HEI HEALTH EFFECTS INSTITUTE

Consequences of Prolonged Inhalation of Ozone on F344/N Rats: Collaborative Studies

Part IV: Effects on Expression of Extracellular Matrix Genes

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Includes the Commentary of the Institute's Health Review Committee

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HEI HEALTH EFFECTS INSTITUTE

The Health Effects Institute, established in 1980, is an independent and unbiased source of information on the health effects of motor vehicle emissions. HEI studies all major pollutants, including regulated pollutants (such as carbon monoxide, ozone, nitrogen dioxide, and particulate materials) and unregulated pollutants (such as diesel engine exhaust, methanol, and aldehydes). To date, HEI has supported more than 120 projects at institutions in North America and Europe.

Typically, HEI receives half its funds from the U.S. Environmental Protection Agency and half from 28 manufacturers and marketers of motor vehicles and engines in the United States. Occasionally, revenues from other public or private organizations either support special projects or provide resources for a portion of an HEI study. For this study, the Institute acknowledges the cooperation and support of the National Toxicology Program (NTP), which consists of four charter agencies of the U.S. Department of Health and Human Services. The NTP sponsored the inhalation component of this project as part of its studies on the toxicologic and carcinogenic effects of ozone. However, in all cases HEI exercises complete autonomy in setting its research priorities and in disbursing its funds. An independent Board of Directors governs the Institute. The Research Committee and the Review Committee serve complementary scientific purposes and draw distinguished scientists as members. The results of HEI-funded studies are made available as Research Reports, which contain both the Investigators' Report and the Review Committee's evaluation of the work's scientific and regulatory relevance.

HEI Statement

Synopsis of Research Report Number 65 Part IV

Genetic Control of Connective Tissue Protein Synthesis After Prolonged Ozone Inhalation

BACKGROUND

The major component of urban smog is ozone, a highly reactive gas that forms when emissions from mobile and industrial sources react chemically in the presence of sunlight. On the basis of scientific data documenting ozone's effects on lung function in humans, the U.S. Environmental Protection Agency set a National Ambient Air Quality Standard for ozone of 0.12 parts per million (ppm). Compliance requires that this level not be exceeded for more than one hour, once per year.

Because ozone can damage cells, prolonged or repeated exposures may constitute a risk factor for lung cancer. Therefore, the National Toxicology Program (NTP) conducted a bioassay of prolonged exposure to ozone to evaluate ozone's carcinogenicity in rodents. Another equally important concern is the potential that prolonged ozone exposure may injure respiratory tissue, leading to the development or exacerbation of chronic lung diseases such as fibrosis or emphysema. The NTP's bioassay presented a unique opportunity for a collaboration between the HEI and the NTP that allowed HEI-funded investigators to study whether prolonged ozone exposure causes or enhances alterations in rat lungs that are characteristic of chronic lung diseases.

Connective tissue is important to the lungs because it confers mechanical strength by providing a supporting framework for cells. However, an excess of connective tissue can lead to fibrosis, which distorts normal lung structure and decreases the lung's efficiency for gas exchange. Also, changes in connective tissue components are believed to be an underlying cause of emphysema. The synthesis of connective tissue proteins is controlled by specific genes. The message encoded in DNA is transferred to a molecule called messenger RNA (mRNA), which in turn transmits information (or coding) to a cell's machinery that synthesizes proteins. This system is highly regulated and can be turned on or off in response to tissue injury.

Dr. William Parks' study of the effects of ozone exposure on the mRNAs that code for connective tissue proteins was one of eight studies in the NTP/HEI Collaborative Ozone Project, and one of three in which researchers examined the effects of ozone on rat lung connective tissue. Other investigations included studies of lung biochemistry, structure, and function, as well as one study of nasal structure and function.

APPROACH

Dr. Parks used molecular biology techniques to measure the levels of mRNAs that code for collagen and elastin, two connective tissue proteins. He compared mRNA levels in the lungs of rats exposed to clean air with those of rats exposed to 1.0 ppm ozone for 20 months. Tissue samples from a separate study of rats exposed for two months to the same ozone levels also were available for comparison.

RESULTS AND IMPLICATIONS

Dr. Parks found low levels of collagen and elastin mRNAs that were comparable in control rats and rats exposed for 20 months to 1.0 ppm ozone. In contrast, when Dr. Parks examined lungs from rats exposed for two months to 1.0 ppm ozone, he found enhanced levels of mRNAs that code for collagen and elastin when compared with control rats. The absence of increased collagen and elastin mRNA levels after 20 months suggests that the genes that code for these proteins were not highly active after prolonged ozone exposure.

Comparing the results of the 2- and 20-month exposures suggests that rats may adapt to prolonged ozone exposure by mechanisms that decrease the activity of genes that code for connective tissue proteins. However, different sets of rats were used for the two exposures, different lung lobes were analyzed, and fewer samples were available for the two-month study. In addition, because each exposure began with rats of the same age, those studied after two months were younger. Therefore, these results must be interpreted with caution and require further experimental validation.

This Statement, prepared by the Health Effects Institute (HEI) and approved by its Board of Directors, is a summary of a research project sponsored by HEI from 1991 to 1993. The inhalation component of this project was supported by the National Toxicology Program as part of its studies on the toxicologic and carcinogenic effects of ozone. This study was conducted by Dr. William C. Parks of the Washington University Medical Center, St. Louis, MO. The following Research Report contains an Introduction to the NTP/HEI Collaborative Ozone Project, the detailed Investigators' Report, and a Commentary on the study prepared by the Institute's Health Review Committee.

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Consequences of Prolonged Inhalation of Ozone on F344/N Rats: Collaborative Studies Part IV: Effects on Expression of Extracellular Matrix Genes

William C. Parks and Jill D. Roby

INTRODUCTION

The National Toxicology Program and Health Effects Institute Collaborative Ozone Project

The NTP/HEI Collaborative Ozone Project was a four-year project with many investigators that was organized to evaluate the effects of prolonged ozone exposure on lung injury in animals. The ozone exposures were conducted by the National Toxicology Program (NTP) at Battelle Pacific Northwest Laboratories. The individual investigators' studies, which addressed the pathologic and physiologic consequences of prolonged ozone exposure, were supported by the Health Effects Institute (HEI). A full description of the NTP/HEI Collaborative Ozone Project and the exposure protocol can be found in the Introduction and Supplement to Research Report Number 65 Part I. This information also will be published in Part VI of Research Report Number 65 that describes the exposure and distribution of the animals.

Briefly, in 1987, the Health Effects Institute entered into a partnership with the National Toxicology Program to evaluate the effects of chronic ozone exposure in rats. The NTP, consisting of four agencies of the U.S. Department of Health and Human Services, coordinates the nation's testing of potentially toxic and hazardous chemicals. The Health Effects Institute, an independent research organization supported by both government and industry, provides unbiased information on the health effects of motor vehicle emissions.

Because of the widespread exposure to ozone and concerns about its potential health effects, HEI and the California Department of Health and Human Services nominated ozone for carcinogenicity and toxicity testing by the NTP. The NTP, recognizing that cancer was only one of the chronic diseases of concern, included additional animals for HEI-supported studies of the pathologic and physiologic consequences of prolonged ozone exposures. The HEI animals were housed in cages that would otherwise have been empty. By developing a partnership, the HEI and NTP were able to leverage their funds to develop a comprehensive research program that extended beyond carcinogenicity endpoints; the HEI-sponsored research focused on the relation between long-term ozone exposure and the pathogenesis of chronic lung diseases, such as asthma, emphysema, and fibrosis. The Health Effects Institute would not have been able to undertake such an expensive project, which requires special facilities and trained personnel, without the NTP's support of the inhalation component and the cooperation of the NTP's contractor, Battelle Pacific Northwest Laboratories.

For the HEI component of the Project, eight studies were selected for funding from proposals submitted in response to the Request for Applications (RFA) 90-1, Health Effects of Chronic Ozone Inhalation: Collaborative National Toxicology Program–Health Effects Institute Studies, Part A: Respiratory Function Studies, and Part B: Structural, Biochemical, and Other Alterations. Because of the complexity of a project with many investigators and many endpoints, the HEI Health Research Committee also funded a Biostatistical Advisory Group to provide assistance with experimental design, animal allocation, and data analyses. Figure 1 presents a diagram of the studies in the NTP/HEI Collaborative Ozone Project and their relations to each other. They include those studies that were part of the NTP bioassay, the eight HEI-funded studies, and the biostatistical study. In addition, HEI engaged Battelle Pacific Northwest Laboratories to provide support services for the HEI-sponsored investigators.

Starting at six to seven weeks of age, male and female F344/N rats were exposed to 0, 0.12, 0.5, or 1.0 parts per million (ppm) ozone, six hours per day, five days per week. These concentrations were selected to include the maximum concentration the animals would tolerate (1.0 ppm), the current National Ambient Air Quality Standard (NAAQS) for ozone (0.12 ppm), and an intermediate concentration. The NTP's carcinogenicity bioassay consisted of a two-year study and a lifetime study in rats and mice, and a study of male rats exposed to 0.5 ppm ozone and two levels of a human pulmonary carcinogen, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK). The design of the HEI studies was directed, to some extent, by the constraints of the NTP protocol. These included ozone exposure concentrations that were set by the NTP, a limit on the sample size (164 rats) to the number of available exposure chambers, and quarantine restrictions that did not allow reentry of animals into the exposure chambers once they had been removed, thus eliminating the possibility of conducting serial tests.

The Biostatistical Advisory Group developed a sample allocation scheme that allowed several researchers to obtain measurements on tissue samples from the same subset of study animals, providing the maximum overlap of animals and tissues among the eight studies while ensuring balance with respect to dose, gender, and time of death. When the ozone exposure of the HEI animals ended (at 20

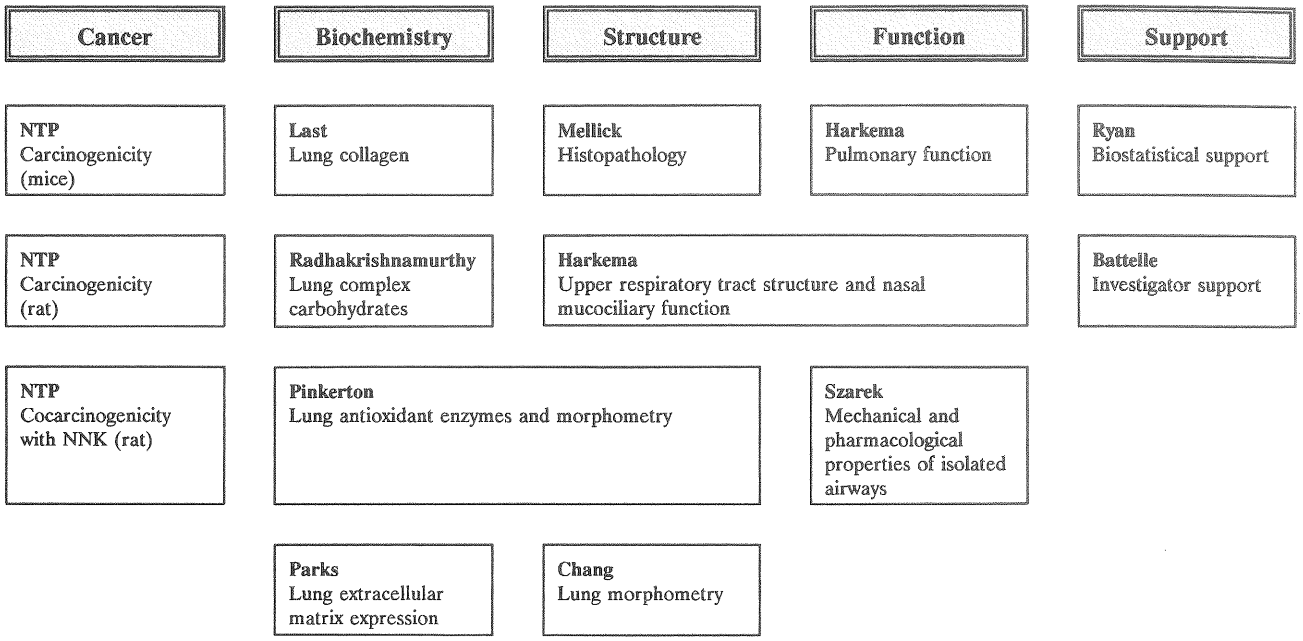

Figure 1. The NTP/HEI Collaborative Ozone Project: individual studies.

months), several investigators traveled to Battelle Pacific Northwest Laboratories to conduct their assays or to obtain samples on site. Battelle personnel prepared the tissues for off-site investigators and shipped them directly to their laboratories.

