Expressions of Laminin-1 in Lung Alveolar Septa after CS gas Exposure in Rats

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CS 가스 흡입이 흰쥐의 폐포막내 Laminin-1에 미치는 영향

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배 경 : Laminin은 생체조직의 세포외기질에 함유되어 있는 당단백질로 특히 기저막내에 특이적으로 분포되어 있다. 저자들은 군중집회 해산용으로 혹은 군사용으로 널리 사용되고 있는 CS 가스(ortho-chlorobenzylidine malononitrile)가 폭로되었을 때 흰쥐의 폐조직내 laminin에 미치는 영향을 밝히고자 하였다.

대상 및 방법 : 본 실험에 사용한 실험동물은 청정동물실에서 사육한 생후 21일령의 흰쥐 (Sprague-Dawley계) 35마리를 사용하였다. 실험동물은 대조군에 5마리, 실험군에 30마리를 배정하였다. CS 가스에 폭로 후 흰쥐를 12시간, 1일, 2일, 3일, 5일 및 7일 경과후 희생시켜 폐장을 절취하여 면역조직염색 및 면역도금법을 시행하였다.

결 과 : 상기와 같이 조직면역반응과 염색을 시행한 후 조직을 관찰하여 다음과 같은 결과를 얻었다. CS 가스 투여후 2일 경과군의 흰쥐 폐포막에는 림파구, 단핵구등이 침투되기 시작하여 3일 경과군에서는 강도의 염증조직이 관찰되었다. 대조군과 CS 가스 투여 12시간군의 폐장의 제2형폐포세포내에 금과립은 층상체주위와 외형질에서 소수 관찰되었다.

결 론 : 이상과 같은 결과로 흰쥐에 CS 가스를 계속 3일간 투여했을 경우 폐포에서 염증조직이 형성되고 이때 기저막내 laminin의 분포는 일시 감소되나 염증조직의 소멸과 함께 다시 증가되는 것을 보아 laminin이 조직재형성에의 역할이 흰쥐 폐포에서도 일어나고 있음을 알 수 있었고, 면역금과립방법을 이용한 전자현미경 검사에서 제2형폐포세포내 laminin의 분포도 증명할 수 있었다. *(Tuberc Respir Dis 2005; 59: 397-405)*

Key words : 라미닌, 폐포중격, CS 최루 가스, 제2형폐포세포

INTRODUCTION

Maturation of the alveolar epithelium in the fetal lung of humans is characterized by the differentiation of alveolar type I and type II cells. When injury occurs to the lung, type II alveolar cells are responsible for the main precursor cells in regeneration of the epithelium[1]. The basement membrane is a specialized extracellular matrix and the major components of the basement membrane include type IV collagen, laminin, entactin, heparan sulfate proteoglycan and fibronectin[2]. Basement membranes are important in regulating various biological processes, such as cellular adhesion and differentiation, the establishment of cell polarity, and morphogenesis. The composition of the basement membrane may be altered during tissue development and basement membrane proteins have much influence on epithelial cell differentiation and biological functions[3].

In basement membrane and extracellular matrix, the general structure of laminin is composed of three polypeptide chains, three types of alpha chain, three types of gamma chain, and two types of beta chain. Furthermore, there are 18 different types of laminin isoforms[4]. Through these three chains of laminin, there are globular and rodlike domains that can be arranged in an extended four

armed, cruciform shape and is well suited for mediating between distant sites on cells and other components of the extracellular matrix[5]. Laminin can be isolated from Mouse Engelbreth Holm Swarm Tumor and from placental tissue[6,7].

There has been many reports of laminin activity found in tumors similar to that found in the developing stages of normal tissue. Laminin-1 has been found to be involved in acinar formation of human submandibular gland cells[8]. Laminin-2 (merosin) activity has been seen in human Schwann cell neoplasms, in the basement membrane of intermediate trophoblast cells of choriocarcinoma and placenta[9,10]. Furthermore, laminin-5 has been found in budding cancer cells in colon cancer and was suggested as a marker for cancer cell invasion[11]. Laminin isoforms have been discovered in activated fibro/myoblasts of oral epithelial hyperplasia and nodular palmar fibromatosis and structurally defective laminin has been discovered in the basement membranes of malignant tumors[12,13].

