Host Defense Proteins of the Male Reproductive Tract

Review

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Innate immunity, the rapidly responsive and phylogenetically ancient system of host defense, is generating increasing interest in part due to the growing appreciation for its remarkable broad spectrum effectiveness. We dwell in a sea of micropredators that are well equipped to colonize and invade our tissues. Yet we coexist with these organisms day after day without chronic inflammation, tissue damage, or even awareness of their presence. Coexistence occurs largely because the innate immune system, which includes antimicrobial proteins, complement, and phagocytes, prevents invasion by destroying or evicting the microbes. Only those maintaining commensal status are allowed to colonize. In addition, evidence is emerging to indicate that antimicrobial proteins stimulate the adaptive immune response. When the innate immune system is overwhelmed and tissue invasion does occur, the T lymphocytes and antibody-producing B lymphocytes of the adaptive system are recruited to eliminate the infection. The success of plants, insects, and other lower animals that lack adaptive immunity affirms the effectiveness of the innate immune system. Today, more than 700 antimicrobial proteins are known in the plant and animal kingdoms. The Antimicrobial Sequences Database lists 752 eukaryotic entries as of February 12, 2002 (Tossi et al, 2002); this is a dramatic increase since the original discoveries in plants 30 years ago (Fernandez-de-Caleya et al, 1972), and in animals 20 years ago (Hultmark et al, 1980; Steiner et al, 1981; Selsted et al, 1983).

Through improved understanding of innate antimicrobial proteins, ways to mimic or enhance their activities may be developed to address the growing need for new therapeutic agents that combat multidrug-resistant pathogens (Gabay, 1994; Ganz and Lehrer, 1999; Cole and Ganz, 2000). One group of mimics, the synthetic cyclic D,L-α-peptides, were recently shown to destroy antibiotic-resistant bacteria by spontaneously forming membrane disrupting nanotubes (Fernandez-Lopez et al, 2001). Despite millions of years of exposure, microbes have not developed resistance against antimicrobial peptides, perhaps because they target components that are integral to bacterial structures and pathogenicity. The need to investigate host defense proteins, particularly in reproductive systems, is brought into focus by the spread of sexually transmitted infections. An estimated 36 million people worldwide were infected with the human immunodeficiency virus (HIV) by the end of 2000 (Anonymous, 2001). Better prevention and control of other infections of the genitourinary tract could also have a major effect on public health.

Host defense proteins have received more attention in myeloid cells, and in the respiratory and digestive tracts than in the male reproductive system, perhaps because of known involvement in disease conditions of these tissues. Examples include resistance to *Porphyromonas gingivalis* conferred by calprotectin (Nisapakultorn et al, 2001), reduced HIV-1 infectivity in the presence of basic, proline-rich salivary gland proteins (Robinovitch et al, 2001), resistance to cationic antimicrobial peptides conferred by a gene product of *Legionella pneumophila* (Robey et al, 2001), a defensin role in inflammatory lung disease (Singh et al, 1998), and the association of mutant NOD2 with Crohn disease (Van Heel et al, 2001). Studies in the male reproductive tract on many of the proteins described in this review are relatively few to nonexistent. Yet this vital system is open to the exterior and vulnerable to invasion. It is our hope that this review will stimulate research into the expression and function of host defense proteins in the male reproductive tract.

This review will examine physical barrier proteins that help prevent pathogen entry and then discuss the defensin and cathelicidin cationic peptides, and the secreted protease inhibitors. Host defense roles of lipocalins, lectins, lactoferrin, and lysozyme will be summarized. The cell surface Toll-like receptors and intracellular NOD receptors, key surveillance proteins that mediate responses to pathogens, will be described. The proteins discussed here, their sites of expression, and their known and suggested functions in the male reproductive tract are summarized in the Table. Immunoglobulins, complement proteins, and

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complement regulatory proteins in the male reproductive tract were previously discussed (Vanderpuye et al, 1992, Beagley et al, 1998, Nonaka et al, 2001) and are not included in this review.

