Bull. Environ. Contam. Toxicol. (1990) 44:700–706

Bulletin of Environmental Contamination and Toxicology

Increased Procollagen Type III Peptide in Serum of Rabbits Exposed to Diesel Engine Exhaust

Takahito Suzuki and Takako Kanoh

Department of Environmental Health, Tokyo Metropolitan Research Laboratory of Public Health, 24-1, Hyakunincho 3 chome, Shinjuku-ku, Tokyo, Japan

Diesel engine usage is expected to increase in many countries, such as Japan and the United States of America, as a result of the pressure exerted by fuel economy measures. Diesel engine exhaust includes both gaseous and particulate components, such as nitrogen dioxide (NO_2), benzo(a)pyrene, etc. (Stenberg et al. 1983). Mohr et al. (1986) reported that most of the rats exposed to diesel exhaust for 2 yr had pulmonary deposits of large amounts of carbonaceous particles phagocytosed by alveolar macrophages and accompanied by severe chronic inflammatory changes characterized by alveolar septal thickening and bronchiolar-alveolar hyperplasia. And also, it has been reported that prolonged exposure of animals to diesel exhaust induces lung tumors (McClellan 1987).

Lung connective tissue is composed of collagen, elastin and proteoglycans. Collagen is the most abundant protein, comprising approximately 11% of normal adult lung and it has been provided at least five polymorphic types in lung (Seyer et al. 1976; Kang et al. 1982). The major collagen types present in the alveolar structures are type I and type III. Normal lung is characterized by a type I to type III ratio of approximately 2 to 1 (Seyer et al. 1976). Collagen-producing cells such as fibroblasts and endothelial cells can synthesize procollagen within the cells. Once procollagen is secreted extracellularly, both amino- and carboxy-terminal peptides of procollagen are cleaved by endopeptidases. These peptides can move from the tissue into the blood stream. Bateman et al. (1983) reported that the proportion of type III collagen increased in early active fibrosis like sarcoid nodules and organising pneumonia. Therefore, the released peptides of type III procollagen may reflect the degree of biosynthesis of type III collagen, and increased peptide level in serum may suggest inflammation or the beginning of

Send reprint requests to T. Suzuki at the above address.

fibrosis.

In this work, we have studied the changes of procollagen type III peptide (P-III-P) levels in sera and bronchoalveolar lavage fluid (BALF) from rabbits exposed to diesel exhaust in order to find a useful biochemical indicator of the effects of diesel exhaust exposure on human health.

MATERIALS AND METHODS

Ten-week-old, male New Zealand white rabbits (Nippon Bio-Supp. Center Co., Tokyo, Japan) were exposed to diluted diesel exhaust containing 4.5 mg/m^3 of particulate matter, 2.2 ppm nitrogen dioxide and 3.2 ppm nitrogen monoxide for 12 wk at 7 hr/d, 5 d/wk. Twenty animals were used for each group. Diesel exhaust was generated by running a 309cc diesel engine (Model NFAD50, Yanmar Diesel Co., Ohsaka, Japan) at 2,400 rpm, diluted with air in dilution tunnel and then drawn into an inhalation chamber. Chamber ventilation was effected with fifteen volume changes/hr. The temperature was at between 23 and 25°C and the relative humidity was varied between 45 and 65% in the chambers with 12 hr dark/light cycle. Control rabbits were exposed to clean air in the same type of chamber under the same conditions. Gravimetric measurements of the particulate matter were conducted daily using automatic β-ray dust mass monitor (Model BAM-102, Shibata Scientific Technology Co., Tokyo, Japan). The concentrations of nitrogen dioxide and nitrogen monoxide were continuously monitored with a chemiluminescent analyzer (Model 8440, Monitor Labs Co., San Diego, CA). The particulate size was determined with a particle fractionating sampler (Andersen Type low pressure impactor LP-20, Tokyo Dylec Co., Tokyo, Japan). More than 90% of the particulate matter in diesel exhaust had a particle size below 0.5 μm.