In this study, when white rats are exposed to CS gas (a type of tear gas), used in the military and for use against civil disturbances, a chemical reaction ensues in tissue and forms malononitrile and is transformed to thiocyanate. This creates cyanide ions, which are responsible for cellular toxicity[14]. The purpose of this study was to identify changes in laminin-1 expression of the air-blood barrier of alveoli structured in basement membrane and patterns of expression of laminin-1 in type II alveolar cells after exposure to CS gas.

MATERIALS AND METHODS

The experimental rats used were 21 day old Sprague-Dawley strain white rats grown in the animal laboratory in the Hanyang University School of Medicine. There were 5 rats used in the control group and 30 rats used in the experimental group. In the experimental group, the rats were exposed to ortho-chlorobenzyldine malononitrile (Hanyoung Chemical. Korea, CS gas) and five rats were sacrificed at 12 hours, 1, 2, 3, 5, and 7 days at each time group after the exposure. A $1m^3$ plastic container was constructed for placement of the experimental rats and 2g of CS gas in a powder form was introduced with the help of a small fan in a uniform manner. The exposure was done for 20 minutes, once per day, for three days and the rats were given an equivalent amount of oxygen during exposure.

The rats were sacrificed 12 hours, 1, 2, 3, 5, and 7 days after CS gas exposure and lung specimens were then fixated in part with a mixture of 0.1% glutaralehype and 4% paraformaldehyde (pH 7.4) solution and in part with a mixture of 4% paraformaldehyde-0.5% glutaraldehyde-3% sucrose (pH 7.4) solution created with caccodylate buffer.

In order to immunohistochemically observe the amount of distribution and formation of laminin-1 in the alveolae of the white rats, paraffin 5μm slices of tissue fixated in a mixed solution of 0.1% glutaraldehyde-4% paraformaldehyde were created and necessary stains made. After deparaffinization and hydration, the specimen was cleansed in phosphate buffered saline solution (PBS) and after allowing to react with 2-3% H_2O_2 methanol for 5 minutes, was applied to phosphate buffered saline and proteinase solution (DAKO Co, USA) at 37℃ for 15 minutes. Primary antibodies, rabbit anti-laminin (Sigma Co. USA), were diluted to a ratio of 1:30, was allowed to react for 90 minutes at 37℃, and cleansed in PBS. Then, after reacting with secondary antibodies, biotinylated goat anti-rabbit IgG (Vectastain Co, USA), for 30 minutes, was again cleansed in PBS. Thereafter, was reacted with ABC (avidin-biotin complex, Vectastain Co, USA) kit

for 30 minutes and cleansed in PBS. The sections were subjected to a color reaction with 3,3- diaminobenzidine tetrahydrochloride (DAB) and after counterstaining with 1% methylene blue, dehydration, washing, and mounting, the specimen was observed through a light microscope. In order to confirm the immunohistologic reaction, staining with reactive solution without the primary antibodies was done for the negative control group.

In order to view laminin-1 by immunogold methods through electron microscopic examination, caccodylate buffer was used and after cleansing and dehydration, the tissue was fixated in a mixed solution of 4% paraformaldehyde-0.5% glutaraldehyde-3% sucrose(pH 7.4) for 3 hours at high temperature. The sections were then embedded and thermopolymerized in LR White (London Resin White Co, Ltd, UK) of moderated intensity and then, ultrathin sections at approximately 800 Å thickness were made and stained. Tris-buffer containing 1% bovine serum albumin inhibited certain reactions, and primary antibodies were used after 20 fold dilution of antibodies used in immunohistochemical studies. Secondary antibodies were used after 20 fold dilution of 12 nm diameter gold particles bound to goat anti-rabbit IgG(Jackson Immuno Research Laboratories Inc. USA). After counterstaining with 2% uranyl acetate for 15 minutes, the tissue was observed through a Hitach 600 (Japan) transmission electron microscope at 800Ky accelerated voltage. In order to verify specificity of the immunogold method, a portion of tissue was reacted with the secondary antibodies, without the primary antibodies, and was observed using the same stains.

