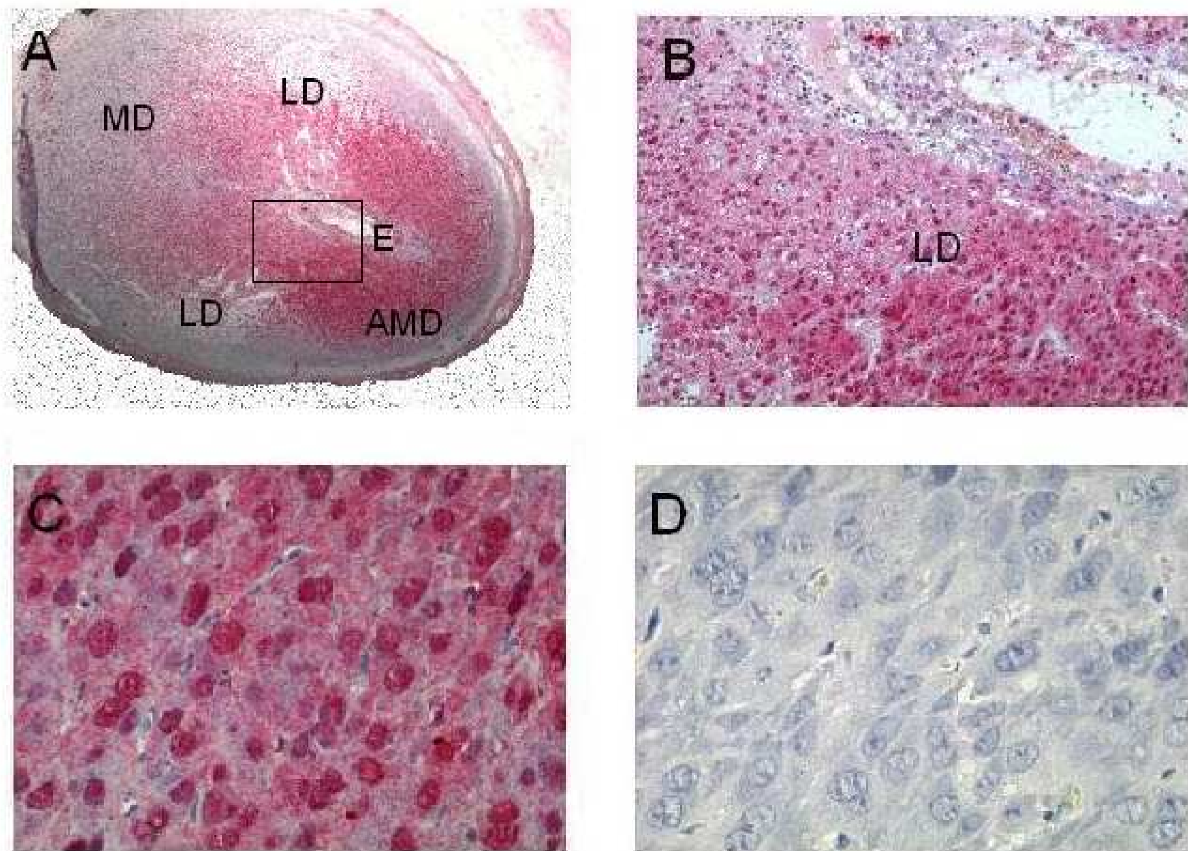

Fig 11. Expression of DcR2 in rat uterus on day 10 of pregnancy. (A) The section is showing positive staining for DcR2, which is present in the antimesometrial, mesometrial and lateral decidua (original magnification x20). (B) Higher magnification shows lateral decidua with typical nuclear staining for DcR2 (original magnification x100). (C) Decidual cells present a positive signalling for DcR2. In the cytosol and in the nuclei the expression is stronger than on day 8 (original magnification x400). (D) Negative control – at a consecutive section with PBS instead of primary antibody (original magnification x400).
AMD – antimesometrial decidua, E – embryo, MD – mesometrial decidua, LD – lateral decidua.

Day 12 – 14 of pregnancy

Between day 12 and 14 the antimesometrial decidua has regressed forming now the decidua capsularis. The mesometrial decidua reaches its maximum development and the placenta starts its formation by invasion of the maternal decidual tissues by the ectoplacental cone. The trophoblast cells make a wave of invasion of maternal arteries (endotrophoblast). The precursors of granulated metrial gland cells become localized to a small area called mesometrial triangle, giving rise afterwards to a structure known as metrial gland.

The immunoreactivity for TRAIL was observable in mesometrial decidua and was restricted to the areas of the decidua near the ectoplacental cone and in the decidual cells surrounding the invaded blood vessels (Fig 12 A and Fig. 14 A). The decidual cells presented the appearance of TRAIL-positive vesicles in the cytosol (Fig. 14 C). Some muscle cells and granulated metrial gland cells in the developing metrial gland had positive staining in their cytoplasm (Fig. 14 B). Some immunoreactivity for TRAIL was observed in the remnant antimesometrial decidual cells.

The expression of DR5 was quite strong in mesometrial decidual cells adjacent to ectoplacental cone on day 12 and in the regions around the trophoblast invaded maternal blood vessels, but became weaker on day 14 of pregnancy (Fig. 12 C and Fig. 14 D). A positive signal for DR5 was detected in the granulated metrial gland, but weaker than TRAIL (Fig. 14 E). The circular muscle layer and the remnant antimesometrial decidua had very low levels of immunoreactivity.

The signal for DcR2 was detected in mesometrial decidua, more pronounced in cells adjacent to ectoplacental cone and was stronger on day 14 than on day 12 of pregnancy (Fig. 13 A and Fig. 15 A). Granulated cells in metrial gland and some cells of the circular muscle layer were positive (Fig. 13 B). Between day 12 and 14 morphological features of apoptosis such as chromatin condensation and apoptotic bodies were found (Fig 15 B).