Because the studies varied in duration from six months to two years, HEI is publishing the reports for each individual study after the Institute's review process for each study is complete. Each Investigator's Report and a forthcoming Integrative Summary Report will be Parts of Report Number 65 of the HEI Research Report series. The present study by Dr. William C. Parks and Ms. Jill D. Roby of the effects of long-term ozone exposure on extracellular matrix gene expression is Part IV. Other investigators in the Collaborative Ozone Project examined the effects of ozone on functional (Harkema, Szarek), structural (Pinkerton, Chang), or biochemical (Last, Radhakrishnamurthy) alterations.

Although some conclusions can be drawn on the basis of the results from each individual study, the interpretation of Dr. Parks' findings will be strengthened when those data are correlated with the outcomes of the other investigators.

The importance of the collaborative NTP and HEI chronic ozone exposure studies is that they provide an unparalleled opportunity to examine the effects of prolonged ozone exposure using a variety of scientific approaches. The interaction of a number of methods to analyze the pathologic and physiologic consequences of chronic ozone exposure is one of this project's unique features. The results of these studies will provide new information about the threshold effects of ozone exposure on lung injury and the type and extent of damage in a well-established animal model. These results may be helpful for evaluating current standards of ozone exposure as they apply to human health and for designing future animal and human studies.

Consequences of Prolonged Inhalation of Ozone on F344/N Rats: Collaborative Studies

Part IV: Effects on Expression of Extracellular Matrix Genes

William C. Parks and Jill D. Roby

ABSTRACT

Increased deposition of lung extracellular matrix in terminal airways is associated with chronic ozone exposure. In situ hybridization was used to assess whether long-term ozone exposure causes elevated and continued expression of genes coding for connective tissue proteins. Accessory lobes were removed from the animals exposed to 0, 0.12, 0.5, or 1.0 parts per million (ppm)* ozone for 20 months as part of the National Toxicology Program (NTP)/HEI Collaborative Ozone Project. The lungs were perfused fixed under physiologic pressure and processed for in situ hybridization. Sections were hybridized with ^{35}S-labeled probes for messenger RNA (mRNA) coding for various matrix proteins, including collagen types I and III, elastin, and fibronectin, and for interstitial collagenase, a matrix metalloproteinase. Fetal rat lung was used as a positive control for hybridization. No signal for any mRNA was detected in terminal airway stromal cells of lungs from animals exposed to ozone for 20 months or control animals breathing clean air. In all samples from animals exposed to ozone for 20 months and control animals, only a very weak signal was seen in occasional cells within the interstitial spaces around large airways and blood vessels. In contrast, a strong signal for matrix-related mRNA was detected in fetal lung tissue. These findings indicate that active or enhanced matrix production is turned off in the adult animals used in the ozone studies, suggesting that the increase in matrix deposition results from a transient and early fibrotic response. Indeed, signal for type I procollagen and tropoelastin mRNAs was seen in alveolar septal cells in lungs of rats exposed to ozone for two months. No signal was seen in alveolar cells of age-matched control animals. (These animals, exposed for two months, and age-matched controls were from earlier studies supported by the HEI.) These findings indicate that ozone mediates a transient fibrotic response that results in a sustained increase in lung extracellular matrix. Confirmation of this hypothesis would require additional studies using animals exposed to ozone for shorter times.

INTRODUCTION

Changes in pulmonary function and morphology associated with ozone exposure indicate that excess connective tissue is produced within the bronchiolar and alveolar interstitial areas (Last 1983; Gross and White 1986). Type I collagen is the principal extracellular matrix protein in the lung interstitium (Clark et al. 1983), and thus, increases in its content reflect a fibrotic response and predict and probably lead to altered lung function (Crouch 1990). As such, many studies on the health effects of ozone have focused on changes in lung collagen and have shown that the mass of deposited collagen increases in animals exposed to relatively high doses (0.5 ppm or more) of ozone. For example, lung collagen content, as determined by hydroxyproline assay, increased about 21% in adult rats exposed continuously or intermittently to 0.96 ppm ozone for 13 weeks; a similar increase was detected in lungs of juvenile monkeys exposed to 0.64 ppm ozone for eight hours per day for one year (Last et al. 1984). Modest increases in lung hydroxyproline content have been reported in various models of rodents exposed to 0.5 ppm or more of ozone for seven days to one year (Hussain et al. 1976a; Last et al. 1979; Last and Greenberg 1980; Myers et al. 1984; Jakab and Bassett 1990). At lower doses (0.5 ppm ozone or less for 12 weeks or longer), however, ozone does not lead to a significant change in total lung collagen accumulation (Costa et

* A list of abbreviations appears at the end of the Investigators' Report.

This Investigators' Report is one part of Health Effects Institute Research Report Number 65 Part IV, which also includes an Introduction to the NTP/HEI Collaborative Ozone Project, a Commentary by the Health Review Committee on the Investigators' Report, and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. William C. Parks, Dermatology Division, The Jewish Hospital of St. Louis, Washington University Medical Center, St. Louis, MO 63110.

This study was supported by HEI funds from the U.S. Environmental Protection Agency and the motor vehicle industry. The inhalation component of this project was sponsored by the National Toxicology Program as part of its studies on the toxicologic and carcinogenic effects of ozone.

al. 1983; Wright et al. 1988). Compared with the marked and potentially rapid increases in total collagen associated with acute respiratory failure (Zapol et al. 1979: Collins et al. 1984), ozone mediates a relatively mild fibrotic response.

The fibrotic response to ozone may vary among species. For example, lung collagen increases approximately 25% in monkeys exposed to 0.25 ppm ozone for eight hours per day during alternating months for 18 months (Tyler et al. 1988). Another potential variable is the method of ozone delivery to the target animals, but long-term studies using continuous (Last and Greenberg 1980; Last et al. 1984), intermittent (eight hours per day) (Reiser et al. 1987), or episodic (every other month) (Tyler et al. 1988) exposures showed similar increases in lung collagen. In fact, similar increases are seen in animals exposed to a pattern of simulated urban ambient ozone (Chang et al. 1992).

The biochemical determinations used in the studies discussed above measure both preexisting and newly deposited matrix in the total lung. Because pulmonary fibrotic changes usually are focal (Crouch 1990), small changes in total lung collagen may reflect more dramatic changes at the sites of injury. Indeed, using morphometric analysis, about a two-fold increase in the connective tissue surrounding bronchioles was seen in rats exposed to 0.5 ppm ozone for six hours per day for 12 months (Hiroshima et al. 1989). Similarly, significant increases in the volume density of the terminal bronchiolar and alveolar extracellular matrices were seen in rats exposed to a daily cycle of 0.06 to 0.25 ppm ozone for up to 78 weeks (Chang et al. 1992). Thus, ozone mediates a localized fibrotic response even at relatively low doses.

Ozone exposure also may influence the production of multiple extracellular matrix proteins. Chang and colleagues (1992) demonstrated that both interstitial and basal lamina matrices, which comprise distinct connective tissue macromolecules, are affected by ozone exposure. Controversy exists, however, as to whether and how ozone affects elastin production. Elastin is a highly insoluble and durable extracellular matrix protein abundant in blood vessels but is a minor, although functionally important component of the alveolar interstitium (Parks et al. 1993). As for collagen, elastin deposition can be altered markedly in fibrotic conditions (Kuhn et al. 1976; Crouch 1990). As reported by Costa and associates (1983), chronic exposure to high doses of ozone (0.8 or 2.0 ppm for six hours per day, five days per week, for 12 weeks) led to increased deposition of elastin; however, at lower doses (0.2 ppm), elastin content decreased relative to that in age-matched, air-breathing control animals. In various other models in which rats were exposed to either low or high doses of ozone (0.25 to 1.5 ppm) for 7 to 78 days, elastin content was not affected or decreased slightly (Dubick et al. 1981b; Myers et al. 1984; Damji and Sherwin 1989; Last et al. 1993b). Because the interpretation of biochemical parameters of whole lung matrix is influenced by numerous factors such as hyperplasia, tissue growth, and localized production (Crouch and Parks 1992), site-specific increases in lung elastin may be hidden by the large background of pulmonary vascular elastin. Indeed, morphometric analysis demonstrates that production of alveolar elastin increases in response to ozone exposure (Chang et al. 1992).

As mentioned, many previous studies of the fibrotic response to ozone have examined total collagen and elastin content of the lung. Because ozone-induced damage may be focal, the true modulation of matrix deposition may have been masked by the abundance of connective tissue in unaffected areas. This concern is particularly relevant for the collagens as they are a heterogeneous group of proteins with tissue-specific deposition (Crouch and Parks 1992). For example, collagen types I and III are found in the interstitium throughout the lung, whereas type II is limited to airway cartilage, and type IV is a component of basal laminae. The many other distinct forms of collagen are not distributed equally among connective tissue compartments in complex tissues, such as lung. Biochemical assays, such as those that measure hydroxyproline, which is uniquely abundant in collagens, or elastin- or collagen-specific cross-links, determine total tissue protein and, hence, may grossly underestimate focal changes in matrix production.

To assess a focal fibrotic response adequately, gene expression must be assessed with techniques that provide cellular resolution; histologic techniques offer the important advantage of allowing discrimination between affected and unaffected areas. For this reason we used in situ hybridization, which detects steady-state intracellular mRNA, to determine whether chronic ozone exposure affects active production of extracellular matrix proteins. Immunohistochemical techniques may demonstrate intracellular localization of matrix proteins, but most matrix immunoreagents cannot discriminate between deposited and newly synthesized protein (Crouch and Parks 1992). This shortcoming precludes accurate identification of active biosynthesis in complex tissues because of the strong signal generated by immunostaining of the surrounding preexisting matrix. In addition, because some matrix products, such as tropoelastin, are rapidly secreted, intracellular localization of the protein may be difficult to assess owing to low intracellular concentrations of the antigen. In contrast, in situ hybridization allows direct determination of the cell type in a heterogeneous tissue that is actively expressing a specific gene transcript. The method relies on autoradiographic visualization of hybridized complexes of cellular mRNA with a radioactive nucleic acid reporter. In addition, in situ hybridization allows for determination of the proportion and distribution of expressing cells in a heterogeneous population. Thus, we

developed a sensitive in situ hybridization assay to assess whether long-term ozone exposure influences the expression of connective tissue proteins.

