Developmental and Comparative Immunology 61 (2016) 48–59

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Localization and developmental expression of two chicken host defense peptides: cathelicidin-2 and avian β-defensin 9

Tryntsje Cuperus [a], Albert van Dijk [a], R. Marius Dwars [b], Henk P. Haagsman [a, *]

[a] Division of Molecular Host Defence, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL, Utrecht, The Netherlands
[b] Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 7, 3584 CL, Utrecht, The Netherlands

ARTICLE INFO

Article history:
Received 19 January 2016
Received in revised form
9 March 2016
Accepted 9 March 2016
Available online 10 March 2016

Keywords:
Chicken
Host defense peptides
Development
Innate immunity
Cathelicidin
Defensin

ABSTRACT

In the first weeks of life young chickens are highly susceptible to infectious diseases due to immaturity of the immune system. Little is known about the expression of host defense peptides (HDPs) during this period. In this study we examined the expression pattern of two chicken HDPs, the cathelicidin CATH-2 and the β-defensin AvBD9 by immunohistochemistry in a set of organs from embryonic day 12 until four weeks posthatch. AvBD9 was predominantly found in enteroendocrine cells throughout the intestine, the first report of in vivo HDP expression in this cell type, and showed stable expression levels during development. CATH-2 was exclusively found in heterophils which decreased after hatch in most of the examined organs including spleen, bursa and small intestine. In the lung CATH-2 expression was biphasic and peaked at the first day posthatch. In short, CATH-2 and AvBD9 appear to be expressed in cell types strategically located to respond to infectious stimuli, suggesting these peptides play a role in embryonic and early posthatch defense.

1. Introduction

During the neonatal period chickens are highly susceptible to infectious diseases that cause relatively few problems later in the life of the animals. Chickens younger than 2 weeks often develop clinically apparent disease and severe symptoms when infected with *Escherichia coli* or *Salmonella* (Gast and Beard, 1989; Goren, 1978; Johnson et al., 2001). This susceptibility is reflected in high antibiotic usage in broilers during the first week of life (GD Animal Health, 2014). When chicks hatch, their immune system is immature and marked development occurs over the first weeks of life. During this period partial protection is offered by maternal antibodies, transferred to chicks via the yolk. Most of these antibodies are depleted by 10 days posthatch (Gharaibeh and Mahmoud, 2013). Though neonatal and even embryonic chicks are able to mount humoral responses of their own, these are generally very low. Antibody titers against orally or intramuscularly administered BSA were higher in vaccinated animals older than 10 days compared to animals vaccinated in the first week posthatch (Bar-Shira et al., 2003; Mast and Goddeeris, 1999). Additionally, at hatch the gut-associated lymphoid tissue is only poorly developed. The numbers of lymphocytes in the intestinal wall are low at hatch, but increase rapidly during the first week (Bar-Shira et al., 2003). Germinal centers in the cecal tonsils are not identified until two weeks posthatch (Gomez Del Moral et al., 1998). Development of the innate immune system in neonatal chicks is less well described, though heterophils of 1 day old chicks show lower phagocytic and bactericidal capacities compared to cells of animals of 4 and 7 days old (Wells et al., 1998).

Host defense peptides (HDPs) are important multifunctional effector molecules of the innate immune system. Two of the main classes of HDPs are cathelicidins and defensins. In mammals, these peptides are believed to be very important in neonatal defense (Kai-Larsen et al., 2014). Expression of the human cathelicidin LL-37 and its murine counterpart CRAMP is higher in neonatal compared to adult skin (Dorschner et al., 2003). In contrast, the expression of intestinal α-defensins is lower in newborns, possibly predisposing neonates to intestinal infections such as necrotizing enterocolitis (Mallow et al., 1996). In the chicken, four cathelicidins and 14 β-defensins have been described. Expression analysis of chicken HDPs showed clear differences over the course of development. Cathelicidin-1, -2 and -3 showed a strongly increased expression in

* Corresponding author.
E-mail addresses: t.cuperus@uu.nl (T. Cuperus), a.vandijk1@uu.nl (A. van Dijk), r.m.dwars@uu.nl (R.M. Dwars), h.p.haagsman@uu.nl (H.P. Haagsman).

http://dx.doi.org/10.1016/j.dci.2016.03.008
0145-305X/

the cecum and tonsils at 4 weeks posthatch (Achanta et al., 2012). Intestinal expression of the β-defensins 1, 2, 4 and 6 decreased in the first week posthatch, but increased again in the second week (Bar-Shira and Friedman, 2006; Crhanova et al., 2011). However, this research was solely based on mRNA levels and did not take into account the cell types expressing these peptides and possible changes herein during development.

In this work we aimed to elucidate the cellular localization and developmental expression pattern of two chicken HDPs, cathelicidin-2 (CATH-2) and β-defensin 9 (AvBD9, previously also named GAL6) at the protein level, hereby increasing the knowledge about the role of HDPs during chicken development.

2. Materials and methods

2.1. Animals and tissue sampling

This animal experiment was conducted in accordance with a protocol approved by the Dutch Animal Experimental Licensing Committee (DEC). Twelve-day incubated Ross 308 broiler eggs were obtained from a commercial hatchery (Lagerwey, Lunteren, The Netherlands) and further incubated at the research facility of the Department of Farm Animal Health (Utrecht University). After hatch, chickens were housed in a ground stable under controlled hygienic conditions. Chickens were fed a commercial broiler diet without antibiotics or coccidostats and were given access to water and food *ad libitum*. At multiple embryonic (ed12, 14, 16, 18, 20) and posthatch (d1, 4, 7, 14, 21, 28) timepoints, eggs/chickens (n = 4/5) were chosen at random for sample collection. Animals were euthanized by cervical dislocation until posthatch day 7 and by electrocution and bleeding at later timepoints.