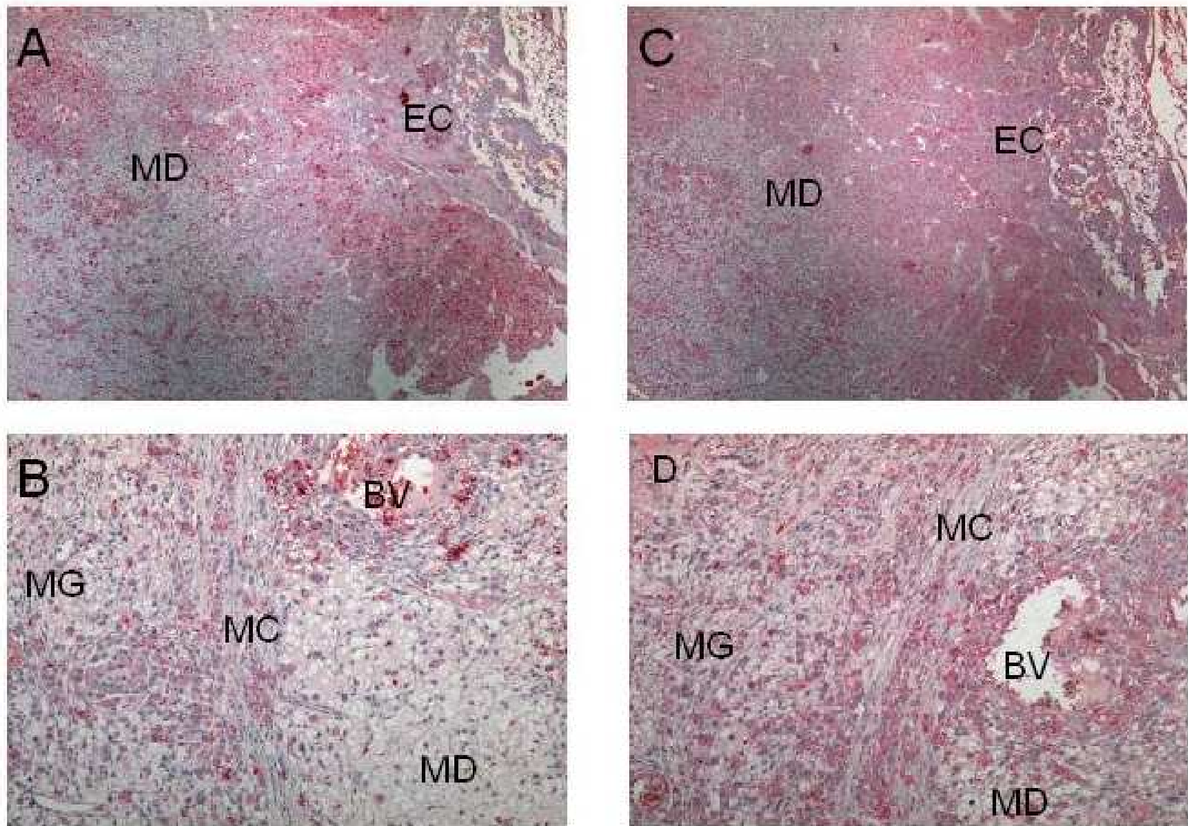

Fig 12. Immunoreactivity for TRAIL and DR5 on day 12 of pregnancy. (A) TRAIL expression was observed in restricted areas in the mesometrial decidua near the ectoplacental cone and the invaded maternal blood vessels (original magnification x40). (B) Higher magnification of (A) showing some cells of the developing metrial gland that have positive signal for TRAIL. The muscle cells in the circular muscle layer have very weak signal (original magnification x100). (C) DR5 expression is present all over the mesometrial decidua. A strong expression was observed near the invaded maternal blood vessels (original magnification x40). (D) Higher magnification from (C) showing the cells in metrial gland and decidua with a positive signal (original magnification x100).

EC – ectoplacental cone, BV – blood vessel, MC – circular muscle layer, MD – mesometrial decidua.

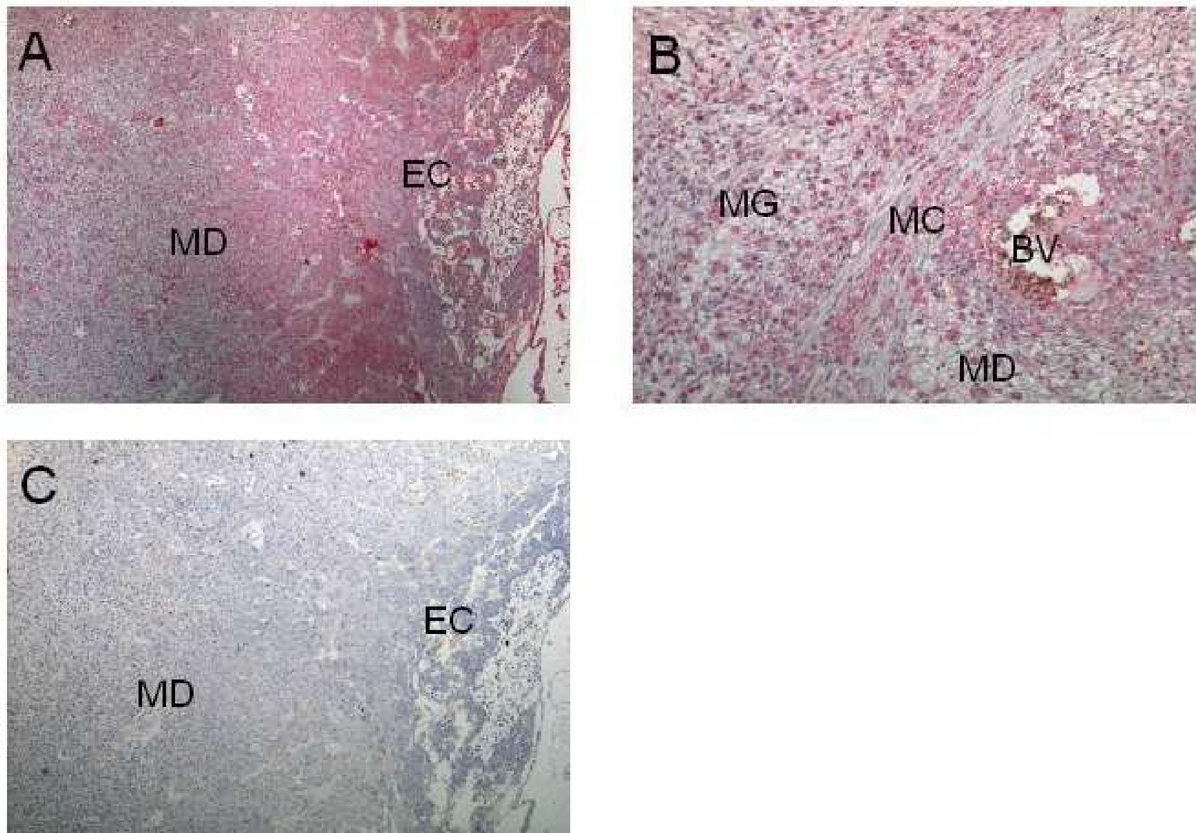

Fig 13. Expression of DcR2 in rat uterus on day 12 of pregnancy. (A) Positive staining for DcR2 in cells adjacent to ectoplacental cone (original magnification x40). (B) Expression in metrial gland cells, mesometrial decidua but not in muscle cells (original magnification x100). (C) Negative control of a consecutive section of the section represented on Fig. A (original magnification x100).

