Ultrastructural study of naturally occuring ovine pulmonary adenocarcinoma in Fars province, Iran

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Summary

Ovine pulmonary adenocarcinoma (OPA) was studied in the lungs of 15 naturally-affected sheep (9 with classical and 6 atypical lesions) by transmission electron microscopy. Two lung samples from normal sheep were used to develop the ultrastructural criteria. Pathologic lesions consisted of focal-multifocal to coalescent nodules or masses in the cranioventral or diaphragmatic lobes. Ultrastructural characteristics of tumor cells in the alveoli and bronchioles allowed categorization of these cells into three groups; neoplastic alveolar type (AT) Π cells in the alveoli contained numerous cytoplasmic lamellar bodies, well developed rough endoplasmic reticulum and glycogen particles; neoplastic clara cells in the bronchioles contained apical electron-dense granules and well-developed smooth endoplasmic reticulum; undifferentiated tumor cells in the alveoli and bronchioles lacked characteristic lamellar bodies or electron-dense granules. Neither complete virions nor viral inclusions were seen in the neoplastic cells. In the present study, there were no ultrastructural differences in the neoplastic cells between classical and atypical forms. It seems that type II pneumocytes and clara cells are the origin of the neoplasia in the alveoli and bronchioles, respectively.

Key words: Ovine pulmonary adenocarcinoma, Atypical, Classical, Ultrastructure

Introduction

Ovine pulmonary adenocarcinoma (OPA) also known as jaagsiekte and previously known as sheep pulmonary adenomatosis and ovine pulmonary carcinoma, is a naturally occurring transmissible contagious pulmonary neoplasm of sheep (DeMartini et al., 1988; Palmarini and Fan, 2001; Fan, 2003; Griffiths et al., 2010). Ovine pulmonary adenocarcinoma occurs in domestic and wild sheep species and affects no other livestock except goats, in which natural cases have been described only in subclinically affected animals (De las Heras et al., 2003). It is caused by a betaretrovirus, jaagsiekte sheep retrovirus (JRSV), which induces oncogenic transformation of alveolar and bronchiolar secretory epithelial cells. The mechanism of oncogenesis of JSRV is still not completely understood (Palmarini et al., 2004; Maeda et al., 2008; Griffiths et al., 2010). Recently, studies in mice and sheep have confirmed

that JSRV Env glycoprotein functions as a viral oncoprotein to stimulate cellular transformation directly, and alone is able to induce lung tumours in vivo (Wootton et al., 2005; Caporale et al., 2006; Chitra et al., 2009).

In addition to its importance as a veterinary problem and providing a new model for fundamental studies on cancer, OPA has a similar pathological and epidemiological feature to bronchiolo-alveolar carcinoma (BAC) in human beings (Palmarini and Fan, 2001; Mornex et al., 2003).

Classical form of OPA has been reported in many sheep-rearing countries including Iran. Fars province of Iran is considered as one of the major sheep-producing areas and this disease occurs in adult sheep with an annual incidence of almost up to 2 percent (unpublished data). In addition to the classical form, an atypical form of OPA has been reported only in Spain, Peru and Iran (De las Heras et al., 1992, 2003; Garcia-Goti

et al., 2000; Khodakaram-Tafti and Razavi, 2010) and there are no reports of its occurrence in other countries where OPA is well recognised, such as the United Kingdom and South Africa (Griffiths *et al*., 2010). To date, there is no report about the ultrastuctural study of these two pathologic forms of OPA in sheep. Therefore, this study was undertaken to obtain further information concerning the ultrastructural features of atypical and classical pulmonary lesions of naturally infected sheep with OPA in Fars province, Iran.

