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BACHELOR THESIS

RAT ALPHA-DEFENSINS: THEIR STRUCTURE, FUNCTION AND EXPRESSION PROFILE

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Affirmation

I hereby declare that I have written this thesis independently, with the use of listed literature.

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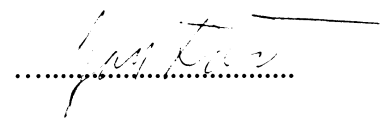

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Abstract

Antimicrobial peptides are gene-encoded antibiotics participating in innate immune response against viruses, bacteria, fungi and parasites. We can find them in plants, invertebrate and vertebrates. Defensins represent a sub-class of this heterogenic family. They are short, cationic, amphipathic peptides, rich on arginine and cysteine residues. Their six cysteins form three disulphide bonds, an arrangement characteristic for this group. Defensin expression is abundant in epithelial tissues and in phagocytic cells where they participate in pathogen destruction. In addition to the most recognized antimicrobial function, involvement of defensins in haematopoietic cell chemoattraction, sperm maturation, cell signalling and coat color determination have been also reported. Recent data demonstrating the secretion of α-defensins into an extracellular environment has significantly increased our interest in defensin's activities with regards to their cytotoxicity, chemotaxis, enhancement of local inflammation and wound repair. However, the wealth of information about the multifaceted role of defensins in immunity and physiology sharply contrasts with the lack of a comprehensive insight into the expression profile of α-defensins across all major tissues in mammals. Our previous analysis revealed α-defensin expression also in the rat thymus, spleen and lymph nodes. This thesis summarizes further continuation of this effort. Its first part gives a brief description of defensin peptides, their classification into subgroups, concise account of current knowledge concerning their gene and peptide structure, mode of action and function. In the experimental section, we briefly report the results of our analysis concerning distribution of all ten so far identified rat α-defensin-specific mRNAs on a panel of rat tissues. We found that their expression is not uniformed but rather tissue-specific. While some of them are detected broadly with varied level of expression across tissues like *NP-1/2* and *NP-3*, others, like *Defa-8* and *Defa-9* are detected in high levels in the duodenum, exclusively. Surprisingly, the thymus is the site of abundant expression of five out of ten α-defensins tested. Immunohistochemistry and confocal microscopy confirmed the presence of NP-1/2 defensin in the thymus. These results are the first to provide an overall picture of α-defensin expression in rat tissues and suggest that the scope of their physiological functions is much broader and tissue-specific than previously thought.

Key words

Antimicrobial peptides, α-defensin, rat, thymus, immunohistochemistry, RT PCR.

Abstrakt

Antimikrobiální peptidy jsou antibiotika kódovaná geny, která se účastní vrozené imunitní odpovědi proti virům, bakteriím, plísním a parazitům. Můžeme je nalézt u rostlin, bezobratlých i obratlovců. Do této různorodé skupiny patří také defensiny. Krátké, kladně nabité, amfipatické peptidy, které jsou bohaté na aminokyseliny arginin a cystein. Právě spojení šesti cysteinů do tří disulfidických můstků vytváří strukturu typickou pro tuto skupinu. Defensiny jsou vysoce exprimovány buňkami epitelií a fagocyty, kde se účastní likvidace patogenů. Kromě této nejdůležitější funkce hrají také roli v chemoatrakci hematopoetických buněk, vývoji spermií, buněčné signalizaci a mohou určovat barvu kožichu některých zvířat. Nejnovější objevy ukazují, že sekrece α-defensinů do extraceluárního prostoru způsobuje cytotoxicitu, chemotaxi, rozvoj lokálního zánětu nebo hojení ran. Velké množství informací, které obecně pojednávají o funkci defensinu z imunitního či fyziologického hlediska, prudce kontrastuje s nedostatkem informací ohledně samotné exprese α-defensinů v savčích tkáních. Naše předchozí experimenty ukázaly, že α-defensiny jsou mimo jiné také exprimovány v thymu, slezině a lymfatických uzlinách. Tato práce doplňuje a rozšiřuje doposud dosažené výsledky. V první části jsou obecně představeny defensiny, rozděleny do podskupin a shrnuty dosavadní poznatky o genové i peptidové struktuře, mechanismu antimikrobiálního působení a funkci těchto peptidů. V experimentální části je pak shrnuto, v kterých tkáních se všech deset doposud objevených α-defensinů exprimuje. Bylo zjištěno, že jejich exprese není uniformní, ale spíše tkáňově specifická. Zatímco některé z nich bylo možné nalézt v širokém spektru tkání například *NP-1/2* a *NP-3*. Další, jako *Defa-8* nebo *Defa-9*, je množné nalézt pouze v části tenkého střeva zvané duodenum. Překvapivě, v thymu je možné nalézt pět α-defensinů z deseti testovaných. Exprese defensinu NP-1/2 v thymu byla navíc potvrzena i imunohistochemií a konfokální mikroskopií. Tyto výsledky jako první shrnují expresi α-defensinů v tkáních potkana a ukazují, že fyziologická funkce těchto peptidů bude zřejmě daleko širší a více tkáňově specifická, než se doposud předpokládalo.

Klíčová slova

Antimikrobiální peptidy, α-defensin, potkan, thymus, immunohistochemie, RT PCR.

CONTENTS

List of abbreviations

AA	amino acid
BM	bone marrow
BR	brain
Cathelin	cathepsin L inhibitor
Cryptdins	crypt defensins
DAPI	4',6-diamidino-2-phenylindole
Defa	defensin alpha
DNA	deoxyribonucleic acid
DU	duodenum
ED	enteric defensin
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HE	hearth
HNP	human neutrophil peptide
KI	kidney
LI	liver
LN	lymph nodes
LU	lung
M	marker
MC1R	melanocortin 1 receptor
MMP-7	matrix metalloproteinase 7, also called metalloproteinase matrilysin
mRNA	messenger ribonucleic acid
NTC	non-template control
NP	neutrophil peptide
ps	pseudogene
rs	related sequence
RTD-1	rhesus theta defensin 1
RT PCR	reverse transcriptase polymerase chain reaction
SP	spleen
ST	stomach
TH	thymus
UTR	untranslated region
qPCR	quantitative real time polymerase chain reaction

1. Introduction

Mammals are daily exposed to potential pathogens through ingestion, inhalation and physical surface contact. To protect themselves against this potentially harmful encounter, mammals have evolved two main systems of defense: (i) non-clonal and non-specific innate immunity and (ii) clonal and antigen-specific adaptive or acquired immunity. The first protective layer of innate immunity, which pathogen must overcome, is a mechanical barrier represented by epithelial cells that are closely joined together with tight junctions. This barrier is complemented by the flow of bodily fluids (blood, urine) and air as well as by the peristaltic movement of gastrointestinal tract. The cellular effectors of the innate immune responses include phagocytic cells (e.g. neutrophils, macrophages), cytotoxic cells (natural-killer cells) and humoral mediators as cytokines, lectins and antimicrobial substances.

Antimicrobial substances comprise microbicidal chemicals – hydrogen peroxide, nitric oxide, hypochlorus acid and a wide variety of host gene-encoded antimicrobial peptides, proteins and multimeric protein complexes, such as complement. Defensins belong to a group of antimicrobial peptides, which are mainly stored in the primary granules of neutrophils and Paneth cells or, alternatively, secreted by epithelial cells. They contribute to innate immunity of the host by causing structural disruption in the pathogen membrane.

Previously, during the assessment of defensin expression in selected rat tissues, our group found that rat myeloid α-defensins neutrophil peptide 1-4 (*NP-1-4*) genes are abundantly expressed in the thymus and spleen. Interestingly, the kinetics of defensin expression in the thymus displayed a regular cycling profile and its expression was independent on the presence of environmental antigens as gnotobiotic rats expressed the same level of α-defensin as rats maintained in a conventional animal facility. Moreover, our analyses conducted previously in collaboration with the research group of Dr. Poussier in Toronto, showed some correlation between the defensin expression in the thymus with the onset of autoimmune disease in rat model of diabetes. While the possible link between defensin expression and diabetes represents a considerable interest and is the subject of ongoing research effort in our lab, we decided to extend our investigation to characterize the expression profile of all 10 rat α-defensins on a panel of rat tissues.

Before we discuss the major experimental outcome of this work, let's briefly describe the current knowledge concerning the antimicrobial peptides in general and mammalian defensins in particular.

