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Original Article

Immunohistochemical Characteristics of Surfactant Proteins A, B, C and D in Inflammatory and Tumorigenic Lung Lesions of F344 Rats

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Abstract: Surfactant proteins (SPs), originally known as human lung surfactants, are essential to respiratory structure and function. There are 4 subtypes, SP-A, SP-B, SP-C and SP-D, with SP-A and SP-D having immunological functions, and SP-B and SP-C having physicochemical properties that reduce the surface tension at biological interfaces. In this experiment, the expressions of SP-A, SP-B, SP-C and SP-D in lung neoplastic lesions induced by N-bis (2-hydroxypropyl) nitrosamine (DHPN) and inflammatory lesions due to quartz instillation were examined and compared immunohistochemically. Formalin fixed paraffin embedded (FFPE) lung samples featuring inflammation were obtained with a rat quartz instillation model, and neoplastic lesions, hyperplasias and adenomas, were obtained with the rat DHPN-induced lung carcinogenesis model. In the rat quartz instillation model, male 10-week old F344 rats were exposed by intratracheal instillation (IT) to quartz at a dose of 2 mg/rat suspended in saline (0.2 ml) on day 0, and sacrificed on day 28. Lung tumorigenesis in F344 male rats was initiated by DHPN in drinking water for 2 weeks, and the animals were then sacrificed in week 30. Lung proliferative lesions, hyperplasias and adenomas, were observed with DHPN, and inflammation was observed with quartz. The expressions of SP-A, SP-B, SP-C and SP-D were examined immunohistochemically. SP-B and SP-C showed strong expression in lung hyperplasias and adenomas, while SP-A and SP-D were observed in mucus or exudates in inflammatory alveoli. These results suggest the possibility that SP-B and SP-C are related to lung tumorigenesis. (DOI: 10.1293/tox.2014–0020; J Toxicol Pathol 2014; 27: 175–182)

Key words: urfactant protein, DHPN, quartz, rat, lung, tumor

Introduction

Surfactant proteins (SPs), originally known as human lung surfactants, are essential to proper respiratory structure and function. They are unique in composition, consisting of about 90% lipids, mostly phospholipids, and 8–10% surfactant-associated proteins[1]. SPs line the alveolar surface, and reduce surface tension at the air–fluid interface[2]. Pulmonary surfactant is stored mainly in type II alveolar epithelial cells in the form of densely packed bilayers called lamellar bodies that are secreted and efficiently transferred to the interface[1, 2]. There are 4 subtypes, SP-A, SP-B, SP-C and SP-D, the characteristics of which are summarized in table 1; the table contents were obtained from the literature and modified for use here[1, 3, 4]. There are many reports regarding the role of SPs in lung inflammation or chronic obstructive pulmonary disease (COPD). However, the expression of SP-A, SP-B, SP-C or SP–D in lung tumors has received only limited attention.

Previously, in order to establish an appropriate bioassay for detection of promoting potential for lung tumor development after intratracheal instillation (IT) of fine particles in rats, sequential histopathological changes up to 30 weeks were examined after initiation of lung tumorigenesis with 0.1% N-bis (2-hydroxypropyl) nitrosamine (DHPN) in drinking water for 2 weeks in male F344 rats[5–7]. It has been reported that activating mutations of the *Kras* gene at codon 12 are detected in almost 50% of rat lung neoplastic lesions induced by a carcinogen[8]. Furthermore, we previously examined the lung toxicity of fine particles of various materials in an *in vivo* bioassay using an IT approach[5, 7, 9]. In humans, coal miners and building construction workers who are exposed to quartz dust demonstrate obstructive and restrictive loss of lung capacity[10], as well as COPD[11, 12]. This is associated with an inflammatory cell responses characterized by alveolitis with recruitment of inflammatory cells, particularly neutrophils, and may result in pulmonary fibrosis and impaired lung function[13]. IT of quartz into rats produces inflammatory reactions followed by his-

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Table 1. Characteristics of SP-A, SP-B, SP-C and SP-D

	SP-A	SP-B	SP-C	SP-D
Synthesis	Hydrophilicity Type 2 cell Clara cell	Hydrophobicity Type 2 cell Clara cell	Hydrophobicity Type 2 cell	Hydrophilicity Type 2 cell Clara cell
Reducing surface tension	−	+	+	−
Innate immune defence	+	−	−	+