MD – mesometrial decidua, MG – metrial gland, MC – circular muscle layer, BV – blood vessel, EC – ectoplacental cone.

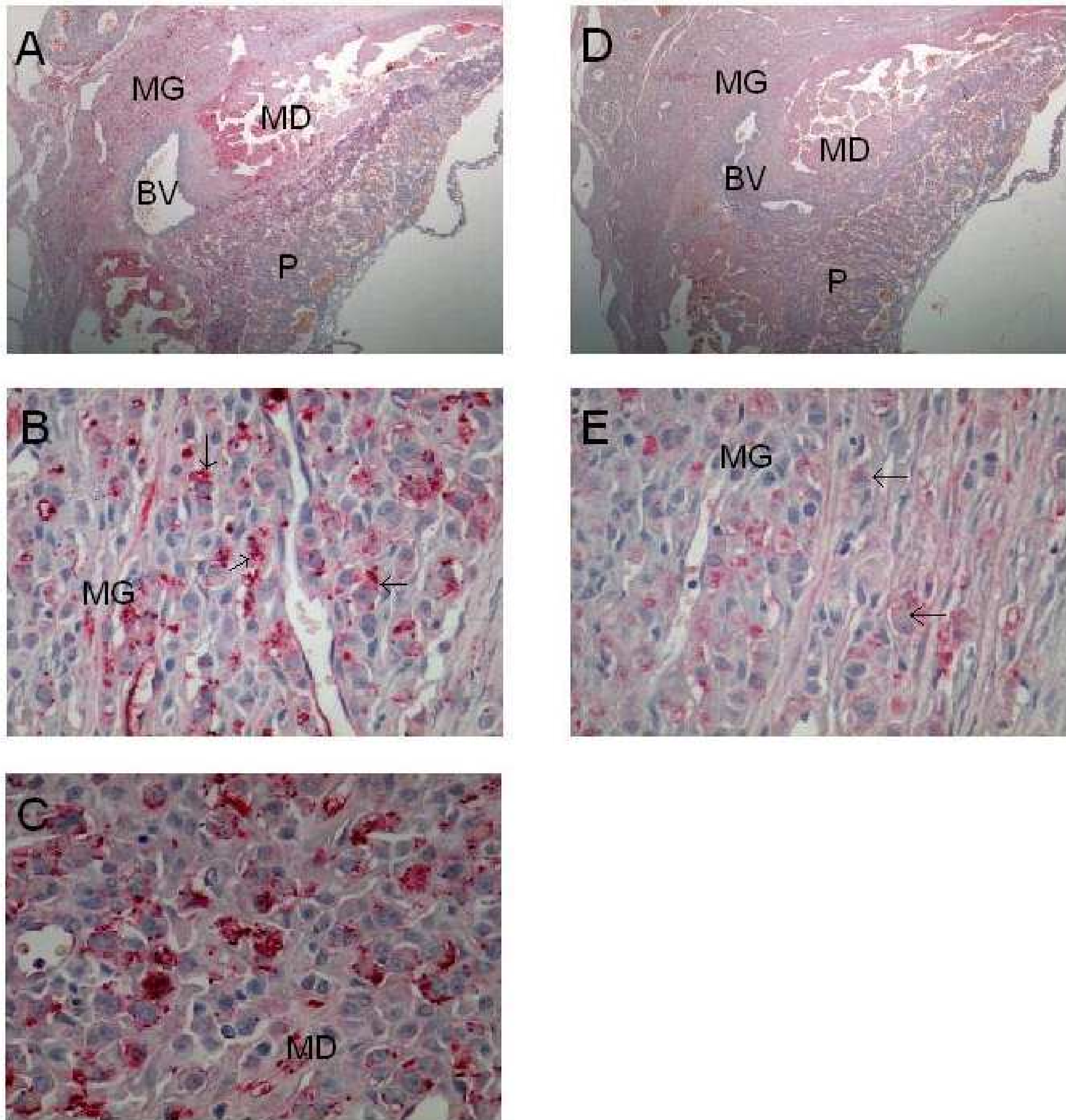

Fig 14. Expression of TRAIL and DR5 in the rat uterus on day 14 of pregnancy. (A) On the picture we can see restricted areas of TRAIL positive staining in mesometrial decidua and metrial gland (original magnification x20). (B) Positive signal for TRAIL in granulated metrial gland cells is marked with arrows. Some muscle cells show positive signal (original magnification x400). (C) Some cells in mesometrial decidua have positive staining inside the cytoplasmatic vesicles (Original magnification x400). (D) The DR5 expression is weaker than TRAIL in mesometrial decidua and metrial gland (original magnification x 20). (E) The arrows show DR5 positive granulated metrial gland cells (original magnification x400).

BV – blood vessel, MD – mesometrial decidua, MG – metrial gland, P – placenta.