1. 1. Brief history of exploration on defensin field

Elie Metchnikoff proposed that phagocytic cells detect and ingest microbes invading the organism. Once the microbes are engulfed in the phagocyte, their destruction ensues. He concluded that ferments (enzymes) are responsible for killing and digestion of microbes. Based on the notion that chemicals are responsible for this action, it was a relatively small step toward identification of chemicals responsible for killing[1]. In the late 1920's Alexander Fleming identified and partially purified lysozyme, first peptide with antimicrobial activity. His famous discovery of penicillin, followed by others who describe other types of antibiotics and sulphonamide-based microbicidals, help at least partially to elucidate mechanism of phagocytic killing[1].

In the end of 1950's James Hirsch found, that extract from human neutrophils contains antimicrobial enzymes, the substance he named phagocytin[2]. Latter on (in 1960's) Zeya and Spitznagel analyzed phagocytin from neutrophils more thoroughly. The most cationic fraction, which migrated more rapidly to cathode than cationic proteins such as lysozyme or ribonuclease, was determined. According to amino-acid (AA) analysis, 25% of protein consisted of basic AAs. Arginine alone represents 15% of the protein. This protein is cationic in nature and has bactericidal activity[3].

In 1985, Robert Lehrer, Tomas Ganz and Michael E. Selsted coined the name "defensin" for very cationic, amphipathic, arginine and cystein-rich, antimicrobial peptides which they identified in granules of human neutrophils. The name of peptide refers to a presumable role in host defense[4]. Other antimicrobial molecules where meanwhile characterized in invertebrates[5] and plants[6]. Furthermore, in late 1980's, Andre Ouellette identified defensins in crypts of mice's intestine. This defensins are expressed by Paneth cells and were called crypt defensins (cryptdins)[7]. Discovery of other type, so called epithelial antimicrobial peptides followed. Gill Diamond founded this kind of defensin called β-defensin in bovine trachea[8]. And as the millennium was coming to the end, θ-defensin were purified from rhesus macaque neutrophils by Yi-Quan Tang. Interestingly this peptide was the first peptide of cyclic structure founded in mammalian body[9]. In the same time, pharmaceutical companies began to focus on potential use of antimicrobial peptides in therapeutics, but this effort is still far from clinical application[1].

2. Antimicrobial peptides

Antimicrobial peptides are defined as short polypeptides with broad range of antimicrobial activity in the host immune system. Its polypeptide chain is no longer than one hundred AAs with some conserved essential structures within individual families. Evolutionary, antimicrobial peptides belong to family of ancient molecular weapons against invading pathogens having defensive role in plants, invertebrate and vertebrates[10]. So far, about nine hundred members of antimicrobial peptides were discovered and described based solely on the general bactericidal definition[11]. Despite of the small similarities in the primary structure of antimicrobial peptides, their polypeptide chain contains high proportion of hydrophobic AAs what ultimately result leading in their net cationic charge. The cationic character of antimicrobial peptides is a prerequisite for interaction with pathogens as their membranes are negatively charged and thus peptides can easily interact with them. The precise mechanism of killing system has not been completely resolved, but several studies published so far revealed the formation of membrane pores in pathogen envelope[12].

Activities of antimicrobial peptides have been reported against broad spectrum of targets including gram-positive and gram-negative bacteria, fungi, parasites such as trypanosomes and plasmodia, and surprisingly, enveloped viruses and cancer cells[12]. Compared with antibiotics produced by bacteria and fungi and synthesized by a complex metabolic pathways, antimicrobial peptides are the products of a single gene[13]. Their antimicrobial activity is dependent on physiological state of the tissues and concentration of effector peptide[12]. It's not surprising that they are potently localized in tissues were natural and frequent contacts with pathogens, for example gastrointestinal, genito-urinary tracts and tracheobronchial tree, occur. Antimicrobial peptides, on the cellular level, are produced not only by phagocytes and lymphocytes, but also by keratinocytes[14].

Classification of antimicrobial peptides based on their chemical structure and AA content was established by H. G. Boman[15] and further reviewed by the same author due to identification of new members of this family. Following five groups of animal antimicrobial peptides were postulated:

1. Linear peptides without cysteine. Members: cecropins, LL-37, magainins.
2. Peptides without cystein and with an over-representation of one or two AAs (proline-arginine, tryptophan or histidine). Members: indolicidin and histatins.
3. Peptides with one disulfide bond. Loop structure with one or two tails. Members: bovine

dodecapeptide, bravenins.

4. Peptides with two or more disulfide bonds. They are mainly or exclusively β-sheets. Members: tachyplesins (two disulfide bonds), defensins (three disulfide bonds).
5. Peptides derived from larger peptides with known function. Members: lactoferricins (digested lactoferrin)[16].

This classification is useful for categorization of antimicrobial peptides according to their primary and secondary structure, but it is not very helpful for sorting antimicrobial peptides according to their biological function(s). Majority of mammalian antimicrobial peptides belongs to families of defensins and cathelicidin-derived peptides[10]. As defensins fit in Boman's group 4, they contain six cysteines linked by three disulfide bonds. On the other hand, cathelilicidin-derived peptides are group with much bigger diversification and belongs to groups 1, 2, 3 and 4[16].

2. 1. Cathelicidin-derived peptides (cathelicidins)

This group of antimicrobial peptides is distinguished by their characteristic protein structure. Protein precursors contain three conserved parts: a N-terminal signal peptide (pre-region), a pro-region, also called cathelin-like domain (cathelin is an acronym from cathepsin L inhibitor), and the variable C-terminal antimicrobial domain containing the mature peptide[17]. Sequence of this cysteine protease inhibitor is similar to the pro-region of cathelicidin.[18, 19]. Figure 1 shows the organization of common cathelicidin precursor.

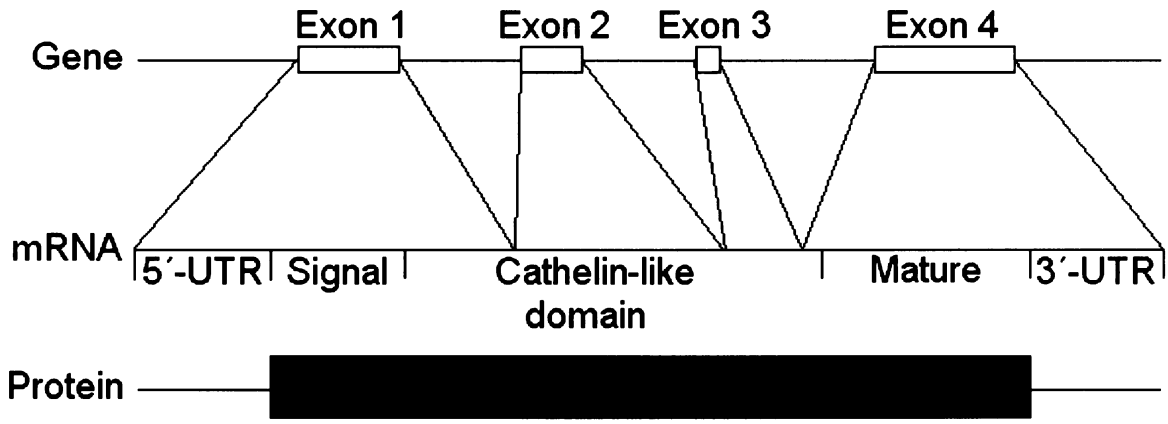


Figure 1. Schematic view of cathelicidin gene, messenger ribonucleic acid (mRNA) and protein structures. Legend: UTR = untranslated region, Signal = signal peptide. Color code: pink = pre-region, green = pro-region, blue = mature peptide. Modified from reference (18).

Interestingly, the pro-region has the capacity to oppose cathepsin L protease action and it also displays an antimicrobial activity. C-terminal antimicrobial peptide has a direct antimicrobial activity: it is able to form ion channels or pores in bacterial membranes. This region is also responsible for chemotaxis of leukocytes, mast cells and monocytes. Cathelicidins can induce expression and/or release of inflammatory mediators and protect mammalian body from septic shock by binding to bacterial lipotechoic acids and endotoxins. Angiogenesis and wound healing can be mediated by cathelicidins as well[17-19].

Cathelicidins are abundantly expressed in mammalian tissues such as bone marrow, thymus, spleen, liver, testis, stomach and intestine. The cellular sources of their expression and production are mostly neutrophils, where cahelicidins are stored in granules. Expression by other cell types has been reported, but it seems to be not significant[18].

In the human body, the only cathelicidin, named LL-37 (derived from pro-peptide hCAP18) has been characterized so far[19]. This protein is coded by *CAMP* gene and its product has been localized in specific granules of human neutrophils[20]. LL-37 antimicrobial peptide sequence starts with two leucine residues and it is 37 AA long. Normally, LL-37 is expressed in neutrophils, lymphocyte, macrophages and epithelial cell and have an impact in protecting the host against bacteria[19]. Its production in keratinocytes is detected only if the skin is injured or damaged. LL-37 binds to deoxyribonucleic acid (DNA) released from injured keratinocytes and mediates endocytosis into the plasmacytoid dendritic cells, where LL-37 binds to toll-like receptor 9. This leads to a massive release of interferons and the activation of myeloid dendritic cells. In psoriatic skin this mode of activation results in T-cell mediated autoimmune inflammation[21].

