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The antimicrobial action of histones in the reproductive tract of cow

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ABSTRACT

An infection of any part of female reproductive tract can severely interfere with fertility and reproduction. The fluids and epithelium from the lumen of the female reproductive tract (uterus, oviduct and ovarian follicle) are a known source of antimicrobial action in several species. In this study, we compared the antimicrobial properties of fluids from the reproductive tract of a cow. After removal of small molecules, we demonstrated that there is an antimicrobial activity connected with a fraction of compounds with a molecular mass range between 3500 and 30,000. The most probable candidates responsible for the observed antimicrobial effect were subsequently identified by mass spectroscopy as histones H2A type 2-C, H2B type 1-K, H3.3, and H4. The antimicrobial role of histone H2B was further confirmed by using an antibody against this histone.

1. Introduction

Even though histones belong to the one of the most studied proteins, all their possible roles in eukaryote organisms are still far from being completely understood. Their principal function is perceived in their interaction with DNA and their participation in the regulation of gene expression. Their occurrence out of nucleus or even out of cell has been often disregarded as a mere artifact of isolation and sample handling or a more or less insignificant consequence of necrotic processes. However, over time, a growing body of evidence has pointed out what a multifunctional group of proteins they really are (for an excellent review see Parseghian et al., 2008 [1]). A number of studies have revealed their active involvement in a broad spectrum of biological processes such as apoptosis [2] (histone H1.2 was identified as a cytochrome c releasing factor from mitochondria), or thyreoglobulin internalization by liver macrophages, where histone H1 serves as a surface plasma receptor [3]. Also, there is a continuously increasing amount of reports on histones and peptides derived from histones as a part of host defense system across the animal kingdom. Not only were histones and their derivatives ascribed with immunomodulatory properties due to their interaction with several crucial proteins (e.g. C-reactive protein [4] or TNF-α [5]) and macrophages [6] as well as their ability to serve as a pattern recognition receptor for LPS [7]. Even more importantly, peptides derived from histones and histones themselves were shown to exhibit pronounced antimicrobial properties. They were found to be a part of antimicrobial defense in hemolycytes of shrimps (H2A, H2B, H4) [8]; in the liver, intestine, stomach, testes, skin, gills and epithelial mucosa of fish (H1 [9], H1-like protein [10], H2B and H1-like protein [11]; parasin -H2A N-terminal residue [12]); in the skin and stomach of amphibians (H2B [13], buforin I -H2A N-terminal residue [14]); in the liver, ovary and oviduct of birds (H2A and H2B [15], H1 and H2B [16]); in the sebocytes (H4) [17], placenta (H2A and H2B) [18]; intestinal mucosa (H1 and its fragments) [19,20], and the amniotic fluid (H2B) [5] of mammals.

Histones, with their rather small molecular size and strong positive charge, fit well in our picture of antimicrobial proteins, an otherwise a very diverse group of molecules with regard to their amino acid composition. The mechanism of their antimicrobial action is still not very clear. Their cationic character enables them to bind negatively charged plasma membrane and there are even reports about their abilities to penetrate the plasma membrane [21]. Then again, there seems to be more to their antimicrobial properties than just a high content of basic residues. Experiments using analogous synthetic peptides derived from histone H1 showed a need for peptidyl-prolyl bonds to be in a cis conformation for these peptides to display their antimicrobial activity [22]. The antimicrobial properties of histones are also exploited in a newly described type of a cell death-an intriguing process of ETosis, during which an extracellular net entrapping and killing Gram-positive and -negative bacteria is formed upon the release of granule proteins and chromatin (containing histones *H1, H2A, H2B, H3, and H4*) from several types of cells of the immune system (neutrophils, eosinophils, mast cells [23–25]).

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Despite the presence of adaptive immune mechanisms in mammals, the innate immune system undoubtedly plays a crucial role in the prevention of infections or suppression and/or elimination of pathogens. In our study, we focused on the antimicrobial properties of fluids from the lumen of the reproductive tract of cow. It is well known fact that all the parts of the female reproductive tract are susceptible to infection by many pathogens and the subsequent inflammation may interfere with reproduction [26–28]. The epithelium lining the lumen of reproductive tract secretes several compounds with antimicrobial properties-especially antimicrobial proteins and peptides: e.g. β-defensins1- 4 have been reported to be expressed in human endometrial epithelium [29] and β-defensin 5 in the human vagina, cervix and oviduct [30] and WAP motif containing proteins (including secretory leukocyte protease inhibitor (SLPI), and elafin) throughout the female genital tract [29,31,32]. In spite of an increasing number of reports on histones' involvement in mammalian innate immunity, the evidence of their presence and role in the female reproductive tract is scarce to the best of our knowledge. The only two reports relate to human placenta and amniotic fluid (H2A and H2B) [18,5].