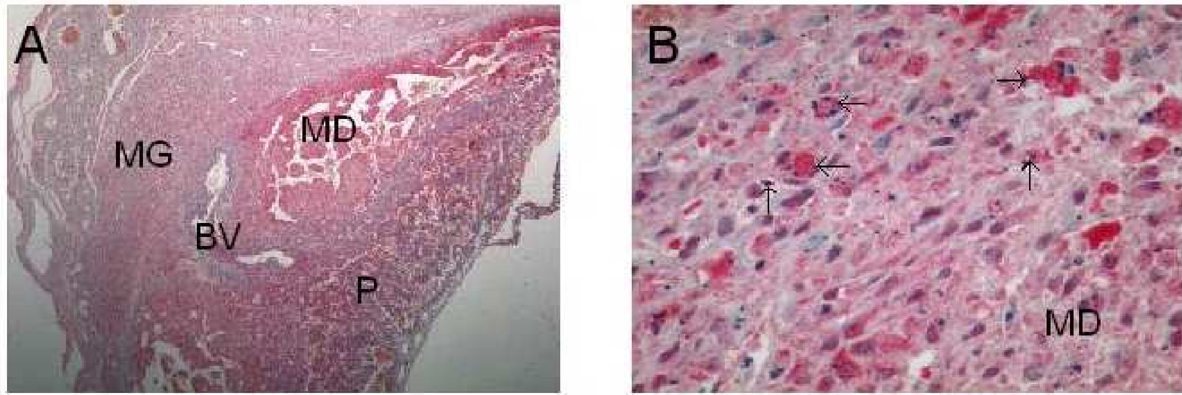

Fig 15. Immunoreactivity for DcR2 in rat uterus on day 14 of pregnancy. (A) Expression of DcR2 is stronger in mesometrial decidua than in metrial gland (original magnification x20). (B) Higher magnification of (A) showing DcR2 positive decidual cells and the presence of apoptotic bodies marked with arrows (original magnification x400).
BV – blood vessel, MD – mesometrial deciduas, MG – metrial gland, P – placenta.

Day 16 – 19 of pregnancy

After day 14 the metrial gland develops while mesometrial decidua regresses giving rise to the decidua basalis. From day 16 till the end of gestation the granulated metrial gland cells degranulate, presenting degenerative changes and near parturition disappear from the uterus.

On day 16 in mesometrial decidua the expression of TRAIL was strong but on day 19 only some cells were expressing the ligand (Fig. 16 A). Some granulated metrial gland cells were positive on day 16, while on day 19 most of granulated cells in metrial gland or their remnants showed positive staining (Fig.16 B). The circular muscle layer was negative.

In this period it was observed a weak staining for DR5 in mesometrial decidua and metrial gland (Fig. 16 C, D). Only some decidual cells and granulated cells were positive. Some muscle cells also showed positive signalling for DR5.

On day 16 DcR2 was expressed in mesometrial deciduas in the cytoplasm and nuclei of the decidual cells, while on day 19 the expression in this area was low (Fig. 16 E). Granulated metrial gland cells (Fig. 16 F) and some fibres in the circular muscle layer were still positive.

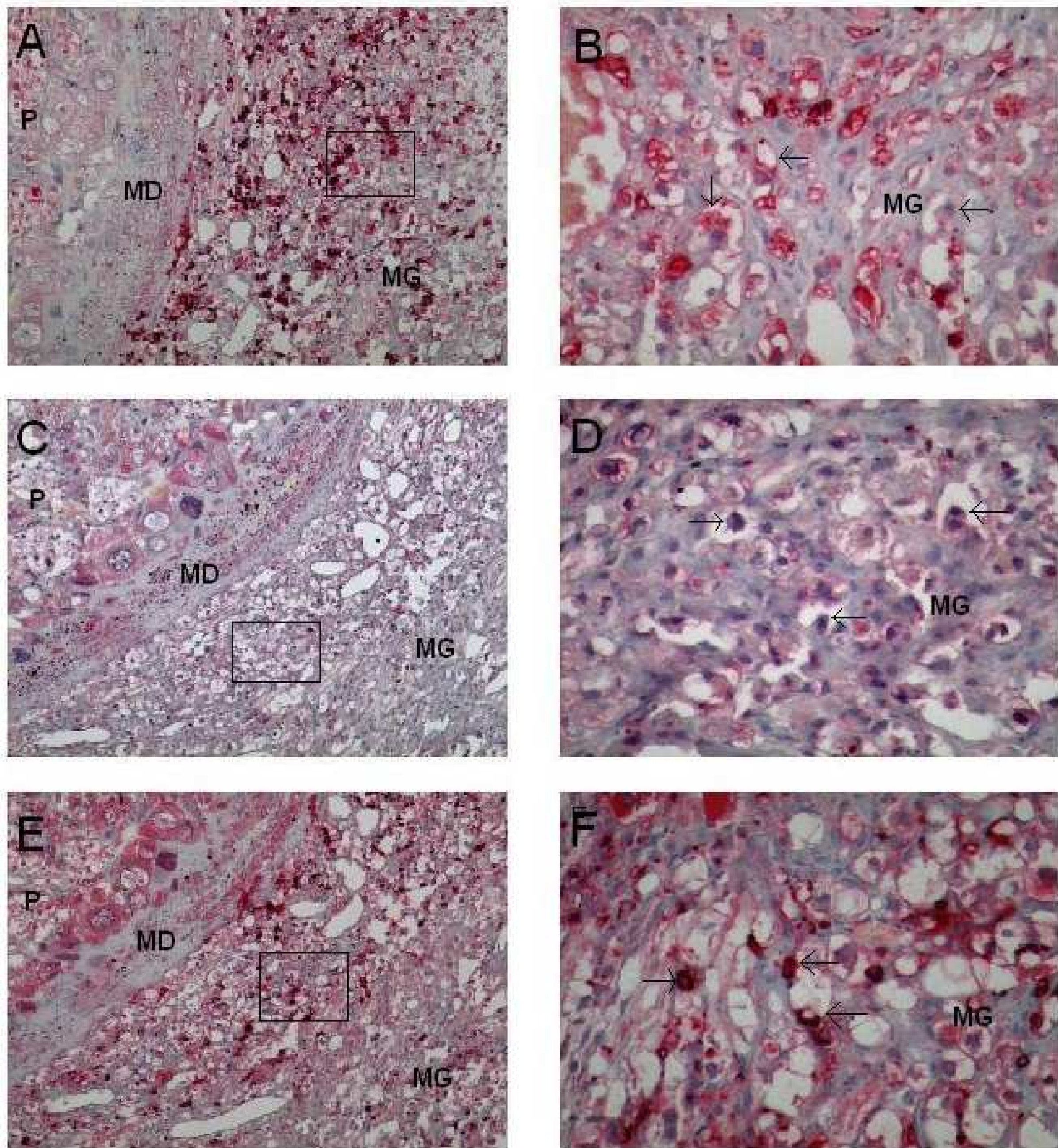

Fig 16. Expression of TRAIL, DR5 and DcR2 in rat uterus on day 19 of pregnancy. (A) TRAIL expression is localized in mesometrial decidual cells and is strong in the metrial gland (original magnification x 100). (B) Higher magnification of the square shown in (A) presenting TRAIL positive granulated metrial gland cells (arrows) or their remnants (empty spaces) (original magnification x400). (C) Weak immunoreactivity for DR5 in metrial gland and mesometrial decidua (original magnification x 100). (D) DR5 expression in granulated metrial gland cells (arrows), the immunoreactivity is lower in comparison with TRAIL and DcR2 (original magnification x 400). (E) Immunoreactivity for DcR2 can be found in mesometrial decidual cells and in most of the metrial gland (original magnification x100). (F) Granulated metrial cells (arrows) have positive signals for DcR2. Some nuclei are also positive (original magnification x400).