Materials and Methods

Lungs from 9400 slaughtered native breed sheep nearly between 7-months to 6-year-old were examined grossly in Fars province, Iran. Out of these, 21 lungs (12 lungs with classical and 9 lungs with atypical form) were diagnosed histopathologically as ovine pulmonary adenocarcinoma (Khodakaram-Tafti and Razavi, 2010). From these, 15 naturally affected sheep (9 lungs with classical type and 6 lungs with atypical type lesions) were selected randomly and ultrastructural characteristics were studied by transmission electron microscopy. Two lung samples from normal sheep were used to develop the ultrastructural criteria. A part of the selected samples were fixed in 4% glutaraldehyde solution (pH = 7.3) concurrently with fixation the same as samples in the 10% neutral-buffered formalin for histo-pathological examination. Then, the tissues were washed in buffer phosphate and cut into pieces less than 1 mm. After this, the tissues were post-fixed with 2% osmium tetroxide, washed briefly in distilled water, dehydrated through graded ethanol and then cleared in propylene oxide and embedded in agar resin. At least three semi-thin sections of 0.5-1 μm thickness from each tumor were obtained using ultramicrotome (Reichert-Jung, Ultracut Austria equipped with glass knives), stained with toluidine blue and were first examined by light microscope to select areas for ultrastructural examination. Ultrathin sections of 60-nm thickness were also cut and collected on copper grids. These sections were stained in aqueous uranyl acetate and lead citrate and were examined with a Philips CM-10 transmission electron microscope.

Results

At gross examination, the lungs of the affected sheep had one or more grayish white firm small nodules to large masses in the cranio-ventral or caudal lobes of the organ. In 9 cases, classical or typical lesions were observed, with solitary or multiple firm and wet masses, usually in the cranioventral portion of the lungs. The cut surface was moist with frothy fluid exuding from the bronchioles of the affected areas. In 6 cases, atypical lesions of OPA were observed with relatively well circumscribed, small hard and dry nodules, usually localized in the diaphragmatic lobe. Microscopically, the lesions in both types were essentially the same, and three growth patterns of well differentiated tumor cells of the alveolar wall including acinar or glandular, papillary (Fig. 1) and solid, and also papillary proliferation of bronchiolar epithelium that filled the lumen were diagnosed (Fig. 2). In some sections desmoplasia associated with the infiltration of mononuclear inflammatory cells in the interstitial tissue of the affected areas and alveoli full of macrophages with vacuolated cytoplasm near to neoplastic foci were seen. Neoplastic cells were well differentiated and organized as a single layer on the basement membrane. These cells were mostly cuboidal with vacuolated eosinophilic cytoplasm. Nuclei were round and located in the basilar portion of the cells (Fig. 3). Mitotic index was not remarkable. Ultrastructural characteristics of tumor cells in both classical and atypical lesions were similar and showed three groups of cells including type Π pneumocytes (ATII), clara cells and undifferentiated cells (Table 1). Neoplastic type II pneumocytes contained numerous cytoplasmic lamellar bodies, prominent well developed rough endoplasmic reticulum and glycogen particles. They had scanty stunted microvilli on the luminal surface, desmosomes and tight junctions between adjacent cells and rested on an intact basement membrane. The nuclei of these cells were oval to round with

a prominent nucleolus and were often deeply indented and the heterochromatin was dispersed in peripheral and central clumps (Fig. 4). Lamellar bodies or secretary surfactants had different sizes, variable shapes and patterns and were irregularly distributed throughout the cytoplasm mostly in the apical portion. Their number appeared to be increased in the neoplastic cells as compared with normal type II cells. Most of these bodies contained concentric or parallel lamellae (lamellated pattern) (Fig. 5) and some were as osmiophilic granular materials with a finger print pattern (Fig. 6). In

Table 1: Ultrastructural characteristics of neoplastic cells in OPA lesions

Feature	ATII cell	Clara cell	UD
Rough endoplasmic reticulum	+++	-	+
Smooth endoplasmic reticulum	-	+++	+
Microvilli	+/-	+/-	-
Desmosomes and tight junctions	++	+	-
Lamellar bodies	+++	-	-
Electron dense granules	-	+++	-
Glycogen granules	++	-	+