Table 2. Details of Antibody and Immunostaining

	SP-A	SP-B	SP-C	SP-D
Antibody	H-148 sc-13977	bs-1034R	FL-197 sc-13979	5F4
Company	Santa Cruz Biotechnology Santa Cruz, CA, USA	Bioss Woburn, MA, USA	Santa Cruz Biotechnology Santa Cruz, CA, USA	Yamasa Tokyo, Japan
Reactivity	Rat, mouse, human	Rat, mouse, human	Rat, mouse, human	Rat, mouse
Antigen retrieval				
Solution	RiboCC	CC1	RiboCC	Target Retrieval Solution
Duration	30 min Ventana Discovery™	30 min Ventana Discovery™	30 min Ventana Discovery™	10 min Microwave
Primary antibody				
Dilution	50×	100×	50×	4000×
Incubation	30 min	Overnight	30 min	60 min

tological changes characteristic of lung fibrosis[14], similar to human conditions. With a dose of 2 mg/rat IT, fine particles of quartz caused toxicity with severe inflammatory changes characterized by neutrophil infiltration and edema after 28 days[6]. In the present study, the expressions of SP-A, SP-B, SP-C and SP-D in lung neoplastic lesions induced by DHPN and in inflammatory lesions caused by quartz were examined and compared immunohistochemically.

Materials and Methods

Animals

Experimental animals were maintained in the Division of Animal Experiments, Life Science Research Center, Kagawa University, according to the Institutional Regulations for Animal Experiments. All were housed in polycarbonate cages with white wood chips for bedding and given free access to drinking water and a basal diet, CE-2 (CLEA Japan Inc., Tokyo, Japan), under controlled conditions of humidity (60 ± 10%), lighting (12-h light/dark cycle) and temperature (24 ± 2°C).

Tissue Samples

Formalin fixed paraffin embedded (FFPE) lung samples featuring inflammation were obtained with the rat quartz instillation model on day 28[9] and FFPE lung samples including neoplastic lesions, hyperplasias and adenomas, were obtained with the rat DHPN-induced lung carcinogenesis model[15]. Briefly, in the rat quartz instillation model, male 10-week old F344/DuCrlCrj rats (Charles River Laboratories Japan, Inc., Kanagawa, Japan) were exposed by IT to quartz with a particle diameter of less than 7 μm (DQ-12, DMT GmbH & Co. KG, Germany) at a dose of 2 mg/rat suspended in saline (0.2 ml) (Isotonic sodium chloride solution, Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) on day 0 and sacrificed on day 28[9]. For the rat DHPN induced lung carcinogenesis model, male 6-week old F344/DuCrlCrlj rats were given 0.1% DHPN (Nacalai Tesque Inc., Kyoto, Japan) in drinking water for 2 weeks and sacrificed in week 30.

At autopsy in both experiments, the lungs were removed, including the trachea and heart, infused through the trachea with 10% phosphate buffered formalin, rinsed in the same fixative and then immersed in the fixative for approximately 48 h. Slices were then routinely processed for embedding in paraffin for histopathological examination.

Histopathological analysis

Lung lesions from the rat DHPN-induced lung carcinogenesis model were categorized as bronchioloalveolar hyperplasia (hyperplasia) and bronchioloalveolar adenoma (adenoma) in accordance with the established criteria given in the International Harmonization of Nomenclature and Diagnostic Criteria (INHAND)[16]. In detail, bronchioloalveolar hyperplasia was diagnosed from the finding of solitary or multiple, segmental (cone-shaped) foci of increased cellularity, a lack of strongly convex or spherical borders, the bronchioloalveolar architecture still being detectable and epithelial cells, usually a single layer, still being dominant and the cause of hypercellularity. Bronchioloalveolar adenomas were frequently located at the lung periphery and usually small in size (less than 3–4 mm in diameter), featuring well-circumscribed areas of high epithelial cell density, usually with a strongly convex border, an underlying alveo-

lar architecture obscured to various degrees, sharp demarcation from the surrounding tissue and relatively uniform neoplastic epithelial cells with mitotic figures rare or absent. Small foci of mild atypia were sometimes present, and occasionally extension into adjacent bronchioles was evident.