MD – mesometrial decidua, MG – metrial gland, P – placenta.

Table 2. Expression of TRAIL, DR5 and DcR2 in rat uterine tissues during pregnancy. AMD – antimesometrial decidua, LD – lateral decidua, MD – mesometrial decidua, CML – circular muscle layer, BV – blood vessels, MG (GC) – granulated cells in the metrial gland; (-) – negative; (+/-) – low levels; (+) – positive; (++) – strong signal.

TRAIL	D8	D10	D12	D14	D16	D19
AMD	+	+	+ / -			
LD	+ / -	+	+ / -			
MD	-	-	+	+ +	+	+ / -
CML	+	-	+ / -	+ / -	+ / -	-
BV	+	-	+ / -	+	-	-
MG (GC)			+ / -	+	+	+ +

DR5	D8	D10	D12	D14	D16	D19
AMD	+ +	+	-			
LD	+	+	+			
MD	+ / -	+ / -	+	+	+	+ / -
CML	+	+ / -	+ / -	+ / -	+ / -	+ / -
BV	+	+ / -	+ / -	-	-	-
MG (GC)			+ / -	+ / -	+	+ / -

DcR2	D8	D10	D12	D14	D16	D19
AMD	+ +	+ +	-			
LD	+	+ +	+ / -			
MD	+ / -	+ / -	+	+ +	+	+ / -
CML	+	+ / -	+ / -	+ / -	+ / -	+
BV	+	+ / -	+ / -	-	-	-
MG (GC)			+ / -	+	+	+ +

5. DISCUSSION

In uterine tissues, remodelling processes, like proliferation, differentiation and cell death, occur simultaneously and at different stages of pregnancy. Decidual cells undergo the differentiation of endometrial stromal cells as a response to the implanting embryo. Decidualization starts on the antimesometrial side spreading to the mesometrial region. Antimesometrial decidua reaches its maximum development by day 10 regressing afterwards by a programmed cell death, while the mesometrial decidua and metrial gland start to regress, respectively, on day 12 and on day 15 of pregnancy. The regression of deciduas, which allows placental development was already studied by many investigators (Bell, 1985; Welsh and Enders, 1991; Correia da Silva et al, 2004). It has been shown that apoptosis plays an important role in this process, however the apoptotic pathways involved in decidual regression are not clear. In some systems induction of apoptosis by TRAIL, a cytokine from TNF family, is believed to be regulated by expression of two death-inducing and two inhibitory (decoy) receptors on the cell surface.

In this work we investigated the expression of TRAIL, the death-inducing receptor (DR5) and the decoy receptor (DcR2) in rat uterine tissues. Our results showed that TRAIL, DR5 and DcR2 were expressed in maternal tissues throughout pregnancy. It is believed that the binding of TRAIL to DR5 can act as an apoptotic factor, while binding of TRAIL to DcR2 can act as a survival factor by competition for TRAIL or via activation of NF-κB. On day 8 of pregnancy ligand and both receptors were expressed in antimesometrial decidua, which regressed after day 10. Since this day the expression of TRAIL and receptors was visible partly in lateral decidua. In mesometrial decidua was observed only some isolated positive cells. On day 10 the decoy receptor was highly expressed in AMD suggesting that some cells are protected against TRAIL mediated apoptosis. On day 12 the staining moves from antimesometrial to mesometrial side and to metrial gland and high levels of TRAIL and DR5 were close to the blood vessels. Till day 12 the expression of TRAIL, DR5 and DcR2 seemed to be on similar level but since day 14 the expression of DR5 was weaker than TRAIL and DcR2 in mesometrial decidua. Some granulated metrial gland cells started to be positive for TRAIL, DR5 and DcR2 on day 12 but since day 14 the expression of DR5 was weaker in comparison to TRAIL and DcR2.

We observed various types of cellular expression of TRAIL and receptors, which differ from each other. TRAIL showed mostly cytoplasmic staining, but in some regions of decidua some cells contained positive vesicles. Cassatella et al. (2005) reported that interferon-

activated neutrophils store a TRAIL intracellular pool and that only minor fraction of the total TRAIL, newly synthetized is released and being the rest retained intracellularly, mainly in secretory vesicles. They demonstrated that the intracellular pool of TRAIL present in INF-pretreated neutrophils is rapidly transferred to the cell surface and can be secreted following exposure to proinflammatory mediators, such as TNFα and others.

Expression of DR5 was cytosolic, but DcR2 had strong nuclear expression in decidua especially on day 10 of pregnancy. Zhang et al. (2000) investigated the localization of TRAIL receptors in human melanoma cells and they found that death receptors DR4 and DR5 are located in the Golgi apparatus, whereas the inhibitory receptors DcR1 and DcR2 are located in the nucleus. After exposure to TRAIL, DR4 and DR5 are transported into endosomes, whereas DcR1 and DcR2 undergo relocation from the nucleus to the cytoplasm and cell membranes. This movement of decoy receptors was dependent on signals from DR4 and DR5, that was shown by blocking with antibodies to DR4 and DR5.

