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Immunohistochemical detection of pro-inflammatory and anti-inflammatory interleukins in the lungs of sheep with jaagsiekte

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ABSTRACT

Objective: In this study, it was aimed to evaluate the levels of interleukins such as IL-1β, IL-6, IL-10 and IL-12β in sheep with Jaagsiekte by immunohistochemical methods. In this way, it will be revealed whether interleukins are effective in the progression of Jaagsiekte and how useful they are in the diagnosis of the disease.

Material-Method: The material of the current study consisted of lung tissues of 26 sheep (Control, n=6 and Jaagsiekte, n=20) brought to the Department of Pathology for routine histopathological diagnosis. Tissue samples taken were fixed in 10% buffered formaldehyde solution. 5 µm-thick sections were taken from the paraffin blocks prepared after routine tissue follow-up procedures. Hematoxylin & Eosin staining was applied to the sections in order to detect histopathological changes. Sections were examined and photographed under a light microscope. The routine streptavidin–biotin peroxidase complex method was used.

Results: In sheep with Jaagsiekte, tumoral foci with large and small acinar or papillary growths were observed in the alveolar and bronchiole lumens. The control group was negative for IL-1β, IL-6, IL-10 and IL-12β immunoreactivity. IL 1β-6-10 and 12β levels were dramatically increased in the Jaagsiekte group compared to the control group.

Conclusion: It was determined that interleukins were produced from tumoral cells and tumor microenvironment elements, and these interleukins showed pro-inflammatory effects, except for IL-10.

Keywords: *Jaagsiekte, Interleukins, Sheep*

INTRODUCTION

Jaagsiekte, also known as ovine pulmonary adenocarcinoma (OPA), is a contagious lung cancer of sheep and goats, caused by Jaagsiekte Sheep Retrovirus (JSRV) (Gomes et al., 2017; Lee et al., 2017). Jaagsiekte occurs worldwide in almost all countries except in New Zealand and Australia (Belalmi et al., 2020). The tumor originates from type 2 pneumocytes and Clara cells and shows many similarities in histological features to human pulmonary adenocarcinomas (Scott et al., 2018; Toma et al., 2020).

Cytokines, such as interleukin (IL), interferon (IFN) and tumor necrosis factor (TNF), which are important signaling proteins act as important mediators of the immune system. Expression levels, polymorphisms or profiles of cytokines and cytokine receptors can affect the pathogenesis of

virus-related diseases (Larruskain and Jugo 2013; Ding et al., 2021).

Interleukin 1 beta (IL-1β), a proinflammatory cytokine, regulates the expression of genes involved in various inflammatory processes and plays a central role in many chronic inflammatory diseases, including lung cancer such as non-small cell lung cancer (NSCLC) (Kim et al., 2013; Bhat et al., 2014). Increased expression of IL-1β in the tumor microenvironment correlates with poor prognosis in human lung adenocarcinomas (Tekpli et al., 2013; Ding et al., 2021).

Interleukin 6 (IL-6), a multifunctional proinflammatory cytokine, is produced by different cells, including immune cells, endothelial cells, cancer-associated fibroblasts, and tumor cells (Islas-Vazquez et al., 2020; Dutkowska et al., 2021). IL-6 is involved in the regulation of tumorigenesis, progression, and metastasis and its overexpression is particularly strongly associated with a poor prognosis for NSCLC (Kiss et al., 2020; Pan et al., 2020; Su et al., 2020).

Interleukin 10 (IL-10), an anti-inflammatory and protumoral effective cytokine, is mostly produced by M2-macrophages, T regulator cells (Tregs), Th2-cells, CD8+ T cells (Vahl et al., 2017; Gao et al., 2020). Il-10 can promote cancer development by suppressing macrophage function and allowing tumors to evade immune surveillance. Also the expression of IL-10 by tumor-associated macrophages (TAMs) correlates with unfavorable prognosis in NSCLC (Hsu et al., 2016; Pang et al., 2017; Hu et al., 2020).

Interleukin 12 (IL-12), an important immune regulatory cytokine, plays a role in stimulating Natural killer (NK) and T cell proliferation, increasing NK and CD8+ T cell cytolytic activity, and inducing various cytokines such as IFN-gamma (Yue et al., 2016). IL-12 exhibits immunostimulating and anti-angiogenic effects in promoting antitumoral immunity (Airoldi et al., 2009; D'Amico et al., 2012).