Physical Barrier Proteins

Intrinsic structural features of epithelial cells throughout the male genital system (Merk et al, 1980; Ortiz and Cavicchia; 1990; Cyr et al, 1999; Fulmer and Turner, 2000; Mitic et al, 2000) impede free movement between the reproductive tract lumina and the blood. In addition, products secreted by and overlying the epithelium are essential to protect the mucosa from primary injury by pathogens. In mucus, the watery gel on the epithelial surface, immune functions can prevent the infectious entry of pathogens and toxin injury (Biesbrock et al, 1991; Cone, 1999; Eggert-Kruse et al, 2000). Mucus gels form complex ecosystems that are filled with cells, bacteria, nutrients, protective factors, and wastes. Secreted mucins are long, fibrous peptides coated with a complex array of oligosaccharides (Lamblin et al, 1991; Gendler and Spicer, 1995), the composition of which varies during development and depends on the host diet as well as the presence and activity of specific commensals and pathogens. Bacteria and viruses bind specifically to mucins (Cohen and Laux, 1995; Tabak, 1995) and thus attachment to the epithelial cell surface can be prevented by the dynamic processes of outward secretion and mucus shedding. Such specific binding of oral mucins to HIV-1 envelope glycoprotein gp120 blocks cellular infection by preventing gp120 complex formation with the high-affinity HIV-1 receptor, CD4 (Bergey et al, 1994). When these mucus clearing processes are impaired in the epididymis, as can occur in patients with cystic fibrosis, blockage resulting in infertility can occur (Dumur et al, 1996).

Defensins

Perhaps the best known innate host defense proteins are the defensin cationic peptides (Lehrer et al, 1999; Hancock and Diamond, 2000). The human defensin family includes at least 6 alpha subfamily members and over 30 in the beta subfamily (Schutte et al, 2002). Alpha defensins are predominantly neutrophil proteins not known to originate in the male reproductive tract, whereas the beta defensins are widely synthesized in epithelial cells. Defensins are synthesized as 93–96 amino acid pre-pro-peptides consisting of a signal peptide, an anionic prosegment, and a C-terminal cationic peptide. Release of the C-terminal peptide from the prosegment by elastase, metalloproteinase, or other proteolytic cleavage activates the antimicrobial peptide. The active peptides are small, typically 3.5–4.5 kd, 29–35 amino acids, and display broad spectrum antimicrobial action against bacteria, yeast, and fungi (Boman, 1995; Ganz and Weiss, 1997). The 3-dimensional structure of defensins is characterized by a cationic β-sheet-rich amphipathic structure stabilized by a conserved 6-cysteine motif. The cysteines in this motif are spaced and paired slightly differently in the α- and β-defensins (Lehrer et al, 1999). The conserved prosegment appears to maintain the protein in an inactive form until it reaches a site where its activity is required either outside the cell or within lysosome-like granules of mature phagocytes (Harwig et al, 1992; Selsted and Ouellette, 1995; Ayabe et al, 2000).

Evidence suggests that defensin oligomers assemble to disrupt microbial cell membranes by forming channels that allow defensins and other host defense molecules to enter (Lichtenstein, 1991), and permit leakage of contents from the perforated cell. Ion gradients are disrupted and the cells die (Lehrer et al, 1989; Wimley et al, 1994). These small cationic peptides preferentially target the negatively charged phospholipids that compose bacterial membranes. Mammalian host cell membranes are less sensitive to these peptides at least in part because their membranes contain electrically neutral zwitterionic phospholipids and cholesterol (Gazit et al, 1995; Matsuzaki, 1999). The activity of defensins is dependent on salt concentration (Goldman et al, 1997). Human and guinea pig neutrophil defensins require low ionic strength for antibacterial activity and their effects are abolished by physiological concentrations (150 mM) of NaCl (Nagaoka et al, 2000). Low sodium concentrations in the rat epididymis ranging from 58 mM in the caput to 15 mM in the cauda (Turner et al, 1977) would be expected to permit defensin activity.

Evidence for regulated expression of defensins and their activating proteases in the male reproductive tract is beginning to emerge. Widespread expression of human beta defensin 1 was detected by reverse transcription-polymerase chain reaction using RNA isolated from testis, prostate, and other organs (Zhao et al, 1996). Rhesus β-defensins 1 and 2 were found in Sertoli cells and seminal vesicle epithelial cells (Bals et al, 2001). Human β-defensin 4 is most abundant in testis (Garcia et al, 2001). Immunoreactivity corresponding to a defensin (cryptdin) was reported in mouse Sertoli cells, especially in stages V-VI, containing elongating spermatids and in Leydig cells but not in testicular macrophages (Grandjean et al, 1997). Neutrophil elastase in semen is also available to activate defensins, and this enzyme increases during inflammation in the male reproductive tract (Zopfgen et al, 2000; Zorn et al, 2000).