TRAIL and its receptors DR5 and DcR2 can have a role in decidual regression as the rat uterine tissues produce both the ligand and receptors and TRAIL can act in an autocrine or a paracrine manner. The expression of TRAIL and its receptors observed, suggests the involvement of these molecules in the apoptotic pathways linked to the physiological decidual regression. It seems that death receptor DR5 is more important in the first half of pregnancy, so apoptosis could be caused by TRAIL pathway. On the other hand we found also expression of the decoy receptor DcR2, which is very strong on day 10, decreasing on day 12 and increasing again from day 14 till day 19, suggesting that there is a competition between DR5 and DcR2, and TRAIL could function as a survival factor in second half of pregnancy.

TRAIL has been shown to induce apoptosis in a wide variety of cancer cells in vitro and to suppress tumor growth specifically without damaging normal cells and tissues in vivo (Kim et al. 2005). Recent studies had shown that DR4 and DR5 are expressed in some types of normal cells, but they are TRAIL-resistant. The explanation could be that most of normal cells are protected from TRAIL-induced apoptosis by decoy receptors which are broadly expressed in normal tissues, while they are only rarely detectable on the surface of tumor cells. So TRAIL and its receptors could be promising targets for cancer therapy. But it was also found that some types of tumor cell lines are TRAIL-resistant, because there is expression of decoy receptors.

In recent studies in this laboratory it was found the presence of TNFα/TNFR1 and FasL/Fas, suggesting that also other molecules of the death receptor pathways are involved in

regression of rat decidua during pregnancy. The combination of cytokines present in the rat uterine environment can determine cell fate for proliferation, differentiation or death. On the other hand it is also possible that the mitochondrial pathway is involved in this process.

6. CONCLUSION

TRAIL is a cytokine which exerts its function by acting as a pro-apoptotic or as pro-survival factor depending on cell type and cytokine environment. The results of this study suggest a role for TRAIL in decidual remodelling and regression during pregnancy, as decidual cells were TRAIL, DR5 and DcR2 positive, though other factors linked to the death receptor pathway, such as FasL/Fas and TNFα/TNFR1 may be involved. Moreover, the granulated metrial gland cells also expressed TRAIL and its receptors. It can be suggested that apoptosis can be induced in decidua and metrial gland through this signalling pathway on certain days and in particular regions of the uterus. However, some cells can be protected from TRAIL induced apoptosis because they expressed the decoy receptor DcR2. The complexity of the process of uterine tissue remodelling and regression is not yet fully understood and for clarifying TRAIL function it will be necessary to study other TRAIL receptors (DR4 and DcR1). The role of many other factors such as inhibitors and activators of apoptosis remains to be revealed.

7. SUMMARY IN CZECH

Apoptóza je důležitý proces během vývoje deciduálních buněk a jejich zániku a je ovlivňována mnoha faktory. Jedním z nich je i TRAIL a jeho receptory. V naší studii jsme za použití imunohistochemie zkoumali časové a lokální aspekty exprese TRAIL, DR5 a DcR2 v maternální tkáni dělohy potkana v průběhu březosti a snažili se objasnit funkci TRAIL a jeho receptorů v reorganizaci této tkáně.

Buněčná smrt je důležitý děj pro udržení buněčné homeostázy v organismu. Existují dva odlišné typy buněčné smrti: nekróza a apoptóza. Nekróza je patologický proces, který vzniká jako důsledek vnějších vlivů, jako jsou fyzikální, chemické a patologické podněty, a je doprovázena zánětlivou reakcí. Apoptóza (neboli programovaná buněčná smrt) je fyziologický děj, charakterizovaný scvrkáváním buněk, kondenzací chromatinu a tvorbou apoptotických tělísek, které jsou následně fagocytovány sousedními buňkami nebo makrofágy. K zánětlivé reakci tu nedochází. Nerovnováha apoptotických dějů v organismu vedoucí buď ke zvýšené nebo k omezené buněčné smrti může způsobit různá onemocnění, včetně neurodegenerativních a autoimunitních chorob a některých nádorových bujení.

Regulace apoptózy je složitý proces a zahrnuje mnoho různých proteinů. Výkonnými proteiny jsou kaspázy (cysteinyl-aspartát specifické proteázy), které se dělí na dvě skupiny: iniciátorové a efektorové. Iniciátorové kaspázy (např. kaspázy 8, 9 a 10) aktivují efektorové kaspázy (např. kaspázy 3, 6 a 7), které mají funkci rozložit buněčné části. Kaspázy jsou produkovány jako inaktivní proenzymy známé jako prokaspázy. Aktivace kaspáz může být vyvolána dvěma různými mechanismy, a to tzv. mitochondriální (vnitřní) cestou nebo death receptorem zprostředkovanou (vnější) cestou. Obě cesty jsou navzájem propojené, ale mají rozdílné iniciátorové kaspázy. Obě se ale sbíhají na úrovni aktivace efektorové kaspázy 3. Aktivita kaspáz je regulována na několika úrovních, počínaje blokádou aktivace kaspáz a konče inhibicí jejich enzymové aktivity.

Mitochondriální cesta je spuštěna uvolněním proteinů z vnitřního mezimembránového prostoru mitochondrie. Dochází ke ztrátě membránové integrity mitochondrie, tvorbě pórů a k uvolnění cytochromu *c*. Za přítomnosti dalšího faktoru Apaf-1 a ATP se vytvoří apoptozom aktivující kaspázu 9, která štěpí efektorovou kaspázu 3. Hlavními mediátory mitochondriální cesty jsou proteiny z Bcl-2 rodiny, které kontrolují integritu mitochondriální membrány a uvolňování mnoha proapoptotických proteinů. Bcl-2 proteiny mohou být proapoptotické,

schopné vyvolat apoptózu i antiapoptotické, inhibující apoptózu a výsledný efekt závisí na vzájemné rovnováze mezi nimi.

Death receptorová cesta spouští apoptózu za pomoci interakce ligand – membránový receptor patřící do TNF (tumor nekrotizující faktor) skupiny. Zatím bylo identifikováno 32 různých receptorů a 19 ligandů. Nejznámějšími death receptory jsou Fas, tumor nekrotizující faktor receptor 1 (TNFR1) a TNF-příbuzný apoptózu indukující ligand (TRAIL) receptory DR4 a DR5. Mohou zprostředkovat proliferaci, diferenciaci nebo apoptózu buněk. Většina receptorů vede také k aktivaci transkripčního faktoru NF-κB, který zajišťuje přežití buňky. Když je aktivace NF-κB potlačena, nastává apoptóza. Pro death receptory je charakteristická přítomnost tzv. death domény (DD) uvnitř buňky, která umožňuje napojení dalších signalizačních proteinů.