The aim of this work was to study and compare the antimicrobial properties of fluids from the reproductive tract of cow and to identify compounds with a molecular mass range between 3500 and 30,000 responsible for their antimicrobial activity.

2. Materials and methods

2.1. Material

Trypsin Gold (Mass Spectrometry Grade) was purchased from Promega Corporation (Madison, WI, USA) and α-cyano-4-hydroxycinnamic acid from Bruker Daltonics (Bremen, Germany). All other chemicals were obtained from Sigma–Aldrich (St. Louis, MO) unless stated otherwise.

2.2. Isolation of bovine oviductal, uterine and follicular fluid

Bovine ovaries, oviducts and uteri from sexually mature Holstein cows were collected from a nearby slaughterhouse and immediately transported to the laboratory on ice in a container filled with pre-cooled phosphate buffered saline (PBS). The ovaries, oviducts and uteri were then cleaned from surrounding tissue and washed three times in PBS.

For protein concentration determination, the content of the lumens of oviducts and uteri was gently squeezed out. The follicular fluid was aspirated from tertiary ovarian follicles. All the fluids were diluted ten times with cooled PBS and centrifuged at 600*g* and 4 °C for 10 min to remove cellular debris. The supernatant was further clarified by centrifugation at 20,000*g* and 4 °C for 15 min and the protein concentration was determined immediately.

For all other experiments, the lumen of oviducts and uteri were washed with cooled PBS (approximately 1 ml in case of oviduct and 50 ml in case of uterus) and the obtained fluids were collected. Follicular fluid was aspirated from tertiary ovarian follicles. The individual follicular, oviductal and uterine samples were pooled together and centrifuged at 600*g* and 4 °C for 10 min to remove cellular debris. The supernatant was further clarified by centrifugation at 20,000*g* and 4 °C for 15 min. All clarified samples were afterwards divided into three parts. The first part was directly lyophilized (non-dialyzed fluid preparations) and the second and third parts were first subjected to dialysis against distilled water using the dialysis membrane with molecular weight cut off (MWCO) of either 3500 or 30,000 (Pierce Co., USA) respectively prior to lyophilization (dialyzed fluid preparations).

2.3. Protein concentration determination

The concentrations of proteins in uterine, oviductal and follicular fluids were determined using the Bicinchoninic Acid Kit (Sigma–Aldrich, St. Louis, MO) according to manufacturer's instructions. Samples of uterine, oviductal and follicular fluids were diluted 10 and 50 times with distilled water and BSA was used as a standard. The concentrations were measured in 96 well plates in duplicates for each dilution. The ratio of diluted samples and BCA Working Reagent was 1:8.

2.4. Antimicrobial properties screening

Antimicrobial properties were assessed by comparison of growth curves of *Escherichia coli K-12* grown in standard LB medium containing serially diluted tested fluid preparations [33]. The final concentrations of all the dialyzed and non-dialyzed follicular, oviductal and uterine fluid samples in LB medium were in the range of 0–50.0 mg/ml (based on absorbance at 280 nm). Pure LB medium served as a negative control (no inhibition) and LB medium with 100 ppm chlortetracycline served as a positive control. The *E. coli* cultures were diluted with a LB medium to the final OD 0.1 at 405 nm and were grown in microplate wells in the total volume of 200 μl of LB medium with tested fluid preparations at 37 °C for 12 h. The OD at 405 nm of the cultures was measured every hour and cell growth curves were constructed.

2.4.1. Inhibition of antimicrobial properties by antibodies against histone H2B

Inhibition studies were carried out using non-dialyzed follicular, oviductal and uterine fluids and their dialyzed (MWCO 3500) preparations, to which polyclonal antibodies against N-terminal part of histone H2B (Santa Cruz Histone H2B antibody (N-20)) were added at a concentration of 1.0 mg/μl. A solution of LB medium with antibodies against histone H2B at a concentration of 1.0 mg/μl served as a negative control. The measurement of antimicrobial activities was performed analogously as described above.

2.5. Protein identification

Antimicrobial proteins were identified using SDS electrophoresis according to Laemmli [34] followed by trypsin in gel digestition and MALDI-TOF/TOF MS analysis and database searching [35].