In our pilot studies for this contract, we detected α1(I) procollagen and tropoelastin mRNAs in alveolar cells in lungs from rats exposed to 1.0 ppm ozone for two months (Parks 1992). Because no signal for these mRNAs was seen in alveolar cells in control lungs, this up-regulated expression was selective for ozone-exposed animals. As summarized in this report and as we reported recently (Last et al. 1993a), no signal for any matrix product was detected in the long-term exposed animals. These results suggest that lung tissue has an adaptive response to chronic ozone exposure. With short-term exposure, an initial fibrotic response is indicated by the detection of tropoelastin and procollagen mRNAs in the animals exposed for two months; however, this response is not sustained with continued exposure. Thus, ozone may stimulate extracellular matrix protein expression only temporarily.

SPECIFIC AIMS

The deposition of extracellular matrix proteins in the lung is increased by exposure to ozone, but identification of the specific matrix molecules involved remains uncertain. In addition, the cell types and lung regions responsive to ozone exposure are unclear. We used in situ hybridization to determine whether matrix gene expression is induced or otherwise influenced in response to chronic ozone exposure. Our survey included assessment of mRNAs coding for abundant and functionally important interstitial fibrillar proteins, type I collagen, type III collagen, and elastin, and for fibronectin, which is deposited in both the interstitium and the basal lamina and often is expressed in response to injury (Ruoslahti 1988). To assess the potential degradation of matrix, we also examined production of interstitial collagenase, a metalloproteinase that catalyzes the rate-limiting step in collagen breakdown (Jeffrey 1986). For these studies, rats were exposed to 0, 0.12, 0.5, or 1.0 ppm ozone for 20 months as part of the NTP/HEI Collaborative Ozone Project. Because elevated matrix gene expression may be dependent on continual ozone exposure, some animals were killed immediately after cessation of ozone exposure and others were killed about one week later (see Table 1). Lungs were removed, perfused fixed at Battelle Pacific Northwest Laboratories, and shipped to us for in situ hybridization.

Table 1. Lung Samples Received for in Situ Hybridization Studies

Rat Number	Gender	Number of Days[a]
0 ppm O_3		
H19	F	6
H22[b]	M	6
H25	F	7
H28	M	7
H31	F	7
H34	M	7
H69	M	0
H73	F	0
H77	M	0
H81	F	0
H85	M	7
H89	F	8
H141	M	0
H145	F	0
0.12 ppm O_3		
H70	M	0
H74	F	0
H78	M	0
H82	F	0
H142	M	0
H146	F	0
0.5 ppm O_3		
H20[b]	F	6
H23	M	6
H26	F	7
H29	M	7
H32	F	7
H35	M	7
H71	M	0
H75	F	0
H79	M	0
H83	F	0
H87	M	7
H91	F	8
H143	M	0
H147	F	0
1.0 ppm O_3		
H21	F	6
H24	M	6
H27	F	7
H30	M	7
H33	F	7
H36	M	7
H72	M	0
H76	F	0
H80	M	0
H84	F	0
H88	M	7
H92	F	8
H144	M	0
H148	F	0

[a] Indicates the number of days after the cessation of ozone exposure when the rats were killed.

[b] These animals died during respiratory function studies, and their lungs were not fixed by perfusion as per our specified protocol. As such, these samples were not included in our studies.

METHODS AND STUDY DESIGN

ANIMALS

F344/N rats were exposed to 0, 0.12, 0.5, or 1.0 ppm ozone for 20 months at Battelle Pacific Northwest Laboratories for the NTP/HEI Collaborative Ozone Project, and animals were studied immediately after cessation of exposure or six, seven, or eight days later (see Table 1). After the rats were used for pulmonary function and other in vivo assays, lungs were removed, and the accessory lobe was fixed by perfusion with 10% formalin buffered with phosphate-buffered saline (PBS) at 25 cm H_2O. (PBS is 10 mM potassium phosphate, 150 mM sodium chloride, pH 7.4.) Tissues were kept in fixative for 24 hours at 4°C, washed twice with PBS, partially dehydrated through sequential changes of 30%, 50%, and 70% ethanol, then shipped to us by overnight courier. After receiving the samples, we performed routine processing for paraffin embedding. Table 1 lists the animals from which the lung samples were taken.

In addition, fixed lungs from three male and three female rats exposed to 0, 0.5, or 1.0 ppm ozone for two months were supplied by Battelle Pacific Northwest Laboratories as part of the Health Effects Institute Ozone Chamber Study conducted in 1989 and sent to us for pilot studies for this project. These lungs were fixed by perfusion with PBS-buffered formalin and stored and shipped in the same fixative solution. When we received the lungs, we removed the right anterior lobe, rinsed it in PBS, and processed it for paraffin embedding. As a positive control for extracellular matrix gene expression, lungs from 17-day rat fetuses were removed immediately after death and fixed by immersion in 4% paraformaldehyde buffered with PBS overnight at 4°C.

SAMPLE PREPARATION FOR IN SITU HYBRIDIZATION

In situ hybridization was performed as previously described in detail (Prosser et al. 1989). Sections were cut at 5 μm, floated onto a sterile water bath, picked up onto glass slides, and dried on a 42°C plate. We used Superfrost Plus slides (Fisher Scientific), which are electrostatically charged to attract tissue sections and offer the advantages for in situ hybridization of low background and nearly absolute tissue retention with no decrease in signal. The fixation and tissue structure of all lung samples were assessed by staining with hematoxylin and eosin.

Sections were deparaffinized in xylene, dehydrated, and rehydrated in PBS. All sections were treated briefly with nuclease-free proteinase K to loosen the constraints of intracellular cross-links caused by fixation, thereby enhancing the diffusibility of probes to their target mRNAs. All sections were washed in a freshly prepared triethanolamine buffer containing 0.25% acetic anhydride to reduce potential nonspecific binding sites (Brahic and Haase 1978).

HYBRIDIZATION

Sections were covered with a sufficient volume (about 25 to 50 μL) of hybridization buffer consisting of 50% deionized formamide, 2× standard saline-citrate (SSC) buffer (SSC: 1× is 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0), 20 mM 2-amino-2-hydroxymethyl-1,3- propanediol (Tris buffer), pH 8.0, 1× Denhardt's solution, 1 mM ethylenediaminetetraacetic acid, 10% dextran sulfate, 100 mM dithiothreitol, 0.5 mg/mL yeast transfer-RNA (tRNA), and 2.5×10^5 cpm of ^{35}S-labeled RNA probe (see below). Slides were incubated in a humidified chamber for 18 hours at 55°C. Sections were covered with a piece of Parafilm to retain the hybridization solution over sections and to prevent evaporation. After hybridization, Parafilm was removed, and the slides were washed twice in 4× SSC at room temperature with gentle stirring. Sections were washed further under stringent conditions (0.5× SSC, then 0.1× SSC, both containing 25 mM dithiothreitol) followed by treatment with RNase A at 50 μg/mL to digested unhybridized probe. Final washes were in 2× SSC and 0.1× SSC followed by dehydration through graded ethanols and air-drying in a dust-free area. For sections hybridized with nonhomologous probes, the final 0.1× SSC was omitted to prevent the potential melting of short hybrids that result after the RNase cleavage at sites of mismatched bases.

AUTORADIOGRAPHY

Washed slides were dipped in NTB-2 emulsion (Kodak, Rochester, NY) prediluted 1:1 with distilled water and equilibrated at 42°C. To minimize stretch artifacts, the emulsion was gelled and dried slowly under conditions of relatively high humidity (Rogers 1979; Prosser et al. 1989). Slides were exposed in a desiccator at 4°C. To ensure adequate autoradiographic exposure, three sections hybridized with antisense RNA probes were processed in separate exposure boxes. After 7 to 30 days, the slides were developed at 15°C in Kodak D19 developer and counterstained with hematoxylin and eosin. Silver grains were visualized by dark-field microscopy. Positive signals should be confined to cytoplasmic areas with few grains overlying nuclei.

CONTROLS INCLUDED IN THE IN SITU HYBRIDIZATION ASSAY

In situ hybridization is prone to numerous autoradiographic and chemographic artifacts that may mimic specific hybridization. Therefore, proper controls were included with each experiment to verify signal specificity. Probe specificity was first confirmed by Northern blot hybridization using rat lung RNA. For Northern blot hybridization, 5 μg of total RNA was denatured in 50% formamide, 1 M formaldehyde, and 50 ng/μL ethidium bromide at 68°C for five minutes, and separated by electrophoresis through a 1% agarose gel containing 1 M formaldehyde. RNA was passively transferred to nylon-reinforced nitrocellulose membranes (Schleicher & Schuell, Keene, NH), and membranes were baked, hybridized, washed, and exposed to x-ray film as described elsewhere (Parks et al. 1988). For Northern blots, antisense and sense RNA probes transcribed in vitro (see below) were labeled with α[^{32}P]uridine triphosphate as previously described (Prosser et al. 1989). As an internal control for autoradiographic and chemographic artifacts (Rogers 1979), sections were processed without labeled probe in each experiment. Also, to assess possible light leakage, plain glass slides were coated with emulsion and exposed with test sections.

PROBE PREPARATION

For these studies we used a rat tropoelastin complementary DNA (cDNA), 124D (Pierce et al. 1990); a human α1(I) procollagen cDNA, Hf677 (Boyd et al. 1988); a human α1(III) procollagen cDNA, T3-Gs (Loidl et al. 1984); a human fibronectin cDNA, pHF6 (Kornblihtt et al. 1985); and a rat collagenase cDNA (Quinn et al. 1990). These probes were supplied in or were subcloned by us into transcription vectors, either pBSKSII+ (Stratagene, La Jolla, CA) or pGEM4Z (Promega, Madison, WI). For example, we isolated a 750 base pair XhoI–EcoR1 fragment from the type III procollagen cDNA and subcloned it into pBSKSII+. This fragment allowed us to make a probe specific for sequences that code for the carboxy terminus, which is homologous among species but unique compared with other collagen genes (Kühn 1987). Insert orientation was determined by restriction enzyme analysis and by partial sequencing.

Plasmids were linearized with the appropriate restriction enzyme to transcribe antisense or sense RNA. RNA transcribed in vitro was labeled with α[^{35}S]uridine triphosphate (α[^{35}S]UTP) (> 1,200 Ci/mmole) using reagents from Promega by a modification of our previously described method (Prosser et al. 1989). To increase the specific activity of the radiolabeled probes, 150 μCi of α[^{35}S]UTP was dried in a microfuge tube under vacuum with gentle centrifugation. This effectively increases the concentration of radiolabeled precursor greater than five-fold compared with our previous protocol. Transcription reagents, five units of the appropriate RNA polymerase, and 1 μg of linearized DNA template were added in a total volume of 20 μL, and the mixture was incubated for one hour at 37°C. At this point, an additional five units of polymerase were added, and the reaction was continued for three hours more. These modifications generally have resulted in a five- to ten-fold increase in the specific activity of the RNA probes.