RESULTS

A strong dark brown colored immunoreaction (+++) for laminin-1 was seen in the alveoli of control rats, where CS gas exposure was absent. A few mononuclear phagocytes, stained in blue, are seen in the alveolar septa and alveolar surface (Figure 1). In the rats at 12 hours after CS gas exposure (12 hour group), strong immuno reactions for laminin-1 were still seen on the alveolar septa and a slightly greater amount of mononuclear phagocytes are observed compared to the control group. Strong immunoreactions for laminin-1 were also seen on the alveolar septa in the rats at 24 hours after CS gas exposure (1 day group) and mononuclear phagocytes and lymphocytes were also observable in small amounts. Lymphocytes and mononuclear phagocytes have increased in the lungs of rats at 48 hours after CS gas exposure (2 day group) and are seen to have partly filled the alveolar interstitium and have also increased in the alveolar septa. Moderate reactions (++) were observed in immunoreactions for laminin-1 on the alveolar septa. Lymph tissue through infiltration of lymphocytes and mononuclear phagocytes has formed in the lungs of rats at 72 hours after CS gas exposure and weak positive reactions (+) for immunoreactions of laminin on alveolar septa are seen (Figure 2). A moderate increase in activity of laminin-1 immunoreactions of alveolar septa in the lung of rats in the 5 and 7 day group after CS gas exposure are seen and a tendency towards gradual decrease of inflammatory tissue composed of lymphocytes and mononuclear phagocytes in the alveolar septa and interstitium is shown (Figure 3).

Several gold particles are seen in the basal lamina of alveolar septa, the blood air barrier, in the rat control group. A small number of gold particles are distributed among endothelial cells of capillaries and type I pneumocytes (Figure 4; Table 1). A substantial decrease in gold particles distributed within the basal lamina, representing laminin-1
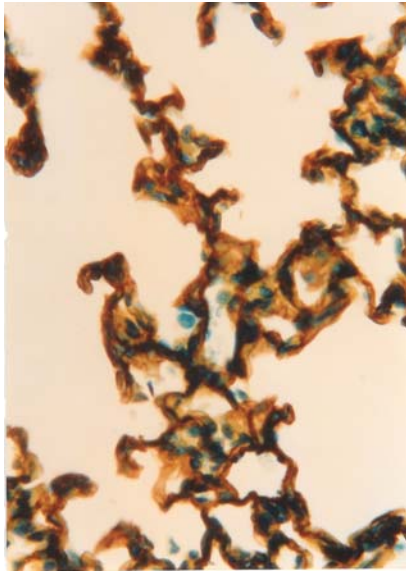
Figure 1. Immunostained photographs of laminin in the alveolar septa of control rats. Strong Immuno-reactions are seen over all alveolar septa. Few mon-onuclear phogocytes are seen in the interstitium of septa (Immunostain and methyl green stain, X400).
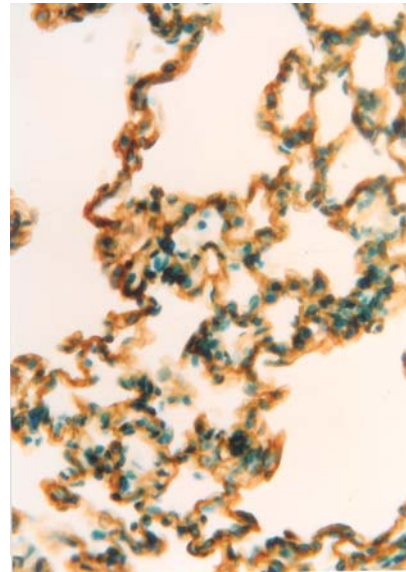

Figure 3. Immunostained photographs of laminin in alveolar septa of a rat at 7 days after CS gas expo-sure. Few mononuclear phagocytes are seen and st-rong laminin reactions are seen (Immunostain and methyl green stain, X400).
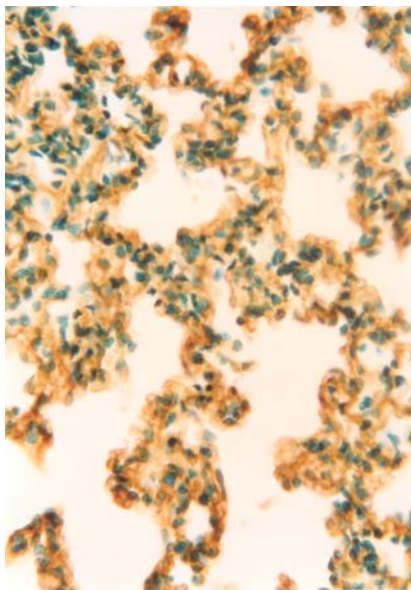