In a recent report, a defensin-like epididymis-specific protein, Bin1b was implicated in epididymis defense against bacteria (Li et al, 2001). Rat Bin1b is a member of the HE2 family of primate epididymal proteins (Osterhoff et al, 1994; Fröhlich et al, 2000). Alternative splicing of human and chimpanzee transcripts from a single

Host defense proteins expressed in the male reproductive tract

Protein	Sites of Expression	Functions	
		Known	Proposed
Mucins	Testis epididymis, prostate	Mobile barrier to prevent entry of bacteria, viruses, toxins	Bind and remove pathogens from the male reproductive tract
β-Defensins	Testis, epididymis, prostate, seminal vesicle	Kill *Staphylococcus, Enterococcus, Escherichia coli, Candida*; chemotaxis	Sperm and epithelial protection
Cathelicidins	Epididymis, neutrophils	Kill *Staphylococcus, E. coli, Pseudomonas*; chemotaxis, sperm-binding	Epithelial cell protection; mobile defense system in male and female reproductive tracts
Bin1b/HE2	Epididymis	Kill *E. coli*; sperm binding	Antimicrobial protection of sperm in male and female tracts
α_2-Macroglobulin	Sertoli cells	Nonspecific inhibition of mammalian proteases	Inhibition of microbial proteases
Secretory leukocyte protease inhibitor	Epididymis, prostate, seminal vesicles	Inhibition of elastase, cathepsin G, and proteases of *Aspergillis*; inhibition of viral infection, kill *E. coli* and *Staphylococcus*	Antimicrobial action in the male reproductive tract
Cystatin C	Sertoli and germ cells, epididymis, prostate, seminal vesicles	Inhibition of mammalian cathepsins, inhibition of group A streptococcal cysteine protease	Antimicrobial action in the male tract; inhibition of microbial proteases
Male reproductive cystatins	Testis, epididymis	Unknown	Antimicrobial action in the male reproductive tract
Tear lipocalin	Germ cells, prostate	Bind and transport small hydrophobic molecules	Host defense in the male reproductive tract
Lung surfactant Protein D (SP-D)	Testis, prostate	Limit lung infection by *E. coli Salmonella, Haemophilus, Pseudomonas, Pneumocystis, Aspergillus*; chemotactic for phagocytes	Protection of the male tract against bacteria and fungi andrecruitment of phagocytes
Lactoferrin	Epididymis	Nutrient sequestering inhibits infection by bacteria, fungi, and viruses	Antimicrobial protection of sperm in male and female tracts
Lysozyme	Testis, epididymis, prostate	Bacterial lysis	Bacterial lysis in the male tract
Toll-like receptors	Testis, prostate	Cell surface mediators of intracellular responses to pathogens	Mediate induction of antimicrobial peptides in response to microbial invasion
NOD1, NOD2	Testis	Intracellular pathogen recognition receptors	Mediate induction of defense pathways in response to viral, bacterial and eukaryotic pathogens

gene generates this family of proteins that consist of signal peptides and propeptides and different predicted elastase-cleavable C-terminal peptides. The transcripts are highly epididymis-specific, androgen-regulated, and the proteins bind spermatozoa (Osterhoff et al, 1994; Hamil et al, 2000; Ibrahim et al, 2001). To date, in this family only Bin1b has been reported to possess antimicrobial activity. However, it is likely that other family members will also prove to have roles in epididymal host defense.

Beta defensins, when tested in vitro in concentrations ranging from 10 to 50 μg/mL, are able to destroy a remarkably broad range of pathogens that can invade the male reproductive tract, including multidrug-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* (Harder et al, 2001) as well as *Escherichia coli* and *Candida* species (Schonwetter et al, 1995, Schroder and Harder, 1999). Human β-defensin-1 killed *Pseudomonas aeruginosa* and reduced infectivity of adenovirus (Gropp et al, 1999). Estimates of physiological defensin concentrations range from 0.01 to 0.1 μg/mL for human beta defensin-1 in voided urine (Valore et al, 1998) to 1–10 mg/mL for defensins in neutrophil granules (Ganz, 1987; Lehrer et al, 1993). The concentration of porcine β-defensin-1 in tongue epithelium was estimated to be 20–100 μg/mL (Shi et al, 1999).

Eukaryotic host cells are not exempt from damage by cationic peptides. Purified defensins kill normal and tumor cells by concentration- and time-dependent mechanisms (Lehrer et al, 1993). A 2-hour pulse treatment of mouse blastomeres with 50 μg/mL of hBD-2 resulted in 70% degeneration after 24 hours and 100% after 48 hours, suggesting usefulness in pregnancy prevention (Sawicki and Mystkowska, 1999). Spermicidal effects of defensins have not been reported.

