doi:10.1017/S0043933909000439

Chicken heterophils: a model for non-oxidative antimicrobial activity

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Chicken heterophils are major components of the innate immune system and constitute the first guard of defence against invading microorganisms. Heterophils in contrast to mammalian neutrophils, lack myeloperoxidase, an essential enzyme of oxidative antimicrobial activity and their antimicrobial activity depends mainly on the non–oxidative mechanism. Heterophils are equipped with very efficient non–oxidative mechanism against microorganisms where the cationic antimicrobial peptides are the most powerful molecules of this killing activity with their broad spectrum activity and the ability to kill microorganisms even with very small concentrations may constitute a new antibiotic class and the basis for new feed additives. These polymorphonuclar cells are the best model for killing activity based on oxygen independent mechanisms. Disorders in the non-oxidative antimicrobial activity induced either by genetic mutations or immunomodulation diseases have direct effect on the killing capacity of heterophils. This review focuses on the heterophils main activity, cationic antimicrobial peptides isolated from chicken heterophils with their remarkable antimicrobial activity and the protective effect of heterophils in different states.

Keywords: heterophils; chicken; antimicrobial; cationic peptides

Introduction

Chicken heterophils are polymorphonuclear leukocytes that constitute the first line of defence against invading microorganisms. They are the most abundant leukocytes that play key roles in the innate immunity which refers to the first-line host defence that serves to limit infection in the early hours after exposure to microorganisms (Hoffmann *et al.,* 1999). These cells are the equivalent of neutrophils in other species; they are the predominant leukocyte in the acute inflammatory response in birds. Heterophils are highly phagocytic cells with broad spectrum antimicrobial activity. They accumulate

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in inflamed tissue, causing tissue damage and forming heterophil granulomas (Harmon, 1998). The initial response of the body to microbial invasion and the events in the first few hours are crucial in determining the outcome of infection (Namavar *et al.*, 1984) and bacterial infections induce recruitment of large numbers of heterophils to the site of infection with marked heterophilia (Kogut *et al.,* 1994; Kogut *et al.,* 1995).

Antimicrobial activity

In contrast to the bactericidal capacities of mammalian neutrophils with their different chemical properties involving proteins, peptides and oxygen-dependent radicals (Hoffmann *et al.*, 1999) generated during the respiratory burst including active oxygen derivatives (superoxides, hydrogenperoxide, hydroxyl radical, singlet oxygen) are essential for intracellular killing activity (Nagahata *et al.*, 1986; Hirabayashi *et al.*, 1985). The release of large amounts of hydrogen peroxide was observed in stimulated neutrophils (Biggar and Sturgess, 1978). However, avian heterophils lack myeloperoxidase, an essential enzyme of the respiratory burst (Brune *et al.*, 1972; Brune and Spitznagel, 1973; Penniall and Spitznagel, 1975; Styrt, 1989; Harmon, 1998) and produce small amounts of oxygen radicals (Conlon *et al.*, 1991). The avian antimicrobial activity of heterophils depends mainly on oxygen-independent mechanisms like cationic antimicrobial peptides and lysozyme (Harmon *et al.*, 1992). Many researchers in the field of natural cationic antimicrobial peptides have been attracted by avian heterophils due to their antimicrobial activity which depends primarily on oxygen-independent mechanisms (Harmon, 1998), and heterophils are useful in studying oxygen independent killing activity.

Heterophils and the cationic antimicrobial peptides

The killing activity of polymorphonuclear leukocytes against bacteria and fungi is conserved in anaerobic conditions and disease where their ability to generate reactive oxygen intermediates is completely abolished (Vel *et al.*, 1984; Ingham *et al.*, 1981; Mandell, 1974). In addition, there appears to be antibacterial activity of the crude extract of the polymorphonuclear leukocytes as well as some granule fractions from the cell homogenates of the polymorphonuclear leukocytes (Zeya and Spitznagel, 1966a; Zeya and Spitznagel, 1966b; Odeberg and Olssen, 1975; Weiss *et al.*, 1978 and Hodinka *et al.*, 1983). These findings suggest that mechanisms other than those dependent on oxygen may have a key role in the killing of some bacteria and fungi, and many investigative studies have been conducted to purify the molecules which contribute to the oxygen-independent antimicrobial activity.