In this study, it was aimed to evaluate the levels of interleukins such as IL-1β, IL-6, IL-10 and IL-12β in sheep with Jaagsiekte by immunohistochemical methods. In this way, it will be revealed whether interleukins are effective in the progression of Jaagsiekte and how useful they are in the diagnosis of the disease.

MATERIALS and METHODS

Ethical Approval

This study was approved by the Kafkas University Animal Experiments Local Ethics Committee (KAU-HADYEK-2021/109).

Animals

The material of the current study consisted of lung tissue samples of 26 sheep (Control, n=6 and Jaagsiekte, n=20) brought to Veterinary Faculty, Department of Pathology for routine histopathological diagnosis.

Histopathological Examinations

Lung tissues were fixed in 10% neutral buffered formalin and routinely processed. Following routine procedures, tissues were embedded in paraffin wax. Paraffin sections of 5 μm thickness were stained with Hematoxylin & Eosin (H&E) to detect histopathological changes. Sections were examined under a light microscope and photographed.

Immunohistochemical Examinations

The routine streptavidin–biotin peroxidase complex method was used according to the manual instructions of the kit (Thermo Scientific Histostain-Plus IHC Kit, HRP, broad spectrum, REF: TP-125-HL). Anti-IL1β antibody (MyBioSource, MBS2026862, Polyclonal, Dilution Ratio: 1/50), anti-IL6 antibody (MyBioSource, MBS2012740, Monoclonal, Dilution Ratio: 1/100), anti-IL10 antibody (MyBioSource, MBS2026258, Polyclonal, Dilution Ratio: 1/200) and anti-12β (MyBioSource, MBS1490500, Polyclonal, Dilution Ratio: 1/50) were used after antigen retrieval and nonspecific protein blocking. The reactions were detected with aminoethyl carbazole (AEC) chromogen (Thermo Scientific, TA-125-HA). Counterstainings were conducted using hematoxylin. After this procedure, glass slides were mounted with Entellan and a coverslip. For control sections, PBS was applied in drops on the sections instead of the primary antibodies.

Prepared slides were examined under a light microscope (Olympus Bx53) and photographed via the Cell^P program (Olympus Soft Imaging Solutions GmbH, 3,4). Analyzes of the images were done with Image J Program (1.51j8). IL-1β, 6, 10 and 12β expressions were analysed by examining ten representative fields of labeled immune positive cells with the 40X magnification. Rating system were designated as negative (-) 0%,

weak (+) 1-10%, mild (++) 11-59% or severe (+++)>60%.

Statistical Analysis

Statistical analysis of the results was performed using the SPSS® (SPSS 26.0, Chicago, IL, USA) program. According to cell infiltration scoring, Kruskal-Wallis H test was used for multiple comparisons of IL-1β, IL-6, IL-10 and IL-12β, and Mann-Whitney U test was used for pairwise comparisons. Obtained results were given as mean ± standard error (SE). $P<0.05$ expression was considered statistically significant in the evaluation of the results.

RESULTS

Histopathological Findings

No pathological lesions were detected in the lung tissue of the healthy control group. In Jaagsiekte cases, tumoral foci with large and small acinar or papillary growths were observed in the alveolar and bronchiole lumens. A large number of alveolar macrophages were detected around these tumoral foci (Figure 1).

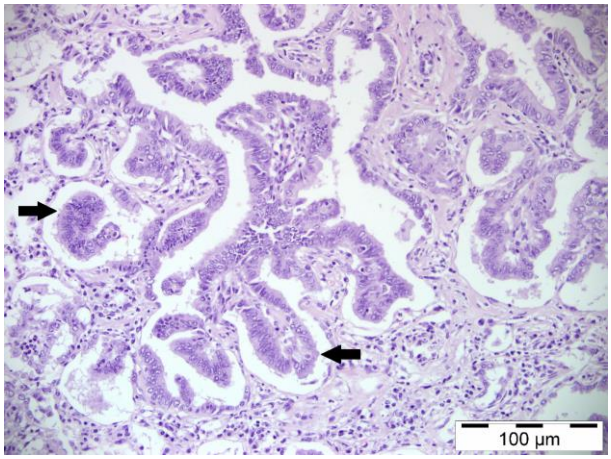

Figure 1. Lung, Neoplastic proliferations (arrows) within the alveolar lumen, H&E, Bar= 100 µm