Defensins serve to link the innate and adaptive immune systems. Defensins contribute to the induction of adaptive antimicrobial immune response through chemotactic mobilization of immunocompetent leukocytes (Yang et al, 1999). Human β-defensins are chemotactic for immature dendritic cells (Yang et al, 2000a). There is evidence that beta defensins recruit dendritic cells and T cells to sites of microbial invasion through interaction with the chemokine receptor 6 (Yang et al, 1999). Beta defensins induce chemotaxis at considerably lower concentrations (0.1–1.0 μg/mL) than are required for antimicrobial effects (Yang et al, 2001).

Cathelicidins

Like defensins, cathelicidins are initially translated as preproproteins containing an N-terminal signal peptide, a middle proregion, and a C-terminal antimicrobial peptide (Gennaro and Zanetti, 2000). The conserved proregion of 100 amino acids is similar to cathelin, a cysteine protease inhibitor (Ritonja et al, 1989). As in the case of defensins, antimicrobial activity is observed only when the C-terminal domain is cleaved from the secreted protein (Scocchi et al, 1992) by proteases such as elastase (Panyutich, 1997) or by proteinase 3 (Sørensen et al, 2001). Cathelicidin active peptides include several structural groups and show great variability in amino acid sequence. Most are primarily alpha helical. LL-37, the active C-terminal peptide of hCAP18, the only known human cathelicidin (Larrick et al, 1995), is a linear peptide that can assume an alpha helical conformation (Gennaro and Zanetti, 2000). The highest antibacterial activity correlates with maximal helical content, whereas intermediate and low activities correspond to low helical content and disordered secondary structure (Johansson et al, 1998). The alpha helical structure of LL-37 is dependent on pH, anion concentration, and its own concentration. Evidence suggests that rather than forming pores, hCAP18 carpets the surface of bacteria initially as monomers and oligomers. Monomers diffuse into the membrane and cause disintegration in a detergent-like manner (Oren et al, 1999).

Developing neutrophils are a major site of synthesis of cathelicidins, but hCAP18 is also produced in human epididymis where expression is highest in cauda and lowest in caput (Malm et al, 2000). CAP18 protein was detected by enzyme-linked immunosorbent assay in seminal plasma at 42–143 μg/mL (Malm et al, 2000) from healthy donors, whereas in serum it was 1.2 μg/mL (Sørensen et al, 1997). An estimated 6.6 million molecules of hCAP18 were reported bound per spermatozoon (Malm et al, 2000), possibly protecting against microbial attack on the way to the ovum and during fertilization. Little or no expression of hCAP18 was detected in testis, prostate, or seminal vesicle in this study.

The antimicrobial C-terminal peptide of many cathelicidins, including LL-37, can bind the lipopolysaccharide of the outer membrane of Gram-negative bacteria (Larrick et al, 1995). Human CAP18 can bind and neutralize the capacity of lipopolysaccharide (also known as endotoxin) to induce endotoxin shock (Larrick et al, 1995). Human CAP18/LL37 was active against urogenital flora (Smeianov et al, 2000). Human CAP18 was tested against *Pseudomonas aeruginosa, E. coli, Staphylococcus aureus,* and methicillin-resistant *S. aureus* (Travis et al, 2000), and at concentrations in the range of 1–15 μg/mL, was active against all test organisms under both low and high salt conditions. The antimicrobial effects of hCAP18 were reduced, but not abolished in physiological saline, as reported for defensin activity (Nagaoka et al, 2000). However, neutrophil defensins together with cathelicidins can act synergistically to kill bacteria even in the presence of 150 mM NaCl. In another report, LL-37/CAP18 and human defensin α-1 synergistically killed *E. coli* and *S. aureus* (Nagaoka et al, 2000). In this study, human defensin α-1 and guinea pig defensin were synergistic with

hCAP18/LL-37 in potentiating the outer and inner membrane permeabilization of *E. coli.*

In vivo studies analyzing the antimicrobial effects of hCAP18 have also yielded promising results. Mice given intratracheal injections of adenoviral recombinant vector expressing hCAP18 had a lower bacterial load and reduced inflammatory response than did untreated mice following pulmonary challenge with *P. aeruginosa.* Mice given intravenous injections of the recombinant adenovirus expressing hCAP18 showed improved survival rates following intravenous injection of *E. coli* or bacterial outer cell membrane constituents lipopolysaccharide and galactosamine (Bals et al, 1999). Galactosamine sensitizes mice, reducing the dose of lipopolysaccharide required for lethality.