As a control for nonspecific hybridization, sections in each experiment were hybridized with ^{35}S-labeled sense RNA transcribed from a bovine tropoelastin cDNA. The validity of this probe as a negative control has been confirmed by Northern blot analysis (Prosser et al. 1989) and by in situ hybridization assays (Saarialho-Kere et al. 1992, 1993a). In experiments that included hybridization for tropoelastin, the sense RNA control was transcribed from the rat tropoelastin cDNA, 124D. For Northern blot hybridization, ^{32}P-labeled RNA probes were prepared as described (Prosser et al. 1989). After transcription, the DNA template was removed by digestion with two units of RNase-free DNase (RQ1, Promega) for 15 minutes. Probes were extracted with phenol–chloroform–isoamyl alcohol (25:24:1), then with chloroform–isoamyl alcohol (24:1). Yeast tRNA (50 μg) was added as a carrier, and transcribed RNAs were precipitated with ethanol, dissolved in 20 mM dithiothreitol, and stored at –70°C.

RNA ISOLATION AND NORTHERN BLOT HYBRIDIZATION

Probe specificity for the appropriate mRNA was assessed by Northern blot hybridization (Parks et al. 1988). Total RNA was isolated from 17-day fetal, 21-day fetal, 4-day neonatal, and adult rat lungs by homogenization in guanidine thiocyanate and isopycnic centrifugation through cesium chloride (Wrenn et al. 1987). Purified RNA was denatured in formaldehyde, resolved by electrophoresis through 1% agarose, and transferred passively to nitrocellulose. Blots were hybridized with 10^7 cpm ^{32}P-labeled RNA probes in 50% formamide, 5× SSC, 25 mM sodium phosphate, pH 6.5, 1× Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), 50 μg/mL salmon sperm DNA, 50% dextran sulfate, and 100 mM β-mercaptoethanol overnight at 65°C. Blots were washed three times, for 10 minutes each, in 2× SSC, 0.1% SDS at room temperature, then three times, for 20 minutes each, in 0.1× SSC, 0.5% SDS at 65°C. Following the last wash, the blots were rinsed in 2× SSC, treated with 50 μg/mL RNase A in the same buffer for 30

minutes at room temperature, then washed at 65°C for 20 minutes in 0.1× SSC, 0.5% SDS. Blots were dried thoroughly and autoradiographed with Kodak XAR-5 film.

STATISTICAL METHODS AND DATA ANALYSIS

By design and application, in situ hybridization is primarily a qualitative technique, and thus, the observations on gene expression answered where, when, and by whom, rather than how much. Although some investigators have quantified in situ hybridization results, their data, expressed as transcripts per cell, were verified by conventional in vitro hybridization assays (Lawrence and Singer 1985; Taneja and Singer 1987). Because we obtained negative data with both the control and ozone-exposed animals, our findings are not amenable to statistical interpretation, nor are they enhanced by such analyses. Because ozone-mediated effects on matrix production are focal, determination of mRNA concentrations by blot hybridization in dissected lung pieces would not provide equivocal or additional information. The accessory lobe of a rat lung is sufficiently small to allow examination of multiple, functionally distinct areas, such as alveolar, bronchiolar, peribronchovascular, and vascular compartments, on a single section. Thus, we can assess connective tissue protein expression in focal areas known to be affected by ozone exposure, such as the central acinar regions, as well as within other matrix compartments.

Although quality control is applied at all steps of the in situ hybridization protocol, variability in signal is present occasionally. Typically, variability is due to unexplained artifacts that occur in some, but not all, slides of an experiment. Because of this, we routinely include three positive slides per tissue and probe for each experiment, and these are exposed separately. In addition, positive experiments are repeated at least three times to verify the reproducibility of the results.

RESULTS

PROBE SPECIFICITY

Blot hybridization was used to confirm the specificity of the various probes used in our studies. Northern blot analysis demonstrated that the α1(I) procollagen (Crouch et al. 1989; Prosser et al. 1989), the α1(III) procollagen (Belknap et al. 1994), the fibronectin (Broekelmann et al. 1991; Botney et al. 1992), and the collagenase (Quinn et al. 1990) probes each hybridized to the appropriate mRNA. Collagen genes share some degree of sequence homology in exons encoding triple helices, but they are distinct in areas coding for noncollagenous domains. To prevent potential cross hybridization with other collagen transcripts, we linearized the α1(I) procollagen construct such that the RNA probe was transcribed from the region corresponding to the unique C-propeptide region (Prosser et al. 1989). Similarly, the α1(III) procollagen probe was complementary to sequences coding for the C-propeptide (see Methods and Study Design).

We needed, however, to assess the specificity of the rat tropoelastin RNA probe. The construct was linearized, and ^{32}P-labeled RNA probes were transcribed with T3 or T7 RNA polymerase. Blot hybridization analysis with rat lung RNA from fetal, and from ambient air–breathing neonatal and adult animals showed that tropoelastin antisense RNA was transcribed from the T7 promoter and that sense RNA was synthesized from the T3 promoter (Figure 1). This experi-

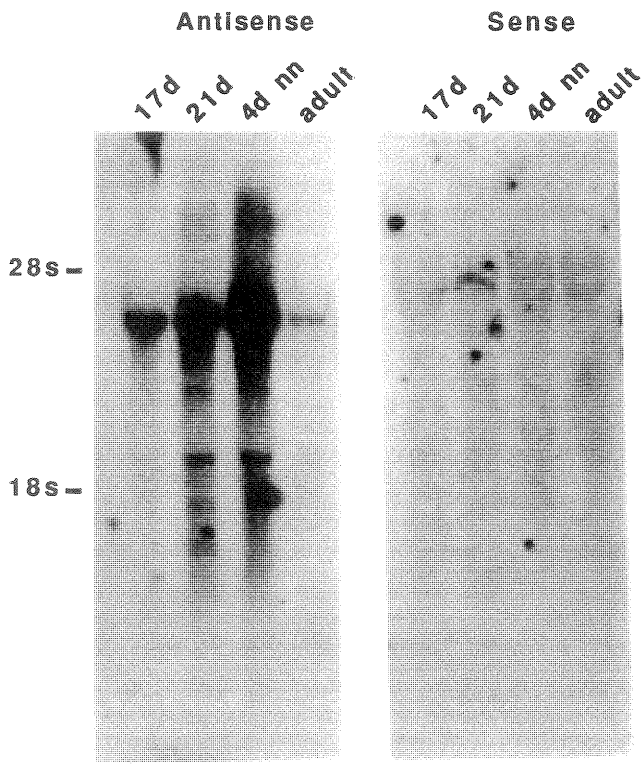

Figure 1. Northern blot hybridization for tropoelastin mRNA. Total rat lung RNA (5 μg per lane) was isolated from 17-day fetal (17d), 21-day fetal (21d), 4-day neonatal (4d nn), and adult animals, and was resolved by formaldehyde agarose electrophoresis. (Neonatal and adult animals breathed ambient air.) After transfer to nitrocellulose, RNA was hybridized with antisense or sense RNA probes transcribed from a rat tropoelastin cDNA and labeled with α[^{32}P]UTP. The migration of the 28 s and 18 s ribosomal RNA subunits is indicated. The results demonstrate specificity of the antisense probe for 3.5 kilobases of rat tropoelastin mRNA, and verify the developmental regulation of lung elastin.

ment demonstrated the specificity of this probe for 3.5 kilobase rat tropoelastin mRNA (Pierce et al. 1990). Furthermore, and in agreement with the findings of others (Bruce 1991; Noguchi et al. 1992), these results showed that tropoelastin expression is developmentally regulated in the lung, with the highest levels of expression reached during late fetal and early neonatal development. By maturity, expression of tropoelastin in the lung had dropped markedly (Figure 1).

IN SITU HYBRIDIZATION OF RAT FETAL LUNG

As a positive control for hybridization, samples of fetal lung were included in all experiments. After a 10-day autoradiographic exposure, fetal rat tissue displayed a strong signal for tropoelastin mRNA in medial cells of developing blood vessels, a moderate signal in the stroma of airways, and a diffuse signal in alveolar stromal areas (Figure 2A and 2B). As expected, the signal for type I procollagen mRNA was expressed by the same cells that were positive for tropoelastin mRNA (Figure 2C and 2D). Fetal tissue probed for α1(III) procollagen mRNA showed a pattern of expression similar to that of type I procollagen mRNA (data not shown). No specific signal was seen in fetal lung sections incubated with the sense RNA probe (Figure 2E and 2F). For fibronectin and collagenase, other tissues were used as positive controls (Botney et al. 1992; Saarialho-Kere et al. 1992).

IN SITU HYBRIDIZATION OF LUNGS EXPOSED TO OZONE FOR 20 MONTHS

For the NTP/HEI Collaborative Ozone Project, most animals were killed about one week after cessation of ozone exposure, emphasizing the evaluation of permanent damage or changes. For assessment of the levels of deposited connective tissue protein this protocol may present no potential problem because many matrix molecules, especially elastin and type I collagen, are highly stable and turn over at a slow rate (Davidson 1990). However, changes in matrix gene expression and the regulation of steady-state mRNA levels often are controlled rapidly (Raghow et al. 1986; Parks et al. 1992). Thus, if ozone enhances the expression of transcripts coding for extracellular matrix proteins, then removal of this stimulus at the cessation of exposure may cause a rapid return to normal levels. Because of this possibility, we requested that about half of the animals we studied be killed immediately at the end of exposure to ozone (Table 1).

Antisense and sense RNA probes were transcribed in vitro in the presence of α[^{35}S]UTP, and sections were processed, hybridized, washed, and prepared for autoradiography. For each experiment, three slides for each specimen were prepared. Two were hybridized with antisense probe and stored in separate, light-proof containers to allow for two autoradiographic exposures. The third slide was hybridized with the sense probe and exposed in the second box. Initially, the shorter exposure was for 7 to 10 days with the second exposure typically ranging between 14 and 21 days. Because we obtained negative results (see below), we extended the initial exposure to 18 to 21 days and developed the second box at 30 days.

No specific signal or evidence of increased expression for the matrix mRNAs examined (tropoelastin, α1[I] procollagen, α1[III] procollagen, fibronectin, and collagenase) was detected in any lung specimens from 20-month, 1.0 ppm ozone-exposed rats and matched controls (Figure 3A and 3B and Table 2). Because many studies have shown a dose response for ozone-mediated increases in lung matrix, we examined first the control samples and those from rats exposed to 1.0 ppm ozone, anticipating that these would reveal the maximal response. Had we obtained positive results with the high-dose samples, we would have repeated these in situ hybridization studies with lungs from the lower-dose animals. However, because we detected no mRNA expression, these other samples were not studied in detail. To assess whether lower doses mediated a prolonged fibrotic response, we probed the samples from rats exposed to 0.12 or 0.5 ppm ozone (killed at day 0) for tropoelastin mRNA, but this experiment was predictably negative (data not shown).

For tropoelastin and α1(I) procollagen mRNAs, two separate in situ hybridization experiments were conducted with all samples, and a third experiment was conducted with samples from rats killed just after cessation of exposure (Tables 1 and 2). In this third experiment, autoradiography was extended to 30 days, but even with this prolonged exposure, no signal for either mRNA was detected in terminal airway areas (Figure 3A and 3B). For α1(III) procollagen, fibronectin, and collagenase mRNAs, in situ hybridization was performed with control and 1.0 ppm ozone samples from rats killed just after cessation of exposure (Tables 1 and 2). The results of these studies essentially were identical to those shown in Figure 3A and 3B. Positive control samples were included in all experiments.