A rat homolog to human LL-37 is called rCRAMP. Mature protein is 43 AA long and forms secondary structure with two amphipathic α-helixes. rCRAMP is more potent gene-encoded antibiotic than LL-37, but it is expressed only by granulocytes. Interestingly this peptide is expressed in thymus. Similar to human LL-37, rCRAMP is the only rat cathelicidin identified so far[22].

2. 2. Defensins

The term defensin was used for the first time in 1985 to describe peptides derived from granules of human neutrophils, which were able to kill bacteria, fungi and viruses and which contain six cysteinyl residues[4, 23]. The new category of antimicrobial peptides has been established.

Currently, three subfamilies of defensins, named α-defensins, β-defensins and θ-defensins, are recognized. This classification is based on arrangement of disulfide bridges between cysteins.

2. 2. 1. Alpha defensins

Alpha defensins are divided to two classes – myeloid and enteric. Major difference relates to the site of their expression (myeloid are expressed by neutrophils and monocytes; enteric by tissues of gastrointestinal tract). Furthermore, while myeloid α-defensins are encoded by three exons (figure 2), enteric only by two[24] (figure 3). These exons are translated to 90–100 AAs long "pre-pro-peptide" composed of the signal peptide (pre-piece ~19 AAs), pro-segment (pro-piece ~39-45 AAs) and mature defensin (~29-35 AAs) [25, 26]. The typical linking of α-defensin's cystein residues in defensins is 1-6, 2-4, 3-6 (figure 2)[12].

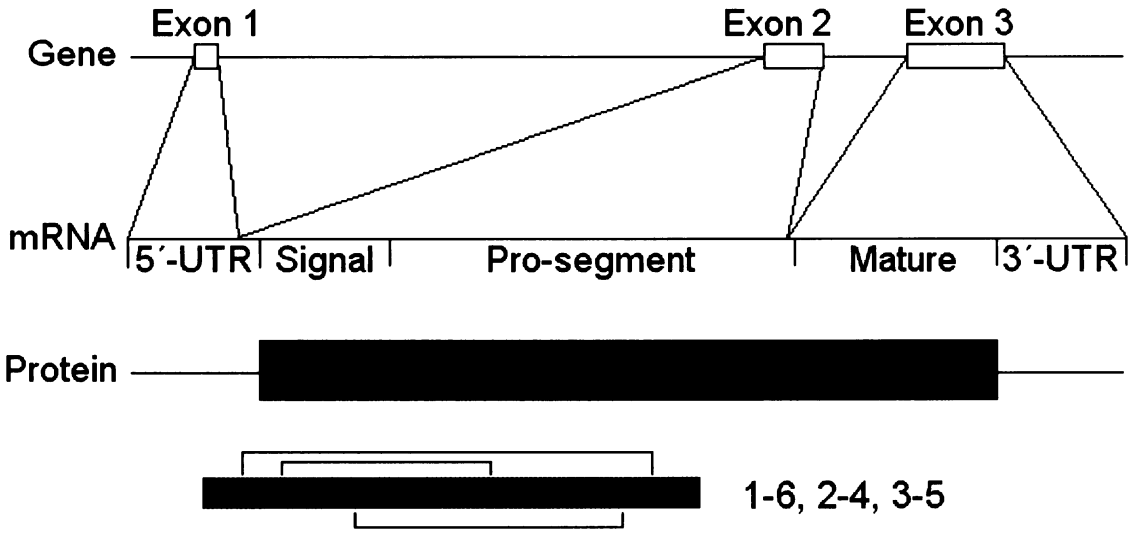


Figure 2. Schematic view of myeloid α-defensin gene, mRNA and protein structures. A typical pairing of cystein residues is shown schematically on the bottom of the figure (pairing of cysteins is the same as in myeloid and enteric α-defensins). Color code as in figure 1. Modified from reference (27).

Myeloid α-defensins are mainly expressed by promyelocytes and early myelocytes stages of neutrophil development in the bone marrow and packed into their azurophil granules (human, rhesus macaques, rat, rabbit, and hamster)[28]. Additional sites of their expression were detected in alveolar macrophages (rabbit), monocytes and natural killer cells (human)[12]. Interestingly, mice neutrophils do not express and produce α-defensins[29].

As enteric α-defensins are expressed constitutively or inducibly by Paneth cells that populate crypts of small intestine, these peptides are often called cryptdins. Cryptdins are also expressed by some epithelial cells in several mammalian species (see table 1 on page 17)[7, 27].

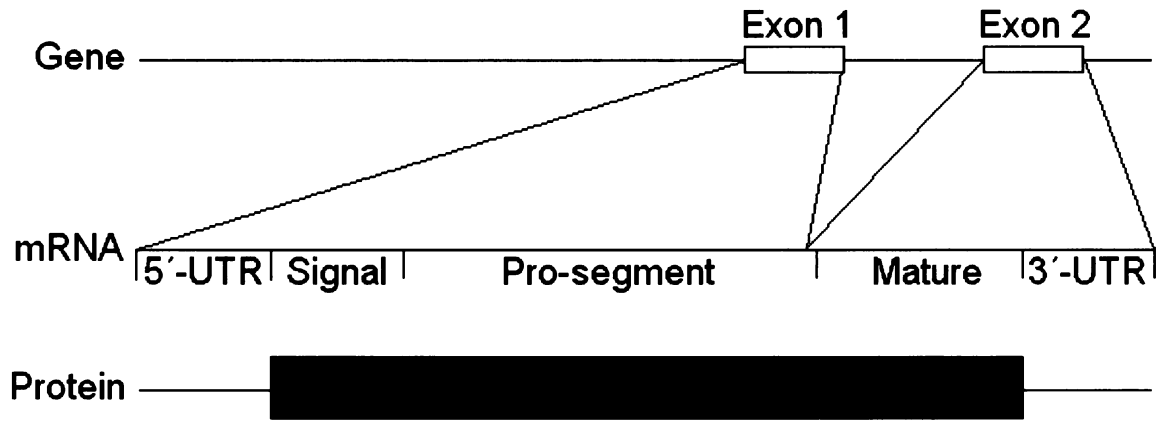


Figure 3. Schematic view of enteric α-defensin gene, mRNA and protein structures. Note, that enteric α-defensin gene is composed only of two exons. Color code as in figure 1. Modified from reference (27).

After synthesis of pre-pro-peptide, the signal peptide is rapidly cleaved. Spliced pro-α-defensin has no detectable antimicrobial activity[26] and displays a neutral net charge compared to a positively charged mature peptide. For example, the number of positively and negatively charged AA residues in rat α-defensins is as follows: NP-1: -11/+12, NP-2: -11/+11, NP-3: -9/+9, NP-4: -9/+9.[28] It is believed that this charged balance might be important to avoid auto-toxicity during post-translational processing[25]. This might be true for a myeloid but not for enteric α-defensins with no or only slightly negative charged pro-piece. For a fully active mature defensin, the pro-piece must undergo a final cleavage mediated by a precise enzymatic mechanism. Till now, only two enzymes with such function were characterized: (i) matrix metalloproteinase 7 (MMP-7)[30] is responsible for generation of functionally active cryptdins in mouse Paneth cells and (ii) some isoforms of trypsin were shown to cleave human defensin 5 and 6[31]. Recently it has been reported that MMP-7 is also able to cleave human neutrophil peptide 1 (HNP-1) into an unusual intermediate, which retains a part of the pro-segment sequence and the mature HNP-1[32].

2. 2. 2. Beta defensins

β-defensins were for the first time isolated from trachea of cow[8] and then localized in various mammalian species for example human, sheep, pig, mouse and rat[33]. β-defensin's gene has only two exons. It is also synthesized as a pre-pro-peptide, where pro-piece part is short or missing and the mature defensin sequence is approximately 38-42 AA long. The enzyme(s) responsible for cleavage of the pro-piece is unknown. Typical pairing of six cysteins residues is 1-5, 2-4, 3-5[12] (figure 4).