Cytotoxic effects of hCAP18/LL-37 have also been reported at higher concentrations. The minimal in vitro inhibitory concentration of hCAP18/LL-37 against *E. coli* D21 was 22 μg/mL (Johansson et al, 1998). At 3–5 times this concentration, the peptide also exhibits cytotoxic activity toward eukaryotic cells, including human blood leukocytes, the T cell line MOLT, and *Trypanosoma cruzi* (Johansson et al, 1998). Thus, hCAP18/LL-37 could cause host cell damage although its cytotoxic activity is inhibited by human serum (Johansson et al, 1998). Some evidence suggests that the spermicidal effects of cationic peptides are reduced in seminal plasma (Edelstein et al, 1991), but systematic studies are needed to determine the influence of seminal plasma and female reproductive tract secretions on mammalian cationic antimicrobial peptide activities.

Like defensins, cathelicidins stimulate the adaptive immune system. LL-37 is chemotactic for neutrophils, monocytes and T cells, but not dendritic cells (Yang et al, 2001). Thus, it has distinct, host-target cell specificity. LL-37 chemotactic action on monocytes is mediated by formyl peptide receptor-like 1, a 7 transmembrane G protein-coupled receptor (Yang et al, 2000b).

Protease Inhibitors

Proteolytic enzymes are essential virulence factors for prokaryotic and eukaryotic parasites during all stages of the infectious process (Armstrong, 2001). Tissue barriers to invasion, including keratinocyte layers of the epidermis, the basal lamina, interstitial extracellular matrix, and blood vessel walls are all susceptible to attack by parasite proteases (Cohen et al, 1991; Sodeinde et al, 1992). Degradation of host proteins provides the invaders with nutrients as well as passage into the host interior. The success of peptide based protease inhibitors against HIV-1 (Tomasselli and Heinrikson, 2000) and *Candida* (Pichová et al, 2001) may serve as a paradigm for developing protease inhibitors as antibiotics against a variety of pathogens.

Proteinase inhibitors are abundant in semen (Starkey and Barrett, 1977; Ohlsson et al, 1995; Kise et al, 2000). The nonspecific inhibitor α_2-macroglobulin and the active site inhibitors, the serpin human seminal inhibitor I and the cysteine protease inhibitor cystatin C, are all expressed in the male reproductive system. Each is known to have antimicrobial activity in other organs, but the conditions for induction and function and the effects of these protective activities have not yet been investigated in the male reproductive tract.

The α_2-macroglobulins enfold the target proteinase, physically blocking access to substrates and conveying the enzyme to a receptor-mediated endocytotic clearance pathway in macrophages and other cells (Van Leuven, 1984). This blanket mechanism enables α_2-macroglobulins to block activity of endopeptidases of diverse origins and catalytic mechanisms including proteases of parasites (Giroux and Vargaftig, 1978; Sottrup-Jensen et al, 1989). In mammals, α_2-macroglobulin is implicated as a key agent that mitigates bacterial elastase-induced septic shock (Khan et al, 1995) and members of the α_2-macroglobulin family have therapeutic potential in conditions in which microbial proteases play a role in disease (Neely et al, 1986), but direct killing of pathogens has not yet been demonstrated. It is possible that further studies on α_2-macroglobulin secreted from Sertoli cells (Cheng et al, 1990; Zhu et al, 1994), and the high concentrations in the rete testis fluid might reveal a role in suppressing pathogen proteases in addition to the host protease regulating functions previously proposed (Aravindan et al, 1997; Peloille et al, 1997).

The inhibitory specificity of serpin active site inhibitors is restricted by the amino acid sequence of the exposed reactive site loop. A protease attempting to cleave this loop becomes covalently bound to the inhibitor, which undergoes a conformational change, resulting in loss of proteolytic capacity of the enzyme (Huntington et al, 2000). Secretory leukocyte protease inhibitor (SLPI) a serpin also known as human seminal inhibitor I or antileukoprotease, was first described in human seminal fluid (Schiessler et al, 1976). It is abundant in epithelial cells and lumina of the prostate, seminal vesicles, and epididymis but not in the stroma of these glands (Ohlsson et al, 1995). It is present in seminal plasma at concentrations of 15–20 μg/mL (Shugars, 1999). Like defensins, SLPI is a low molecular weight (11.7 kd) cationic, nonglycosylated protein (Seemuller et al, 1986). SLPI inhibits mammalian neutrophil elastase and cathepsin G (Thompson and Ohlsson, 1986). It also inhibits proteases of *Aspergillis fumigatus* and cell detachment caused by this fungus (Tomee et al, 1997). SLPI inhibits infection of T cells and monocytes by HIV-1 (McNeely et al, 1995). SLPI is active against *E. coli* and *S. aureus* at concentrations above 20 μg/mL (Hiemstra et al, 1996) and is found

in secretions at concentrations ranging from 10 to 80 μg/mL (Cole et al, 1999). Through inhibition of a trypsin-like protease, SLPI inhibits the infectivity of influenza A and Sendai viruses (Kido et al, 1999). Other functions of SLPI relevant to innate host defense include anti-inflammatory activity (Mulligan et al, 2000) and promotion of wound healing (Ashcroft et al, 2000).