In some samples, weak autoradiographic signal for type I procollagen and tropoelastin mRNAs was seen in occasional cells in the interstitial areas of large airways and vessels, but the signal strength and pattern did not differ between ozone-exposed and control animals (Figure 3C and 3D). These results indicate that the mRNA in the NTP/HEI samples is intact and is hybridizable and that the in situ

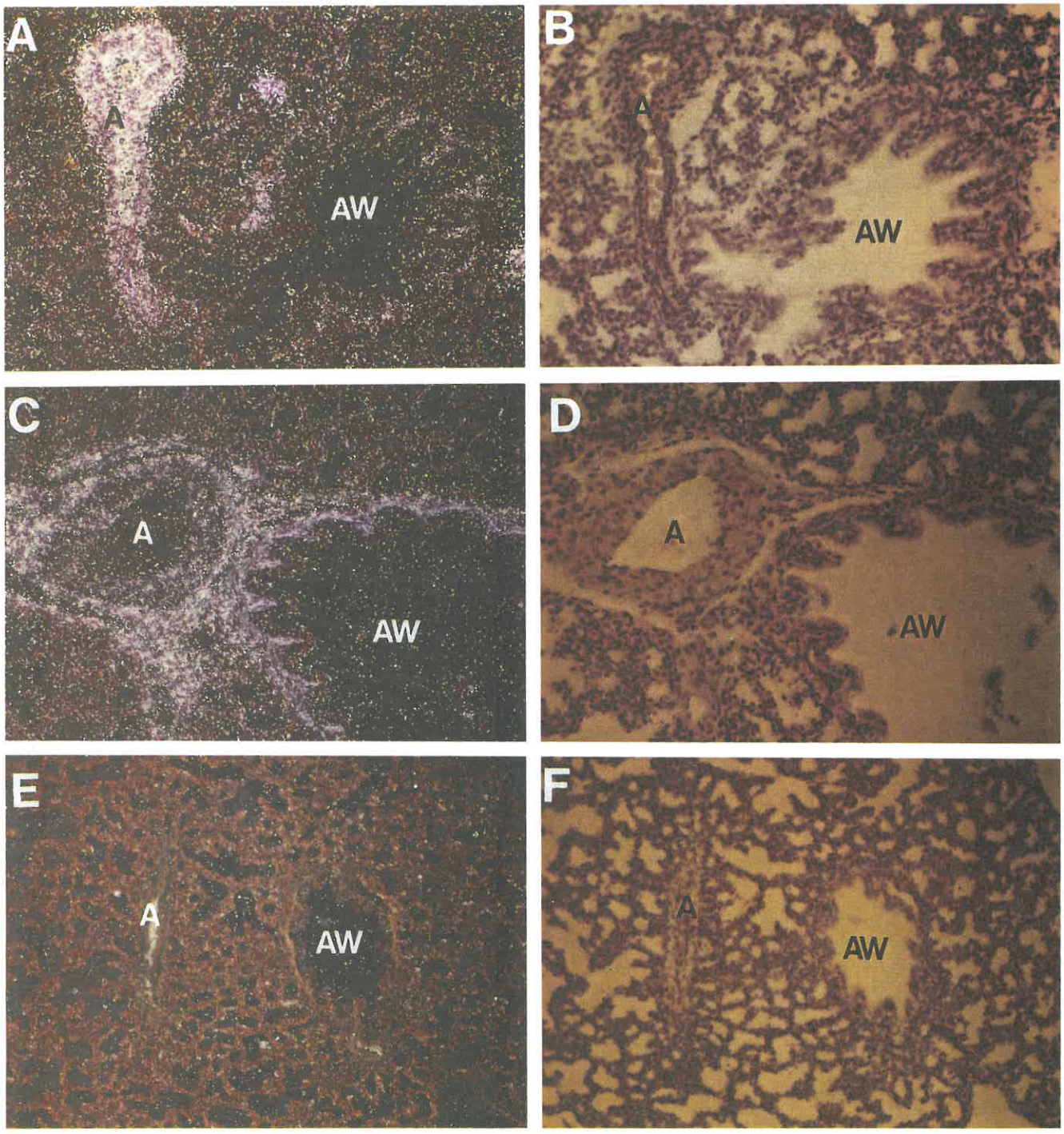

Figure 2. In situ hybridization of fetal rat lung for tropoelastin and α1(I) procollagen mRNAs. Fetal rat lung (17 days of gestation) was fixed and processed for in situ hybridization. Panels A and B: Paired dark-field and bright-field photomicrographs of sections hybridized with antisense ^{35}S-labeled RNA probe for tropoelastin mRNA. Strong signal was seen in developing arteries (A), and moderate signal was detected in subepithelial stromal cells of an airway (AW). Diffuse signal for both antisense probes was seen throughout the lung, probably representing fetal expression of elastin in alveolar stroma. Panels C and D: As for tropoelastin mRNA, strong signal for α1(I) procollagen mRNA was seen in fetal arteries (A) and airways (AW). Panels E and F: No specific signal was detected in sections hybridized with sense ^{35}S-labeled RNA probe. Autoradiographic exposure was for 10 days, and sections were counterstained with hematoxylin and eosin. Magnification = × 200.

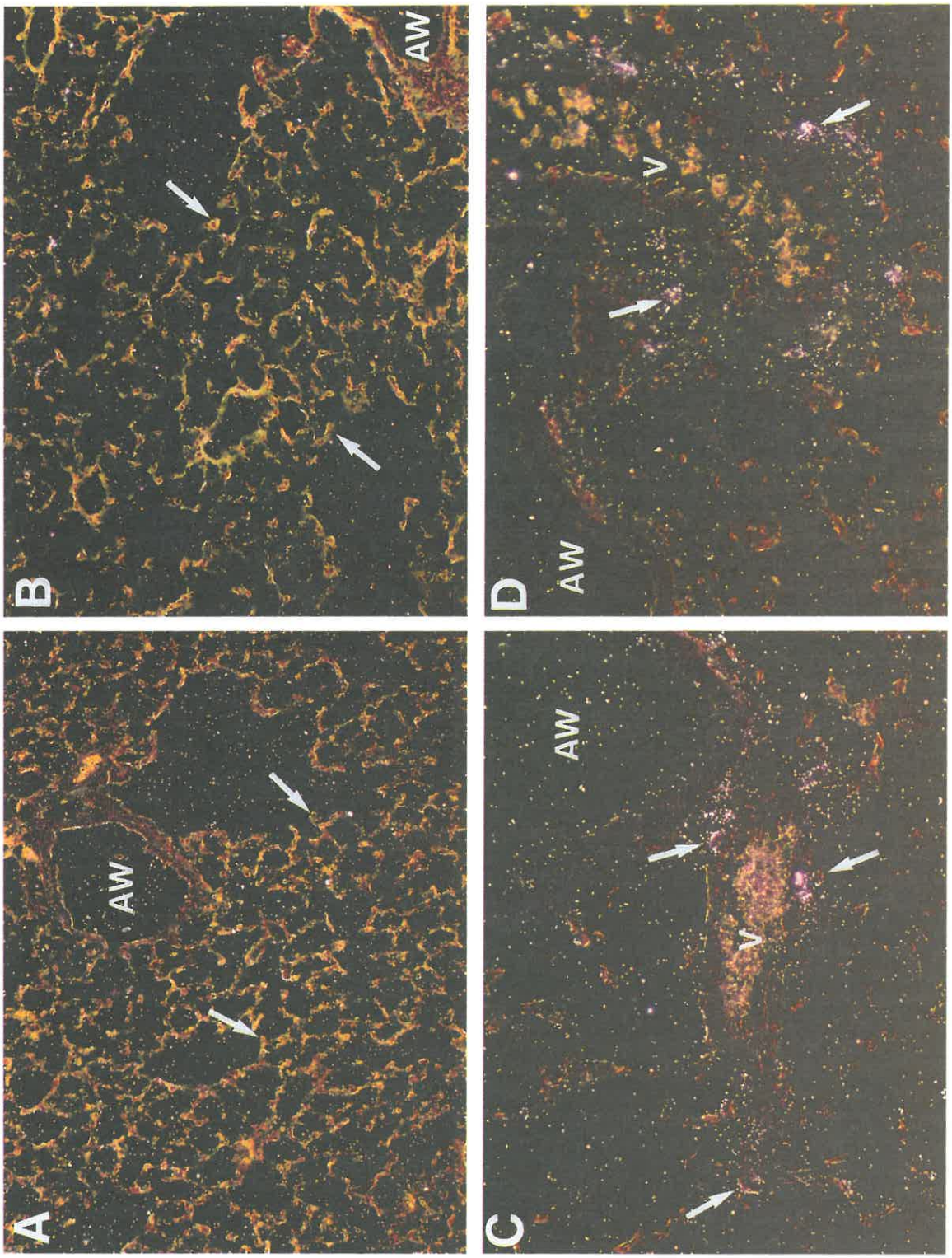

Figure 3. In situ hybridization of rat lung exposed to ozone for 20 months. Panels A and B: Sections of lungs from rats exposed to ozone (1.0 ppm for 20 months) and killed after cessation of exposure were hybridized with antisense ^{35}S-labeled RNA (A) for α1(I) procollagen mRNA or (B) for tropoelastin mRNA. No signal for either mRNA was detected. Arrows mark a few alveolar tips where signal for these mRNAs was detected in animals exposed for two months (see Figure 4). Similar negative findings for all mRNAs were found in all samples from rats exposed to 1.0 ppm ozone for 20 months regardless of when the animals were killed (see Table 2). Autoradiographic exposure was for 30 days. Magnification = × 100. Panels C and D: In about half of the samples examined, whether control or ozone-exposed, signal for α1(I) procollagen mRNA was detected in occasional interstitial cells (arrows) surrounding blood vessels (V) or large airways (AW), and the signal strength was quite similar in both (C) control lungs and (D) lungs exposed to ozone for 20 months. Autoradiographic exposure was for 30 days. Magnification = × 200.

Table 2. Summary of in Situ Hybridization Results[a]

Gene Product	Fetal Lung[b]	Pilot Experiment[c]		20-Month Exposure	
		Control	1.0 ppm Ozone	Control	1.0 ppm Ozone
Tropoelastin	+++	–	+	–[d]	–[d]
α1(I) Procollagen	+++	–	+	–[d]	–[d]
α1(III) Procollagen	+++	ND[e]	ND	–[f]	–[f]
Fibronectin	+	ND	ND	–[f]	–[f]
Rat collagenase	ND	ND	ND	–[f]	–[f]

[a] +++ = strong responses; + = weak response; and – = no response.

[b] An autoradiographic exposure of 7 to 10 days was used to detect an mRNA signal in fetal samples.

[c] Six samples (three males and three females) were used in all these studies. Autoradiographic exposure was from 14 to 21 days.

[d] Control rats and rats exposed to 1.0 ppm ozone (14 per group) were killed one week after the cessation of exposureand two sets of tissues were fixed (experiments 1 and 2). Tissue samples from rats killed immediately after cessation of exposure (see Table 1) were processed by in situ hybridization (experiment 3). In experiments 1 and 2, autoradiographic exposure was from 14 to 21 days; in experiment 3, it was from 21 to 30 days.

[e] ND = not determined.

[f] Samples from the rats killed immediately after cessation of ozone exposure (see Table 1) were hybridized for these mRNAs. Autoradiographic exposure was from 14 to 30 days.

hybridization assay is sufficiently sensitive to detect low levels of expression in occasional cells. Procollagen mRNA in these sections was confined to stromal cells within the adventitia of conducting arteries and the loose connective tissue surrounding large airways or blood vessels (Figure 3C and 3D). Unlike the fetal samples (Figure 2), an extended autoradiographic exposure (30 days) was required to detect procollagen signal in these areas. Because the fibrotic changes in ozone-exposed animals occur in the peri-alveolar stroma (Chang et al. 1992), expression in these large interstitial areas probably represents continued basal production of collagen.