For SP-A, SP-B and SP-C, all immunohistochemical staining processes from deparaffinization to counterstaining with hematoxylin were performed automatically using the Ventana Discovery™ staining system (Ventana Medical Systems, Tucson, AZ, USA). For SP-D, immunostaining was performed by hand with an EnVision™ kit (DAKO, Tokyo, Japan). Antigen retrieval was performed with 3 methods using RiboCC solution (lot. D02647, Ventana Medical Systems, Tucson, AZ, USA) for SP-A and SP-C, CC1 solution (lot. D06855, Ventana Medical Systems, Tucson, AZ, USA) for SP-B and Target Retrieval Solution (DAKO, Tokyo, Japan) for SP-D. The details of the SP-A, SP-B, SP-C and SP–D antibodies used in the experiments are summarized in Table 2. The expressions for each marker, SP-A, SP-B, SP-C and SP-D, were evaluated as none (–), weak (+) and strong (++) compared with the histopathological normal area without inflammation or a proliferative lesion in the same specimens.

Results

Inflammatory lesions were observed in the lungs with the rat quartz instillation models on day 28. The main findings were neutrophil infiltration in the walls and spaces of the alveoli, pulmonary edema, pulmonary fibrosis, alveolar macrophage infiltration and restructuring of the alveolar walls and granulation-like changes with giant cells and macrophages in the alveoli. For evaluation of the expressions of the SPs, the histopathological normal area of the lungs treated with quartz was used as a control (see Figure 1-D, E, F, I and J). The lesions were observed to be strongly positive for SP-A in the mucus in alveoli (see Figure 1-B), weakly positive for SP-B in the mucus and strongly positive for SP-B in the alveolar epithelial cells and bronchial epithelial cells (see Figure 1-C), weakly positive for SP-C in the mucus and strongly positive for SP-C in the alveolar epithelial cells (see Figure 1-G), and strongly positive for SP-D in the mucus and partially positive for SP-D in the alveolar epithelial cells (see Figure 1-H). The bronchiolar epithelial cells after treatment with quartz were strongly positive (++) for SP-B and weakly positive (+) for SP-A, SP-C and SP-D (see Figure 2). DHPN-induced proliferative lesions, hyperplasias and adenomas, in F344 rat lungs were weakly positive (+) for SP-A (see Figure 3-B), strongly (++) positive for SP-B (see Figure 3-C), strongly positive (++) for SP-D (see Figure 3-G), and almost negative (–) for SP-D, although the normal alveolar epithelium was also positive (see Figure 3-H). For evaluation of the expressions the SPs, the histopathological normal area of the lungs treated with DHPN was used as a control (see Figure 3-D, E, F, I and J). The findings for the expressions in the control areas were similar (Table 3).

The immunohistochemical results are summarized in Table 3. The expressions of SP-A and SP-D were strongly positive in the mucus in the alveoli with inflammatory changes. SP-B and SP-C showed strong positivity in the proliferative lesions, hyperplasias and adenomas, whereas the expressions of SP-A and SP-D were weak and lacking, respectively.

Discussion

In the present experiment, the expressions of SP-A and SP-D appeared to be similar, with strong positivity in the mucus in alveoli of inflammatory lesions induced by quartz. SP-A and SP-D both play significant roles in surfactant homeostasis and pulmonary immunity and are known to bind to a range of microbial pathogens that invade the lungs and target them for phagocytic clearance by resident alveolar macrophages. They are also involved in the removal of apoptotic and necrotic cells and subsequent resolution of pulmonary inflammation[17, 18] *In vivo* studies have shown that SP-A regulates responses involved in initiation and potentiation of inflammation by regulating the production of proinflammatory cytokines, such as tumor necrosis factor α (TNF-α), in response to lipopolysaccharide (LPS) or by accelerating the clearance of a variety of pathogens[19–21]. Alveolar macrophages are thought to play a critical role in host defense in the lung[22]. *In vitro*, both SP-A and SP-D can stimulate alveolar macrophages to generate oxygen radicals, as measured by chemiluminescence[23, 24]. As hydrophilic surfactant proteins, they participate in the innate immune response by binding to bacterial and viral pathogens and activating alveolar macrophages[2]. The strong expression of SP-A and SP-D in the present experiment suggests activation of macrophages to deal with instilled quartz particles.

