ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

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International Journal of Poultry Science 7 (2): 129-133, 2008
ISSN 1682-8356

Histone H1 Accumulation in the Avian Bursa of Fabricius Damaged by Infectious Bursal Disease Virus

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Abstract: The present study addresses the localization of histone H1 in tissues sustaining highly apoptotic and necrotic tissue induced by a viral infection of the Bursa of Fabricius as evidence of a possible extranuclear and extracellular movement beyond the nuclei. This potential mechanism of release could be related to previous reports of extranuclear biological activities of this protein. Detection of HH1 by *in situ* immunostaining was demonstrated as strong and moderate immunoreactivity in the BF of specific pathogen free chickens challenged with Infectious Bursal Disease Virus. The histone H1 immunoreactivity is apparently reduced and principally limited to single or multiple immunoreactive foci when high levels of virus particles are detected. Additionally, the moderate observed immunostaining appears to be associated with cellular aggregates in multiple small localized foci. In virus-infected tissue, those aggregates that display strong and moderate immunoreactivity clearly indicate HH1 immunoreactivity beyond the nuclei and possibly release for dissemination. These results suggest that high tissue damage, through even relatively common viral infections involving the avian humoral immune system, could activate extranuclear and extracellular HH1 release.

Key words: Histone H1, bursa of Fabricius, apoptosis, extranuclear, extracellular and Infectious bursal diseases

Introduction

In addition to the very well known role of the unique avian Bursa of Fabricius (BF) regarding the B-lymphocyte ontogeny (Glick *et al*., 1956; Cooper *et al*., 1966; Lydyard *et al*., 1976), additional *in vitro* and *in vivo* studies have postulated an endocrine role associated with the immune-gonadal axis (Jolly and Pezard, 1928; Riddle, 1928; Kirkpatrick and Andrews, 1944; Glick 1956, 1957; Meyer *et al*., 1959). The putative molecule responsible for such a mechanism is still unidentified. However, one candidate molecule extracted from the BF exhibiting potent anti-steroidogenic and anti-proliferative bioactivities on several mammal and avian cells, called bursal anti-steroidogenic peptide/protein (BASP) (Byrd *et al*., 1993; Caldwell *et al*., 1998, 1999) has an indistinguishable relationship to the HH1 family (Garcia-Espinosa *et al*., 2002).

The histone H1 (HH1) family is a heterogeneous and ubiquitous group of basic nuclear proteins targeting chromatin (Ramakrishnan, 1997), which can also be observed on the whole cellular body of cortical and medullary cells from the normal BF (Garcia-Espinosa *et al*., 2003). This observation may suggest a possible extranuclear and extracellular presence of HH1, which in turn is consistent with previous hypotheses suggesting that histones could serve as secondary messenger signal molecules (Bab *et al*., 1992; Hiemstra *et al*., 1993; Bolton and Perry, 1997; Brown *et al*., 1997; Brix *et al*., 1998; Garcia-Espinosa *et al*., 2002; Wang *et al*., 2002).

Although it is unknown how HH1 is released, we hypothesized that damaged tissue could be a mechanism for HH1 release based on previous *in vitro* studies which have demonstrated that apoptosis may be one such mechanism (Holers and Kotzin, 1985; Bell *et al*., 1990; Franek and Dolnikova, 1991; Emlen *et al*., 1994). Furthermore, a similar phenomenon has also been demonstrated *in vivo* associated with some autoimmune diseases such as Lupus (Rumore and Steinman, 1990). Both apoptosis and necrosis are common tissue events associated with the pathogenesis of some infectious diseases, providing the potential for a similar release mechanism. Presently, we examined the microanatomical presence of immunoreactive HH1 within the avian BF during the clinical phase of a RNA virus infection using *in situ* immunostaining. In the present study we utilized Infectious Bursal Disease Virus (IBDV) as a model, which is known to induce a high ratio of cellular apoptosis and necrosis in the follicles of the BF (Sivanandan *et al*., 1986; Tanimura and Sharma, 1998).