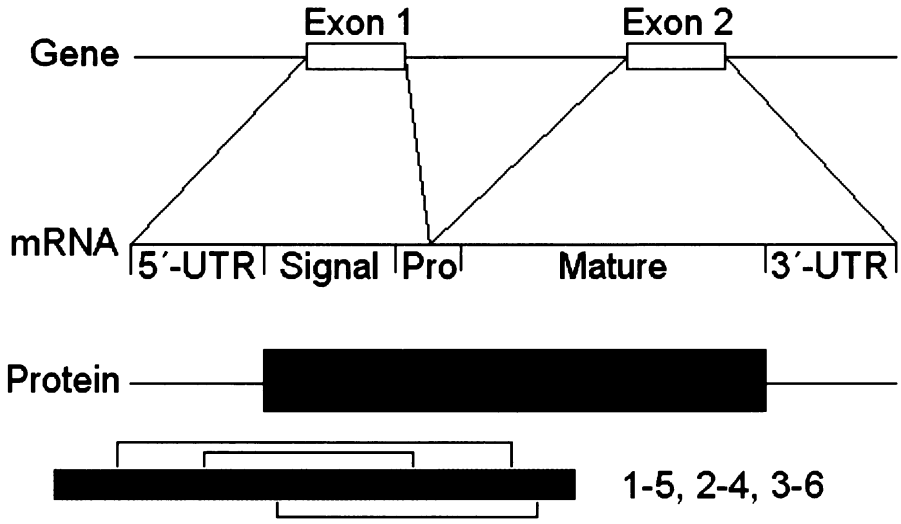


Figure 4. Schematic view of β-defensin gene, mRNA and protein structures with a typical pairing of cystein residues (bottom). Color code as in figure 1. Modified from reference (27).

β-defensins are expressed by various epithelial tissues (e.g. gastrointestinal tract, pulmonary tract) and by cow's and chicken's neutrophils[12].

2. 2. 3. Theta defensin

In 1999, a discovery of 18 AA long antimicrobial peptides by analysis of rhesus macaque leukocytes was reported. Surprisingly this peptide has a cyclic structure and it is named rhesus theta defensin 1 (RTD-1).[9]

RTD-1 is encoded by two genes *RTD-1a* and *RTD-1b* (93% similarity) each of them have 3 exons (same as myeloid α-defensins). Pre-pro-peptide have 76 AAs, containing 20 AAs signal peptide, 44 AAs pro-segment and 12 AA long mature peptide RTD-1a or RTD-1b. The transformation of the two linear precursors to 18 AAs cyclic peptide requires proteolytical cleavage to two nonapeptides and their head-to-tail ligation. Final cyclic peptide has three disulfide bonds where cysteins are linked together by following scheme: 3-16, 5-14, 7-12. Very close relationship with α-defensins is supported by 88% nucleotide sequence similarity of RTD-1 with human α-defensin pseudogen[9].

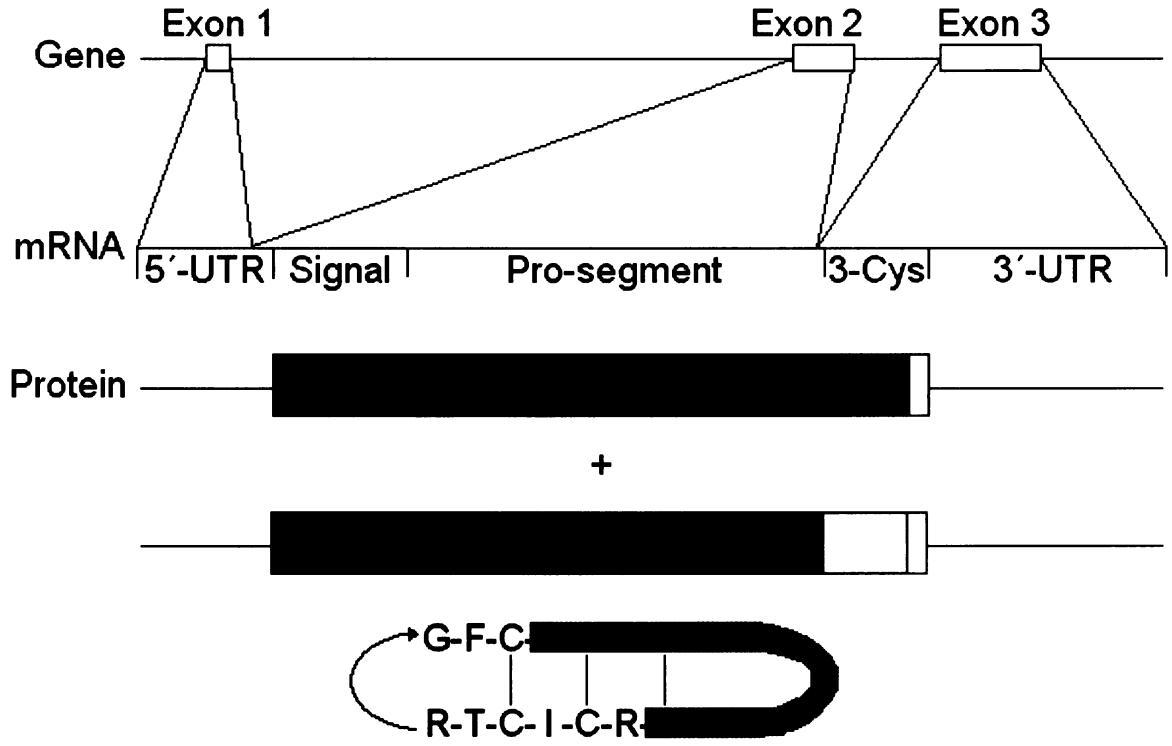


Figure 5. Schematic view of θ-defensin gene, mRNA, protein structures. Typical pairing of cystein residues is shown on the bottom. Color code as in figure 1. Modified from reference (27).

Theta defensins are expressed by rhesus neutrophils and monocytes. Interestingly, in humans, θ-defensins mRNA was founded, but it is not translated into the protein due to a stop-codon mutation in the peptide signal sequence[9, 34].

2. 2. 4. Summary

Alpha and beta subfamilies of mammalian defensins differ in the length of mature peptide sequence and the pairing of cystein residues[12]. However, they share the same tertiary structure, with a structural motive termed defensin-like fold, consisting of antiparallel β-sheets linked by three disulfide bridges[14]. In contrast θ-defensin peptides, made from two truncated α-defensins by enzymatic ligation, display a cyclic backbone[9]. Although different in their structure, all defensins contain six cystein residues paired together. From evolutionary point of view, mammalian β-defensins are the oldest one (conserved). α-defensins may evolved from two separate β-defensins ancestors (they are forming specific clusters)[35] and finally, θ-defensins are formed by ligation of two incomplete α-defensins chains (α-defensin paralogs)[9, 35]. Differences related to the sites of expression of defensins in several animal species are highlighted in table 1.

	α	α	α and β
	α and θ	α	β
	None	α	α and β
	α	α	β
	β	none	β
	β	?	β

Table 1. Expression of defensins in relevant cell populations in selected animals. Modified from references (12 and 36).

2. 3. Mechanism of antimicrobial action and other functions of defensin

Antimicrobicidal activity of the members of defensin family are restricted towards bacteria and fungi[12]. Some of them are also active against enveloped viruses, including human immunodeficiency virus[37], and parasites, even though all these activities has been observed only in *in vitro* conditions. In physiologically optimal testing condition, such as low concentration of salt and plasma proteins, antimicrobial activities of defensins were observed at concentration of 1-10 μg per ml[12].

Permeabilization of a pathogen membrane is the most important mode of action related to defensin's antimicrobial activities. Defensins are able to form channels in membrane bilayers[38], especially if this bilayer is negatively charged[39]. Lehrer *et al.* reported that treatment of *Escherichia coli* by human α-defensins resulted in the membrane permeabilization for small molecules such as trypan. Furthermore, bacterial DNA, RNA and protein synthesis were also inhibited, presumably due to a loss of metabolic intermediates and altered ionic cellular environment[40].

Mechanism of permeabilization of plasma membranes by HNP-2 was studied quite intensively. This defensin forms stable, 25Å in diameter pores. Formation of such a large pore requires interaction of six HNP-2 dimmers[41].

Based on these results and studies conducted on small antimicrobial peptides such as magainins, a model of action of antimicrobial peptides has been postulated. Antimicrobial peptides display on their surfaces positively and negatively charged AAs. The positively charged AAs interact by electrostatic and hydrophobic forces with negatively charged phospholipids (which are present in bacterial membrane in inner and outer leaflets, but in animal cells only in the inner leaflet). These interactions might contribute to the specificity of these peptides towards bacteria. After initial binding to the outer leaflet, defensins penetrate deeper into the plasma membrane where the pore-forming process occurs. This action usually leads to disruption of the plasma membrane, diffusion of peptides into intracellular space, efflux of cellular fluids followed by a growth arrest and often a death[42, 43].

