Investigation of mannose-binding lectin, surfactant protein B and heat shock protein expression in bovine respiratory syncytial virus infection

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SUMMARY

Bovine respiratory syncytial virus (BRSV), after which the disease was named, is an important pathogen of the respiratory tract in cattle. Animals in the range of 15 days to 18 months of age, and especially those aged 2-4.5 months, are more susceptible to the infection. Paraffin-embedded blocks of 50 animals' lungs previously histopathologically diagnosed with BRSV and 10 control animals were used in this study. Mannose-binding lectin (MBL), surfactant protein B and heat shock protein (HSP) expression release from type-2 pneumocytes were studied on tissue sections using immunohistochemical methods. Cases divided as acute and chronic according to duration of the disease. Increased MBL and HSP immunopositivity and poorer surfactant protein B (SFPB) immunopositivity were observed during acute course of the disease, whereas decreased MBL and HSP immunopositivity and increased SFPB immunopositivity were observed during chronic and less severe course of the disease. The lack of a significant increase in SFPB during the acute phase indicates that it is not involved in acute inflammatory reaction. On the other hand, the increase in SFPB in chronic cases indicates that it has a role in the regeneration phase.

Keywords: Cattle, Respiratory syncytial virus, Heat Shock Protein, Mannose Binding Lectin, Surfactant Protein B

RÉSUMÉ

Expression de protéines inflammatoires au cours de l’infection par le virus respiratoire syncytial bovin

Le virus respiratoire syncytial bovin (BRSV) est un pathogène important des voies respiratoires chez les bovins. Les animaux âgés de 15 jours à 18 mois, en particulier ceux âgés de 2 à 4 mois et demi, sont plus sensibles à l’infection. L’effet cytopathogène (CPE) du virus sur les pneumons et la libération de médiateurs à partir des tissus affectés fournissent des informations importantes sur le mécanisme de développement et l’inflammation locale. Des blocs de poumons inclus dans la paraffine de 50 animaux précédemment diagnostiqués histopathologiquement comme atteints de BRSV et 10 animaux témoins ont été utilisés dans cette étude. Une analyse immunohistochimique a été effectuée pour mesurer l’expression de la lectine liant le mannose (MBL), la protéine B du surfactant (SFPB) et les protéines de choc thermique (HSP) à partir des pneumocytes de type 2. Une immunoposivité accrue à la MBL et à la HSP et une immunoposivité plus faible pour la SFPB ont été observées pendant l’évolution aigue et sévère de la maladie, tandis qu’une diminution de l’immunoposivité MBL et HSP et une immunoposivité accrue de la SFPB ont été observées dans les formes chroniques et moins sévères. L’absence d’augmentation significative de la SFPB dans les formes aiguës suggère qu’elle n’est pas impliquée dans la réponse inflammatoire aigue. D’autre part, l’augmentation de la SFPB dans les cas chroniques suggère son implication dans la phase de régénération.

Mots-clés : Bovins, Virus respiratoire syncytial, inflammation, immunohistochimie

Introduction

BRSV infection is an important respiratory tract condition in cattle, which is epizootic and enzootic and characterised by severe clinical and pathological changes [3, 4, 5]. Several factors such as transport, stress, poor care and feeding conditions, lack of any received colostrum and environmental factors play an important role in disease development. In the studies conducted in Turkey, it has been reported that BRSV infection plays an important role in bovine pneumonia [6, 17, 24]. The disease was named after interstitial pneumonia signs and syncytial cell formations developing as a result of its cytopathic effect on the lungs [2, 5]. The main pathological lesion of the disease is interstitial pneumonia. However, it usually transforms to bronchointerstitial pneumonia because of the involvement of secondary bacteria such as Pasteurella spp. and Mycoplasma spp. This condition is called ‘respiratory disease complex’ [5, 10].