Figure 2. Immunostained photograph of laminin in alveolar septa of a rat at 3 days after CS gas expo-sure. Many mononuclear phagocytes are infiltrated into the interstitium of alveolar septa and moderate laminin reactions are seen (Immunostain and methyl green stain, x400).
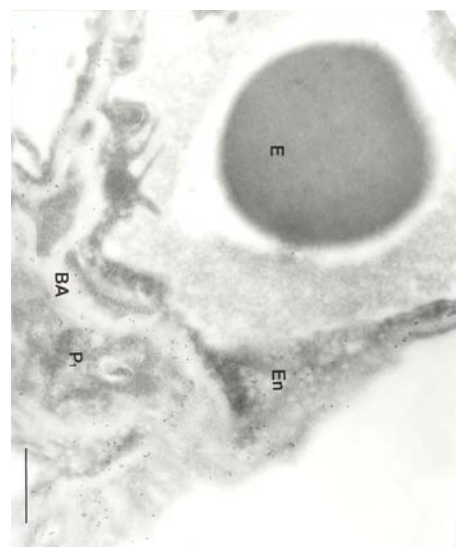

Figure 4. Immunogold electron micrographs of alve-olar septa of a control rat. A few gold particles are seen in the basal lamina(BA), endothelial cells(En), and type I pneumonocytes(P_1). Round erythrocytes(E) in the lumen of capillaries (Uranyl acetate stain and immunogold reaction).

within the basal lamina, in the 12 hour and 1 day group after CS gas exposure (Figure 5). The dis-tribution of gold particles in the 2 and 3 day group after CS gas exposure have recovered and gradu-ally resemble those in the control group and the amount of gold particles in basal lamina of alve-olar septa in the 5 and 7 day group after CS gas
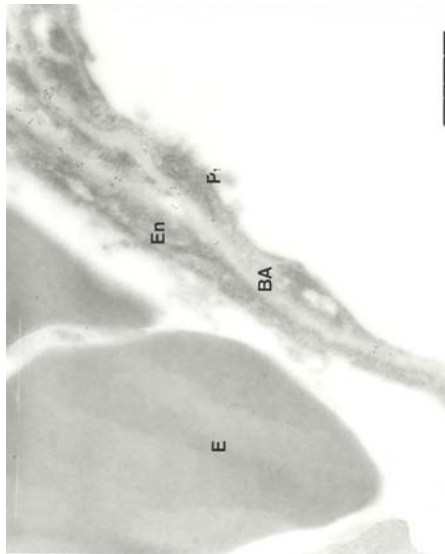
Figure 5. Immunogold electron micrographs of rat alveolar septa at 1 day after CS gas exposure. Gold particles are decreased in basal lamina(BA), endothelial cell(En,) and type I pneumonocytes(P₁) (Uranyl acetate stain and immunogold stain).
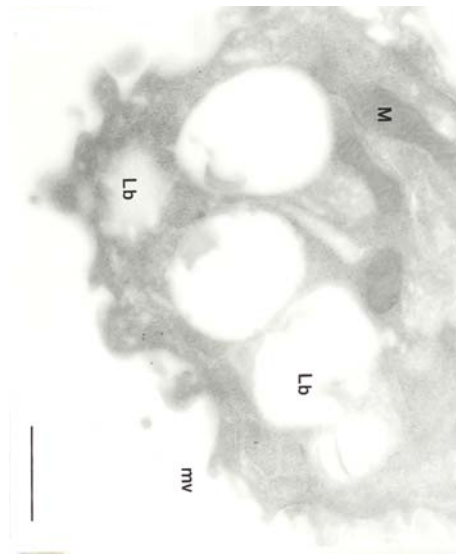