Expression of SP-A was weak in the proliferative lesions in the present experiment, and this was noteworthy because SP-A has been used as a serous marker for human lung cancer in clinical examinations in Japan. Lung cancer is the leading cause of cancer-related deaths worldwide in both developing and developed regions[25, 26]. In one study, SP-A was expressed in approximately 49% of primary non–small cell lung carcinomas[27]. It was found to be closely associated with some specific subtypes of adenocarcinoma[28, 29]. Some studies have suggested that SP-A might have a role in suppressing lung cancer progression, and SP-A expression by tumor cells leads to recruitment and activation of NK cells and tumor-associated macrophages[19]. In another study, activating mutations of the *Kras* gene at codon 12 were reported in 47% of lesions induced by DHPN in the F344 rat[8]. Overexpression of oncogenic *Kras* leads to activation of the NF-κB pathway, which appears to be required for the development of tumors in a mouse model of lung adenocarcinoma[30]. SP-A has been shown to inhibit LPS-induced NF-κB, TNF-α production, inducible NO synthase protein expression or activity and, in the presence of diverse stimuli, NADPH oxidase in immunocompetent cells[31]. In the present experiment, only hyperplasias and adenomas, not adenocarcinomas, were observed as neoplastic lesions,

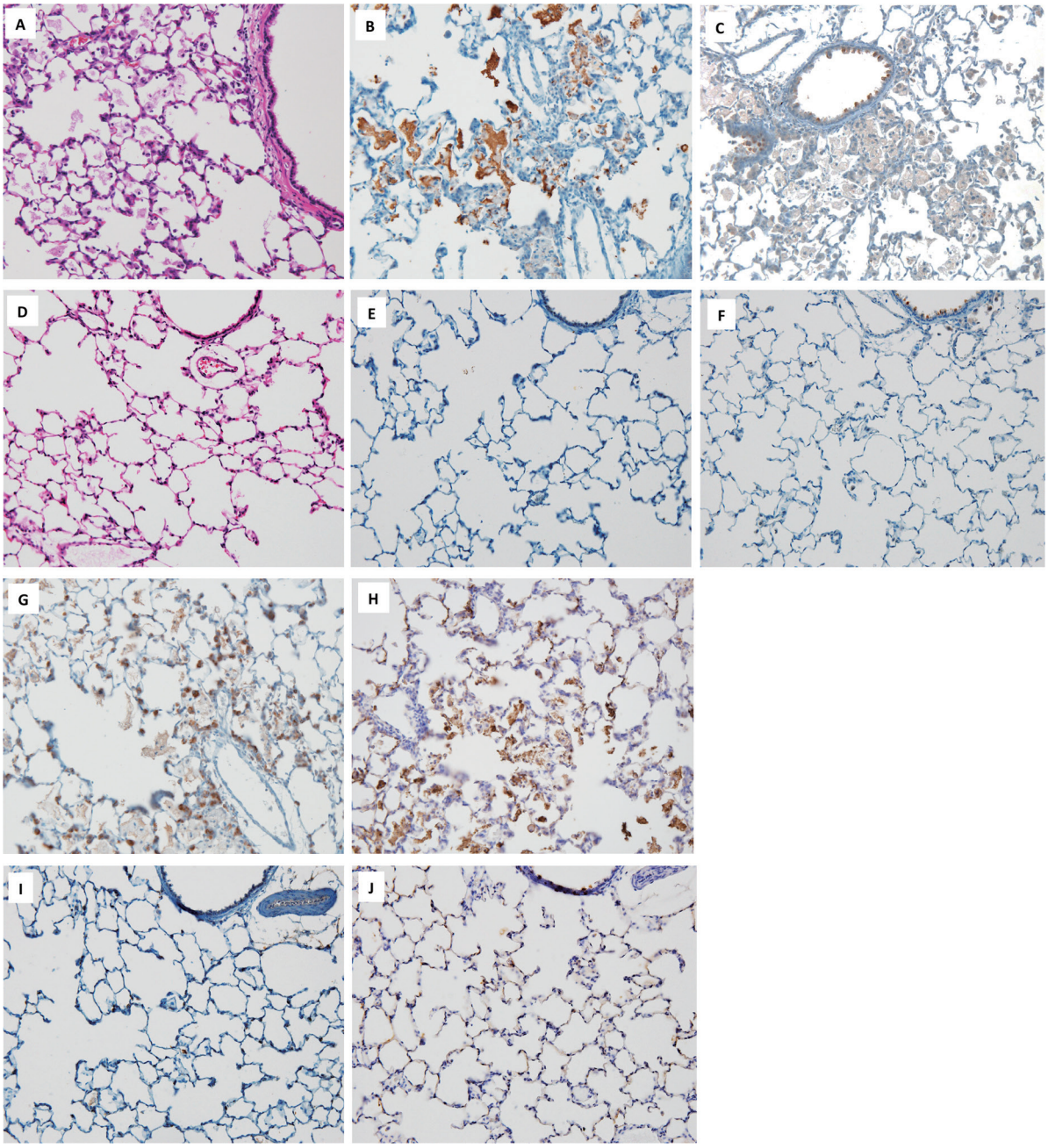