Organs in contact with the external environment (intestine, lung, skin) and a selection of lymphoid organs (spleen, bursa) were chosen for analysis. Additional organs (yolk, pancreas, thymus, kidney) were also sampled, but only perfunctory analysis on the absence or presence of CATH-2 and AvBD9 expression was performed for this study. During the experiment no symptoms of disease were noticed in live birds or at post-mortem. Birds were free of infectious bronchitis virus based on serology at d28.

2.2. Antibodies

Rabbit polyclonal antibodies against CATH-2 and AvBD9 were generated at Biogenes (Berlin, Germany). CATH-2 antibody production was previously described (van Dijk et al., 2009a). For the production of AvBD9 antibody, two short peptides derived from the AvBD9 sequence (CSFVASRAPSVD and LASRQSHGSC) were conjugated to *Limulus polyphemus* hemocyanin (LPH) at the cysteine residue and the mixture of peptides was used to immunize two rabbits. Antiserum was collected from the immunized animals and purified by affinity chromatography on CNBr-Sepharose columns coated with the peptides. Monospecific IgG was eluted from the columns with 0.2 M Glycine-HCl buffer (250 mM NaCl, pH 2.2), neutralized with 1 M Tris-HCl (pH 7.5) and centrifuged to remove remaining debris. Antibodies were aliquoted and stored at −80 °C. For the staining of enteroendocrine cell (EEC) products, commercial rabbit antibodies were used which had previously been shown to work on chicken tissue samples: anti-glucagon-like peptide 1 (GLP-1, GA1176, Enzo, Farmingdale, USA), anti-gastric inhibitory peptide (GIP, T-4340, Peninsula Laboratories, San Carlos, USA) and anti-serotonin (5-HT, 20080, Immunostar, Hudson, USA). Antibodies were used in the following dilutions: 1:50 for anti-AvBD9, 1:100 for anti-CATH-2 and anti-5-HT, 1:200 for anti-GLP-1 and 1:500 for anti-GIP.

2.3. Tissue processing and sectioning

Tissue samples were fixed in 4% paraformaldehyde (w/v) in phosphate buffer (pH 7.2) for 24 h and subsequently paraffin-embedded. Sections (5 μm for single or sequential staining or 2 μm for serial sections) were mounted on glass slides, deparaffinized and rehydrated.

2.4. Immunohistochemistry

For antigen retrieval, sections were boiled in citrate buffer (pH 6.0) for 10 min (AvBD9, GLP-1 and GIP staining). Endogenous peroxidase activity was blocked by incubating the sections for 30 min in 1% H_2O_2 in methanol. Subsequently, sections were blocked with 10% normal goat serum and 2.5% BSA for 1 h before incubation with the primary antibody (see section 2.2). Incubation times of the primary antibodies were as follows: CATH-2, 1 h; AvBD9, GLP-1, GIP and 5-HT overnight (16–20 h). Immunostaining with rabbit serum or in absence of the primary antibody served as the negative control. Sections for CATH-2 staining were then incubated with the Horse Radish Peroxidase labelled anti-rabbit polymer from the EnVision + system (Dako, Glosstrup, Denmark). All other sections were incubated with a biotinylated goat-anti-rabbit antibody (1:250, Vector Laboratories, Burlingame, USA) for 30 min followed by incubation with ABC reagent (Vector Laboratories). Staining was visualized by incubating the sections with diaminobenzidine (DAB) for 5–10 min. Finally, sections were counterstained with hematoxylin, dehydrated and mounted with Pertex.

2.5. Sequential stain with HE or Giemsa

Sections were sequentially stained with hematoxylin and eosin (HE) followed by anti-AvBD9 or Giemsa followed by anti-CATH-2 to elucidate morphology of AvBD9 and CATH-2 expressing cells. Routine HE staining was performed in a Leica Autostainer XL (Son, The Netherlands). For Giemsa stain, tissue sections were incubated for 1 h in 20% Giemsa's Azure Eosin Methylene Blue solution (Merck, Darmstadt, Germany) and differentiated in diluted acetic acid for 15 min. Subsequently, differentiation was stopped by dipping sections in ethanol 96% followed by incubation in aceton. Photographs of distinctive cells were taken and subsequently sections were incubated overnight in fresh xylene to remove the cover slips. Sections were then destained by incubation in acidified ethanol (27 ml HCl 37% and 973 ml ethanol 70%) for 5 min. After rehydration, sections were stained with antibodies against CATH-2 or AvBD9 as described above (section 2.4).

2.6. Silver staining

Fontana-Masson silver stain was performed on gastro-intestinal tissue sections as previously described (Grimelius, 2004). Briefly, sections were incubated for 1 h in prewarmed ammoniacal silver solution (5% $AgNO_3$ with NH_4OH) and rinsed in Milli-Q water. Sections were counterstained with Nuclear Fast Red (Vector Laboratories, Burlingame, USA), dehydrated and mounted with Vecta-Mount (Vector Laboratories). Silver stained sections were compared with serially cut sections stained with antibody against AvBD9.

2.7. Quantitative analysis

Five to ten random photographs were made of each tissue section using an Olympus BX51 microscope with movable table at 400x magnification (Field size 0.09 μm^2). Images were analyzed