Blood samples were collected from the marginal ear vein at 1, 2, 3, 4, 8 and 12 wk for the determination of serum levels of P-III-P. At 4 and 12 wk, ten animals of each group were anesthetized with intravenous sodium pentbarbital (50 mg/Kg), the chest was opened and the trachea was then cannulated with a 19 gauge Teflon catheter. Forty mL of physiological saline solution at room temperature was twice injected gently into the catheter and withdrawn to gather the lavage fluids. From each animal, the recovered lavage fluids were pooled and centrifuged at 1,000 x g for 10 min at 4 °C. The cell-free supernatants were concentrated by approximately 1/100 at 1,000 x g, 4 °C with Centriflo® (CF25 Ultrafiltration, Amicon Co., Danvers, MA). Sera and the concentrated BALF were stored at -20 °C until assayed. The P-III-P levels in sera and BALF were determined

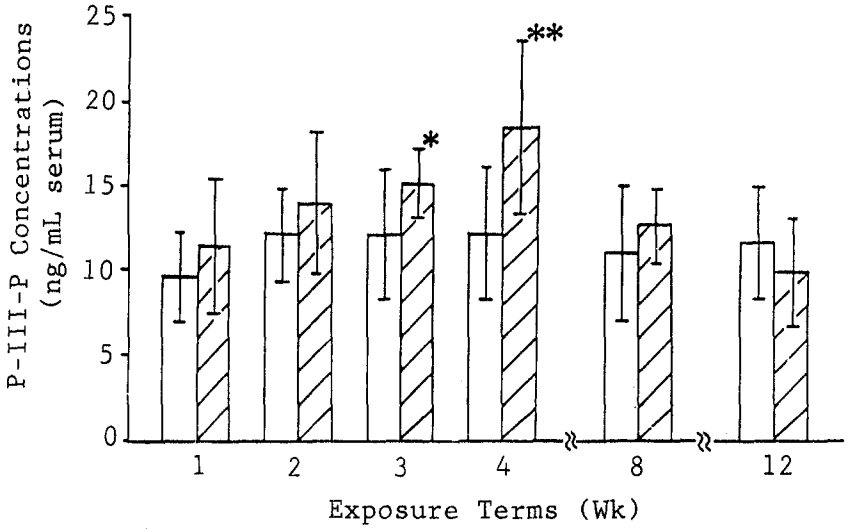

Figure 1. Changes in serum P-III-P concentrations of rabbits exposed to diesel exhaust. Data are expressed as the mean ± S.D. (n=10). Symbols indicate the significant difference from control by the Student t test (*: $P<0.05$, **: $P<0.02$).
□ : Control, ▨ : Diesel Exhaust

using radioimmunoassay kits (RIA-gnost® Procollagen-III-Peptide, Behringwerke AG, Marburg, Germany) according to the method of Rohde et al. (1979). The kits include specific antibody against bovine P-III-P, unlabeled bovine P-III-P standard and ^{125}I-labeled P-III-P. The serum and concentrated BALF of each rabbit were diluted to four different concentrations (1/4, 1/10, 1/20, 1/40), incubated with excess specific antibodies to form antigen-antibody complex and the remaining antibodies reacted with the known ^{125}I-labeled antigen. Then, both unlabeled and ^{125}I-labeled immune complexes were precipitated after the addition of separating reagent, Tachisorb®, followed by the centrifugation. The supernatant of the remaining ^{125}I-labeled antigen was discarded. The precipitate of ^{125}I-labeled immune complex was counted by scintillation counter (Model LS-5801, Beckman Instruments Inc., Fullerton, CA). Thus, we obtained the inhibition binding curve for each serum and BALF and calculated the P-III-P concentration from the standard inhibition curve using a 50% intercept method. The levels of P-III-P were expressed in ng of P-III-P per mL of serum or BALF.

One gram of the residual lungs were homogenized with 10 mL of acetone, defatted and dried succesively with 10 mL of chloroform-methanol (3:1, v/v) solution and 10 mL of ethyl ether for determination of collagen contents. Two

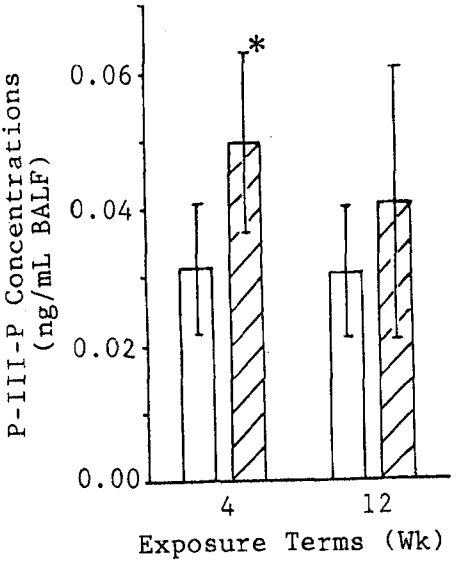

Figure 2. Changes in P-III-P concentrations of broncho-alveolar lavage fluid (BALF) of rabbits exposed to diesel exhaust. Data are expressed as the mean ± S.D.(n=10). Symbol indicates the significant difference from control by the Student t test (*: $P<0.05$). ☐ :Control, ▨ : Diesel Exhaust