Figure 7. Immunogold electron micrograph of type II pneumonocytes in control rat alveolar septum. Several mitochondria(M), lamellar bodies(Lb), and microvilli (Mv) are seen in the type II cells and there are few gold particles in the ectoplasm (Uranyl acetate stain and immunogold reaction).
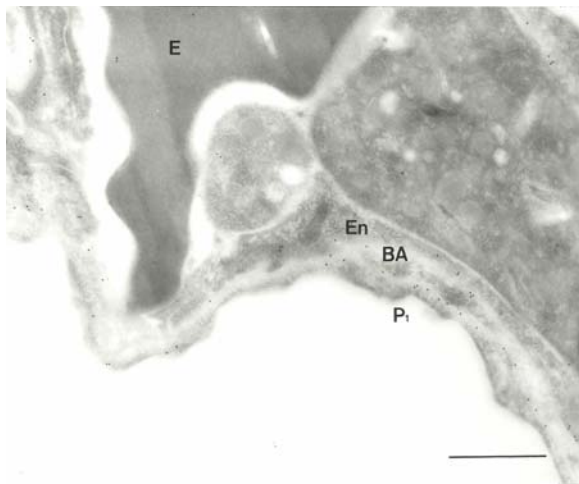

Figure 6. Immunogold electron micrographs of rat alveolar septa at 5 days after CS gas exposure. Gold particles are seen in the alveolar septa similar to that of control group rats (Uranyl acetate stain and immunogold reaction).

Figure 8. Immunogold electron micrographs of type II pneumonocytes. At 2 days after CS gas exposure. No gold particles are seen in the cytoplasm of the type II cell(Lb=lamellar bodies, P₁=type I pneumonocyte) (Uranyl acetate stain and immunogold reaction).

exposure have exceeded that of those in the control group (Figure 6, Table I).

There was a small number of gold particles observed in the alveolar septa of capillaries and type I pneumocytes of the normal rat contr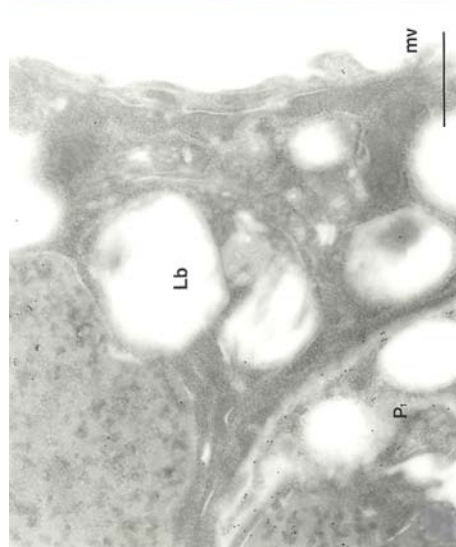ol group (Figure 4). Although gold particles were barely seen in the 12 hour group and 1 day group after CS gas exposure (Figure 5), gold particles were seen in small amounts in the 2 and 3 day group after CS gas exposure and a greater increase was

Table 1. Gold particle expressions of lung alveolar septum in CS gas exposed rats

Group/Tissue	Endothelial cell	Basal lamina	Type I cell	Type II cell
control	+	+++	++	+
12 hours	+-	+	+	+
1 day	+-	+	+-	±
2 day	+	++	+	−
3 day	+	++	+	±
5 day	+	+++	+	+
7 day	+	+++	++	+

Note : strong reaction : +++   moderate reaction : ++   mild reaction : +   trace reaction : ±
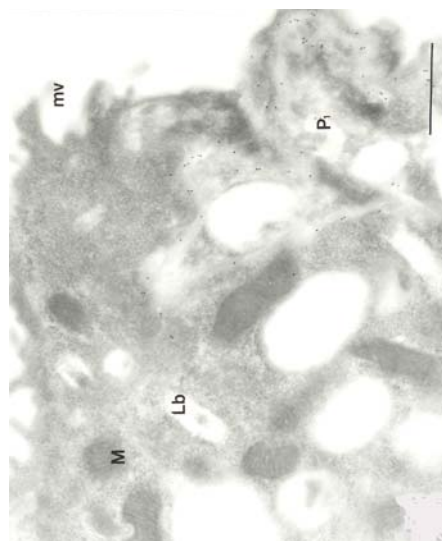negative reaction : −   CS gas : o-chlorobenzylidene malononitrile

Figure 9. Immunogold electron micrographs of type II pneumonocytes of rat alveolar septa at 7 days after CS gas exposure. Few gold particles are seen in the ectoplasm of the type II cells. (Lb=lamellar bodies, M=mitochondria, P_1=type I pneumonocytes and mv=microvilli) (Uranyl acetate stain and immunogold reaction).

evident in the type I pneumocytes. A small amount of gold particles are seen in endothelial cells of the 5 and 7 day group after CS gas exposure and a greater amount of gold particles appear over the perinuclear cytoplasm rather than the cytoplasm margin (Figure 6; Table 1).