The release of proteinaceous substances from affected tissues as a result of the cytopathic effects observed during the disease course provides valuable information regarding the development mechanism and effects of inflammation [5]. One of these substances is mannose-binding lectin (MBL), which is a plasma glycoprotein that consists of collagen and collectin, and is primarily secreted by the liver. MBL is an important component of the innate immune system. It engulfs microorganisms that cause inflammation such as bacteria, virus, and fungi, enables macrophages to recognise and expose these microorganisms to phagocytosis, or activates the lectin pathway of the complement system [8, 9, 13, 14, 16, 23].
Cells synthesise a protein known as heat shock proteins (HSP) in response to sudden increases in temperature, anoxia, reactive oxygen metabolites, and changes in glucose levels [7]. Many stress factors such as decomposition of toxic components and oxidation result in HSP synthesis in all cells. Therefore, these proteins are also called stress proteins. Stress proteins are antigens that play a role in the induction of an immune response by many antigenic agents in the host [18]. Stress proteins induced by pathogenic microorganisms can constitute a common target for immune response by exhibiting the common activity of various infections. Thus, immunity developed against a stress protein induced by a pathogen may have a protective role against another pathogen [20].

Surfactant protein B is a protein with phospholipid structure and it is released from alveolar type-2 pneumocytes. It is responsible for maintaining the alveolar surface tension as well as helping the surfactant create a barrier against foreign factors (bacteria, viruses and allergens) after it joins the surfactant structure [22]. Previously, it has been established that the release of surfactant protein B decreases along with other surfactant proteins in hyaline membrane disease, which is a congenital disease of genetic origin previously seen in humans [15]. The signs of BRSV infection include alveolar proteinosis, interstitial cell infiltration and hyperplasia of type-2 pneumocytes. Similar findings are also found in viral interstitial pneumonia characterised by fibrosis, interstitial cell infiltration and typical hyaline membrane foetalisation. However, surfactant protein B has not been previously identified and evaluated in the lungs during BRSV infection [15, 21, 22].

Several pneumonia agents can cause lung lesions in cattle and most of them related viral agents but pathogenesis of lung reactions is unknown [5]. The objective of this study is to investigate the histopathological and immunohistochemical findings as well as pathogenetic mechanisms of BRSV infection in cattle in detail.

Materials and Methods

The materials used in the study were paraffin-embedded blocks prepared from 50 calf lungs previously diagnosed histopathologically in the Pathology Department of the Faculty of Veterinary Medicine. According to necropsy notes about diseases duration, samples divided in 3 groups as acute cases (<7 days, n = 33), chronic cases (>8 days, n = 17), controls (n = 10). In this study 33 acute, 17 chronic cases and 10 control lungs were examined. Sections of these paraffin-embedded blocks with 5-µm thickness were placed on normal and poly-l-lysine coated slides. The diseased and control lung sections were stained with polyclonal anti-MBL-2 [Anti-Mannan Binding Lectin antibody (ab203303) ], anti-surfactant protein B (SP-B) [Anti-Pro + Mature Surfactant Protein B antibody (ab40876) ] and anti-heat shock factor 2 binding protein (HSP) [Anti-Heat Shock Factor 2 Binding Protein antibody (ab26149) using standard commercially available avidin–biotin complex peroxidase (ABC-P) kit [EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC kit (ab80436)], 3,3-diaminobenzidine (DAB) was used as chromogen. For negative controls primary antiserum step was omitted. All examinations were performed on blinded samples.

Paraffin-embedded blocks of the lungs obtained from 10 calves that were at the same age and did not exhibit pneumonia signs were used as the control group after confirming the absence of pneumonia histopathologically. Same sections were obtained from the paraffin-embedded blocks of the lungs of the animals in the control group, stained with the specific primary antibodies and compared to the animals with the disease, then cells that exhibited positive staining for MPB, HSP and surfactant protein B were counted using a commercial analysis software (Bap bs 2000 Pro) and compared with the control group. In addition, the localisation and intensity of MBL and HSP immunopositivity in lung tissues were evaluated in the scope of the manuscript.

To evaluate the severity of the immunohistochemical reaction of lungs with markers, semiquantitative analysis was performed using an arbitrary visual scale with a grading score ranging from (−) to (+++) as follows: (−)=negative, (+)=weak staining, (++)=mild staining, (+++)=strong staining.

Statistical analysis

Data were analyzed statistically by one-way analysis of variance (ANOVA). Bonferroni tests were performed. The SPSS software program (version 11.0, SPSS, Chicago, IL, USA) was used for all statistical analyses at a significance level of P<0.05. All results are expressed as means±standard deviation.