IN SITU HYBRIDIZATION OF LUNGS EXPOSED TO OZONE FOR TWO MONTHS

If ozone mediates an early and transient fibrotic response, then increased matrix gene expression should be detectable at earlier exposures. Indeed, we detected selective matrix gene expression in animals exposed to ozone for two months. Samples from lungs of rats exposed to 0 or 1.0 ppm ozone for two months were obtained for our pilot studies and were reexamined with ^{35}S-labeled sense and antisense probes for tropoelastin and α1(I) procollagen mRNAs. Interestingly, individual septal cells in many alveoli of ozone-exposed lungs were strongly positive for tropoelastin mRNA (Figure 4A, 4B, 4C, and 4D). No signal was detected in alveolar cells in lungs from control rats (Figure 4E), but a few vascular wall cells were positive for tropoelastin mRNA (Figure 4F). Although the signal in vessel wall cells was quite weak, it nonetheless provided an internal positive control for hybridization. As for tropoelastin mRNA, individual septal cells in many alveoli of ozone-exposed lungs were strongly positive for α1(I) procollagen mRNA (Figure 5A), and no signal was detected in alveolar cells in lungs from control rats (Figure 5B). In both control lungs and rat lungs exposed to ozone for 20 months, weak signal was detected for procollagen mRNA in vascular wall cells and in the stroma surrounding airways (Figure 5B). No specific signal was detected in sections of ozone-exposed lungs hybridized with sense probe (findings not presented).

DISCUSSION

As discussed in the Introduction, chronic ozone exposure mediates an increase in lung connective tissue within the alveolar and terminal bronchial septa. In our studies, however, we detected no active expression of mRNAs for collagen types I or III, elastin, or fibronectin in these areas in lungs from rats exposed to 1.0 ppm ozone for 20 months. Weak signal was detected in occasional interstitial cells around large airways and blood vessels in some samples, both ozone-exposed and control, indicating that the samples were prepared properly and that the in situ hybridization assay is sufficiently sensitive to detect low levels of mRNA. As is discussed below, matrix expression is very

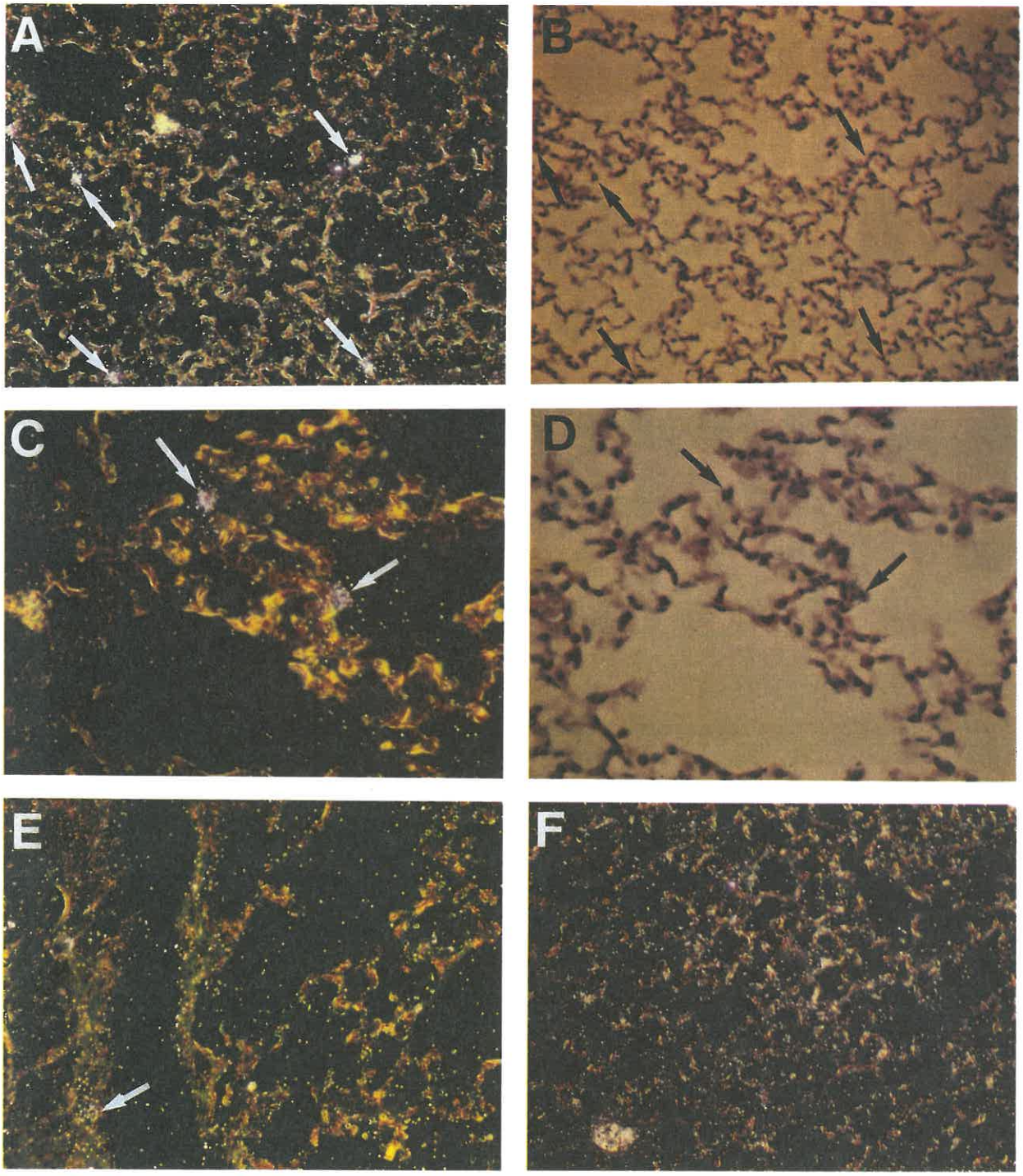

Figure 4. In situ hybridization for tropoelastin mRNA in lung samples from control rats and rats exposed to ozone for two months. Panels A and B: Lungs from ozone-exposed rats (1.0 ppm, two months) were hybridized with antisense ^{35}S-labeled RNA specific for tropoelastin mRNA. Specific signal for tropoelastin mRNA was detected in individual cells (arrows) of some alveoli. Magnification = × 100. Panels C and D: Higher magnification views showing two cells with positive signal for tropoelastin mRNA. Magnification = × 200. Panel E: In sections from age-matched control rats, weak signal for tropoelastin mRNA was seen in a few cells in the wall of an artery. Magnification = × 100. Panel F: No specific signal for tropoelastin mRNA was seen in alveolar areas in lungs from age-matched control animals. Magnification = × 100. Autoradiographic exposure was 10 days for all sections, and sections were counterstained with hematoxylin and eosin.

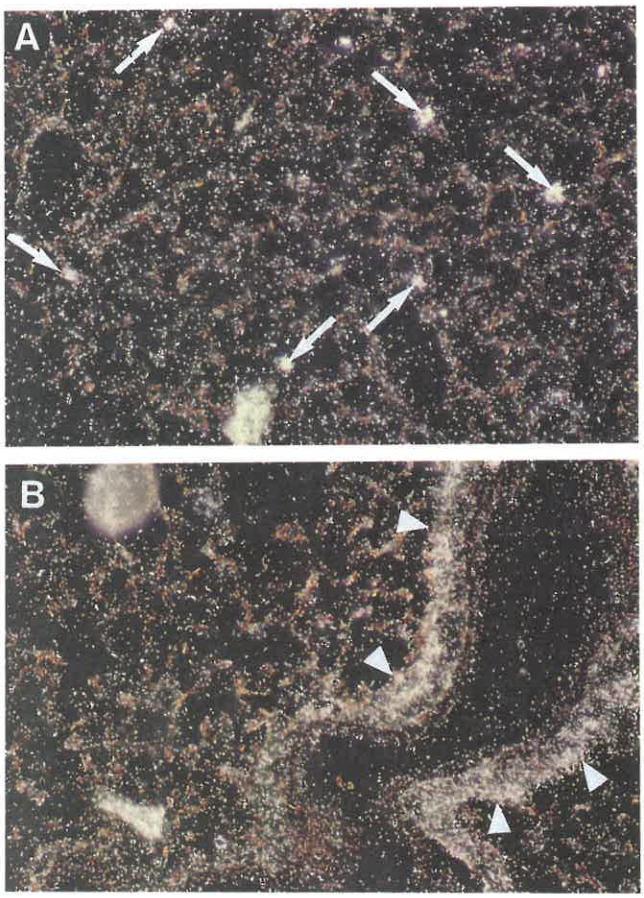

Figure 5. In situ hybridization for α1(I) procollagen mRNA of control rats and rat lungs exposed to ozone for two months. Panel A: Lungs from ozone-exposed rats (1.0 ppm, two months) were processed for in situ hybridization with antisense ^{35}S-labeled RNA probe for α(I) procollagen mRNA. Specific signal for procollagen mRNA was detected in individual alveolar cells (arrows). Magnification = × 100. Panel B: No signal was detected in alveolar areas, seen on the left half of the micrograph, of lung sections from control rats, but weak positive signal was evident in airway stromal cells (arrowheads). Magnification = × 100. Autoradiographic exposure was for 21 days, and sections were counterstained with hematoxylin and eosin.

low in mature, nonremodeling tissues. In light of findings from previous studies showing increased deposition of matrix in terminal airways in response to long-term ozone exposure (Hiroshima et al. 1989; Chang et al. 1992; Pinkerton and Mercer 1992), we interpret our findings to indicate that increased matrix production is an early and transient response to ozone and that at some time before 20 months, enhanced or renewed connective tissue protein biosynthesis had ceased.

The production of essentially all structural matrix molecules is limited to periods of tissue growth, and cellular expression typically is maximal during fetal and neonatal development (Clark et al. 1983; Davidson 1990; McGowen 1992; Parks et al. 1993). By maturity, synthesis has ceased, yet the deposited proteins, which usually are insoluble and resistant to proteases, remain in place and turn over at a minimal rate if at all (Dubick et al. 1981a; Davidson 1990). Hence, in adults, continued synthesis is not required to replace the extracellular matrix, and gene expression of extracellular matrix proteins is either very low or nonexistent. Thus, because basal levels in mature tissues are minimal to undetectable (Last 1983; Crouch and Parks 1992), increased focal production in response to injury may be detected biochemically in the whole lung. Using such strategies, investigators have shown that active collagen biosynthesis increases significantly in response to ozone (0.5 to 2.0 ppm), but in all studies, the response is transient, peaking as early as 3 days of exposure (Hussain et al. 1976a) and returning to control levels by 7 to 30 days of exposure (Hussain et al. 1976a; Last et al. 1979, 1984; Last and Greenberg 1980; Jakab and Bassett 1990). Although increased lung collagen content persists with chronic exposure, the production of this new matrix seemingly occurs during early stages of exposure.