Interakce Fas ligandu s Fas receptorem vede k napojení adaptorového proteinu FADD přes DD a dojde tvorbě death indukujícího signalizačního komplexu (DISC). FADD obsahuje kromě DD ještě death efektorovou doménu (DED), přes kterou se napojí prokaspáza 8 svojí DED. Následně dojde k její aktivaci a vzniku kaspázy 8, která aktivuje efektorové kaspázy. FasL-Fas má funkci hlavně v imunitním systému.

TNFα (tumor nekrotizující faktor α) svůj účinek uplatňuje spojením s TNFR1, přítomným v mnoha typech buněk a TNFR2, který se nachází hlavně v leukocytech a endoteliálních buňkách. Po spojení ligandu a TNFR1 dochází k interakci s adaptorovým proteinem TRADD a k aktivaci prokaspázy 8. Na druhou stranu, TNFα může fungovat také jako antiapoptotický faktor, a to když po napojení TRADD dojde k interakci s RIP, který reaguje s TRAF2 a dále aktivuje NF-κB. TRAF2 se může také přímo napojit na TNFR i bez přítomnosti adaptorových proteinů, což vede k aktivaci NF-κB.

TRAIL indukuje apoptózu pomocí svých death receptorů DR4 a DR5. Dále má také dva decoy (klamné) receptory DcR1 a DcR2, které postrádají funkční DD a nenavozují apoptózu. Dále TRAIL interaguje s rozpustným receptorem osteoprotegerinem (OPG), který má funkci v procesu osteoklastogeneze, ale může zde dojít ke kompetici o TRAIL pokud je OPG v nadprodukci. TRAIL se, jako ostatní ligandy, vyskytuje ve formě trimeru, hlavně v buňkách imunitního systému, hepatocytech a nádorových buňkách. Cytoplazmatické části DR4 a DR5 obsahují DD. Po navázání TRAIL na death receptor dochází k trimerizaci molekul receptoru a následně k interakci s adaptorovým proteinem FADD, formaci DISC a k aktivaci prokaspázy 8 a spuštění kaspázové kaskády. V buňkách typu I je aktivace kaspázy 3 kaspázou 8 dostatečná pro vyvolání apoptózy, kdežto v buňkách typu II je zapotřebí ko-aktivace mitochondriální cesty. K tomu dojde štěpením jednoho z členů Bcl-2 rodiny – Bid kaspázou 8

a následně se tvoří tBid, který navodí uvolnění cytochromu *c* z mitochondrie. Cytochrom *c* způsobí vznik apoptozómu a aktivaci prokaspázy 9, která obratem štěpí prokaspázy 3 a 7. TRAIL má schopnost způsobovat apoptózu u mnoha druhů nádorových buněk, ale šetří většinu normálních buněk. Proto se v současnosti provádí hodně experimentů s TRAIL jako potenciálním protinádorovým léčivem a testuje se také jeho využití v terapii autoimunitních onemocnění. Některé nádorové linie ale nejsou na TRAIL citlivé. Je to přisuzováno právě decoy receptorům, které chrání buňku před apoptózou mechanismem kompetice o ligand nebo aktivací NF-κB. V důsledku toho se začaly provádět experimenty s monoklonálními protilátkami proti DR4 a DR5 a nebo kombinace TRAIL s chemoterapie nebo ozařování. Avšak funkce TRAIL za fyziologických podmínek nebyla ještě zcela objasněna.

Děloha potkana se skládá ze tří morfologicky odlišných vrstev – endometria, myometria a perimetria. Myometrium je složeno z longitudinální a cirkulární svalové vrstvy. Endometrium se skládá z luminálního a glandulárního epitelia a z fibroblastických stromálních buněk. Tyto stromální buňky jsou schopny přeměny v deciduální buňky v brzkém stadiu březosti.

Decidualizace je reakce dělohy na implantující embryo. V děložních tkáních je možné identifikovat tři typy buněk: antimesometriální deciduální buňky, mesometriální deciduální buňky a granulované buňky metriální žlázy.

Blastocyt implantuje asi 5. den po početí v antimesometriální oblasti a dává vzniku antimesometriálním deciduálním buňkám (AMD). Jejich maximální rozvoj nastává 8.den březosti, 11.den dochází k regresi AMD v tzv. decidua capsularis. Krátce po rozvoji AMD se decidualizace šíří k mesometriálnímu pólu a vznikají mesometriální deciduální buňky (MD). Nejdříve se MD buňky objevují v centrální části, maximálního rozvoje dosahují 12. den, poté degenerují tvoříce decidua basalis. Decidua basalis se dále formuje a tvoří součást definitivní placenty.

Třetím typem buněk jsou granulované buňky metriální žlázy (GMG). Tyto buňky obsahují cytoplasmatická granula s perforinem a granzymem B. objevují se již od raného stadia březosti (8.den) rozptýlené v MD, později jsou lokalizované v mesometriálním trianglu, z kterého se následně vyvíjí metriální žláza (MG) (11.-12.den). Maximálního rozvoje dosahují ve dnech 13-15, později vykazují apoptotické rysy a zanikají. Jejich hlavní úloha je udržování rovnováhy a vzájemné tolerance materiálních a fetálních tkání.

Dospělé samice potkanů byly oplodněny samci a nález spermií ve vaginálním stěru příští den ráno byl počítán jako 1.den březosti. V den 8, 10, 12, 14, 16 a 19 byli potkani usmrceni

cervikální dislokací. Děložní rohy byly vyjmuty, fixovány a zpracovány do parafinových bločků. Poté byly nařezány na mikrotomu na 4μm tenké řezy a naneseny na mikroskopická sklíčka. Pro studium obecné morfologie dělohy jsme použili hematoxylin/eosin barvení.

Detekce TRAIL, DR5 a DcR2 byla provedena imunohistochemickou metodou za použití polyklonálních ovčích protilátek. Deparafinizované a hydratované řezy jsme inkubovali s normálním sérem zvířete, ve kterém byla vytvořena sekundární protilátka, pak s primární protilátkou, sekundární protilátkou a Vectastain ABC-AP (avidin-biotin – alkalická fosfatáza) činidlem. Reakce byla vizualizovaná za použití Sigma Fast Red TM tablet. Řezy jsme obarvili hematoxylinem. Jako negativní kontrolu jsme použili PBS místo primární protilátky.