Peptides and derivatives that mimic the protease binding site of another active site inhibitor, cystatin C, block the growth of group A streptococci apparently by inhibiting a cysteine proteinase specific to these bacteria (Björck et al, 1989). Such peptides and whole cystatin C protein are active against other bacteria (Blankenvoorde et al, 1998; Kasprzykowski et al, 2000) and viruses (Björck et al, 1990; Cimerman et al, 1996). Plant cystatins are also involved in host defense (Aoki et al, 1995, Urwin et al, 1997; Koiwa et al, 2000). Cystatin C is present in Sertoli cells and germ cells of the testis (Tsuruta et al, 1993), as well as in epididymis, prostate, and seminal vesicles (Cole et al, 1989). Cystatin C inhibits mammalian cysteine proteases including the lysosomal cathepsins B, L, and H, all of which are expressed in the male reproductive tract and other organs. Of these, cathepsin L is a major secretory product of Sertoli cells (O'Brien et al, 1993); it is expressed in the caput epididymis (Tomomasa et al, 1994; Okamura et al, 1995; Jervis and Robaire, 2001) and prostate (Shuja et al, 1991), and is found in sperm (McDonald and Kadkhodayan, 1988).

In addition, four cystatins expressed primarily or exclusively in the male reproductive tract have been reported. Cystatin-related epididymal and spermatogenic proteins (CRES or cystatin 8; Cornwall et al, 1999), testatin (cystatin 9; Töhönen et al, 1998), cystatin T (Shoemaker et al, 2000), and CRES/cystatin-related protein ESC13 (cystatin 11; Liu et al, 2001) have been reported, but their functions have not been fully defined. CRES is located in the sperm acrosome, a site of high hydrolytic and proteolytic activity where a role in regulating acrosomal enzymes is likely (Syntin and Cornwall, 1999). However, the reactive sites of the male reproductive cystatins are unique and distinct from that of cystatin C and are consistent with the potential to inhibit different proteases. Cystatin 11 can kill *E. coli* (Hamil et al, 2002). It is possible to investigate and it should be investigated whether the functions of cystatins in the male reproductive tract extend beyond inhibiting native proteases and whether those functions include inhibiting pathogen proteases.

Lipocalins

Tear lipocalin, also known as tear prealbumin and von Ebner gland protein (Lögdberg and Wester, 2000) is abundant in tears where it constitutes 20% of total protein. The majority of proteins in tear fluid, including secretory immunoglobulin A, lysozyme, lactoferrin, and cystatin are well known to protect the eye against pathogens (Gachon and Lacazette, 1998). Tear lipocalin is also expressed in prostate (Holzfeind et al, 1996) and male germ cells (Syed et al, 1999) where its functions have not been investigated. Lipocalins are small, barrel-shaped proteins whose physiological functions are not well understood beyond their ability to bind and transport small hydrophobic molecules (Flower, 1996). Tear lipocalin contains peptides that are homologous to the papain binding domains of cystatins. These tear lipocalin peptides exhibit cysteine protease inhibitory potency comparable to that of cystatins (van't Hof et al, 1997). One of these peptides contains a motif previously shown to block bacterial growth (Björck et al, 1989) and the replication of herpes simplex virus (Björck et al, 1990). These reports raise the possibility that tear lipocalin also protects against pathogens; however, direct demonstration of antibacterial or antiviral effects has not been reported. Other lipocalins abundant in the male reproductive system, including prostaglandin D synthase, (Urade and Hayaishi, 2000), probasin (Kasper and Matusik, 2000), epididymal retinoic acid-binding protein (Ong et al, 2000), and native glycodelin (Seppala et al, 1997; Seppala et al, 1998; Keil et al, 1999) have not been reported to display antimicrobial activity.

Lectins

The collectins are secreted lectins, or carbohydrate-binding proteins that act collectively (Holmskov et al, 1994; Hoffmann et al, 1999). Discrimination between self and non-self carbohydrate structures is accomplished through the specificity of the collectin carbohydrate-recognition domains and the spatial arrangement of multiple such domains in oligomers (Gadjeva et al, 2001). These multimeric structures mediate microbial agglutination in vitro (Kuan et al, 1992). The collectin and lung surfactant protein-D, (SP-D) acts locally to limit lung infection and is also expressed in testis and prostate (Madsen et al, 2000). Microbial targets of SP-D include *E. coli, Salmonella minnesota, Haemophilus influenzae, P. aeruginosa, Pneumocystis carinii, A. fumigatus,* and others (Clark et al, 2000). SP-D also acts as a potent chemotactic agent for phagocytes (Cai et al, 1999). A role in protecting the male reproductive tract for collectins and other lectins has not been reported.