Results

**HISTOPATHOLOGICAL FINDINGS**

In the evaluation of the sections using light microscopy in histopathological examinations, the main pattern in the lung tissue was bronchointerstitial pneumonia. This manifested alongside signs of bronchopneumonia induced by possible secondary infections as well as the signs of interstitial pneumonia seen in viral pneumonias. In the microscopic examinations, interalveolar, interlobular and interlobar veins were hyperaemic. Interalveolar septal tissue, interlobular septa and interlobar septa were enlarged because of mononuclear cell infiltrations and oedema. In acute cases, pink-uniformly coloured oedema fluid and sporadic fibrin filaments were observed in bronchi, bronchiolar and alveolar lumens. Epithelial cells shed into the alveolar lumen were filled with neutrophil, leukocyte and macrophage infiltrations in some cases. Syncytial cell formations, which are characteristic of the disease and caused by the syncytium of 3–5 shed epithelial cells, were observed in some alveolar...
lumens. Type-1 pneumocytes were mostly degenerated whereas type-2 pneumocytes exhibited distinct hyperplasia. Alveolar walls were lined by type-2 pneumocytes, wherein a manifestation was present, which is intrinsic to viral pneumonias and called ‘epithelisation’ or ‘foetalisation’. In severe cases, shed epithelial cells along with neutrophil, leukocyte and macrophage infiltrations were observed also in the bronchial and bronchiolar lumens. In cases with severe infection, uniformly pink and large necrotic areas in the form of amorphous material were observed in the lung parenchyma. These areas were surrounded by dense inflammatory cell infiltration. Syncytial cell formations, which give the disease its name and was caused by the syncytium of shed epithelial cells, were observed in some bronchiolar lumens. Although degradation continued partially in bronchi and bronchioles, epithelial cell hyperplasia was observed concurrently indicating the reparative efforts of the body. In many bronchi and bronchioles, a manifestation of ‘peribronchial cuffing syndrome’ caused by mononuclear cells of lymphoid origin associated with severe hypertrophy of bronchoalveolar lymphoid tissue structures associated with lymph follicle hyperplasia was observed. Spindle-shaped or fusiform connective tissue proliferations with medium or dense collagen texture were observed in interstitial tissue especially in chronic cases. It was observed that alveoli surrounding these regions were flattened and underwent atelectasis. Regions with emphysema were observed in the adjacent alveoli. Intracytoplasmic ‘inclusion bodies’ with a uniformly pink appearance were observed in some affected bronchial and bronchiolar epithelial cells. Interlobar and interlobular septa were present along with oedema, fibrin accumulation, neutrophil and macrophage infiltrations on the pleura, particularly in cases with bronchointerstitial pneumonia (Figure 1).

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IMMUNOHISTOCHEMICAL FINDINGS

In immunohistochemical staining with the anti-BRSV polyclonal antibody for confirming the presence of the agent, immunopositive areas were observed in the shed epithelium in bronchi, bronchiolar epithelial cells, and bronchiolar lumens as well as macrophage and lymphocyte cytoplasm. Immunopositive areas in the form of a thin film layer were observed in bronchiolar and alveolar lumens in some regions. Dense immunopositive areas were also observed in syncytial cell formations, macrophage and lymphocyte cytoplasm, shed cells and inflammatory exudate in alveolar lumens. Apart from these, there were also immunopositive areas in macrophage cytoplasm and in the free form in interstitial tissue. There was no positive staining in control group tissues (Figure 2).

The activity of MBL, which triggers inflammation via the lectin pathway by the opsonisation of agents, was determined using anti-MBP antibody. Immunopositivity was observed in all areas with inflammation during microscopic examination. Immunopositivity was observed particularly in the exudate in bronchi, bronchiolar epithelial cells and alveolar lumens; interstitial tissue; in areas containing shed cell accumulations in bronchi, bronchiolar epithelial cells and lumens and alveolar lumens, and in macrophage cytoplasm and syncytial cell formations. Immunopositive areas were also observed in the periphery of interalveolar, interlobar and interlobular veins and peribronchial glands. In cases that progressed to necrotic bronchointerstitial pneumonia, immunopositivity was denser in necrotic areas than in other areas (Figure 3).