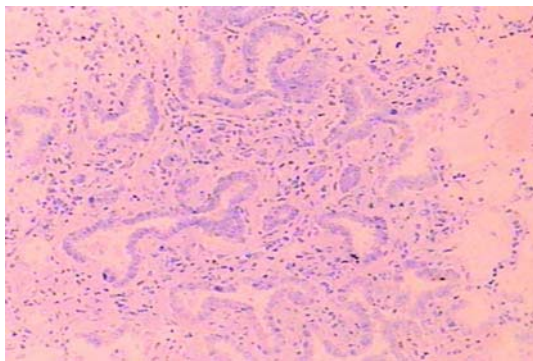UD: Undifferentiated cell, - = absent, +/- = occasional, + = few, ++ = moderate, and +++ = abundant

Fig. 1: Acinar and papillary proliferations of neoplastic cells of pulmonary alveoli, (Toluidine blue, ×160)
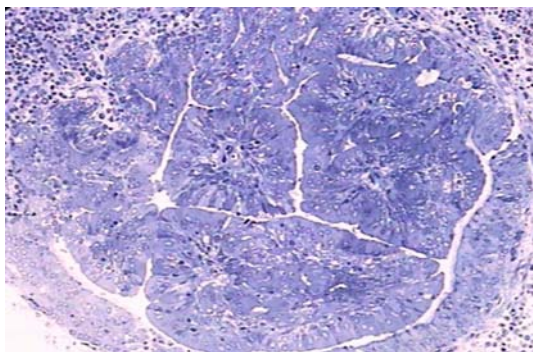

Fig. 2: OPA. Severe papillary proliferation of bronchiolar epithelium that filled the lumen, (Toluidine blue, ×160)

affected bronchioles, clara cells were most abundant and protrude into the bronchiolar lumen. Neoplastic clara cells were columnar and contained electron-dense apical granules and abundant well-developed smooth endoplasmic reticulum (Fig. 7). The nuclei
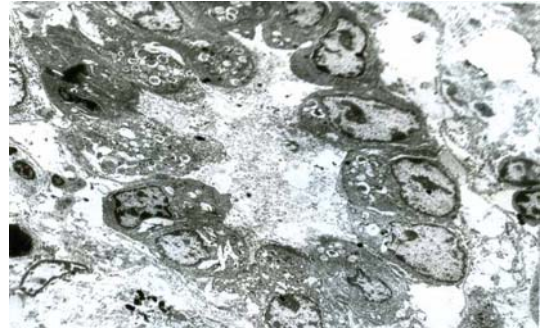

Fig. 3: Neoplastic ATII cells with numerous intracelluar lamellar bodies lining the alveolar wall. Uranyl acetate and lead citrate, (×2200)

Fig. 4: The nucleus of a neoplastic ATII cell shows irregular indentations and the heterochromatin is dispersed in peripheral and central clumps. Uranyl acetate and lead citrate, (×8900)
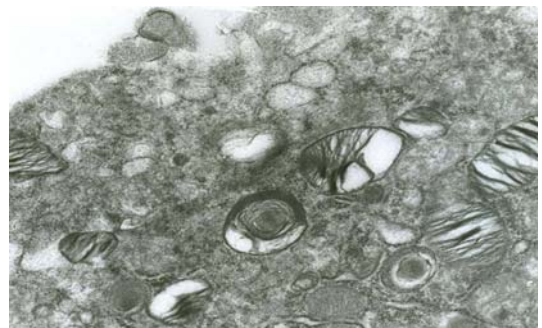

Fig. 5: Apical part of a neoplastic ATII cell. Intracytoplasmic lamellar bodies contained concentric or parallel lamellae (lamellated pattern). Uranyl acetate and lead citrate, (×21000)