Fig. 1. Histopathological and immunohistochemical findings for inflammatory lesions induced by quartz in the F344 rat lung (×200). A and D, H.E.; B and E, SP-A; C and F, SP-B; G and I, SP-C; H and J, SP-D. A, B, C, G and H, inflammatory lesions; D, E, F, I and J, noninflammatory lesions (control). Note the strong positive reactions in the mucus in the alveoli in Fig. 1-B, strongly positive staining of alveolar epithelial cells and bronchial epithelial cells in Fig. 1-C, alveolar epithelial cells in Fig. 1-G and mucus in the alveoli in Fig. 1-H.

and this suggests that *Kras* mutations and activation of NF-κB might be associated with later stages of malignancy.

SP-C, which is a specific marker of type II epithelial cells in the lung[3], showed the strong positivity in proliferative lesions in the present experiment. SP-B and SP-C have physicochemical properties that reduce the surface tension of biological interfaces[4]. In the lung tumorigenesis model induced by a mixture of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and benzo[*a*]pyrene (BaP) in A/J mice, pulmonary SP-A, SP-B, and SP-C were identified in the mouse lung, but only the SP-C levels increased in carcinogen-treated versus untreated mice[32].

In the present experiment, SP-B was also positive in neoplastic lesions. The main function of SP-B is to accelerate the formation of a surface-active film composed of phospholipids at the air-water interface by increasing the adsorption rate[33, 34]. SP-B has anti-inflammatory properties and may be involved in protecting the lung against oxida-