mg of the defatted dry tissues were dissolved in 0.2 mL of 1N sodium hydroxide and neutralized by 12N hydrochloric acid, and then hydrolyzed in 2 mL of 6N hydrochloric acid for 90 min at 120 °C with an autoclave. The contents of collagen in the lungs were determined by the method of Bergman and Loxley (1963). The collagen contents were expressed in μg of hydroxyproline per mg of defatted dry tissues.

RESULTS AND DISCUSSION

As shown in Figure 1, serum P-III-P levels in the rabbits exposed to diesel exhaust increased at wk 3 and 4, and they returned to the control levels from the 8th wk on. A significant increase of P-III-P level in BALF from rabbits exposed to diesel exhaust was also observed at wk 4, and it returned to the control level at wk 12 (Figure 2). As shown in Figure 3, the collagen contents of the lungs increased at wk 12 in the rabbits exposed to diesel exhaust.

Bateman et al. (1983) reported that collagen content in fibrotic lung increased markedly in the interstitium of alveolar and capillary walls and the areas of early active fibrosis are characterized by an increased ratio of type III collagen to type I collagen. They suggest

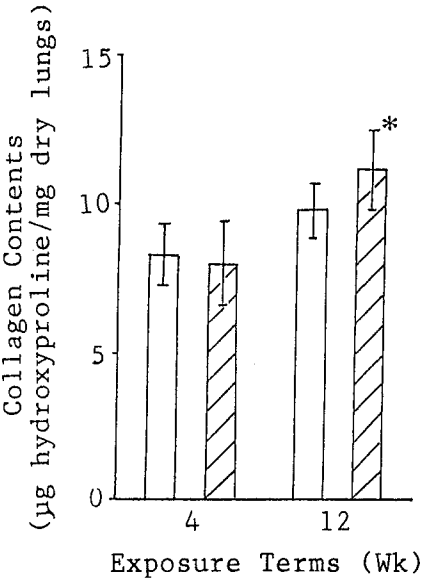

Figure 3. Changes in lung collagen contents of rabbits exposed to diesel exhaust. Data are expressed as the mean ± S.D. (n=10). Symbol indicates the significant difference from control by the Student t test (*: P<0.05). ☐ : Control, ▨ : Diesel Exhaust

that type III collagen reflects the early reversible stage of fibrosis in patients with fibrosing alveolitis. Experimental paraquat poisoning is widely used as an established model for an acute fibrotic process. Nerlich et al. (1984) also reported that in the sheep treated with paraquat the release of P-III-P into lymph fluid increased markedly in the early fibrotic stage, although the pathological changes in the lungs were slight and the total lung collagen content was within the normal range. They suggest that the observed increase in the release of P-III-P is an early indicator for a beginning pre-fibrotic tissue disarrangement which was already proposed by Rhode et al. (1979) in human liver disease. On the other hand, it was reported that the serum P-III-P levels did not always increase in chronic fibrotic patients (Okazaki et al. 1983; Low et al. 1983). Seyer et al. (1976) demonstrated that in chronic fibrotic lung the relative content of type III collagen diminished markedly, while the content of type I collagen increased. Namely, the serum P-III-P levels in the fibrotic diseases do not reflect the accumulated collagen contents and indicate the rate of type III collagen biosynthesis. In the present study, the serum P-III-P levels of rabbits exposed to diesel exhaust increased significantly at wk 3 and 4, and they returned to the control levels from the 8th wk on (Figure 1). A

significant increase of P-III-P level in BALF was also observed at wk 4, and it returned to the control level at wk 12 (Figure 2). No significant change was observed in the collagen content of the lung from rabbits exposed to diesel exhaust at wk 4, but it increased at wk 12 (Figure 3). From these phenomena, it was speculated that the fibrotic tissue disarrangement in the rabbits exposed to diesel exhaust began at wk 3 and the lungs might be in a chronic fibrotic state from the 8th wk on. Low et al. (1983) reported that the levels of P-III-P in BALF increased in patients with active and clinically progressive idiopathic pulmonary fibrosis and sarcoidosis. Watanabe et al. (1985) also demonstrated that a significant rise in P-III-P levels was observed in BALF from the bleomycin treated rabbits in the early reversible stage of fibrosis. These data are in agreement with the observation that the levels of P-III-P in BALF increased in the rabbits exposed to diesel exhaust. These results suggest that the determination of P-III-P levels in BALF would prove to be useful in assessing the process of connective tissue change in interstitial lung diseases.