Lactoferrin

Lactoferrin is a major epididymal secretory protein that binds sperm (Jin et al, 1997). The best known mechanism of host defense by lactoferrin is that of restricting the availability of iron (Weinberg, 1984). A 78-kd single-chain transferrin-like glycoprotein, lactoferrin can bind 1 or 2 molecules of ferric iron as well as copper, zinc, manganese, and other metal ions (Levay and Viljoen, 1995).

It is abundant in secreted fluids, including semen, tears, and breast milk. However, in addition to nutrient sequestering, lactoferrin can permeabilize bacterial membranes and disperse lipopolysaccharides through a cation-mediated process (Ellison, 1994). It is active against a broad range of bacteria, fungi, and viruses. An N-terminal peptide derived by proteolytic cleavage of lactoferrin, known as lactoferricin B, is several times as active against bacteria as intact lactoferrin and uses a nonferrochelating mechanism (Bellamy et al, 1992). Concentrations of lactoferricin B required to completely inhibit growth of diverse bacteria ranged from 0.3 to 150 μg/mL (Bellamy et al, 1992). Although estrogen regulation of lactoferrin has been described in uterus (Das et al, 1997), steroid hormone regulation in the male reproductive tract has not been investigated and the role of lactoferrin in the epididymis and on sperm surface is unknown.

Lysozyme

Lysozyme is widely distributed in biological fluids and tissues (Jolles and Jolles, 1984), including epididymis, prostate, testis and seminal fluid (Tauber et al, 1976). Lysozyme catalyzes lysis of cell walls of Gram-positive bacteria by cleaving the bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in the peptidoglycan. Lysozyme is less effective against Gram-negative bacteria despite its ability to interact strongly with and disrupt their lipopolysaccharides (Ohno and Morrison, 1989). Destructive effects of lysozyme on Gram-negative bacteria are not all dependent on enzymatic activity and can be increased by heating that results in loss of activity (Ibrahim, 1998). A pentadecapeptide derived from lysozyme that lacks muramidase activity permeabilizes the outer bacterial membrane, inhibits bacterial RNA and DNA synthesis, and causes bacterial death (Pellegrini et al, 2000). Testicular macrophage secretion of lysozyme is suppressed by lipopolysaccharide, but is unaffected by follicle-stimulating hormone, luteinizing hormone, and testosterone (Wei et al, 1988). It is intriguing that expressed sequence tags predicting proteins related to lysozyme are reported in GenBank exclusively from testis and germ cell libraries. The significance of these lysozyme-related expressed sequences and whether their role in the male reproductive tract includes host defense has not been reported.

The antimicrobial peptides on mucosal epithelia exist in heterogeneous mixtures and can exhibit cooperative antimicrobial action. In a recent study, lysozyme was synergistic with lactoferrin or secretory leukocyte protease inhibitor, and lactoferrin had synergistic effects with secretory leukocyte protease inhibitor. The triple combination of lysozyme, lactoferrin, and secretory leukocyte protease inhibitor showed even greater synergy in killing *E. coli* (Singh et al, 2000).

Pathogen Recognition and Response

The Toll signaling pathway mediates extracellular pathogen recognition and stimulates intracellular responses (Imler and Hoffman, 2001). Toll transmembrane receptors were originally described in *Drosophila,* in which they mediate dorso-ventral axis establishment in developing embryos as well as pathogen-induced antimicrobial peptide expression in larvae and adults (Anderson, 2000). Toll-like receptors (TLRs) (Akira, 2001) were more recently discovered in mammals and share evolutionarily conserved amino acid sequence and structural features with the *Drosophila* Toll receptors. Mammalian TLRs are expressed in many tissues, including testis and prostate (Rock et al, 1998), but little is known of their functions in the male reproductive tract. TLRs individually and as dimers mediate cellular recognition of conserved pathogen-associated molecular patterns such as lipopolysaccaride, CpG-rich bacterial DNA, and flagellin that are shared by many families of microorganisms, but are absent from vertebrate cells (Medzhitov and Janeway, 1997; Ozinsky et al, 2000; Hemmi et al, 2000). Toll and TLR proteins contain varying numbers of N-terminal leucine-rich repeats and cysteine-rich motifs that form the extracellular pathogen-detection domain. A single hydrophobic transmembrane domain joins the intracellular C-terminal region called the Toll/interleukin receptor (TIR) domain based on homology with the interleukin-1 receptor. The extracellular leucine-rich domains require other host proteins including CD14 to interact with pathogens (Aderem and Ulevitch, 2000). Following activation by microbial antigen complexes, TLRs initiate intracellular responses through interaction of their TIR domains with intracellular factors, including myeloid differentiation primary response protein 88 (MyD88). These interactions lead to activation of NF-κB. Activated NF-κB can translocate to the nucleus and stimulate transcription of responsive genes. NF-κB is required for increased human β defensin-2 messenger RNA after *Salmonella dublin* or enteroinvasive *E. coli* infection (O'Neil et al, 1999). NF-κB also mediates *Helicobacter pylori*-stimulated transcriptional regulation of human β defensin-2 (Wada et al, 2001). Bacterial lipopolysaccaride stimulates human β defensin-2 production (Birchler et al, 2001).