The findings of many studies examining diverse parameters support the idea that ozone mediates early and transient responses in the lung. Lung permeability and wet weight and the influx of inflammatory cells increase in mice within five days of exposure to 0.5 ppm ozone (Jakab and Bassett 1990). In rats, alveolar cell proliferation, neutrophil and macrophage accumulation, and the activity of enzymes associated with energy-generating pathways were markedly enhanced after three days of continuous exposure to 0.75 ppm ozone (Bassett et al. 1988b). Indeed, many changes occur quite rapidly. In human volunteers, increased alveolar neutrophils and decreased forced expiratory volume in one second were seen after a one-hour exposure to 0.3 ppm ozone (Schelegle et al. 1991), and lung permeability increased in healthy, male nonsmokers exposed to 0.4 ppm ozone after just two hours with intermittent intense exercise (Kehrl et al. 1987). In fact, as determined by increased levels of neutrophils, prostaglandin E_2, alpha-1-proteinase inhibitor, fibronectin, and interleukin-6 in lavage collected from healthy men (Devlin et al. 1991), a dose as low 0.08 ppm ozone for 6.6 hours is sufficient to mediate an inflammatory reaction in the lung.

Because cytokines, such as transforming growth factor β1, released locally by migratory cells, mediate fibrosis and stimulate type I collagen production (Roberts et al. 1986; Crouch 1990; Davidson 1990; Broekelmann et al. 1991), the

rapid inflammatory response mediated by ozone may in turn mediate an enhanced matrix response soon after the start of exposure. Indeed, active collagen biosynthesis increases significantly by one day after exposure to 0.5 ppm ozone (Hussain et al. 1976a,b). Furthermore, the data presented in Figures 4 and 5 indicate that subchronic ozone exposure (1.0 ppm for two months) stimulates tropoelastin and procollagen gene expression in alveolar septal cells (Parks 1992; Last et al. 1993a). In these studies, we detected α1(I) procollagen and tropoelastin mRNAs in occasional alveolar cells. Because no signal for these mRNAs was seen in alveolar cells in age-matched control lungs, this expression was selective for ozone-exposed animals. Interestingly, the specific induction of tropoelastin and procollagen gene expression was seen in the same sites where excess deposition has been noted in recent morphometric studies (Chang et al. 1992).

Our findings and the studies by others discussed above support the idea that ozone mediates a transient yet sustained fibrotic response. In time course studies, the fibrotic response to ozone plateaus by 30 days of exposure and parameters of active collagen biosynthesis return to control levels, yet increased collagen mass is maintained at all subsequent points (Hussain et al. 1976a; Last et al. 1979, 1984; Last and Greenberg 1980; Jakab and Bassett 1990). This pattern of deposition suggests an adaptive response to ozone, which prevents progressive lung injury. Indeed, studies by Bromberg and coworkers (1991) and Hackney and associates (1977) indicate that ozone-induced changes in epithelial permeability and airway reactivity are reversed with chronic or repeated exposure.

Total matrix deposition is a balance of synthesis and degradation, and ozone may inhibit matrix turnover, thereby sustaining increased matrix levels during exposure. Because elevated levels of matrix deposition are maintained in chronically exposed animals, the newly formed connective tissue apparently is not turned over to any great extent. In fact, although the influx of inflammatory cells and altered lung function mostly are reversed during recovery from ozone, increased collagen content and morphologic changes, such as alveolar thickening, persist well after exposure has ceased (Gross and White 1986, 1987; Reiser et al. 1987; Bassett et al. 1988a). Consistent with these findings are our observations that interstitial collagenase was not expressed in the lungs of ozone-exposed animals. This metalloproteinase catalyzes the rate-limiting step in collagen breakdown, and hence, its absence suggests no appreciable turnover of lung collagen (Jeffrey 1986). This, however, is an equivocal conclusion because some cells store proteinases and do not actively transcribe them (Ståhle-Bäckdahl and Parks 1993). Thus, immunohistochemistry and enzyme activity assays would be needed to address the role of proteinases in ozone-exposed and recovered lungs adequately. Other groups have shown that ozone exposure is associated with an inactivation of serine protease inhibitors and an increase in protease activity (Johnson 1987; Pickrell et al. 1987a,b). Because these reports do not include a description of the nature or location of the proteolytic activities detected, it would be overly speculative to suggest a role of altered proteinase/antiproteinase balance in controlling lung connective tissue content in response to ozone.

Our studies to date mainly have focused on fibrillar collagens and elastin, but ozone may mediate a persistent upregulation of other extracellular matrix molecules. In response to our application for this project, the HEI Research Committee recommended that we examine expression of the α2 chain of type I collagen to assess differential expression of collagen subtypes. However, the type I collagen produced in vivo during fibrosis is qualitatively unchanged from that made during development (Crouch 1990). Thus, examination of α1(I) is a reliable predictor of expression of the α2(I) gene (Crouch and Parks 1992). Although the cross-link composition of newly deposited collagen in ozone-exposed lungs is different from that in control tissue (Reiser et al. 1987), this apparently is not due to variations in the relative amount of type I collagen chains or to differential expression of type III collagen (Last et al. 1984). Because basement membranes are thickened in ozone-exposed animals (Chang et al. 1992), proteins such as fibronectin, type IV collagen, entactin, and laminin may be influenced by chronic exposure. Although we found no evidence of increased expression of fibronectin mRNA in animals exposed for 20 months, additional studies will be needed to address the temporal response of basement membrane proteins adequately. However, we predict that fibrosis in all extracellular matrix compartments occurs early in response to ozone exposure.

Although our findings are consistent with an early and transient fibrotic response, there are other possible explanations for our negative results. Because RNAs are susceptible to degradation by intracellular and exogenous RNases, transcripts for matrix mRNAs could have been destroyed during the early stages of tissue processing. However, we were able to detect weak signals for tropoelastin and α1(I) procollagen mRNAs in occasional cells within the interstitium of airways and blood vessels in both control and ozone-exposed animals, and this sustained low level of expression in occasional cells is consistent with findings using normal rat and human adult lung tissue (Bruce 1991; Botney et al. 1992; Noguchi et al. 1992). Another potential criticism is that in situ hybridization is not sufficiently sensitive to detect low levels of mRNA, but in actuality, this is a reliable and

sensitive technique and reportedly can detect as little as one transcript per cell (Singer and Ward 1982; Lawrence and Singer 1985; Singer et al. 1986). For example, we have seen in various models a positive signal in just a few cells within normal tissues (Saarialho-Kere et al. 1992, 1993a,b), and using oligomeric probes, which have about a 100-fold lower specific activity than do transcribed RNA probes (Singer et al. 1986; Taneja and Singer 1987), we have been able to detect rare, alternatively spliced mRNA variants after a 14-day autoradiographic exposure (Parks et al. 1992). Thus, we are confident that our negative results reflect that elevated matrix production is not sustained with chronic ozone exposure. Still, alveolar interstitial cells may express matrix mRNAs below the level of detection of our assay, but the biologic significance of such low expression and its ability to contribute significantly to matrix deposition is questionable. Because minimal expression is maintained in areas that probably are not affected by ozone exposure, sensitive techniques, such as RNase protection and reverse transcription/DNA polymerase amplification, applied to isolated RNA would provide equivocal quantitative results and no information on the site of expression.

Although formalin perfusion would rapidly preserve cellular macromolecules, stress during the in vivo procedures performed before fixation may have led to enhanced mRNA degradation. However, in a bovine model of hypoxic pulmonary hypertension, in which the animals undergo extensive and invasive physiologic monitoring before lung fixation (by the same perfusion protocol used in these ozone studies), we have seen little evidence of significant mRNA degradation (Mecham et al. 1987; Stenmark et al. 1987; Parks et al. 1989; Prosser et al. 1989). Thus, we believe that our in situ hybridization results indicate that lung extracellular matrix expression is turned off or is maintained at undetectable levels in both the control and ozone-exposed rats. Although Northern blot hybridization may verify these negative findings, we do not have a source from which to extract RNA. Also, it is difficult, if not impossible, to isolate intact RNA from paraffin-embedded tissues.

Another possibility for our negative results is that the fibrotic response to ozone is not mediated by an increase in the steady-state levels of matrix mRNAs. In support of this idea, intracellular degradation of collagen is inhibited after short-term ozone exposure (Pickrell et al. 1987a,b), but seemingly this occurs in the presence of enhanced collagen biosynthesis (Hussain et al. 1976b; Last and Greenberg 1980; Jakab and Bassett 1990). Because type I collagen production is controlled primarily at the level of the gene (Slack et al. 1993), increases in active biosynthesis indicate that the steady-state levels of $\alpha 1(I)$ procollagen mRNA are up-regulated as well. Similarly, increases in elastin deposition and synthesis correlate with changes in the levels of tropoelastin mRNA (Parks et al. 1993). Our findings indicate that matrix mRNA levels are increased at early stages of ozone exposure, then are repressed at some later time. We cannot, however, determine the mechanisms involved in controlling stimulated matrix deposition during periods of active synthesis, and we do not have the appropriate samples to temporally correlate mRNA levels with matrix synthesis and deposition. Because no mRNA was detected in affected areas in the samples from animals exposed for 20 months, we can safely assume that no protein was being translated at this time point.

In conclusion, the findings from our studies suggest the hypothesis that ozone inhalation alters lung matrix gene expression. Our findings also indicate that ozone mediates a transient and early fibrotic response in the lung. Additional studies using animals exposed to ozone for shorter periods would be required to address this hypothesis.

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Dr. William C. Parks received his Ph.D. in anatomy and cell biology in 1982 from the Medical College of Wisconsin. He had postdoctoral training in carcinogenesis at Michigan State University with Drs. Veronica Maher and Justin McCormick, and in connective tissue biology at Washington University with Dr. Robert Mecham. Currently, Dr. Parks is an Assistant

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PUBLICATIONS RESULTING FROM THIS RESEARCH

Parks WC. 1992. Localization of tropoelastin and type I procollagen expression in lungs after short-term ozone exposure. In: New Methods in Ozone Toxicology: Abstracts of Six Pilot Studies, pp. 12–14. HEI Communications Number 1. Health Effects Institute, Cambridge, MA.

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ABBREVIATIONS

cDNA	complementary DNA
cpm	counts per minute
FEV_1	forced expiratory volume in one second
mRNA	messenger RNA
NTP	National Toxicology Program
^{32}P	phosphorus-32
PBS	phosphate-buffered saline
ppm	parts per million
^{35}S	sulfur-35
SDS	sodium dodecyl sulfate
SSC	standard saline citrate
Tris	2-amino-2-hydroxymethyl-1,3-propanediol
tRNA	transfer RNA
$\alpha[^{35}S]UTP$	$\alpha[^{35}S]$uridine triphosphate

INTRODUCTION

Clinical and epidemiologic studies provide evidence that young adults or children who are exposed for short time periods to ozone while exercising experience transient decrements in lung function (reviewed by Lippmann 1993). Whether repeated or prolonged inhalation of ozone produces long-term decrements in lung function or aggravates existing chronic lung disease is not known. Because of substantial uncertainties regarding the health risks of extended exposure to ozone, and the widespread exposure to this pollutant, the Health Effects Institute (HEI) and the National Toxicology Program (NTP)* entered into a collaboration (described briefly in the Introduction to this Research Report) to evaluate the effects of prolonged exposure of laboratory animals to ozone.