In addition to their antimicrobial activities, defensins are involved in the chemotaxis of certain haematopoietic cells. Defensins mobilize and attracts macrophages, T-cells and mast cells. In case of mast cell, it has been documented that defensins can induce histamine secretion[44]. Exposure to defensins led to the recruitment and maturation of dendritic cells as well[45]. Defensins has also been found in a nuclear fraction of lymphocytes what suggests that they could play a role in the nuclear regulatory processes of these cells[46]. They also inhibit the function of protein kinase C and phosphorylation of some endogenous proteins[47]. Taken together, it seems that defensins could be somehow involved in immune response modulations as signalling molecules. However, the precise role and mechanism of their action remains still unknown[48].

Human β-defensins are chemotactic for immature $CD34^+$ dendritic cells expressing chemokine receptor CCR6. Due to their similarity to chemokine CCL20, defensins are able to interact with this receptor. It has been suggested that defensin-CCR6 interaction might play a role in

the recruitment of immature dendritic cells and memory T-cells to the site of microbial invasion on mucosal epithelium[49]. HNPs also induce interleukin-8 release, using $P2Y_6$ seven-transmembrane domain, G-protein coupled receptor. Detail signalization mechanism and the specific site of binding to $P2Y_6$ has not been mapped so far[50].

Interesting interaction between β-defensin, G-protein coupled seven-transmembrane domain receptor and receptor-associated molecule was described in the dogs. In this case, the binding of the melanocortin 1 receptor (MC1R), expressed on melanocyte, to the agouti protein determines the color of the coat. In this scenario, association of defensin with MC1R regulates MC1R-agouti interaction. Specifically, the color of the coat depends whether dominant or recessive allele of β-defensin gene binds to MC1R. If the MC1R functions properly and binds to a dominant allele variant of β-defensin, agouti is not recruited to the receptor and the coat retains the black color. However, if recessive form is present and is bound to MC1R, agouti is able to bind to the same receptor what results in the yellow color[51]. It has been reported that the same mechanism governs the coat color in grey wolfs and coyotes[52].

Defensins protective function in sperm maturation in reproductive tract of man, rat and mouse have been reported. In rat reproductive organ, and specifically within seminiferous tubules and Sertolli cells, the expression of several α- and β-defensins at relatively high levels was detected. Antimicrobial peptides were also found in the ejaculated spermatozoa and seminal plasma. As these peptides exhibited a potent antimicrobial activity it has been postulated that they play an important role in innate organ defense against pathogenic invaders[53-55].

As mentioned previously, we have recently detected the α-defensin expression in the thymus and spleen. As α-defensin expression in the thymus and secondary lymphoid organs is quite unusual and has not been described in the literature before, our effort is focused on experiments that could provide an insight into their tissue-specific function and physiology. For this reason, in the next chapter we briefly summarize the current knowledge concerning α-defensins in the rats.

3. Rat alpha defenins

In the rat, fourteen α-defensin's genes have been identified up-to-date. As illustrated in figure 6, all these genes are clustered on chromosome 16 (16q12.4-12.5), spanning approximately 310 kb. Three of those genes are defensin alpha (Defa) pseudogenes (ps), namely *Defa12-ps*, *Defa13-ps*, and *Defa14-ps*. These pseudogenes have a stop-codon at AA position 34, 29 or 84, respectively. Analysis of the rat genome also revealed one α-defensin related sequence (rs) (namely *Defa1-rs*). It has a conserved pre-sequence (signal-peptide) and pro-sequence. However, it contains only five cysteine residues and no characteristic α-defensin motif. Some authors speculate that Defa1-rs might play a role in innate immunity of gut by forming functional homodimers with antimicrobial activity[35].

As a general scenario, we can divide rat α-defensins into two groups: the myeloid and the enteric one.

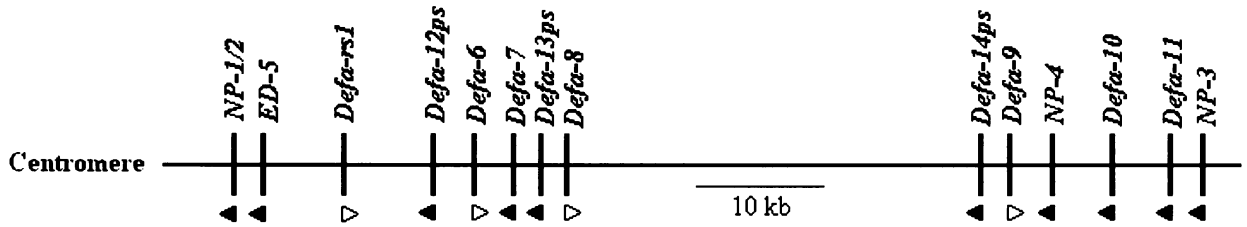


Figure 6. Rat defensin locus on chromosome 16. Position of each gene is marked by a vertical bar; triangles indicate the direction of transcription. Modified from reference (35).

3. 1. Rat myeloid alpha defensins

Group of the rat myeloid α-defensins is represented by several members with the typical myeloid α-defensin motif as described in chapter 2. 2. 1[35]. These are NP-1, NP-2, NP-3, NP-4, DEFA-7, DEFA-10, DEFA-11.

In 1989, Robert Lehrer *et al.* reported that his group purified three defensins from rat's neutrophils. Those were the first defensins found in the rat: NP-1, NP-3 and NP-4. Each of these three defensins has a relatively high content of cystein and arginine residues. Due to their net positive charge (NP-1 (the most cationic) > NP-3 > NP-4) these defensins exhibit a strong

antimicrobial activity against bacteria (tested on *Escherichia coli* ML-35 strain), fungi and enveloped viruses[56].

NP-1-4 are abundantly and predominantly expressed in bone marrow, however, their low expression is detectable also in spleen and small intestine. Because myeloid defensin genes are transcribed only in early phases of neutrophil differentiation and afterwards are stored in the azurophil (primary) granules[28], α-defensin expression in the later stages of neutrophil development is hardly detectable.

DNA sequence of *NP-3* and *NP-4* contains a unique polypurine tract in 3′ UTR. In the case of *NP-3* it is GAA repeat, which occurres approximately 28 times and in the case of *NP-4* it is AG-rich, 45-nucleotide long tract, without any apparent sequential consensus[28]. In this sense, it is also interesting to compare the coding sequence of other two related α-defensins genes *NP-1* and *NP-2*. The gene *NP-2* differs from *NP-1* in a single base, leading to replacement of arginine to serine in NP-2 peptide. As NP-2 has the same activity as NP-1, share the same expression profile and they localize to the same spot on a defensin genetic locus on chromosome 16 (see figure 6), it has been proposed that *NP-1* and *NP-2* might be two alleles of the same gene[57].

Interestingly, NP-1 has also stimulatory effect on the regeneration of the damaged sciatic nerve in rats even though the mechanism of this action is unknown. Treatment of injured animals with defensin NP-1 also helps to restore the function of nerves[58].

Our knowledge about *Defa-7*, *Defa-10* and *Defa-11* defensins is limited to their expression profile. They are expressed in bone marrow (not very abundantly though) and in lesser extend in small intestine[35].

3. 2. Rat enteric alpha defensins

Members of this group of defensins are: *enteric defensin 5 (ED-5)*, *Defa-6*, *Defa-8*, and *Defa-9*. According to the published data all of them are exclusively expressed in small intestine[35].

ED-5 is expressed inducibly by Paneth cells localized at the base of the crypts of Lieberkühn in small intestine and proximal colon. Mechanism of defensin production and their release from Paneth cells is not completely understood[59], but it seems that it is constitutive[27].

3. 3. Rat alpha defensins sequence alignment

The following sequences were taken from the Pubmed protein database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=Protein&itool=toolbar). A multiple alignment of α-defensin's sequences was performed using ClustalW2 program (version 2.0.11, http://www.ebi.ac.uk/Tools/clustalw2/index.html). Default settings by EMBL-EBI program were used for sequence alignment and phylogram tree construction. Results of these analyses are presented in figure 7.

