The Investigation of Cell Adhesion Molecules in the Lung Tissues of Cattle with Cystic Echinococcosis

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Abstract: Cystic echinococcosis is a zoonotic disease with worldwide distribution caused by Echinococcus granulosus, represents a substantial global health problem. Hydatid cyst (Echinococcus) has a remarkable negative effect on the health of people and the economic development of the country. The objective of this study was to investigate the CD68, nicotinamide nucleotide adenylyltransferase 3 (NMNAT 3), Neuregulin 1 (NRG1) and Neuregulin 2 (NRG2) expressions in bovine lungs infected with E. granulosus and to identify whether they have any correlation with pulmonary pathology. For this purpose, 30 bovine lung tissues were used between January 2016 and December 2016 collected in Kirikkale slaughterhouse. In histopathologic examinations, proliferation of fibrous connective tissue and infiltration of mononuclear cells were detected in the lung tissues of the bovine. Most of the cysts were seen to be quite thick capsule. There was also a cellular line rich in abundant fibroblasts and mononuclear cells. The cyst wall was found to be an eosinophilic laminar structure. There was infiltration with lymphocytes and macrophages, especially eosinophils and giant cells. Immunohistochemically, CD68 positivity was seen around the bronchi, bronchioles and cystic matter. However; NMNAT 3, NRG1 and NRG2 showed no positive reactions in macrophages, bronchi, bronchioles and alveolar epithelium. These results indicate that NMNAT 3, NRG1 and NRG2 pathways were not used in pulmonary pathology. Therefore, it is the most important result of the study that the adhesion molecules in pulmonary pathology are not originating from NMNAT 3, NRG1 and NRG2.

Key words: CD68, NMNAT 3, Neuregulin 1, Neuregulin 2, Pathology

Introduction

Cystic echinococcosis (CE), also known as hydatid cyst, is a zoonotic disease caused by Echinococcus granulosus and is widely observed in our country and in the world. The definitive host of E. granulosus is varies where there are many wild carnivores except the dog and red foxes. Human and animals such as sheep, cattle, and swine are the intermediate host of the parasite. The definitive hosts are infected by taking the infected organs by the alimentary}\n
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route. Adult parasites are consist of the released protoscolexes. The final host contaminates the environment with pregnant rings thrown from adult parasites [19].

Clinical findings of hydatid cyst may not be seen for years due to the slow development of the cyst and its importance in organelle localization. The size of the lesions is directly proportional to the location of the cyst in the host [21]. Molecular characterization studies have shown that there are genetic differences among E. granulosus isolated from different hosts and countries [29]. Therefore, the control of CE is important for revelation of the strains seen in endemic regions, and it is important to control the disease [8].

NAD is the coenzyme that involved in many metabolic enzymatic reactions. Nicotinamide nucleotide adenyltransferase 3 (NMNAT-3) regulates the mitochondrial NAD level in the mitochondria in cells. Previous studies have shown that NMNAT-3 is expressed in lung tissue [3]. However, there were no studies found about this molecule in domestic animals in CE cases. Neuregulin 1 (NRG1) is a trophic factor that is indicative of an epidermal growth factor (EGF) signaling by inducing ErbB receptor tyrosine kinases [10,28]. Neuregulin 2 (NRG2), an insert variant of NRG1, is a transmembrane protein that assists in the regulation of cell proliferation, cellular differentiation and survival [4, 5]. As shown by the studies, NRG1 and NRG2 were expressed in the lung epithelial tissues [22]. It has been reported, these molecules found especially in alveolar, bronchial and bronchiolar epithelia. In recent years, the effectiveness of these molecules has been investigated in lung cancers and inflammatory cases. It has been concluded that it has anti-inflammatory action in inflammatory cases. Cluster of Differentiation 68 (CD68) is a protein that is excreted in large amounts from tissue macrophages in tissues and monocytes in the blood [16].