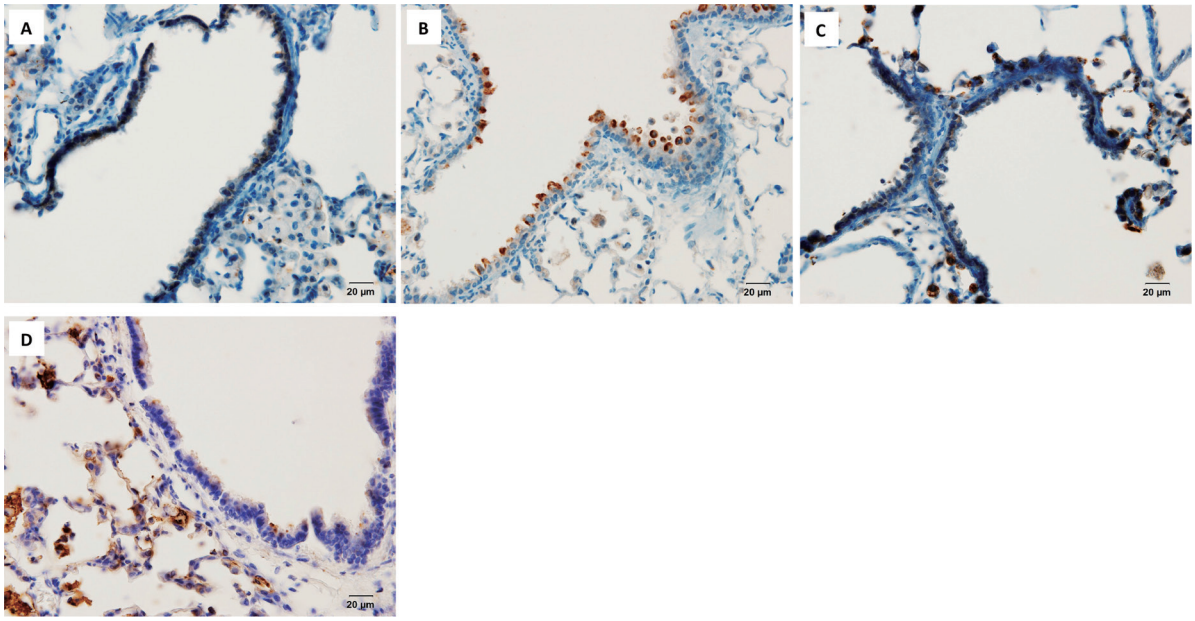

Fig. 2. Immunohistochemical findings for bronchiolar epithelial cells in the F344 rat lung after quartz exposure (×200). A, weak positivity (+) for SP-A; B, strong positivity (++) for SP-B; C, weak positivity (+) for SP-C; D, negative for SP-D.

tive stress[33, 35, 36] and circulating pro–SP-B is reported to be a biomarker for early detection of non–small-cell lung cancer (NSCLC)[37]. On synthesis, pro-SP-B quickly undergoes proteolytic cleavage by cysteine proteases in the endoplasmic reticulum, resulting in the production and secretion of a 9-kD noncollagenous hydrophobic SP-B, which is the functional mature form[38]. Lung tumor cells, especially adenocarcinomas, have dysregulated SP-B synthesis, leading to the overexpression of pro-SP-B with a muted ability to posttranslationally modify the precursor into the mature hydrophobic form[39, 40].

As described in the introduction page, SPs are stored in the lung type II epithelial cells, and in the present experiment, the lung proliferative lesions, hyperplasias and adenomas, were also positive for SPs. This result suggests the lung hyperplasias and adenomas would have their origin in type II alveolar cells.

In our previous experiment, napsin A was determined to be a good marker for detection of hyperplastic lesions linked to actual neoplasia[41]. Napsin A is an aspartic proteinase involved in the maturation of SP-B[42] and is expressed in the cytoplasm of type II pneumocytes and Clara cells in the lung and also in the proximal tubular renal epithelium and exocrine pancreas[43, 44]. Human clinical studies suggest that napsin A may be a highly specific marker for adenocarcinoma in the lung[45, 46]. However, when there is a need to rule out lung metastasis from other organs, for example, from renal, thyroid or endometrial carcinomas, implementation of other biologically specific markers should be considered[45]. In the present experiment, the positive area of SP-B seemed not to correspond completely with that of napsin A.

In conclusion, SP-B and SP-C showed strong expression in lung hyperplasias and adenomas and expression of SP-A and SP-D was observed in mucus or exudates in inflammatory alveoli. These results suggest the possibility that SP-B and SP-C are linked to lung tumorigenesis.

Table 3. Summary of the Expressions of SP-A, SP-B, SP-C and SP-D

	SP-A	SP-B	SP-C	SP-D
Inflammatory lesions				
Mucus in the alveoli	++	+	+	++
Type II alveolar cells	−	++	++	+
Macrophages	++	+	+	++
Proliferative lesions				
Hyperplasia	+	++	++	−
Adenoma	+	++	++	−
Control area*				
Type II alveolar cells	−	+	+	+
Bronchiolar epithelial cells	+	++	+	−

Negative, −; weakly positive, +; strongly positive, ++. * Histopathological normal area of the lungs treated with DHPN or quartz.