Fig. 6: Apical part of a neoplastic ATII cell. Intracytoplasmic secretary surfactant bodies contained variable sized osmiophilic granular materials with a finger print pattern. Uranyl acetate and lead citrate, (×11500)

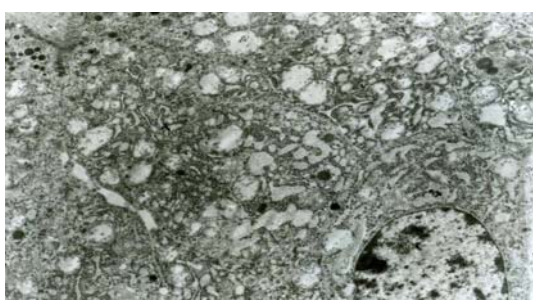**Fig. 7: Neoplastic clara cell with abundant SER and electron dense apical granules. Uranyl acetate and lead citrate, (×5200)**
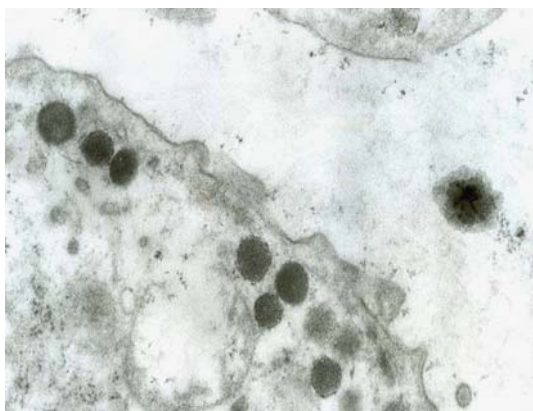

Fig. 8: Upical intracytoplasmic electron dense granules in a neoplastic clara cell. Uranyl acetate and lead citrate, (×28500)

were round, centerally or eccentric situated and usually without prominent indentations. The apical cytoplasm of most cells contained variable numbers of round osmiophilic electron dense bodies (Fig. 8). Undifferentiated tumor cells lacked characteristic lamellar bodies or electron-dense granules in the affected alveoli and bronchioles. Neither complete virions nor viral inclusions were seen in the neoplastic cells.

Discussion

A definitive diagnosis of OPA in an individual animal always requires the identification of the characteristic gross and histopathological findings by post mortem examination. Electron microscopy has been used to document the early growth of the tumor, to identify ultrastructural characteristics of neoplastic cells and to support the histological findings.

In typical cases, infection with JSRV is clinically silent until the tumor is sufficiently advanced to compromise respiration or to cause the animal to lose condition. Therefore, in many infected animals OPA lesions never develop within a normal or commercial lifespan (Salvatori *et al*., 2004; Caporale *et al*., 2005). Importation of such apparently healthy animals may facilitate the inadvertent introduction of the disease into naïve flocks and to new countries (Griffiths *et al*., 2010). The absence of JSRV-specific antibodies in infected sheep has precluded the use of serological tests for infection and greatly hindered the development of diagnostic assays (Summers *et al*., 2002). The JSRV was detected by RT-PCR in all 10 lungs from OPA affected sheep and in 40% of lungs from in contact-sheep without gross or microscopic lesions (Khodakaram-Tafti *et al*., 2009). PCR and RT-PCR tests on blood samples are informative for epidemiological studies and for identifying infected flocks. The most successful method for identifying early OPA has been PCR testing of bronchoalveolar lavage samples collected from live animals (Voigt *et al*., 2007). PCR and RT-PCR on saliva and nasal secretions were negative for JSRV (Griffiths *et al*., 2010).

Two pathologic forms of OPA are described, classical and atypical. In the present study, the morphologic characteristics of these two forms are compatible with those reported by other researchers (De las Heras *et al*., 1992; Garcia-Goti *et al*., 2000; De las Heras *et al*., 2003).