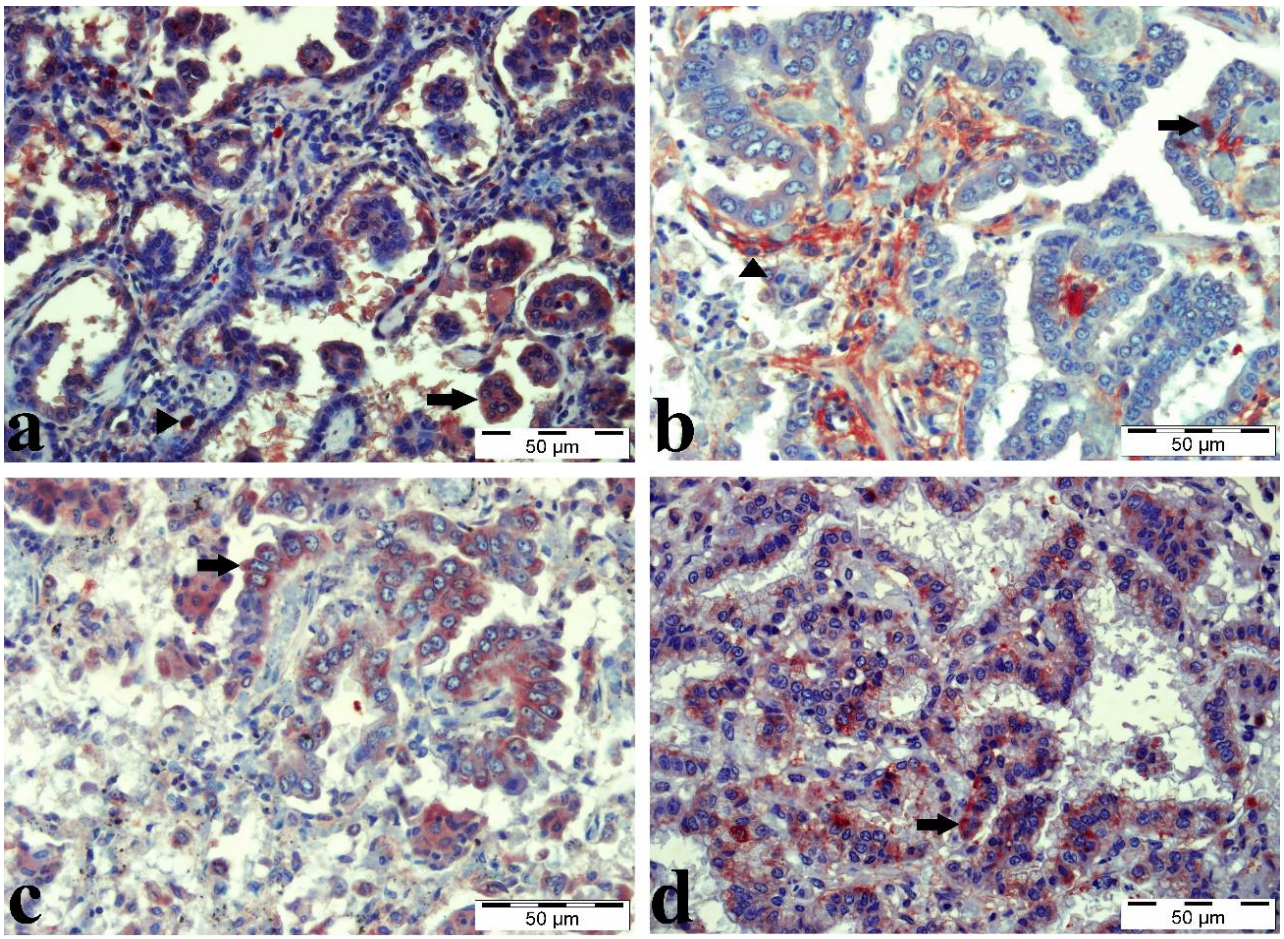

Figure 2. Lung, IHC, AEC, **a:** IL-1β immunopositive reactions in acinar structures (arrow) and lymphocytes in tumor stroma (arrowhead), **b:** IL-6 immune positive reactions in the cytoplasm of neoplastic cells forming papillary extensions (arrow) and tumor stroma (arrowhead), **c:** Intracytoplasmic IL-10 expressions in papillary structures (arrow) inside alveolar lumens **d:** IL-12β immunoreactivity in the cytoplasm of finger-like proliferations (arrowhead) in tumoral foci.