In this study, the presence of NMNAT 3, NRG1, NRG2 and CD68 expressions were investigated in E. granulosus in naturally infected bovine lungs. In addition, it was aimed to investigate the role of inflammatory response of these molecules in lung in CE. Thus, it has been try to determine whether there is a relationship between pulmonary pathology and the expressions of these markers.

Materials and Methods

Materials

Materials were collected from Kirikkale slaughterhouse. Regular checks were conducted between January 2016 and December 2016. During this time, 45 lung tissues with cysts hydatid were collected. The infected lung tissues of these 45 animals were classified as firstly macroscopic and then histopathological according to their severity. Macroscopically, a total of 30 bovine lung tissues with at least one cyst in all lobes of the lung were included in the study (Figure 1 A-B). However, in order to examine the entire cyst structure in a histology slide, cystic tissues smaller than 1 cm in diameter were processed histopathologically.

Methods

Histopathological Method

After the fixation procedure, the lung tissues were subjected to alcohol, xylol and paraffin wax respectively. Subsequently the tissues were cut at 5 µm thickness of sections by microtome, glued to slides and examined under light microscope, after stained by haematoxylin and eosin staining [18].

The following criteria were evaluated semi-quantitatively for histopathological scoring of the cases. Cyst diameter (mm), inflammatory cell and fibrous tissue infiltrations (No lesion: 0, Mild: +1, Moderate: +2 and Severe: +3). Data were statistically described in terms of mean and standard deviation (mean±SD) for histopathological scoring.

Immunohistochemical Method

Indirect immunoperoxidase method was used for immunohistochemical results. For this purpose, 5 µm thick sections of paraffin wax were adhered to positive charged slides. The sections were dried in a 60 °C oven for 15 min. Then these sections were subjected to 3 times for 5 minutes xylene and then 96%, 90%, 80%, 70% and 50% alcohols for the deparaffinization procedure. At the end of this process, sections were boiled in citrate buffer solution for 20 min for antigen retrieval procedure. Then 3% H₂O₂ was added to the sections to remove peroxidase activity and incubated for 7 min. After this process, the block solution was added for 5 min and the primary antibodies [Anti-NMNAT-3 antibody
(Santa Cruz Biotechnology, inc. sc-390433), Anti-Neuregulin-1 antibody (Santa Cruz Biotechnology, inc. Sc-57384), Anti-Neuregulin-2 antibody (Santa Cruz Biotechnology, inc. Sc-398594) and Anti CD68 antibody (Santa Cruz Biotechnology, inc. sc-514937)] were incubated for 2 hours. At the end of the incubation, biotinylated secondary antibody and streptavidin solution were added at 15 minute intervals. The sections were finally stained with aminooethyl carbazole (AEC). After counter-staining with hematoxylin, slides were closed by coverslips and evaluated under a light microscope. The negative control slides were also stained according to the same procedure. However, PBS was used instead of the primary antibody.

**Results**

Histopathological lesion scores were given into the table 1. Histopathological examination revealed fibrous connective tissue proliferation and infiltration of mononuclear cells in lung tissues of cattle (Figure 2A). Most of the cysts had a thick capsule. In most cases, the cyst wall showed an eosinophilic laminar structure. In the inflammation sites, lymphocytes, macrophages, eosinophil granulocyte and foreign body giant cell infiltrations were seen (Figure 2B). Alveolar canals were enlarged and an inflammatory infiltrate was found in some peribronchial and peribronchiolar areas (Figure 2C). In some cases, bronchiolar epithelial cell atrophy and emphysema areas were remarkable.

| N=30 | Cyst diameter (mm) | Inflammatory cell infiltrations score | Fibrous tissue score |
|------|--------------------|--------------------------------------|---------------------|
| Mean + Std error of Mean | 5.85 ± 0.539 | 1.95 ± 0.169 | 1.5 ± 0.211 |
| Std Deviation | 2.412 | 0.759 | 0.945 |