Pro každý ze zkoumaných dnů březosti jsme použili tkáňové řezy minimálně ze tří různých zvířat.

8.den březosti byla pozorována pozitivní reakce na TRAIL a silnější signál pro receptory v AMD, částečně LD, CML, BV ale ne v MD. V den 10 byl rovněž signál pro TRAIL v AMD a mizel v CML a BV. Exprese DR5 v AMD, CML a BV klesla, kdežto u DcR2 je velice silná exprese v AMD s dobře patrným jaderným barvením. V den 12 se objevila exprese ligandu i receptorů v MD, u některých svalových vláken, některých BV a také některých granulovaných buněk ve tvořící se MG. 14. den březosti se zvýšila exprese TRAIL a DcR2 v MD, zatímco DR5 klesla. Byl patrný rozdíl v expresi TRAIL a receptorů: TRAIL byl více lokalizován okolo EC a BV, receptory-pozitivní buňky byly rozptýleny po celé MD. MG vykazovala TRAIL a DcR2 imunoreaktivitu, ale méně u DR5. BV byly TRAIL pozitivní ale DR5 a DcR2 negativní. Některá svalová vlákna byla pro ligand i receptory pozitivní. V den 16 byl zaznamenán pokles exprese TRAIL a DcR2 v MD, exprese ve svalových vláknech zůstala na stejné úrovni imunoreaktivity a zmizel signál pro ligand i receptory v BV. U MG vzrostla exprese DR5; TRAIL a DcR2 zůstala pozitivní. V den 19 klesla exprese ligandu i receptorů v MD. V MG vzrostla exprese TRAIL a DcR2 a klesla DR5. CML byla TRAIL negativní ale u některých svalových vláken byla pozorována pozitivita pro DcR2 a slaběji pro DR5. BV byly TRAIL, DR5 a DcR2 negativní.

Deciduální buňky se rozvíjejí jako reakce na implantující embryo. Vývoj a následná regrese těchto buněk byla studována mnohými vědci a zjistilo se, že důležitou roli v tomto procesu hraje apoptóza.

V naší studii jsme detekovali TRAIL, DR5 a DcR2 v materiální tkáni potkana v průběhu březosti. AMD vykazovala expresi TRAIL v den 8 a 10 Exprese receptorů v den 8

byla silná, ale DR5 v den 10 poklesl, zatímco DcR2 měl velice významný signál. V MD se signál objevil od 12. dne, zesílil v den 14 pro TRAIL a DcR2 a poté postupně klesal. V MG se exprese objevil od 12. dne a s postupující březostí rostl u TRAIL a DcR2, zatímco DR5 kolísal a nebyl tak výrazný.

Pozorovali jsme odlišné typy buněčné exprese. TRAIL vykazoval cytoplazmatické barvení a v některých buňkách byly přítomny pozitivní cytoplazmatiké vehikuly, což demonstroval i Cassatella et al. (2005) ve své studii. DR5 měl cytoplazmatickou expresi. DcR2 měl jadernou i cytosolickou expresi, což bylo patrné hlavně 10. den březosti. Ve studii Zhanga et al. (2000) bylo zjištěno, že DR4 a DR5 jsou lokalizovány v golgiho aparátu, kdežto decoy receptory jsou umístěny v jádře. Po interakci buňky s TRAIL jsou receptory transportovány na cytoplazmatickou membránu.

Nalezli jsme rozdíly v intenzitě exprese TRAIL i jednotlivými receptorů. Zdá se, že v určitých dnech má decoy receptor DcR2 významnější úlohu než DR5, protože jeho exprese je silnější. Může to znamenat, že určité buňky jsou tak chráněny před TRAIL způsobenou apoptózou a na regresi deciduálních buněk se podílejí i další faktory jako např. TNFα a jeho receptor, FasL a Fas nebo Bcl-2 proteiny. Ostatní TRAIL receptory DR4 a DcR1 zůstavají neprozkoumány a jejich role v tomto procesu by také měla být studována.

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8. APPENDIX

1. Phosphate buffer saline

NaCl	8,0g
KCl	0,2g
Na_2HPO_4	1,44g
KH_2PO_4	0,24g
Distilled water	ad 1000 ml

Dissolve the substances in distilled water and complete the volume to 1000 ml.

2. Buffered neutral formalin

Formaldehyde 40%	100 ml
$Na_2HPO_4.2H_2O$	8,15g
$NaH_2PO_4.2H_2O$	4,0g
NaCl	8,0g
Tap water	ad 1000 ml

Dissolve the substances one by one in tap water. Immediately before use add formaldehyde and complete the volume to 1000 ml.

3. Preparation of blocks

Fixation

- Buffered neutral formalin 10%(v/v) 24 to 48 hours at room temperature

Dehydratation

- Alcohol 70% 90 min.
- Alcohol 96% 90 min.
- Absolute alcohol 90 min.
- Benzol 30 min.
- Paraffin: benzol (1:1) 60 min.
- Paraffin (56°C) 60 min.

Paraffin inclusion

4. Coating slides

- Immerse the slides in 1% Extran (alkaline detergent) 30 min.
- Wash in running tap water 30 min.
- Wash in ultra pure water 2x5 min.
- Wash in 96% ethanol 2x5 min.
- Air dryer in front of fan 10 min.
- Coat slides in a freshly prepared 2% solution of 3-aminopropylethoxysilan (Sigma ® A3648) in dry acetone 5 seconds
- Rinse twice quickly in dry acetone
- Rinse twice in ultra pure water
- Air dry 42°C overnight
- Store at room temperature in dust free environment

5. Process of deparaffinization

- Xylen 10 min.
- Xylen 10 min.
- Absolute alcohol 5 min.
- Absolute alcohol 5 min.
- Ethanol 96% 5 min.
- Ethanol 96% 5 min.
- Ethanol 70% 5 min.
- PBS 10 min.

6. Staining with hematoxylin and eosin

- Tap water 5 min.
- Mayer´s Hematoxylin solution 4 min.
- Tap water 5 min.
- 1% eosin solution 7 min.
- Tap water 1 min.
- Dehydratation through alcohols and xylen
- Mounting with DePeX (BHD) mounting medium