Well-developed lamellar bodies are distributed in groups in the ectoplasm and several rod-shaped mitochondria are seen in the type II pneumocytes of the rat control group.

A small amount of rough endoplasmic reticulum was seen distributed around lamellar bodies. A small number of gold particles appearing in the area of laminin-1 are seen distributed in microvilli within the ectoplasm (Figure 7). A small number of gold particles were seen between the ectoplasm and lamellar bodies of type II pneumocytes of the 12 hour group rats after CS gas exposure. Gold particles are seen only on the surface of the cell membrane of type II pneumocytes in the 1 and 2 day group rats after CS gas exposure (Figure 8). Although a few gold particles are seen distributed in a portion of the cytoplasm of type II pneumocytes of the 3 day group rats after CS gas exposure, gold particles can only be seen on the surface of cytoplasm and the cell membrane of type II pneumocytes of the 5 and 7 day group rats after CS gas exposure (Figure 9; Table 1).

DISCUSSION

Laminin is known to function in adhesion, contact, migration, and signal transmission of materials within tissue and is a major important component of basement membrane. Laminin has several structural isoforms and may aid in histopathologic diagnosis of various diseases.

The laminin-1 heterotrimer is a cross-shaped molecule, a basic structure of all laminins with some modifications. A distinct feature of the alpha

chains is the C-terminal extension of five laminin globular modules. Laminin beta and gamma chains lack these modules. Domains I and II of alpha, beta, and gamma chains contain a triple alpha-helical coiled-coil forming the long rod-like structure. Domains III and V contain several laminin epidermal growth factor like modules which form rigid rod-like structures, and separate laminin globular domains IV from laminin N-terminal globular domains VI. The elastase fragments E3 and E8 contain major cell recognition sites. The greatest expression of laminin α1 is seen during early epithelial development and epithelial basement membranes in the adult in tissues such as in the placenta, kidney, liver, eyes, and male and female reproductive organs[15].

The major in-vivo role of laminin-1 is to influence epithelial cells. Laminin-1 has roles in epithelial development in four distinct domains, the short arm N-terminal domains involved in laminin polymerization, the nidogen-binding site of laminin γ1, the cell-binding E8 fragment, and the α1 chain specific E3 fragment. Antibodies against the E3 fragment have been shown to inhibit epithelial tubulogenesis of embryonic kidneys in vitro. The E3 fragment was also shown to influence breast epithelial cells in vitro. Active Akt-1 was shown to significantly enhance synthesis of laminin-1 and collagen IV and its overexpression can have important roles in malignancy by increasing survival of tumor cells[16]. Diabetics may develop abnormal basement membranes, which have been found to thicken in the late stages of diabetes and may be due to aberrant Akt activation[15].

The differentiation to certain laminin isoforms may aid in the diagnosis of diseases such as blistering genetic dermatoses or muscular dystrophies[17]. Rannels and his colleagues suggested that laminin may be required for differentiation of type II pneumocytes as shown in their experiments with rats[18]. Laminin is secreted from the extracellular matrix in epithelial cells of mammals and promotes differentiation functions of the cell[19,20]. Laminin within basement membrane is thought to have a similar structure to the blood-air barrier in alveolar septa and this basement membrane, during injury to the alveolar septa or after infection, may have an indirect role in its regeneration. In vivo research done on cells binding laminin have shown laminin to be produced in the mesenchymal cells of alveolar septa of the fetus and neonate[21]. Although, it has already been reported that laminin is synthesized in alveolar type I cells and vascular endothelial cells, it has only been suggested that laminin is produced from alveolar type II pneumocytes[22]. In situ hybridization of mRNA studies of cells in monoculture indicated that both epithelium and mesenchyme produce complete laminin molecules and a gradual increase in the 3 polypeptide chains of laminin mRNA in epithelial cells was found during development[23]. It is the general opinion that alveolar type II cells can differentiate into alveolar type I cells of the lung at the end of its life-span or when it has been injured and thus, laminin is thought to be produced from type II pneumocytes[1]. In this study, immunoresponse by using immunogold methods is seen as a few gold particles bound to antilaminin in the cytoplasm and cell surface membrane of the extracellular matrix in the 12 hour, 5, and 7 day group rats after CS gas exposure. This is thought to be laminin produced in or laminin bound to the cell membrane of the type II pneumocyte, which is enough proof that laminin-1 is produced from type II pneumocytes.