Materials and Methods

Animals: Fifteen fertile specific pathogen free (SPF) eggs were obtained from SunRise Farms, Catskill N.Y., incubated and hatched at the University of Arkansas Poultry Health Laboratory. Chickens were housed in two negative pressure Horsfal isolation chambers on day of hatch. All birds received a nutritionally balanced ration and water *ad libitum* throughout the study.

Virus preparation: The enteric derived IBDV RB-4 strain (Patent 6, 406, 843B1) is classified into molecular group six and was originally isolated from the proventriculus of commercial broiler chickens exhibiting proventriculitis syndrome (Snyder *et al.*, 1988). The virus was propagated in SPF embryonated eggs and the titer was adjusted to a concentration of $10^{3.2}$ ID_{50}/ml in tryptose phosphate broth.

Bird challenge: All SPF chickens were maintained in Horsfal isolation cages until they reached 17 days of age. Chicks in one isolator were challenged by bilateral eyedrop, with each chick receiving 30µl/eye ($10^{3.2}$ ID_{50}/ml). Chicks within the second isolator served as controls.

Tissues samples
Five days post-challenge, three SPF chickens from both the challenged and negative control groups were euthanazied by CO_2 asphyxiation and subjected to necropsy. The bursa was dissected, collected and evaluated for the presence of gross lesions. Individual tissues from each bird were incised and placed in 10% buffered formaldehyde and stored at 4°C for 40 hours. Tissues were cut and embedded in paraffin according to standard protocols. Cross sections (5µm) were deparaffinized, hydrated and prepared for standard or immunohistochemical staining procedures as described below.

Histology: Tissues sections were stained with hematoxylin and eosin following a standard protocol (Humason, 1972).

Antibodies: To detect histone H1 we utilized mouse IgG2a monoclinal antibody (Clone AE4) which recognizes histone H1 in the nucleus of animal and plant cells (ICN Biomedical) and the doublet band of purified calf thymus histone H1 and BASP/Histone H1 purified from the chicken BF (Garcia-Espinosa *et al.*, 2002). Infectious bursal Disease virus was detected with a mouse monoclinal antibody anti-consensus epitope of IBDV strains (Clone B29, produced in ascitic fuid and purified by precipitation[1]). Mouse isotype-matched IgG (Sigma, St. Louis, MO) as primary antibody and biotinylated horse IgG (Vector Laboratories, Burlingame, CA) were utilized as a negative controls.

Immunohistochemical staining procedure: All tissue sections were treated with 1% hydrogen peroxide in distilled water for 10 minutes and washed with phosphate buffered saline (PBS). Immunostaining was performed with a commercially available biotin-avidin-peroxidase kit (Vectastin Elite ABC kit; Vector laboratories, Burlingame CA). Briefly, tissues were blocked using 2% normal horse serum for 4 hours followed by incubation of either 1:100 dilution of monoclinal anti-HH1 antibody, 1:100 dilution of monoclinal antibody anti-IBDV, or 1:50 dilution of negative control antibodies for 2 hours, washed, incubated with 1:50 dilution of secondary biotinylated antibody for 30 minutes, washed and incubated with ABC reagent for additional 30 minutes. Color development was carried out with VIP (Vector VIP; Vector Laboratories) following the manufacturer's protocol. Tissue sections were washed, dehydrated and coverslips were mounted in non-aqueous mounting media (Vector mount, Vector laboratories) and slides were evaluated microscopically.