The heterophils' cationic arginine-rich proteins were first demonstrated by Brune and Spitznagel (1973) and the existence of such cationic proteins was proved using the ammoniacal silver reaction (Macrae and Spitznagel, 1975). The cationic antimicrobial peptides generally have two to nine excess, positively charged amino-acids, either arginine or lysine (Hancock and Chapple, 1999). Three cationic antimicrobial peptides have been isolated from chicken leukocytes. They are cysteine-rich molecules with 36 -39 amino acid residues and with sequence homology to the human β-defensins and nine invariant residues (six cysteines, two glycines and one proline) common to bovine β-defensins, named 'gallinacins': Gal-1α, Gal-1, Gal-2, (Harwig *et al.*, 1994). In the same year, two antimicrobial peptides were isolated from the granule extract of the chicken heterophils and named chicken heterophil peptides (CHP1 and CHP2). They were

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cationic and rich in cysteine, arginine and lysine; CHP1 was characterized and consists of 39 amino acid residues with a molecular weight of 4481 (Evans *et al.*, 1994). The gallinacins, like the cationic antimicrobial peptides of mammalian species, are stored in the granules of chicken heterophils (Evans *et al.*, 1994). The gallinacin-3 expression was found to be strongly prominent in the chicken tongue, bursa of Fabricius and trachea and was also moderately expressed in the skin, oesophagus and air sacs; weaker expression of GaL-3 was seen in the large intestine, kidney and ovary. In contrast to GaL-3, the expression of GaL-1 (and –1α) and GaL-2 was limited to bone marrow cells (Zhao *et al.*, 2001).

Antibacterial activity of chicken cationic peptides

The cationic antimicrobial peptides have a wide killing activity; they are active against enveloped viruses, gram-negative and/or gram-positive bacteria, fungi, parasites and even cancer cells (Hancock and Diamond, 2000). Bacterial killing occurs in minutes, and, in most cases, requires bacterial cell growth (Huttner and Bevins, 1999). The avian cationic peptides isolated from heterophils, like those of mammals, were found to have antibacterial activity against either gram-positive or gram-negative bacteria.

Quantitative assays reveal that CHP-1 kills 80% of *E. coli* at 2.7 μg/ml, while 80% of the *S. aureus* was killed at only 0.7 μg/ml. The antibacterial activity of CHP-2 tested by the radial diffusion method indicated that it was bactericidal against both *S. aureus* and *E. coli.* The turkey heterophils peptide-1 (THP-1) is less potent than chicken heterophil peptides, and kills 80% of *S. aureus* at 2.6 μg/ml. Killing of *E. coli* required higher concentrations and 80% of the *E. coli* was killed at 5.3 μg/ml. The other two turkey heterophil peptides (THP-2 and THP-3) failed to kill *E. coli* in vitro at a concentration of 21 μg/ml and killed 80% of the *S. aureus* at 10.5 μg/ml (Evans *et al.*, 1994).

The antimicrobial activity of CHP-1 and CHP-2 was tested against a variety of microorganisms including avian pathogens and human pathogens which are harboured by birds. Both peptides were effective against all the bacteria tested at a concentration of 16 μg/ml, except for *Pasteurella multocida* serotype A:3, strain 1059, also the THP-3 has no effect on *Bordetella avium*, *E. Coli*, and *Salmonella typhimurium* at the maximum concentration of 16 μg/ml and the peptides were unable to neutralize infectious bronchitis virus (IBV) in experimental conditions (Evans *et al.*, 1995).

Tested against *E. coli* ML-35P, *Listeria monocytogenes* EGD and *C. albicans* by the ultrasensitive radial diffusion method, Gal-1 and Gal-1α were powerful against *E. coli* and were 10 fold more potent than HNP-1. Gal-2 (CHP-2) expressed less efficiency against *E. coli* but far exceeded that of HNP-1. All three gallinacins have almost equal antibacterial activity against *L. monocytogenes.* Gal-1 and Gal-1α killed *C. albicans,* whereas Gal-2 was ineffective even at a concentration of 400 μg/ml (Harwig *et al.*, 1994). It has been found that components of lysed heterophils were sufficient to kill *E. coli* and S. aureus (Harmon *et al.*, 1992). Gallinacins 4, 7 and 9 have antibacterial activity against *salmonella* serovars (Milona *et al.,* 2007).

Mode of action

The mode of action of cationic antimicrobial peptides has been extensively studied in human neutrophil peptides. Usually the cationic antimicrobial peptides interact directly by attraction between the positively charged peptides and the negatively charged bacterial

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surfaces; causing the loss of membrane integrity and increased membrane damage leading to lysis of bacteria (Gudmundsson and Agerberth, 1999).