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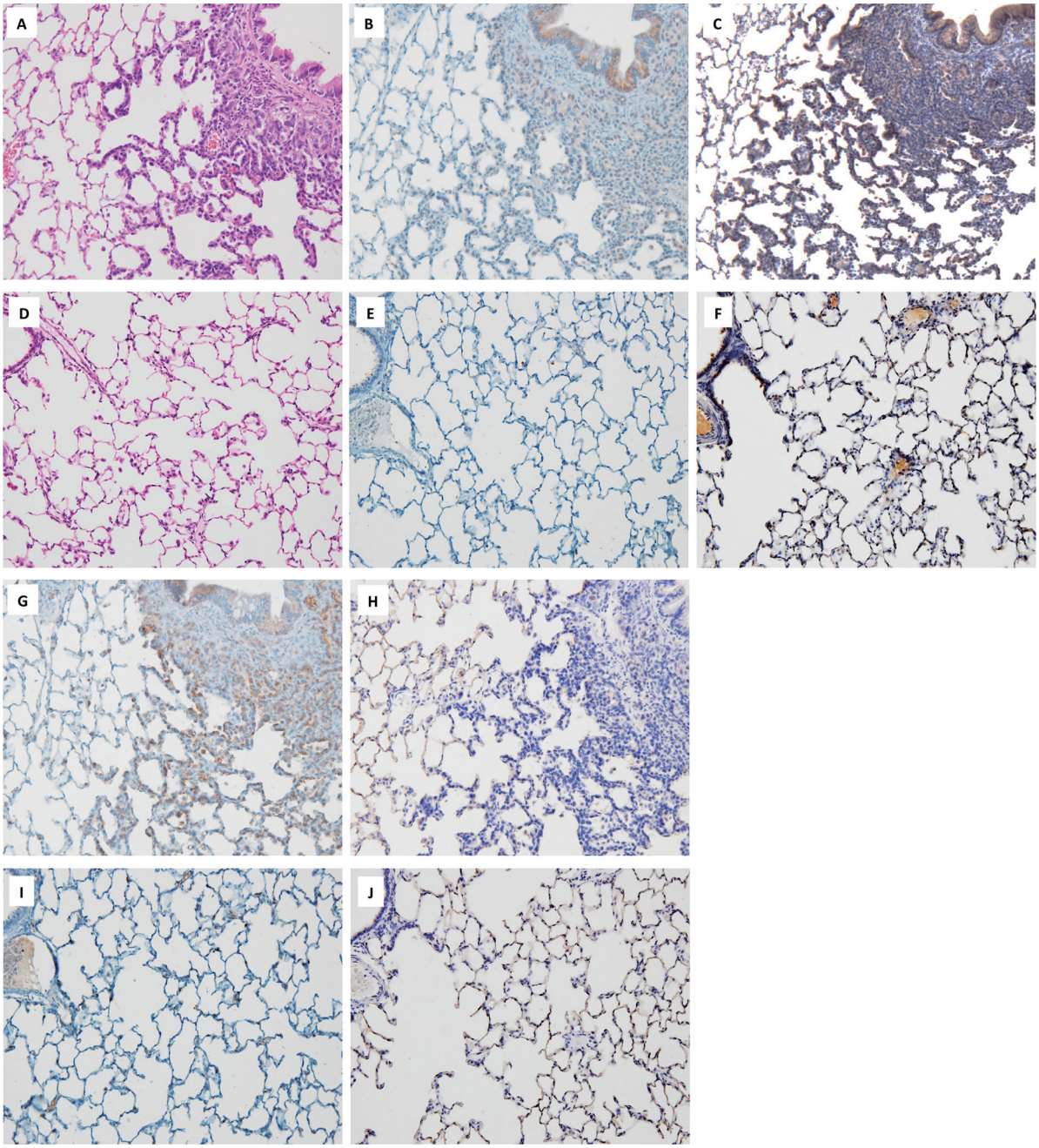

Fig. 3. Histopathological and immunohistochemical findings for DHPN-induced proliferative lesions, hyperplasias and adenomas, in the F344 rat lung (×200). A and D, H.E.; B and E, SP-A; C and F, SP-B; G and I, SP-C; H and J, SP-D. A, B, C, G and H, proliferative lesions; D, E, F, I and J, nonproliferative lesions (control). Note the weak positivity (+) for SP-A in Fig. 1-B, strong positivity (++) for SP-B in Fig. 1-C, strong positivity (++) for SP-D in Fig. 1-G and almost negative (−) staining for SP-D in Fig. 1-H.

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