Immunohistochemical Findings

Immune positivity scores of all groups are shown in Table 1. The control group was negative for IL-1β, IL-6, IL-10 and IL-12β immunoreactivity. IL 1β-6-10 and 12β expressions were statistically increased in the Jaagsiekte group compared to the control group. In the Jaagsiekte group, IL-1β, IL-6, IL-10 and IL-12β expressions were mostly detected in the cytoplasm of cuboidal-columnar tumoral cells with acinar or papillary growths. Immune positive reactions were also observed in alveolar macrophages localized around tumoral foci.

Table 1. Immunpositivity scores of all groups

Groups	IL-1β	IL-6	IL-10	IL-12β
Control n=6	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
Jaagsiekte n=20	2.18±0.18[b]	2.25±0.16[b]	1.36±0.15[b]	2.45±0.16[b]
p value	<0.001	<0.001	<0.001	<0.001

[a-b] represents the statistical difference between the groups ($p<0.05$).

In addition, there was positive interleukin staining in the tumoral stroma, especially in lymphocytes and connective tissue cells. IL 1β, 6 and 12β immunoreactivities were detected especially in areas where the inflammatory reaction was severe. On the other hand, IL-10 positive staining was more pronounced in areas where the severity of the inflammatory reaction decreased (Figure 2).

DISCUSSION

IL-1β, a pro-inflammatory interleukin, synthesized by macrophages acts as an alarm cytokine and regulates chronic inflammation (Bhat et al., 2014; Li et al., 2020). IL-1β is involved in many stages of malignant processes such as initiation and promotion of carcinogenesis; tumor development, metastasis and invasion and is correlated with tumor progression in patients with NSCLC in many studies (Tekpli et al., 2013; Li et al., 2020; Ding et al., 2021). Petrella et al., (2012) noted that IL-1β is an important pro-invasive factor in NSCLCs. Kim et al., (2013) emphasized that IL-1β is an important prognostic marker for patients with advanced NSCLC. Bhat et al., (2014) determined that the polymorphism in the Il-1β gene was significantly associated with an increased risk of NSCLC. In a similar study, Ding et al. (2020) reported that IL-1β was associated with poor long-term prognosis in early lung adenocarcinoma patients. In another study, Li et al. (2020) found that both the incidence of lung cancer and mortality rates were significantly reduced by inhibition of IL-1β. There are very few studies evaluating various interleukin levels in OPA, which is an important chronic respiratory disease of sheep (Larruskain et al., 2012; Larruskain et al., 2015; Karagianni et al., 2019). As a result of their RNA-Seq analysis, Karagianni et al. (2019) revealed that IL-1β expression did not change significantly between experimentally infected sheep and normal sheep. In the current study, it was determined that IL-1β expression in naturally infected sheep with JSRV was statistically increased compared to healthy control group sheep. IL-1β immunoreactivity was higher in the Jaagsiekte group. It was interpreted that IL-1β, which increased in correlation with the severity of inflammation similar to the literature data in human medicine (Petrella et al., 2012; Kim et al., 2013; Tekpli et al., 2013; Bhat et al., 2014; Li et al. 2020; Ding et al., 2021), may contribute to the progression of Jaagsiekte on the basis of chronic inflammation.