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In anti-HSP staining which demonstrates tissue damage and oxidative stress, dense immunopositive areas were observed particularly in interstitium and necrotic regions, and immunopositive areas with variable density were observed in the mucus and inflammatory exudate in alveolar wall and lumen, and in bronchial and bronchiolar epithelial cells and lumens. There was no positive staining in control group tissues (Figure 4).

Semi-quantitative analysis of immunohistochemical staining showed a significant increase in MBL, HSP and SFPB levels in comparison with control groups especially during the acute exudative phase when the inflammation was active and desquamation and oedema were distinct. Comparing the release of proteinaceous substances in question, it was observed that MBL and HSP levels were higher than the SFPB level. Analysis results of immunohistochemical scores were shown in Table I.

Discussion

BSRV infection is a contagious and fatal disease especially in young cattle [5, 10, 11]. There are research studies regarding the pathology, serology, seroprevalence and vaccination for BRSV infection [1, 6, 12, 17, 24]. However, there are no studies regarding the pathogenetic mechanisms of the disease. In this study, the pathomorphological and immunohistochemical signs of BRSV infection seen in cattle in Burdur area and the pathogenetic mechanisms of the disease were investigated in detail.
The treatment options for viral infections of the respiratory tract are limited. The virus causes an infection by entering the body directly or through the epithelial barrier, which is weakened or irritated because of predisposing factors such as immunodeficiency, poor care and poor nutrition. With mucosal barrier breakdown, opportunistic superinfections with bacteria in the inhaled air make disease more severe. In such cases, the phagocytic defence system is activated in the body and enables the phagocytic cells to recognise the agents [5]. The process of marking agents is called opsonisation. In opsonisation, various proteins engulf the agent, stimulate the complement system and start inflammation with a series of reactions. One of these proteins is MBL [5, 8-10]. In the study, most pneumonia cases were of bronchointerstitial pneumonia type, which is an indicator of mixed infection. In addition, there were anti-BRSV-positive areas in the affected regions and there was dense MBL staining in BRSV immunopositive areas especially in acute phase pneumonia cases, which were all consistent with previously published data.

Past studies have reported that congenital MBL deficiency leads to fatal respiratory syndrome. In addition, MBLs' role in opsonisation during inflammation has been demonstrated in the studies conducted on humans [9, 13, 14]. This study investigated the activity of MBL in BRSV infection, which causes a severe respiratory tract infection in cattle. In immunohistochemical staining, immunopositive areas were observed in epithelial cells shed in bronchi, bronchiolar and alveolar lumens and epithelial cells affected by the virus, in alveolar macrophages, syncytial cell formations intrinsic to the disease and in the oedema fluid. The lack of immunostaining in the control group indicates that the release of MBL increases in affected cases. Dense immunopositivity particularly in the necrotic regions in parenchyma and lighter immunopositivity in chronic areas, where infection severity is decreased, indicate that the release of this proteinaceous substance is directly proportional to disease severity.

Many protein secretions that appear in oxidative stress and tissue damage act as chemotactic agents in inflammation of the affected region. One of these substances, i.e. HSP, is released from affected tissues in many diseases. The importance of stress proteins is based on their capability to associate with other proteins and change their functions and structures [19]. Stress proteins are of vital importance in all phases of cell metabolisms including cell growth, differentiation, division and even death. Stress proteins are antigens that play a role in the induction of an immune response by many antigenic agents in the host [18]. Stress proteins triggered by pathogenic microorganisms can constitute a common target for immune response by exhibiting the common activity of various infections. Therefore, immunity developed against the stress protein triggered by a pathogen can have a protective role for another pathogen [20]. In this study, HSP immunopositivity was observed in all areas with inflammation. Strong immunopositivity observed particularly in necrotic areas in severe cases indicated that HSP release increased in a directly proportional manner to inflammation severity. In addition, the presence of strong HSP-positive staining in regions that also exhibited strong anti-MBL-positive staining indicated that defence mechanisms against the disease were triggered by HSP release and these two had a cooperative relationship.