3. Results

3.1. Antimicrobial activity of the bovine reproductive tract fluids

All tested fluids from uterus, oviduct and ovarian follicle demonstrated significant antimicrobial action against *E. coli* (Fig. 1) at a protein concentration well within their physiological range. Even the least potent inhibitor, which was the uterine fluid, was able to diminish the growth rate of *E. coli* by half. Removal of compounds with a molecular mass smaller than 3500 by means of dialysis generally led to a decrease in antimicrobial activity of all fluids by 20–50% (Table 1). Further removal of compounds smaller than 30,000 practically abolished any observable antimicrobial activity of studied fluids. Adding polyclonal antibodies against N-terminal part of histone H2B also had a detrimental effect on the antimicrobial activity of studied fluids. When added to dialyzed fluid samples (MWCO 3500), the antimicrobial activity dropped below detection limit and when non dialyzed fluid samples were used, the antimicrobial activities were decreased by half as compared with antimicrobial activity of fluids without the antibodies.

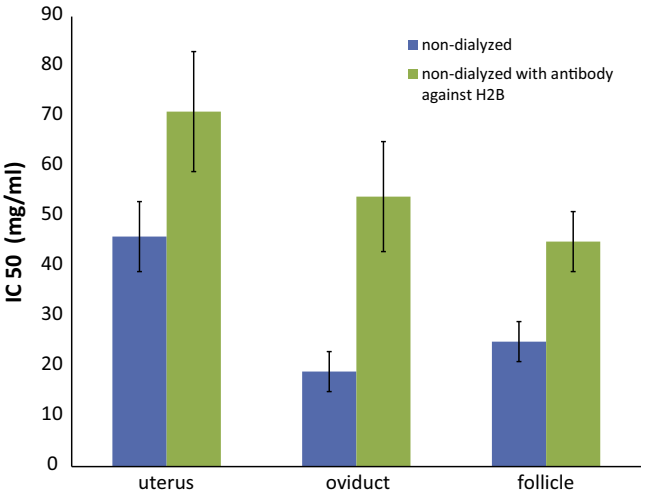

Fig. 1. Comparison of antimicrobial properties of fluids from reproductive tract of cow. The first bar in each group represents the half maximal inhibitory concentration (IC_{50}) values of non-dialyzed fluid samples, the second bar represents IC_{50} values of non-dialyzed fluids, to which antibody against N-terminal part of histone H2B was added at a concentration of 1.0 mg/ml.

Table 1
Comparison of antimicrobial activities of non-dialyzed and dialyzed fluids (MWCO 3500 and 30,000) from the reproductive tract of cow and the effect of polyclonal antibody against the N-terminal part of histone H2B. The antimicrobial effects are compared using the half maximal inhibitory concentration (IC_{50}).

Sample	Ic_{50} (mg/ml)
Non-dialyzed uterine fluid	46 ± 7
Non-dialyzed oviductal fluid	19 ± 4
Non-dialyzed follicular fluid	25 ± 4
Dialyzed uterine fluid MWCO 3500	85 ± 11
Dialyzed oviductal fluid MWCO 3500	34 ± 7
Dialyzed follicular fluid MWCO 3500	32 ± 9
Dialyzed fluids from reproductive tract MWCO 30,000	>>100
Non-dialyzed uterine fluid with antibody against histone H2B	71 ± 12
Non-dialyzed oviductal fluid with antibody against histone H2B	54 ± 11
Non-dialyzed follicular fluid with antibody against histone H2B	45 ± 6
Dialyzed fluids from oviduct MWCO 3500 with antibodies against histone H2B	91 ± 16

3.2. Protein concentration in fluids from the cow reproductive tract

Total protein content in fluids from the reproductive tract of cow was determined by bicinchoninic acid assay. The fluids differ significantly in their protein concentrations (see Table 2). The follicular fluid is the most concentrated, with a protein concentration of 126 ± 45 mg/ml, which is about three times more than the least concentrated uterine fluid. These values give a rough estimation of relevant values of protein concentration from a physiological point of view.

3.3. SDS electrophoresis of fluids from reproductive tract of cow and mass spectrometry analysis and protein identification from oviductal fluid

Results of SDS polyacrylamide gel electrophoresis of dialyzed fluids from reproductive tracts (MWCO 3500) revealed four intense bands in the region of relative molecular weight between 14,400, 16,800, 17,300 and 18,000 in the case of oviductal fluid. The same bands occur in fluids from the uterus and follicle (picture is not shown). Proteins bands were analyzed by MALDI-TOF mass spectrometry. The protein band with the highest mobility yielded peptides homologous to bovine histone H3.3, protein bands with lower mobility produced peptides homologous to histones H4, H2A type 2-C, and H2B type 1-K.