A

```
                Pre-piece                       Pro-piece                                   Mature defensin
NP-1     MRTLTLLLTALLLLALHTQAKSPQGT--AEEAPDQEQLVMEDQDISISFGGDKGTALQDADVKAG-VTCYCRRTRCGFRERLSGACGY-RGRIYRLCCR----------
NP-2     MRTLTLLLTALLLLALHTQAKSPQGT--AEEAPDQEQLVMEDQDISISFGGDKGTALQDADVKAG-VTCYCRSTRCGFRERLSGACGY-RGRIYRLCCR----------
NP-3     MRTLILLLTTLLLLALHTQAESPQGS--TKEAPD------EEQDISVFFGGDKGTALQDAAVKAG-VTCSCRTSSCRFGERLSGACRL-NGRIYRLCC----------
DEFA-10  MRTLTLLLTALLLLALHTQAESPQGS--PKEAPDQEQLDMEDQDISVFFGGDKGTALQDA---AG-STCSCRIGTCVSGEWLSWVCRI-NGRIYRLCCR----------
NP-4     MRTLTLLLITLLLLALHTQAESPQER--AKAAPDQD-MVMEDQDIFISFGGYKGTVLQDAVVKAG-QACYCRIGACVSGERLTGACGL-NGRIYRLCCR----------
DEFA-7   MRTLTLLLTTLLLLALHTQAESPQGSRSTEEALDQEQLVMEDQDISISFGGDKGTALQDADVKSG-VTCYCRLLSCQFGERLAGSCRS-GGVTYPLCCH----------
DEFA-11  MGTLTLLLTTLLLLALHTQAESPQGC-----TEDQKLLIMEDQDISISFTGDKGTALQDADVKSG-VICNCRPRGCSFKERVYGECGY-NGHILRLCCQPLNTMNKKSPSVI
DEFA-6   MKTLVLLSALVLVAYQVQADPIQGA--EEETKTEEQPSDEDQDVSVSFEGPEASALQDFEIGRPVRRCRCR-ANCGPKEYATAFCAQGPFKQFKFCCT----------
DEFA-9   MKTLVLLSALVLLAFQIQADPIQEA--EEETKTEEQPADEDQDVSVSFEGPEPSALQNLEIRWPWKRCHCR-SFCRPYENATSFCAQGLFKQHKFCCLETWPPRMK-----
DEFA-8   MKTLVLLSALVLLAFQVQADHIQEA--EEETKTEEQPADEDQDMSVSFEGPEASALQNLEIGWPLKQCHCR-KFCRPYEKAEGSCRPGLFIKRKICCIQQWTPGRT-----
ED-5     MKKLVLLSALVLLALQVEAEPTPKT--DEGTKTDEQPGKEDQVVSVSIEGQGDPAFQDAVLRD--LKCFCRRKSCNWGEGIMGICKK-RYGSPILCCR----------
         * .* ** :*:*:* : :*.          :        *:* : : : *   ..:*:         * **   *   *     *         :**
```

B

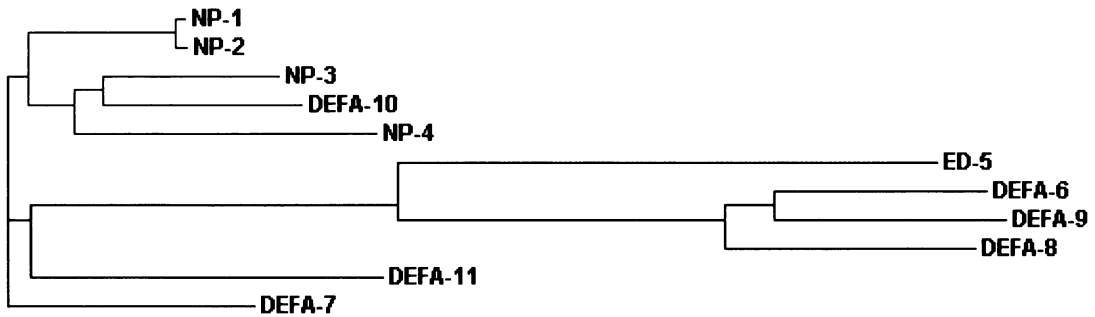


Figure 7. Panel A. A multiple alignment of rat α-defensin's sequences. Color code: red color marks small, hydrophobic AAs, acidic AAs are in blue, basic AAs are pink and hydroxyl or amine containing AAs are green. Evolutionary conserved AAs in all sequences are marked by asterisks (*). The colon (:) indicates a "conserved" substitution of one AA by another AA with the same characteristics. The dot (.) means that semi-conserved AAs substitutions have been observed. **Panel B.** Phylogram tree analysis. The figure shows that defensins cluster into three major groups. First one containing myeloid defensins is marked by red color. Among these, NP-1 and NP-2 share the highest degree of sequence similarity among all rat α-defensins. The second cluster includes ED-5, DEFA-6, DEFA-8 and DEFA-9 and represents the group of rat enteric defensins, marked by blue color. DEFA-11, is expressed in myeloid cells but sequentially is closer to this group of enteric defensins. Finally, DEFA-7, the myeloid defensin, seems to be sequentially dissimilar to all other α-defensins, marked by green color.

4. Experimental part

4. 1. Main objectives

I. An assessment of α-defensin expression on a panel of rat tissues.
II. Verification of α-defensin expression in thymus using immunohistochemistry approach.

4. 2. Materials and methods

Animals:

Rattus norvegicus (Rattus sp. strain Wistar) from breading colony in Institute of Physiology AS CR, v.v.i. was used for all experiments. 15 days old animals of both genders were sacrificed by cervical dislocation and extracted tissues were processed immediately.

Reverse transcriptase polymerase chain reaction (RT PCR):

Total RNA was isolated from several tissues using NucleoSpin RNA II kit (Macherey-Nagel; German, Düren). In case of RNA isolation from blood samples, RNeasy protect animal blood kit (Qiagen, Germany) was used. The RNA quality was checked by gel electrophoresis. The quantity of RNA was estimated by using Nanodrop spectrophotometer (Nanodrop Technologies; U.S.A., Wilmington). Whole RNA (400 ng) was reverse transcribed into cDNA using Superscript II (Invitrogen; U.S.A., Carlsbad) and random hexamers (Promega; U.S.A., Madison). All steps were done according to manufacture's instructions. Primers were designed by using Roche assay design centre (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=UP030000) and Primer 3 program (http://primer3.sourceforge.net/). Specifity of all primers were tested by BLAST search, using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). All primers sequences are listed bellow in the table 2. PCR reactions using Combi Taq DNA polymerase (Top-Bio; Czech Republic, Prague) were firstly normalized on housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and secondly on selected α-defensins. All reactions were performed on XP cycler (Bioer technology; China, Binjiang). The PCR products were visualized by ethidium bromide staining (2.0% agarose gel, Lithium/Borate buffer). As a marker FastRuler ultra low range DNA ladder (Fermentas; Canada, Burlington) were used.

AATGTATCCGTTGTGGATCTGA	GCTTCACCACCTTCTTGATGT	76
TCCTTTGGAGGGGATAAAGG	GCGTGTGCGTCTGCAATA	81
CTCCAGATGAGGAGCAGGAT	TCTCCAAAACGACAGCTTGA	124
CTCCCCAAGAAAGAGCTAAGG	GCTTGACCTGCCTTCACAAC	127
CAAGCTGAACCTACCCCAAA	ATCCCCATAATGCCTTCTCC	188
CCAGGATGTGTCTGTCTCCTT	TCCTCACTGGCCTTCCTATC	77
TCCTTTGGAGGGGATAAAGG	GACAGCTGAGGAGTCTGCAA	85
CAGGATATGTCTGTCTCCTTTGAA	TGCTTTAATGGCCATCCTATCT	77
CAGCAGATGAGGACCAGGAT	CTTCCATGGCCATCTGATCT	89
TTTCCGTATTCTTTGGAGGAGA	TCTGCAAGAGCAGGTCGAG	71
CTCTGCTCACCACCCTTCTC	TAACCGCATTCCCCATAGAC	229

Table 2. Sequences of primers used for PCR reactions.

Immunohistochemistry:

Fresh thymi were fixed in 4 % paraformaldehyde in phosphate buffered saline and immersed in 30 % sucrose overnight. Tissues were embedded into a tissue freezing medium (Leica Microsystems; Germany, Nussloch) and frozen at -80°C. Thymi were sectioned (5-8 μm) using cryotom and subsequently subjected to immunofluorescent staining according to the protocol available on the following web site: (http://www.cellsignal.com/support/protocols/IF.html). Briefly, slides were fixed in 4 % paraformaldehyde and preincubated with primary antibodies (diluted 1:500) followed by secondary antibodies conjugated with fluorochrome (Alexa Fluore 488). The primary polyclonal rabbit antibody against rat NP-1/2 was kindly provided by Michael E. Selsted, University of California[28]. The slides were mounted by Vectashield containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories; Canada, Burlingame) onto a coverslip. Specimen was examined under Leica DMI6000 epifluorescence microscope or Leica sp5 confocal microscope (both Leica Microsystems; Germany, Wetzlar). Visualization of defensin signal covering entire area of thymic section was completed by the Leica software. This software enables to build large area image from individual smaller pictures.