Results and Discussion

Fig. 1 shows the recognition of histone H1 in the cortex and medulla of normal follicle of the BF by the antibody anti-histone H1, peroxidase-VIP reaction and its classical architecture in haematoxylin and eosin, in contrast with Fig. 2, which exhibits the recognition of histone H1 in the follicle of IBDV-infected BF by the antibody anti-histone H1, peroxidase-VIP reaction and detection of the virus by the anti-IBDV in a sequential tissue section. The ubiquitous nuclear protein HH1 was clearly observed in most of the cells of the follicle from normal BF tissues (Fig. 1). However, immunoreactive HH1 was observed much less frequently in tissue derived from IBDV-infected chicks (Fig. 2). In these tissues, virus antigen expression was much more commonly detected as compared to that of HH1 (Fig. 2). This relationship was consistently observed in all sections examined from each of the 3 IBDV-infected chicks (data not shown). In tissues derived from IBD-infected chicks, areas of high immunoreactivity were consistently associated with foci which frequently appear to represent either individual cells or cell aggregates expressing HH1 (Fig. 3). Additionally, a lower but moderate immunoreactivity staining for HH1 was observed in the apparent cell aggregates in IBDV-infected tissues as compared to normal BF tissue (Fig. 3). Non-specific immunoreactivity was not detected with either of the negative control antibodies (data not shown).

The nuclear protein HH1 plays a significant role in chromatin remodeling and allows for gene expression. Although this protein was first associated with nuclei, it can be found in the cytoplasm when cells are undergoing mitosis (Richardson *et al.*, 2000), arrested in G1 phase of the cell cycle (Chiu and Marzluff, 1982), and if the cell is programmed to die (Rosen *et al.*, 1995). The high ratio of apoptosis and necrosis induced by infection with IBDV, is consistent with the intense cellular loss that often occurs during IBDV infection and likely explains why we observed less frequent HH1 immunoreactivity in IBDV-infected versus normal tissue. In the present study, localized HH1 immunoreactivity

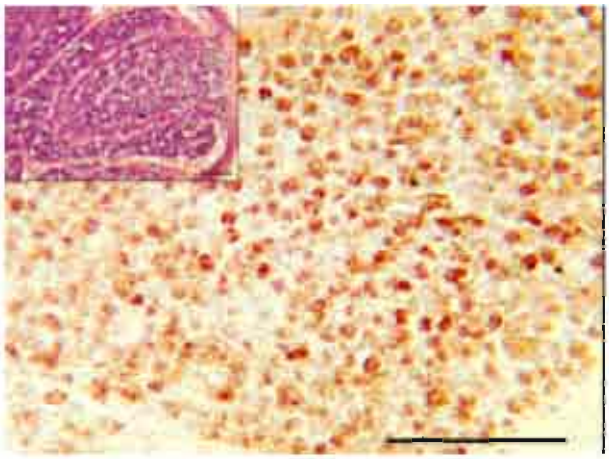

Fig 1: Recognition of histone H1 in the cortex and medulla of normal follicle of the BF by the antibody anti-histone H1, peroxidase-VIP reaction and its classical architecture in haematoxylin and eosin at left upper corner of the photograph, bar=50µm.

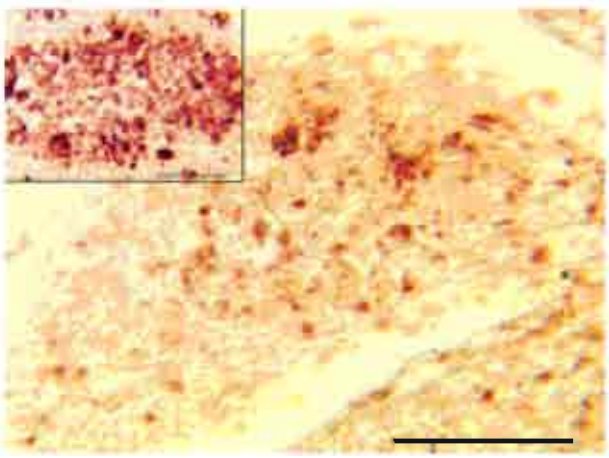

Fig. 2: Recognition of histone H1 in the follicle of IBDV-infected BF by the antibody anti-histone H1, peroxidase-VIP reaction and detection of the virus by the anti-IBDV in a sequential tissue section (peroxidase-DAB reaction) at left upper corner of the photograph, bar=50µm.