Table 2
Concentration of proteins in fluids from the reproductive tract of co as determined by bicinchoninic acid assay.

Sample	Protein concentration (mg/ml)
Uterine fluid	40 ± 12
Oviductal fluid	80 ± 19
Follicular fluid	126 ± 28

4. Discussion

Antimicrobial proteins and peptides represent essential components of the host immune system across the plant and animal kingdoms. They usually constitute a quick albeit often less specific response to infections and have the capacity to inhibit the growth or proliferation of a broad spectrum of pathogens ranging from viruses and bacteria to fungi or protozoa [36,37].

In our study, we examined an antimicrobial effect of fluids from the female reproductive tract of cow on a culture of *E. coli* as a model prokaryotic organism. We tested the antimicrobial properties of fluids from uterus, oviduct and ovarian follicles and compared them with the same samples after removing the compounds of relative molecular mass of less than 3500 by means of dialysis. We found that all dialyzed fluids are capable of diminishing the growth of *E. coli*, even though the effect of dialysis was pronounced and led to decrease in antimicrobial activity by 20–50% in all three dialyzed fluids. This may be explained by the removal of small antimicrobial peptides and fragments of antimicrobial proteins, which can still exhibit potent antimicrobial properties [14]. An alternative explanation could be a depletion of the possible antibiotic residue contaminants from the animal tissue. Nevertheless the protein concentrations of dialyzed fluids necessary for 50% inhibition of growth of *E. coli* (IC_{50}) were still well within the physiological range (IC_{50}~33 mg/ml), with the possible exception of dialyzed uterine fluid (IC_{50} = 85 mg/ml) as was shown by protein concentration determination by bicinchoninic acid assay.

The subsequent increase of dialysis membrane cut off at 30,000 led to practically no observable antimicrobial activity of all studied fluids. Obtained results indicate that fluids from the reproductive tract of cow contains compounds with a molecular mass between 3500 and 30,000 with pronounced antimicrobial properties.

To identify these antimicrobial compounds, an SDS electrophoresis of dialyzed fluids (with MWCO 3500) was carried out. Obtained electrophoreogram revealed four intense bands in the region of a relative molecular mass between 14,000 and 18,000. These bands from the oviductal fluid were subsequently identified by MALDI-TOF/TOF MS analysis as histone H2A type 2-C, histone H2B type 1-K, histone H3.3, and H4. Although the sequence coverage was about 60%, there was no peptide fragment which would not belong to any of the detected histones. Surprisingly, not even traces of histone H1 were detected even though this is the histone very often connected with antimicrobial activity elsewhere [9,16,19,20,38]. It is important to point out that the absence of histone H1 lends weight to the argument that the presence of the other histones is not accidental and their source is not probably from damaged or necrotic tissue. It also raises a question of how these histones are transported out of the cells and by what means is the histone composition of fluids from reproductive tract regulated.

To confirm the antimicrobial role of histones in fluid from reproductive tract, an antihistone antibody was used as an inhibitor of antimicrobial properties of fluids. The idea behind this was

that antibodies bound to the epitopes on the surface of histone would interfere with its antimicrobial action. Polyclonal antibodies against the N-terminus of H2B histone were chosen based on the facts that antimicrobial histone-like peptides are usually derived from this region [14,22] and also because the H2B histone appears to be the most abundant, although far from being the only detected histone in these fluids. The adding of the described antibody had a profound negative effect on the antimicrobial properties of fluids of reproductive tract of cow. In the case of dialyzed fluids (MWCO 3500), the antimicrobial activity was reduced beyond the detection limit and when non-dialyzed fluids were tested, the antimicrobial activity decreased by about half after adding the antibody against the histone H2B.

5. Conclusion

In summary a comparison of antimicrobial properties of dialyzed and non-dialyzed fluids from uterus, oviduct and ovarian follicle of cow exhibited the presence of compounds with a pronounced antimicrobial properties with a molecular mass range of 3500–30,000. These compounds were further identified as histone H2A type 2-C, histone H2B type 1-K, histone H3.3, and histone H4. The antimicrobial effect of histone H2B was subsequently confirmed by experiments using the antibody against this histone, which when added to the tested fluids, interfered with their antimicrobial properties. The observed presence of extracellular histones in the reproductive tract of cow and their antimicrobial action here is a novel discovery and it may help to our understanding of histone roles in the innate immunity of mammals.

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