The bactericidal activity of human neutrophil peptide against *E. coli* ML-35 is due to sequential permeabilisation of the outer membrane and inner membrane, with the latter appearing to be the lethal event (Lehrer *et al.*, 1989). For Gram-positive bacteria, only a single membrane is involved (Hancock, 1997). The cationic antimicrobial peptides disrupt the normal barrier property of the outer membrane which permits the passage of a variety of molecules, including the uptake of the peptide itself (Hancock, 1997; Hancock and Chapple, 1999).

Antiendotoxinic activity of cationic antimicrobial peptides

Septicemia is usually caused by the release of lipopolysaccharides from the outer membrane of Gram-negative bacteria and the toxicity of this endotoxin (LPS) is contained within its lipid A portion. Gram-positive septicemia is also presumed to be caused by the release of bacterial cell wall components (Hancock and Scott, 2000). In contrast to most antibiotics which induce endotoxinaemia by releasing LPS, some cationic peptides have the ability to neutralize LPS (Hancock, 1997). Most antimicrobial peptides have the ability to block the activation of macrophages by LPS and this reduces or prevents the LPS-induced inflammatory responses (Scott *et al.*, 2000). Also the cationic antimicrobial peptides inhibit the production of cytokines such as tumour necrosis factor TNF-α and IL-6 by macrophages stimulated with LPS (Hancock and Scott, 2000). Other small α-helical cationic peptides, MBI-27 and MBI-28 derived from cecropin and bee melittin have the ability to overcome lethal endotoxemia, and they bind to LPS and prevent its ability to induce TNF response, this latter is a major mediator in endotoxinic shock (Gough *et al.,* 1996).

Action on mammalian cells

Beside their role as natural antibiotics, the cationic antimicrobial peptides have cytotoxic effects on the host cells. In contrast to bacteria, the outer leaflet of the cell membrane is less negatively charged in eukaryotic cells and this may partially explain the different effects of the peptides on prokaryotic cells compared to eukaryotic cells, whose membrane contains a mixture of negatively charged and zwitterionic phospholipid (Gudmundsson and Agerberth, 1999).

The cytotoxic activity was demonstrated in assays of cytotoxicity against various cell types especially erythrocytes. The chicken peptides gallinacins 4, 7 and 9 showed less than 10% haemolysis with their microbial killing concentrations (Milona *et al.,* 2007).

The haemolytic activity is strongly dependent on the conditions under which the haemolytic assay is performed. Histatin and magainins were strongly active against *C. albicans* and human erythrocytes only in low ionic strength buffer (Helmerhorst *et al.,* 1999).

The cytotoxic peptides found in antimicrobial secretions function primarily by providing a first line of defence against invading microorganisms, whereas some cytotoxic peptides present in venoms (*e.g.* bee, scorpion or spider) function as toxins and are used for defence against predators (Kourie and Shorthouse, 2000).

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Other roles of cationic antimicrobial peptides

It was suggested that higher concentrations of defensins in the airways of mice may play an important role in the pathogenesis of inflammatory lung diseases (Zhang *et al.,* 2001) and defensins from human neutrophils and other species have histamine releasing activity and degranulate rat peritoneal mast cells (Befus *et al.,* 1999).

The leukocyte defensin has a role in lipid metabolism and causes increased binding of Lp(a) to endothelial cells and to smooth muscle cells. Defensin caused also a comparable increase in the internalization of Lp(a) by each cell type without concomitant increase in degradation and it was suggested that defensin may contribute to the development of atherosclerosis (Higazi *et al.,* 1997).

Cationic peptides in pathologic states

The epithelial Gal-3 was found in the trachea and its tracheal expression was increased in response to experimental infection with *Haemophilus paragallinarum* whereas, its expression in the other organs was unaffected (Zhao *et al.,* 2001). Cationic peptides provide protection against colibacillosis in chickens (Jiang *et al.,* 2005) and give higher protection against bacterial pathogens with immunostimulation of chicken heterophils when used as feed additives mainly during the first week post-hatch where the innate immune response is inefficient (Kogut *et al.,* 2007). Heterophils participate in chicken protection against salmonella infection by releasing another potent cationic antimicrobial peptide, cathelicidin-2 (Van Dijk *et al.,* 2009). Thus heterophils form a major barrier against invading microorganisms either by phagocytosis or by their effective non-oxidative antimicrobial activity depending on cationic antimicrobial peptides within cytoplasmic granules and they constitute the best model for studying the oxygen independent mechanisms.

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