4. 3. Results

4. 3. 1. Expression of α-defensins in selected tissues

For the assessment of α-defensins expression by RT PCR we used a panel of rat tissues which included: thymus (TH), lymph nodes (LN), bone marrow (BM), spleen (SP), brain (BR), stomach (ST), lung (LU), liver (LI), duodenum (DU), kidney (KI), hearth (HE) and blood. These tissues were examined for the presence of ten α-defensins: *NP-1/2, NP-3, NP-4, ED-5, Defa-6, Defa-7, Defa-8, Defa-9, Defa-10* and *Defa-11* by RT PCR.

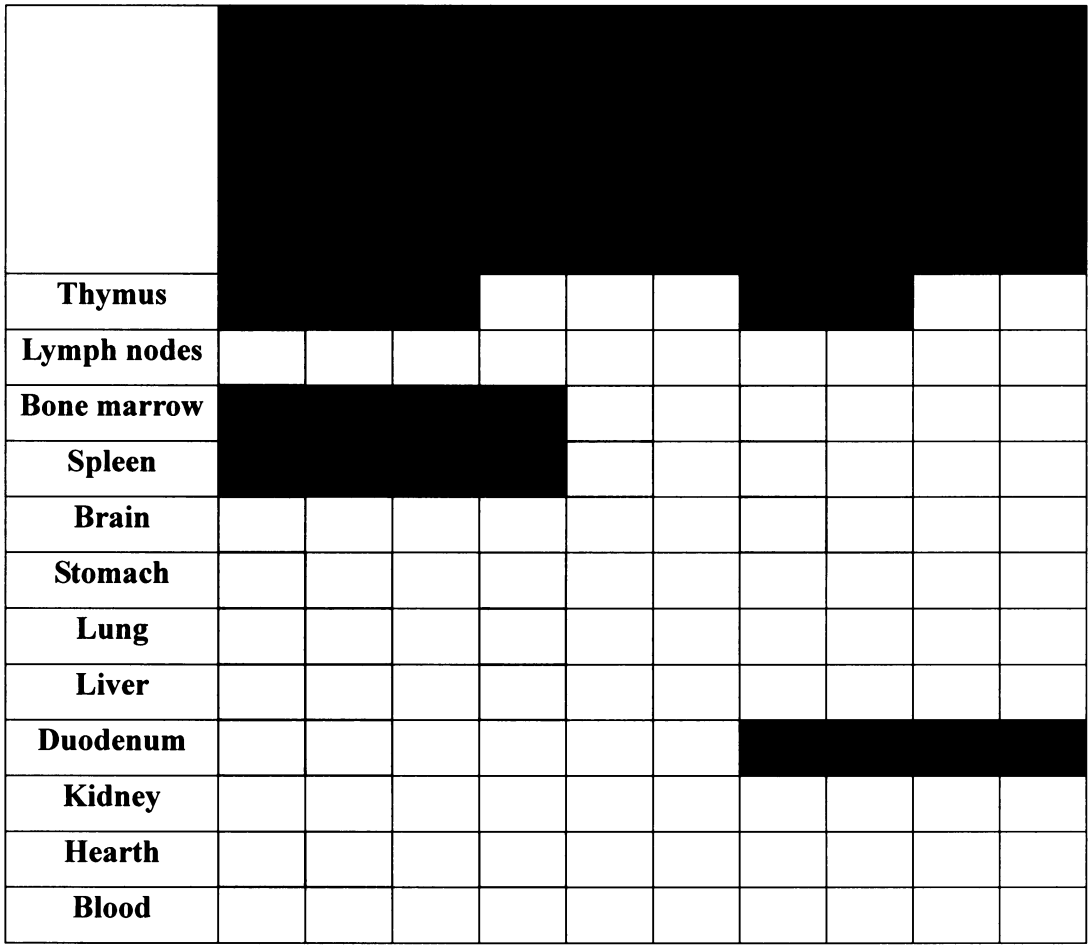
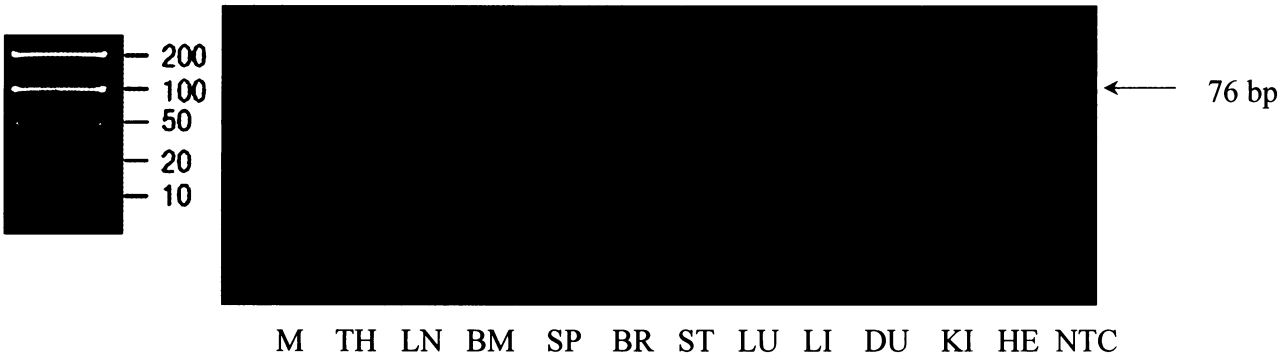


Table 3. Expression of rat α-defensins in selected tissues. Color code: Myeloid α-defensins are marked by orange color, enteric α-defensins by green. Expression levels: red color indicates a significant expression, yellow color – intermediate expression, white color – no expression was detected.

All results generated from individual gel analyses are summarized in table 3. We provide figure 8 to illustrate the process of assessment of expression of individual defensins using RT PCR

followed by the agarose gel electrophoreses analysis of endpoint PCR products. Marker is indicated as M and NTC stands for a non-template control representing the negative control (PCR reaction mix with no template). Blood cDNA was assessed for the presence of defensins in a separate experiment, but there was no detectable expression for any of the defensins tested.

GAPDH after 29 cycles:



ED-5 after 37 cycles:

Defa-11 after 38 cycles:

Figure 8. Assessment of α-defensin expression on a panel of rat tissues using agarose gel electrophoresis of RT PCR endproducts. House-keeping gene *GAPDH* and defensins *ED-5* and *Defa-11* are used as examples of approximation of expression levels. Specifically, the middle panel illustrates an assessment of *ED-5* expression levels and shows a significant expression in the thymus and duodenum (marked by red color in table 3) while the expression is only intermediate in the bone marrow, spleen and brain (marked also by yellow color in table 3). Picture highlighting *Defa-11* expression profile (bottom panel) exhibits the significant expression levels in bone marrow and spleen (marked by red color in table 3) and lower expression in stomach, lung, liver and heart (marked by yellow color in table 3). Remaining samples in all three pictures shown are negative and they are marked in table 3 by white color.

4. 3. 2. Verification of α-defensin expression in thymus using Immunohisto-chemistry approach

Anti NP-1/2 immunofluorescence of cryosectioned thymus revealed the presence of positively stained cells clustered in certain regions across the thymus. Control samples, where primary antibody was replaced with a non-immunized rabbit serum, appeared to be negative (data not shown).