Unlike some other studies, metastasis to regional lymph nodes was not observed here (Cutlip and Young, 1982; Hunter and Munro, 1983; Verwoerd et al., 1985), but resembled those reported by Verwoerd (1990) and Bouljihad et al. (1996). Distant metastases have been reported in the liver, kidney, heart and skeletal muscle but are generally rare (Hunter and Munro, 1983). Also, the renal and cardiac metastases of jaagsiekte-like tumor in a goat has been reported (Al-Dubaib, 2005).

Ultrastructurally, the neoplastic cells of OPA lesions have been studied previously in experimentally-induced or naturally infected cases (Payne and Verwoerd, 1984; Angus et al., 1985; DeMartini et al., 1988; Sharp and Angus, 1990; Bouljihad et al., 1996), but there is no report about comparative ultrastructure changes between classical and atypical forms. In the present study, ultrastructural characteristics of tumor cells in the affected alveoli and bronchioles allowed categorization of these cells into three groups; neoplastic alveolar type (AT) II cells in the alveoli, neoplastic clara cells in the bronchioles and undifferentiated neoplastic cells in the alveoli and bronchioles. Similarly, Platt et al. (2002) described a heterogenous population of tumour cells in most cases, and suggested that such heterogenecity may be consistent with the simultaneous virus-induced transformation of multiple cell types, or a retrograde, trans- or continued differentiation of type II pneumocytes or clara cells following neoplastic transformation. In their study, ultrastructural features of 82, 7 and 11% of tumor cells were consistent with type 2 pneumocytes, clara cells and undifferentiated cells, respectively.

These researchers compared the neoplastic lesions in naturally occurring and experimentally induced cases and found a few differences, including the earlier onset of clinical disease and a large number of small widely disseminated tumor nodules in experimentally inoculated animals. They have concluded that these differences may be related to the age of onset of lesions or delay in diagnosis in the natural disease. There was no difference in the proportion of specific cell types found in naturally and experimentally induced tumors. In the present study, ultrastructural characteristics of tumor cells were in agreement with the Platt et al. (2002) results. Neoplastic clara cells in the alveoli and neoplastic AT II cells in the bronchioles were not observed in the present study. Undifferentiated tumor cells lacked characteristic lamellar bodies or electron-dense granules, but in the alveoli ultrastuctural features of these cells indicate they may be immature type II pneumocytes and in the bronchioles, as immature clara cells. In agreement with findings of other workers, neither complete virions nor viral inclusions were seen in the neoplastic cells of the two forms of OPA in the present study (Cutlip and Young, 1982; Bouljihad et al., 1996; Garcia-Goti et al., 2000). On the basis of the results of this study, it seems that type II pneumocytes and clara cells are the origin of the neoplasia in the alveoli and bronchioles, respectively. In the present study, there were no ultrastructural differences in neoplastic cells between the classical and atypical forms. Garcia-Goti et al. (2000) by immunohistochemical study of OPA lesions, has found fewer JSRV-positive cells in atypical tumours than in the classical form. It seems the atypical form is an early or limited feature of the tumour and occurs only as a subclinical finding in abbatoir studies or when the animal is autopsied for unrelated reasons. An important question in understanding the pathogenesis of OPA is the identification of the target cells within the lung that is initially infected and transformed. The presence of undifferentiated cells suggest that a progenitor cell, possibly a stem cell, may be the actual target for transformation (Wootton et al., 2006). Several groups have described candidate stem cells in the lung as clara cells, type II pneumocytes, brochioloalveolar stem cells (BASCs) and additional epithelial cell types in the bronchiolar epithelium (Kim et al., 2005; Teisanu et al., 2009). Developing a transgenic mouse that expresses a B-galactosidase receptor gene under the control of the JSRV promoter exhibited B-galactosidase expression only in type II pneumocytes, but not in BASCs. This suggests that BASCs may not be a target for JSRV (Dakessian and Fan, 2008). However,

further investigation for determination of the target cell or cells that are initially infected and transformed in the ovine lung is warranted.

Acknowledgements

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