The substances which caused the increased P-III-P levels in serum and BALF from rabbits exposed to diesel exhaust are not explainable, because diesel exhaust includes both gaseous and particulate components, such as NO_2, benzo(a)pyrene, nitropyrene and nitroarene etc., and the inhalation of these substances induces pulmonary fibrotic changes (Kleinerman 1979; McClellan 1987).

For detecting the early fibrotic tissue changes, the various biochemical indicators, such as serum prolyl hydroxylase and urinary hydroxyproline etc., have been investigated in many diseases, but these indicators for estimation of the biosynthesis and/or degradation of collagen decreased with growth and age (Prockop et al. 1979). On the other hand, serum P-III-P levels did not fluctuate with age, and this method of serum P-III-P assay was useful for detecting the progressive interstitial pulmonary fibrosis at its early stage (Okazaki et al. 1983). The present study suggests that serum P-III-P levels can be used as convenient biochemical indicator of the effects of diesel exhaust exposure on human health, because the serum P-III-P level would be clinically applicable in the prediction of increased collagen biosynthesis at an early stage in lung.

REFERENCES

Bateman ED, Turner-Warwik M, Haslam PL, Adelmann-Grill BC (1983) Cryptogenic fibrosing alveolitis: Prediction of fibrogenic activity from immunohistochemical studies of collagen types in lung biopsy specimens. Thorax

38:93-101
Bergman I, Loxley R (1963) Two improved simplified methods for the spectrophotometric determination of hydroxyproline. Anal Chem 35:1961-1965
Kang AH, Seyer JM, Postlethwaite AE, Hibbs MS, Mainardi CL (1982) Collagen polymorphism in lung fibrosis. J Univ Occup Environ Health 4(Suppl):95-107
Kleinerman J (1979) Effects of nitrogen dioxide on elastin and collagen content of lung. Arch Environ Health 34:228-232
Low RB, Cutroneo KR, Davis GS, Giancola MS (1983) Lavage type III procollagen N-terminal peptides in human pulmonary fibrosis and sarcoidosis. Lab Invest 48:755-759
McClellan RO (1987) Health effects of exposure to diesel exhaust particles. Ann Rev Pharmacol Toxicol 27:279-300
Mohr U, Takenaka S, Dungworth DL (1986) Morphologic effects of inhaled diesel engine exhaust on lungs of rats. In Ishinishi N, Koizumi A, McClellan RO, Stober W (ed) Carcinogenic and mutagenic effects of diesel engine exhaust, Elsevier Science, Amsterdam, p459
Nerlich AG, Nerlich ML, Langer I, Demling RH (1984) Release of amino-terminal procollagen peptides in paraquat-induced acute pulmonary fibrosis. Exp Mol Pathol 40:311-319
Okazaki I, Maruyama K, Okuno F, Suzuki H, Aoyagi K, Sera Y (1983) Serum type III procollagen peptide in patients with pneumoconiosis. J Univ Occup Environ Health 5:461-467
Prockop DJ, Kivirikko KI, Tuderman L, Guzman NA (1979) The biosynthesis of collagen and its disorders. N Engl J Med 301:77-85
Rohde H, Vagas L, Hahn E, Kalbfleisch H, Bruguera M, Timpl R (1979) Radioimmunoassay for type III procollagen peptide and its application to human liver disease. Eur J Clin Invest 9:451-459
Seyer JM, Hutcheson ET, Kang AH (1976) Collagen polymorphism in idiopathic chronic pulmonary fibrosis. J Clin Invest 57:1498-1507
Stenberg U, Alsberg T, Westerholm R (1983) Applicability of a cryogradient technique for the enrichment of PAH from automobile exhaust. Environ Health Persp 47:43-51
Watanabe Y, Yamaki K, Yamakawa I, Takagi K, Satake T (1985) Type III procollagen N-terminal peptides in experimental pulmonary fibrosis and human respiratory diseases. Eur J Respir Dis 67:10-16
Received October 21, 1989; accepted November 20,1989.