Mammalian cells also express intracellular pathogen-recognition receptors NOD1 and NOD2 that possess modular structure similar to the TLRs. They contain an amino-terminal caspase recruitment domain, a central nucleotide binding domain, and carboxyl-terminal leucine-rich repeats. The leucine-rich repeats of NOD1 and NOD2 mediate bacterial lipopolysaccharide-induced activation of NF-κB (Inohara et al, 1999, 2001, 2002). NOD1 and NOD2 mediate activation of NF-κB differentially in response to different lipopolysaccharide preparations from

different bacteria (Inohara et al, 2001). NOD2 proteins mutated in the leucine-rich repeat and adjacent regions are associated with Crohn disease, a chronic inflammatory disorder of the gastrointestinal tract (Hugot et al, 2001; Ogura et al, 2001a). NOD1 and NOD2 are remarkable examples of evolutionary conservation, and are structurally and functionally similar to certain R plant disease-resistance proteins that contain a TIR domain, a nucleotide binding domain, and carboxyl-terminal leucine-rich repeats (Baker et al, 1997). These R-protein, leucine-rich repeats are postulated to mediate detection of a wide range of viral, prokaryotic, and eukaryotic pathogen components (Ellis et al, 2000).

Although only NOD1 and NOD2 have been investigated thus far, estimates suggest the human genome may contain more than 30 NOD gene homologs (Staskawicz et al, 2001). Northern blot analyses reveal NOD1 (Inohara, 1999) and more weakly, NOD2 (Ogura et al, 2001b) expression in testis, and there are NOD1 expressed sequence tags in GenBank from the Soares testis library 303. However, studies on NOD1 and NOD2, their expression, and function in the male reproductive tract are lacking.

Closing Comments

Over the last decade, discovery driven investigations into male reproductive tract gene expression have identified a number of novel proteins of unknown function (Kirchoff et al, 1990; Naaby-Hansen et al, 1997; Fouchecourt et al, 2000; Liu et al, 2001). Considering the diversity of protein classes that exhibit antimicrobial activity, novel host defense proteins seem likely to emerge from these studies. Among many candidate antimicrobial proteins specific to the male system, ESP13.2 (Perry et al, 1999), a primate epididymis-specific protein of unknown function, contains a cysteine array in the amino terminal region similar to such arrays in horseshoe crab defensin. The epididymis-specific primate ESC42 (Liu et al, 2001) contains a similar defensin-like cysteine array in the amino terminus and may have a host defense role. Other candidates, the epididymis-specific human HE3 (Kirchhoff et al, 1994) and macaque ESC461 (Liu et al, 2001) show similarities to secreted ribonucleases of the RNase A family. Functional studies on these novel ribonuclease-like proteins have not yet been reported. However, their relatedness to the eosinophil ribonucleases, RNase 2 and RNase 3, which show antibacterial, antiviral, and antiparasitic activity (reviewed in Rosenberg and Domachowske, 1999) raise the possibility of roles in host defense.

Several proteins implicated in host defense are found on the sperm surface, including hCAP18, matrilysin, lactoferrin, CST11, and HE2. A major unanswered question concerns what role, if any, these proteins have in fertility. In particular, the presence of male-reproductive tract-specific defensin-like HE2 proteins on sperm reinforces the notion that these host defense proteins may be involved in the production of fertilization-competent sperm. Additional possibilities are that they may have roles in sperm survival in the female reproductive tract and in sperm-egg interaction. Investigations into the mechanisms of action of male reproductive tract antimicrobial proteins may also lead to new methods for the prevention of sexually transmitted infections.

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