Laminin also has roles in the morphogenesis of the lung. Schuger found that anti-laminin antibodies altered normal lung morphogenesis and presented a marked inhibition of branching morpho-

genesis and a distortion of the bronchial tree in the fetal mouse[24]. Durham and Snyder suggested that changes in localization and accumulation of laminin that occurred during alveolar type I and type II epithelial cell differentiation implies an important role in mediating the differentiation of these cell types during rabbit fetal lung development[21].

In this study, the rapid decrease in laminin expression in the basement membranes in the 12 hour and 1 day group rats after CS gas exposure and the reappearance of laminin in the 2 and 3 day group rats after CS gas exposure were due to the toxic effects of the degradation product of CS gas, cyanide, causing inhibition in the production of laminin in the endothelial, alveolar type I, and mesenchymal cells. In the 3 day group rats after CS gas exposure, the inflammation of alveolar septa by invasion of mononuclear phagocytes is thought to cause the decrease in the expression of laminin in the basal lamina of the extracellular matrix. Furthermore, in the 5 and 7 day group after CS gas exposure, reappearance of laminin occurred when the inflammation disappeared from the alveolar septa and soon a recovery to levels seen in the control group were observed. Thus, CS gas exposure has had consistent influence over laminin expression, which has shown to possess important roles in the differentiation and functions of the lung in alveolar septal tissue.

SUMMARY

Background :

Laminin-1 is known to have regular functions in the development and course of differentiation of the lungs. The morphogenesis and distribution of laminin-1 still remains as a mystery and its distribution and changes in the molecular structure of laminin-1 in the pathogenesis of the lung still is a subject of great controversy. In this study, experiments were done to delineate the distribution and changes in the amount of laminin-1 after inducing inflammation of the lungs by exposing experimental animals to CS gas and especially, to find compositions of laminin-1 within type II pneumocytes.

Materials and Methods :

The experimental subjects of study were newborn rats and the extracted tissue from the experimental rats were viewed under light microscope and electron microscope after the sections were treated with immunohistochemical methods and immunogold reaction methods using bounded gold particles.

Results :

1) Lymphocytes and mononuclear phagocytes invaded the alveolar septa in the 2 day group rats after CS gas exposure and intense interstitial inflammation was seen in the 3 day group. 2) Laminin immunoreactions decreased to a moderate degree in the 2 and 3 day group rats after CS gas exposure and strong laminin immunoreactions were seen again in the 5 and 7 day group rats. 3) Gold particles in basal lamina of the lung blood-air barrier decreased and in the type I pneumocytes decreased in the 2 and 3 day group rats after CS gas exposure. 4) Gold particles were seen only on the surface of the cell membranes of type II pneumocytes of the 1 and 2 day group rats after CS gas exposure. 5) Few gold particles around the lamellar bodies and cytoplasm of type II pneumocytes in the control rat group and at 12 hours after CS gas exposure. Gold particles are seen only on the surface of type II pneumocytes of the 1 and 2 day group rats after CS gas exposure and are evenly distributed in small amounts in the cells of the 3 day group after CS gas exposure.

Conclusion :

CS gas exposure in the rats caused inflammation

of lung alveolar septa and also induced a decrease in laminin-1 in basal lamina and loss of laminin-1 in the cytoplasm of type II pneumonocytes. As the inflammatory cells disappeared, an increase in the distribution of laminin-1 occurred. This reflects tissue regeneration functions of laminin-1 in the pneumocytes of rats and the distribution of laminin-1 in type II pneumocytes can be seen through the electron microscope using immunogold methods.

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