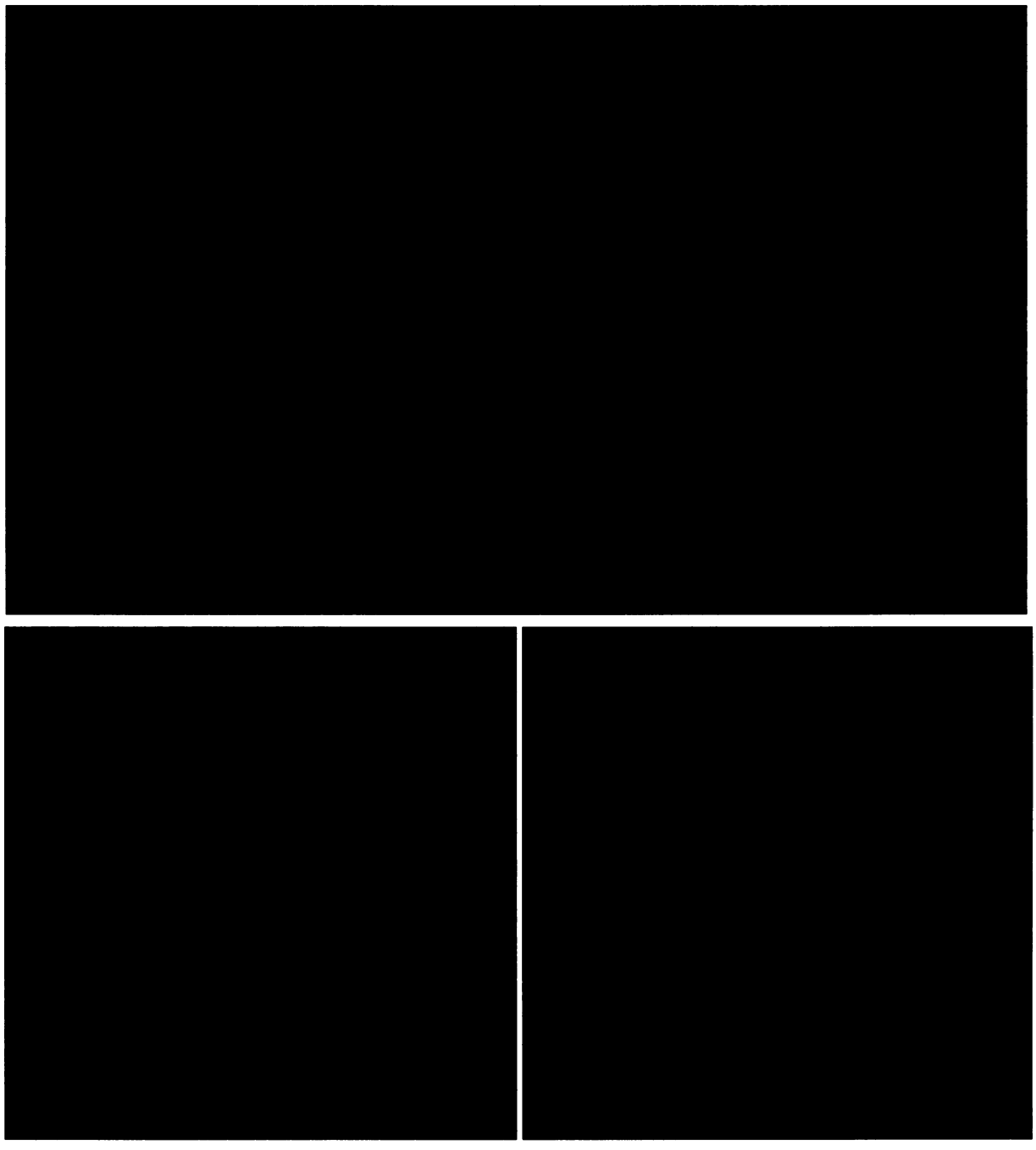


Figure 9. Epifluorescence (top panel) and confocal microscopy (button panels) of immunofluorescently stained thymus using anti-NP-1/2 antibody. Top panel shows whole

area of thymic section, (magnification 400x). Bottom pictures feature individual NP-1/2 positive cells (magnification 1000x). Blue color marks the cell nuclei stained by DAPI and green Alexa Fluor 488 represents NP-1/2 protein.

4. 4. Discussion

We have examined defensin expression on mRNA level in selected rat tissues. Even though we were mainly focused on α-defensin expression in the thymus, examination of other rat tissues represented a significant point of interest. This was due to a paucity of information concerning tissue-specific expression profile of rat α-defensins.

Search of the literature showed that the only defensin which expression is detected in the thymus by RT PCR is *NP-3*[35]. In contrast to this finding by Patil *et al.*, we detected a relatively abundant thymic expression of *NP-1/2, NP-4, ED-5* and *Defa-6*. Reason for this discrepancy is not clear, however, we can offer two possible explanations: (i) we used newly self-designed primer sets that could exhibit a higher sensitivity of detection and (ii) an age difference between used animals. Specifically, in contrast to Patil's group which used 2-months old rats, we utilized the thymic tissues derived from 15-days old rats. It is of note however, that the analysis performed by Patil's group is outstanding from the bioinformatics point of view as they were able to identify new members of α-defensin family by a computer search.

To confirm our data, Northern blot analyses and quantitative real time PCR (qPCR) performed on RNA isolated from the total thymic tissues showed marked expression of *NP-1-4* (Neuwirth and Filipp, unpublished data). Moreover immunohistochemistry data, showed herein in the figure 9, provided clear evidence for NP-1/2 expression on the protein level within the thymus. Surprisingly, we were not able to detect any α-defensin expression in the RNA sample derived from the whole peripheral blood. This might be due to the fact reported previously that synthesis of α-defensin mRNAs occurs only in early developmental stages of neutrophils and ceases in mature neutrophils[28].

An overarching goal of this study was to obtain an insight into the expression profile of all ten so far identified α-defensins across broad spectrum of rat defensins. RT PCR is a suitable approach for such assessment as it can provide not only a qualititive (yes or no answer) but also a semiquantitative estimate of expression levels. However, a precise quantification of defensin

expression cannot be achieved by this approach. Thus, the next step in our investigation will be the quantitative analysis of α-defensins mRNA levels in rat tissues using qPCR. This method should provide an accurate evaluation of expression levels and will also enable a direct comparison of expression levels among all α-defensins. Still, we have to be very cautious in the interpretation of these results, as what really matters is the expression of defensins on the protein level. Proteosynthesis is strictly regulated and complex process, and thus the mRNA levels might not always correlate with the protein levels. The very same scenario could be applied to the situation in the thymus. More notably, although we detected considerable mRNA levels of five α-defensins in this organ, whether or not these expressions correlate with their protein levels, remains to be determined. In this sense, we are certain that at least one of thymic α-defensins, NP-1/2, is indeed expressed detectably on protein level. However our protein level analysis in regards of other thymic defensins is largely limited by unavailability of antibodies to these peptides. Other important questions concern the cellular source of defensin protein expression, their cellularity (number of cells per organ), level of expresssion per cell, mode of protein expression (constitutive or inducible) and whether stored intracellularly or secreted. All these questions await further evaluation and experimentation and will be the main objective of our future work.

The importance of thymic architecture to support biological processes confined to this organ is well established. Distinct thymic regions like cortex, medulla and juxta-medullary junctions, to mention just a few, have been described in details[60]. We performed immunohistochemistry on thymus section to localize NP-1/2 signal and observed that majority of cells expressing NP-1/2 clustered into distinct areas in thymus (see figure 9, the top). While this result is encouraging, in order to associate these NP-1/2 positive cellular cluster-like formations with certain thymic regions, we will perform a co-localization experiment. In this way, parallel double staining of thymic sections with anti-NP-1/2 antibody with antibody specific for cortico-medullary markers and/or counterstaining of slides with haematoxylin-eosin will be preformed. Insight provided from this experiment will allow us to position NP-1/2 positive cells within the inner architecture of the thymus and thus suggest it possible role in its physiology and biology.

The most important information related to the function of α-defensins in the thymus pertains to the possible cellular source of individual α-defensins. Towards this end, we will employ two strategies: firstly, FACS cell sorting of thymic cell subpopulations followed by individual defensin qPCR analysis, and secondly, an immunohistochemistry approach with anti-NP-1/2 antibody on sorted thymic populations.

Expression profiles of α-defensins *NP-1/2*, *NP-3* and *NP-4* exhibit a similar tissue specific expression profile. Notably, there are abundantly expressed in the thymus, bone marrow and spleen (table 3). However, other two myeloid defensins, namely *Defa-7* and *Defa-11*, are not expressed in the thymus. The last myeloid defensin – *Defa-10* is not detectable in any of the tissues tested. Interestingly, all enteric α-defensins have the same strong expression in duodenum (part of small intestine), but *ED-5* and *Defa-6* are also abundantly expressed in thymus. Moreover *ED-5* is expressed on intermediate level the in bone marrow and spleen. Question remains, whether expression of these enteric defensins in the thymus, bone marrow and spleen fulfills the same or similar role as in the case of myeloid α-defensins expressed in the same organs and also whether enteric defensins are expressed by the same type of cells or not.

4. 5. Conclusions

In aggregate, these results are the first to provide an overall picture of α-defensin expression in rat tissues. We found that expression of α-defensins is not uniformed but rather tissue-specific. While some of them are detected broadly with varied level of expression across tissues like *NP-1/2* and *NP-3*, others, like *Defa-8* and *Defa-9* are detected in high levels in the duodenum, exclusively. Surprisingly, the thymus is the site of abundant expression of 5 out of 10 α-defensins tested: 3 of them are myeloid and two enteric. Certainly, these results open a whole new area for investigation of the physiology and function(s) of α-defensin in mammalian immunity as well as in non-immune processes and suggest that the scope of their physiological functions is much broader and tissue-specific than previously thought.

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