Congenital surfactant protein B deficiency in humans and animals is a known disease with severe outcomes. Many studies have been published regarding infection development and lack of sufficient alveolar dilation because of surfactant B deficiency in such cases [15, 21]. Studies mostly reported immature or pro-surfactant protein B deficiency and focused on the association of the condition with genetic predisposition [21]. In addition, studies involving mature surfactant protein B mostly focused on its high activity in neoplastic or hyperplasic tissues [19], but its association with inflammation in terms of opsonisation was not studied in detail. In our study, surfactant protein B release associated with acute inflammation was lower in infection group than in the control group. Furthermore, stronger positive staining in bronchi, bronchiolar epithelial cells and type-2 pneumocytes in subacute or chronic cases indicated that mature surfactant proteins were playing a role to re-establish the epithelial barrier by providing homeostasis and recovery, while the body was fighting in affected regions. In addition, light positive staining of surfactant protein B in animals in the mature control group indicated that none of the animals that developed the disease had hereditary surfactant protein B deficiency.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Score</th>
<th>Area (%)</th>
<th>Score</th>
<th>Area (%)</th>
<th>Score</th>
<th>Area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute (n=33)</td>
<td>2.51±0.71A</td>
<td>19.15±1.83A</td>
<td>2.78±0.41A</td>
<td>19.69±1.59A</td>
<td>0.33±0.17A</td>
<td>2.69±0.38A</td>
</tr>
<tr>
<td>Chronic (n=17)</td>
<td>1.29±0.46A</td>
<td>11.64±1.57B</td>
<td>1.00±0.61A</td>
<td>11.70±1.72B</td>
<td>2.29±0.68B</td>
<td>12.05±1.56B</td>
</tr>
<tr>
<td>Control (n=10)</td>
<td>0.40±0.05C</td>
<td>0.63±0.20C</td>
<td>0.20±0.04C</td>
<td>0.31±0.18C</td>
<td>1.40±0.51C</td>
<td>6.20±2.82C</td>
</tr>
</tbody>
</table>

*: Values are presented as means±SD. The relationships between groups and results of immunohistochemical scores are assessed by one way ANOVA. **: The differences between the means of groups carrying different letters in the same column are statistically significant (P< 0.001).

Table I: Statistical analysis results of immunohistochemical scores and percentage/area distribution of immunohistochemical markers.
Anti-surfactant protein B, known as the ‘anti-atelectasis factor’, lines the alveolar lumen as a film layer and prevents alveoli from adhering to each other. Moreover, there are reports indicating increased release of this protein in lung inflammation [15, 21, 25]. Yokohiro et al. [25] identified in a study conducted on rat lungs that surfactant protein B staining was poor in inflammatory exudates in bronchiolar and alveolar lumens. In addition, they observed strong positive staining in tumours and bronchi, bronchiolar epithelial cells that underwent hyperplasia, and in peribronchial glands in hyperplastic and neoplastic cases of the lung. In our study, surfactant protein B staining was very poor in regions that exhibited strong anti-MBL and anti-HSP staining, whereas surfactant protein B-positive staining was strong in hyperplasic bronchial and bronchiolar epithelial cells and type-2 pneumocytes in the alveolar wall. According to this result, surfactant protein B does not have an important function in triggering the acute inflammation but plays an active role in chronic inflammations. In addition, light immunopositivity observed in alveolar lumens was considered as the breakdown and accumulation of surfactant film layer in the lumen because of alveolar damage. However, light staining of phagocytic cells raises the question that whether it helps the digestion of affected proteins or the opsonisation of agents. Increased MBL and HSP immunopositivity and poorer SFPB immunopositivity observed during acute and severe course of the disease, and decreased MBL and HSP immunopositivity and increased SFPB immunopositivity observed during chronic and less severe course of the disease, indicate that the staining during the acute phase is the result of the residues from SFPB breakdown and that SFPB is not involved in opsonisation. On the other hand, increase in SFPB in chronic cases indicates that it supports the regeneration phase. Further studies are needed to determine whether or not it induces fibrosis in interstitium.

There are pathological, virological and immunological studies conducted on humans and animals with BRSV virus infection. There are also natural and experimental studies in different fields regarding MBL, HSP and SFPB, all of which were employed in this study. However, no study investigating and evaluating markers such as MBL, HSP and SFPB together in cases of BRSV infection in cattle were identified in our literature review. Therefore, this study is the first on this subject.

Conflict of interests

None.

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References