The high serum concentration of IL-6, an important tumor-enhancing cytokine, is associated with tumor stage, size, metastasis, and poor survival in many types of human cancer, including NSCLC (Islas-Vazquez et al., 2020; Ke et al., 2020; Su et al., 2020). In veterinary medicine, there is only one study evaluating IL-6 levels in OPA, and in that study, IL-6 was upregulated in the experimentally infected group compared to the control group (Karagianni et al., 2019). Islas-Vazquez et al. (2020) found that human lung cancers had very high levels of IL-6 compared to healthy subjects. They reported that the level of this cytokine decreased in the group with a high overall survival rate after treatment. Dutkowska et al., (2021) observed increased expression of IL-6 in both tumoral and tumor-adjacent tissue of patients with NSCLC. They interpreted this increase can promote inflammatory processes in lung carcinogenesis. Pan et al., (2020) demonstrated that IL-6 increased the epithelial-mesenchymal transition, which plays a vital role in tumor invasion in lung adenocarcinoma cells. Su et al., (2020) suggested that IL-6 is an important therapeutic target for NSCLC metastasis and a highly promising prognostic marker for the disease. Kiss et al. (2020) reported that IL-6 increased cellular migration and proliferation in lung adenocarcinomas. In the current study, IL-6 expression was significantly increased in the OPA group compared to the control group, as

previously reported by Karagianni et al. (2019). Parallel to the IL-1β results, IL-6 immunopositivity was higher in the Jaagsiekte group. This suggested that, similar to the results of different researchers in human medicine (Islas-Vazquez et al., 2020; Ke et al., 2020; Su et al., 2020; Dutkowska et al., 2021), IL-6 may be a remarkable marker in lung cancers of animals, especially Jaagsiekte, as it is in humans. The results obtained from this study led to the conclusion that the inflammatory process in the tumor microenvironment may play a role in the development of Jaagsiekte.

IL-10, an important anti-inflammatory cytokine, regulates autoimmunity, cellular proliferation, survival, apoptosis and angiogenesis (Vahl et al., 2017). IL-10 is mostly produced by tumor-associated macrophages (TAMs, M2 macrophages) in the tumor microenvironment, as well as by CD4, Tregs or CD8 T cells and even tumor cells themselves (Pang et al., 2017). In particular, TAMs increase tumor growth and angiogenesis by releasing various cytokines and promote tumor invasion and metastasis by degrading the extracellular matrix (ECM) (Yang et al., 2019; Hu et al., 2020). The clinical effect of IL-10 in cancers is not fully understood (Hsu et al., 2016). Patients with NSCLC expressing high levels of IL-10 have a poor prognosis. Conversely, high levels of IL-10 produced by tumor-infiltrating CD8+ cells indicate a favorable prognosis (Gao et al., 2020). Larruskain et al. (2015) determined that there was a polymorphism in the IL-10 gene in sheep with OPA. Parallel to IL-1β results, Karagianni et al. (2019) revealed that IL-10 expression did not change significantly between experimentally induced OPA infection sheep and normal sheep. Similar to previously reported (Pang et al., 2017; Vahl et al., 2017), IL-10 expressions in this study were mostly observed in tumor-associated macrophages and tumor cells themselves. Contrary to Karagianni et al. (2019), in the current study, IL-10 expression was significantly increased in the Jaagsiekte group compared to the control group, the increase was particularly much higher in areas with less inflammatory reaction. These findings also supported the anti-inflammatory properties of IL-10 (Vahl et al., 2017).

IL-12 is mostly produced by monocytes, macrophages, and other antigen-presenting cells, and it activates NK cells by targeting them (Arango Duque and Descoteaux, 2014; Turner et al., 2014). IL-12, which is an important regulator of the immune response, has various anti-tumoral effects (Airoldi et al., 2009; D'Amico et al., 2012). IL-12 is a useful predictive and prognostic marker for patients with lung adenocarcinoma (Bugalho et al., 2016). Larruskain et al. (2012) found that the IL-12 microsatellite was the least polymorphic with 7 alleles in OPA. In another study, Larruskain et al. (2015) reported that, together with IL-2 and IL-4, IL-10 is one of the important interleukin genes involved in OPA. Similar to the results of IL-1β and IL-6 immunoreactivities in this study, IL-12β showed a significant increase in the OPA groups compared to the control group, in parallel with the severity of the inflammation.

CONCLUSION

In conclusion, it was determined that various interleukins were produced from tumoral cells and tumor microenvironment elements, especially TAMs, and these interleukins showed pro-inflammatory effects, except for IL-10. More detailed analyzes are essential to determine whether these interleukins have a direct effect on the progression of Jaagsiekte.

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