was occasionally observed in localized foci in IBDV-infected tissue (Fig. 3). These foci could represent cells undergoing the apoptotic process, late-phase apoptotic cells undergoing necrosis, or just the nuclei of bystander cells. However, the clear presence of diffuse or apparently spreading HH1 (moderate immunoreactivity) in the immunoreactive foci of IBDV-infected tissue may indicate recruitment of phagocytic cells surrounding apoptotic and necrotic cells (Fadok *et al.*, 1992), where HH1 and other nuclear compounds may be subjected to

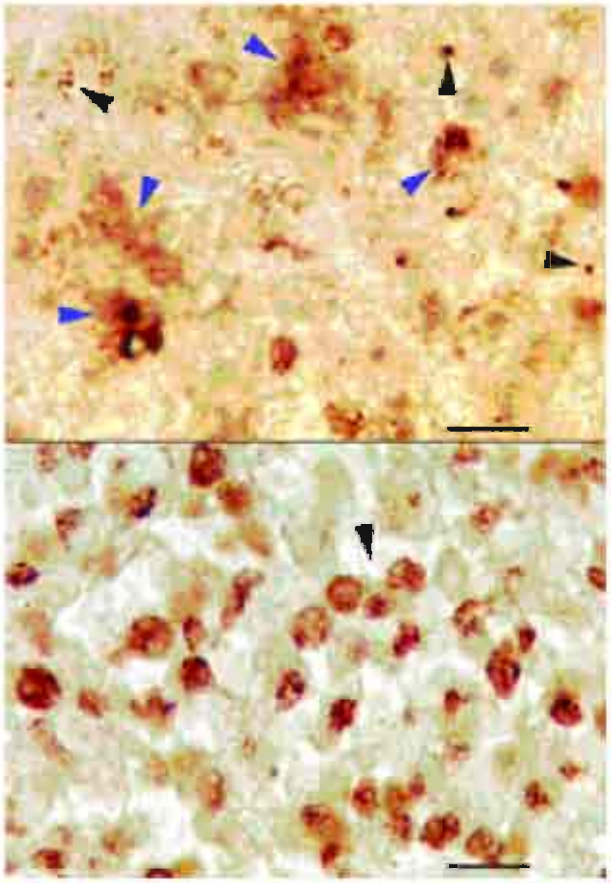

Fig. 3: Recognition of histone H1 in the follicle of IBDV-infected BF or normal tissue (upper and below photograph respectively) by the antibody anti-histone H1. Black and blue head arrows (upper) show strong and moderate immunoreactivity respectively. Notice the multiple immunoreactive complex (blue head arrow). In contrast, normal BF display the protein preferentially in the whole cellular body of medullar cells (arrow head). Peroxidase-VIP reaction was used. Bar=10µm.

degradation (Desai-Mehta *et al.*, 1995) or might be a simple virus-associated apoptosis/necrosis site. This diffuse HH1 immunostaining may include HH1 anchorage to the cellular surface membrane as previously described in Sindbis virus-infected cells (Rosen *et al.*, 1995).

Additionally, the HH1 foci observed in this study suggest that HH1 movement beyond the nuclei can be variable depend on the disease, examples of this are the prion-associated and Alzheimer's pathologies, where a HH1 isoform was detected in the whole cellular body of isolated affected neurons and astrocytes (Bolton *et al.*, 1999).

The architecture and cellular loss of the follicle in the IBDV-infected BF, in parallel to the HH1 accumulation, clearly indicates potential movement of HH1 beyond the nuclei. The lack of monoclinal antibodies against different regions and subunits of HH1 make it difficult to establish weather or not HH1 or its fragments can be present in the circulation during pathogenesis. However, detection in circulation of nucleosomes in lupus (Rumore and Steinman, 1990) or the 14 amino acids histone H4 fragment called Osteogenic Growing Peptide present in both bone and bone marrow injury in mammals (Bab *et al*., 1992), support the hypothesis that tissue damage can be the source of such histone release.

The results of the present study suggest that the biological activities of HH1 reported *in vivo* and *in vitro* could have a more important role in pathologies where tissue damage is involved.

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[1]Produced by Dave Snyder, University of Delaware and provided by Intervet, Inc.