**Figure 1.** A. Gross appearances of cysts in the lung (arrows). B. Close up view of cysts (arrows).

**Figure 2.** A. Mononuclear cell infiltrations (blue arrow) around the cyst wall (black arrows) and fibrous connective tissue (red arrow). X40. HE. B. Cyst wall (black arrows) and foreign body giant cell infiltrations (arrowheads). X400. HE. C. Enlargement of the alveoli (A), hyperemia in the blood vessels (Hv) and inflammatory cell infiltrations (arrows) around the bronchiolar (Br) area. X40. HE.
As a result of immunohistochemical investigations, NMNAT 3, NRG1 and NRG2 expressions were not observed (Figure 3A-C). CD68 expressions were found to be positive for intracytoplasmic reactions in macrophages in the inflammatory cell surrounding the cyst and in some cases macrophages in the peribronchial and peribronchiolar areas (Figure 3D-E).

Figure 3. A-C. Immunonegative reactions in the lung tissue of NMNAT-3, NRG1 and NRG2. X100. AEC. D-E. intracytoplasmic positive immunostaining in the macrophages in the inflammatory cell infiltrations (arrows). X400. AEC.

Discussion and Conclusion

Cystic echinococcosis is a serious zoonotic disease in countries where environmental health and preventive medicine measures are not cared enough. It has a wide spread due to its presence all over the world. It is widely seen all over the world. Dogs and wild canines have a major role in the transmission of disease [14]. In this context, it is very important to reveal the pathological changes and molecular reactions occur in intermediate hosts such as cattle, sheep and pigs. In this study we investigated the expression of NMNAT 3, NRG1, NRG2 and CD68 cytokines in bovine lung with CE. The main aim is to determine the pathways of the pathogenesis of the disease by the molecular level.

Some researchers have focused on specific cases on CE and they investigated the where the disease is localized [1,11,26]. In one hand, some previous investigators emphasize the molecular characterization of the agent [9,24]. On the other hand, some other researchers emphasized the importance of nad1 / cox1 gene expression by genetically locating / expressing [12,13]. In addition, a large proportion of scientist focused on the diagnosis of the disease [6,27]. Eckert et al. [7] stated that the necropsy findings are the best method for the diagnosis of CE. However, Larrieu et al. [17] explained that they could not find a statistical difference between necropsy findings and histopathological examination in their study. In the present study, all cases in the study had both grossly and histopathological findings to confirm CE. Findings of the present study are consistent with the results of previous studies [2,15,22,25].

The studies on the pathogenesis of the disease are very limited and there are still unexplored points. Yin et al. [33] studied on the expression of TGF-β related to determine the pathogenesis of the CE. In a pathogenesis study investigating the expression of interleukin (IL) in the lungs in experimentally CE infections, the expression of IL-10 increased while IL-5 and IL-17A expression decreased [32]. Vismarra et al. [31] observed immunohistochemically positive reactions of FOXP3 around the cyst, most of which were CD3 positive. Sakamoto and Cabrera [23], obtained CD8 positive reactions in the inflammatory area around the cyst in the cattle lungs and stated that these cells could originate from lymph nodes draining the lungs. Vatankhah [30] reported CD68 positive immune reactions in inflammatory cells around the cyst in human liver tissues with CE. In this study, CD68 positive immunostaining were found in the macrophages in...
the peribronchial and bronchiolar areas with associated with the inflammatory cells around the cyst in the cattle lungs. Although there is no previous study with cytokines such as NMNAT-3, NRG1 and NRG2, the expressions of these molecules were not determined in CE lung tissues.

In conclusion, the findings obtained from this study showed that NRG1 and NRG2 expressions that show a similarity between Neuregulin derivatives in the pathogenesis of CE do not make sense. Additionally, another important finding was that transferase activation (NMNAT 3) was not used as an important pathway by using nicotinamide mononucleotide-mediated energy (ATP). Therefore, it is thought that antihelmintics will not play a role in the mechanism of action and the development of new therapies. However, CD68 expressions were firstly demonstrated in the cattle lung with natural CE infections.

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