Dr. William C. Parks, of the Washington University Medical Center, St. Louis, MO, was one of six investigators selected to test the feasibility of their methods in pilot studies on rats exposed to ozone for two months (Parks 1992). After completing his pilot study, Dr. Parks submitted an application in response to RFA 90-1, Effects of Chronic Ozone Inhalation: Collaborative National Toxicology Program/Health Effects Institute Studies, Part B: Structural, Biochemical, and Other Alterations. The title of his application was "Extracellular Matrix Expression in Ozone-Exposed Lungs." The HEI Research Committee viewed a study of extracellular matrix as forming an important component of an integrated program of studies of ozone's effects on lung structure, function, and biochemistry. They approved the two-year study, which began in June 1991, at a total cost of $183,877. Dr. Parks' final report was received at HEI in September 1993, and his revised report was accepted for publication by the HEI Review Committee in April 1994.

During the review of the Investigators' Report, the Review Committee and the investigator had the opportunity to exchange comments and clarify issues in the Investigators' Report and the Review Committee's Commentary. The following Commentary is intended to serve as an aid to the sponsors of HEI and the public by highlighting both the strengths and limitations of the study. Other reports from investigators in the NTP/HEI Collaborative Ozone Project present the biochemical, structural, and functional data, and discuss their implications for human health.

* A list of abbreviations appears at the end of the Investigators' Report for your reference.

REGULATORY BACKGROUND

The U.S. Environmental Protection Agency (EPA) sets standards for oxidants (and other pollutants) under Section 202 of the Clean Air Act, as amended in 1990. Section 202(a)(1) directs the Administrator to "prescribe (and from time to time revise) . . . standards applicable to the emission of any air pollutant from any class or classes of new motor vehicles or new motor vehicle engines, which in his judgment cause, or contribute to, air pollution which may reasonably be anticipated to endanger public health or welfare." Sections 202(a), (b)(1), (g), and (h), and Sections 207(c)(4), (5), and (6) impose specific requirements for reductions in motor vehicle emissions of certain oxidants (and other pollutants) and, in some cases, provide the EPA with limited discretion to modify those requirements.

Section 109 of the Clean Air Act provides for establishing National Ambient Air Quality Standards (NAAQS) to protect the public health. Ozone's potentially harmful effects on respiratory function led the EPA to promulgate the NAAQS for ozone of 0.12 parts per million (ppm), a level not to be exceeded for more than one hour once per year. Section 181 of the Act classifies the 1989 nonattainment areas according to the degree that they exceed the NAAQS and assigns a primary standard attainment date for each classification.

The current ozone standard relies heavily on data derived from controlled human exposure studies that have demonstrated lung dysfunction after short-term exposure of human subjects to ozone while exercising. These studies do not address the issue of potential long-term health effects, such as chronic lung injury resulting in interstitial fibrosis. Determining the appropriate standards for emissions of oxidants and their precursors depends, in part, on an assessment of the risks to health that they present. Therefore, research into the effects on the lungs of prolonged exposure to ozone, such as that supported by the NTP/HEI Collaborative Ozone Project, is essential to the informed regulatory decision-making required by the Clean Air Act.

SCIENTIFIC BACKGROUND

The extracellular matrix of connective tissue is an organized meshwork of macromolecules that serves as a universal "glue" to hold and support cells (Alberts et al. 1983). In the lungs, connective tissue provides tissue integrity and elasticity, and serves as a barrier against potentially harmful agents.

Lungs undergo large changes in volume during each respiratory cycle. This unique function requires a special-

ized structure for the connective tissue components. For example, the walls of the alveoli must be thin enough to allow the exchange of gases, such as oxygen and carbon dioxide, between the air spaces and the oxygen-carrying blood cells; yet they also must be firm enough to support the alveolar cells, and flexible enough to cope with the changes in lung volume during breathing. The connective tissues that lie between the different cell types of the lung fulfill the requirement for these properties (reviewed by Juul et al. 1991).

The major protein components of lung connective tissue are collagen, elastin, and fibronectin. The study by Dr. Parks and Ms. Roby addressed ozone's effects on the expression of genes that provide information (or coding) so that these three proteins can be synthesized. This Background provides a short introduction to each of these proteins; it continues with a discussion of fibrosis and emphysema, including the changes in connective tissue proteins that may influence the development of these diseases; and summarizes current information concerning the effects of ozone on fibrosis and emphysema. The Background section concludes with a discussion of the advantages of using in situ hybridization, as the investigators did, to estimate the level of synthesis of specific proteins at different time points.

COLLAGEN

The collagens are a family of fibrous proteins that contain three polypeptide chains wound around each other to form a triple-helical molecule called procollagen. Fourteen collagen types have been described (reviewed by van der Rest and Garrone 1991). Of these, types I and III collagen account for the majority of collagen in the lung interstitium in all mammalian species, and are produced by interstitial fibroblast cells and smooth muscle cells (Crouch and Parks 1992).

After procollagen is secreted from the cell, large collagen fibrils form extracellularly by spontaneous aggregation of individual procollagen molecules. Finally, the fibrils are strengthened by forming crosslinks both within and between the constituent collagen molecules. Collagen fibrils can ultimately aggregate into larger bundles, called collagen fibers, that can contain both types I and III collagen (Weibel and Crystal 1991). It is this mature collagen that confers tensile strength to lung tissue.

ELASTIN

Elastin is one component of elastic fibers found where elastic recoil is critical to tissue mechanics as in the lungs (Mecham et al. 1991; Weibel and Crystal 1991). The elastin precursor, tropoelastin, is secreted from the cell as a single polypeptide chain. However, in a manner similar to the formation of collagen fibrils, the final steps in elastin synthesis occur extracellularly by the formation of fibers containing individual tropoelastin molecules crosslinked to one another. In addition to elastin, elastic fibers contain a matrix of glycoproteins that are believed to serve as a scaffolding for the deposition of newly formed elastin (Crouch and Parks 1992).

FIBRONECTIN

Fibronectin is found in the lung interstitium and basement membranes (highly specialized extracellular matrix structures that lie beneath the epithelial and endothelial cells and provide a structural framework to which these cells attach). The precise biological role of fibronectin in the lung is not fully established; however, experiments conducted in vitro have demonstrated fibronectin's ability to influence cellular and extracellular events. For example, specific regions of fibronectin bind to glycosaminoglycans and collagen; in this way fibronectin helps to organize matrix components into a cohesive meshwork (Alberts et al. 1989). Other regions of fibronectin bind to different cell types. Thus, fibronectin's capacity to bind to both cells and extracellular matrix constituents facilitates the cells attaching to the matrix. Most cells in culture need to be "anchored" to extracellular matrix components to survive and grow. Therefore, fibronectin's binding properties may stabilize cells and enhance critical cellular events such as proliferation and differentiation (reviewed by Roman and McDonald 1991).

CHANGES IN CONNECTIVE TISSUE PROTEINS IN FIBROSIS AND EMPHYSEMA, AND POSSIBLE RELATIONS WITH OZONE EXPOSURE

Pulmonary Fibrosis

As discussed above, connective tissue is vital for maintaining structural integrity; however, an excess of lung connective tissue can distort normal lung structure and decrease the lung's efficiency for gas exchange. When a tissue responds to injury and heals its wounds, inflammatory cells (such as macrophages) accumulate at the site of injury. Macrophages release proteins, called growth factors, that stimulate connective tissue cells to proliferate and create a connective tissue scar. According to Crystal and colleagues (1991), the biologic basis of pulmonary fibrosis is similar to wound healing. However, whereas dermal wounds are localized and self-limited, lung injury is a diffuse, ongoing process that progresses as long as the stimulus to injury is present.

In chronic lung injury, inflammatory cells (such as macrophages) are recruited to the lung and become activated. As discussed above, they release growth factors to make scars. On the other hand, they also release elevated amounts of oxidant species that damage cells in the alveolar wall, and protease enzymes that degrade matrix constituents such as collagen and elastin. In addition, because activated macrophages produce more growth factors than do normal macrophages the excess production of matrix proteins that characterizes pulmonary fibrosis is augmented.

The concept of pulmonary fibrosis evolved from the observation of increased numbers of collagen fibers in the thickened alveolar walls of patients with fibrotic diseases (Crystal et al. 1991). By staining with antibodies for types I and III collagens, it was determined that type III collagen predominated in the early stages of pulmonary fibrosis; however, type I collagen predominated in the later stages of disease (Bateman et al. 1981; Raghu et al. 1985).

Animal models of lung fibrosis also have demonstrated changes in the ratio of types I and III collagen (Quinones and Crouch 1986), in type I procollagen gene expression (Kelley et al. 1985; Raghow et al. 1985), and in types I and III collagen biosynthesis (Reiser and Last 1981). Because type I collagen is less compliant than type III collagen, an increase in type I collagen may be responsible for the fibrotic lungs' increased stiffness and decreased ability to expand. Both the content of elastin and elastin gene expression increased in an animal model of lung fibrosis (Starcher et al. 1978; Raghow et al. 1985); however, the significance of elevated elastin levels in pulmonary fibrosis is unclear.

The fibronectin found in tissues of healthy adults is produced predominantly in the liver and secreted into the blood for transport to tissues such as the lung (Roman and McDonald 1991). However, animal models of lung fibrosis have shown increased fibronectin gene expression and fibronectin accumulation in the lung (Kelley et al. 1985; Raghow et al. 1985; Bray et al. 1986). An interaction between fibronectin and type I procollagen was suspected after Kuhn and colleagues (1989) examined lung biopsy specimens from patients with chronic idiopathic fibrosis. Because fibronectin was localized to the area of type I procollagen synthesis, and the level of fibronectin and procollagen I gene expression paralleled each other in experimental fibrosis (Kelley et al. 1985; Raghow et al. 1985), Kuhn and colleagues (1989) proposed that fibronectin is involved in assembling collagenous matrices.

Ozone is a powerful oxidant with the potential to damage lung cells. The inflammatory cells recruited to the lung as a response to ozone exposure can increase the damage initiated by ozone by releasing their content of oxidant species and degradative enzymes (Mustafa 1990). Because the lungs' predominant inflammatory cell response to ozone is the recruitment of alveolar macrophages, an increased level of macrophage-derived growth factors could cause an elevated deposition of collagen and other connective tissue components.

Biochemical studies of ozone's effects on lung collagen content, type, rate of biosynthesis, and degree of crosslinking in rats and monkeys were discussed in the HEI's Review Committee Commentary to Part I of this Research Report (Last et al. 1994). The results of several investigations are contradictory because some investigators found increases in collagen content and biosynthetic rate (Last et al. 1979, 1984; Reiser and Last 1981), and others did not (Filipowicz and McCauley 1986; Wright et al. 1988). Because fibrotic changes in lungs are often focal, methods that can distinguish localized changes, such as histochemistry and electron microscopic morphometry, may be more useful in detecting ozone-induced matrix alterations. The results of three such studies suggesting that collagen deposition increased as a response to prolonged exposure to ozone (Chang et al. 1992; Last et al. 1994; Chang et al. 1994) are discussed below in the section Methods to Determine Ozone's Effect on Connective Tissue Components. (The studies of Last and colleagues [1994] and Chang and associates [1994] were part of the NTP/HEI Collaborative Ozone Project.)

Little information is available regarding ozone's effect on fibronectin content. McKinnon and colleagues (1992) observed that human bronchial epithelial cells in culture secreted fibronectin in response to ozone exposure.

Emphysema

Emphysema, the major cause of chronic obstructive airway disease, is characterized by the permanent enlargement of the alveolar air spaces, destruction of alveolar walls, and the absence of fibrosis (reviewed by Snider et al. 1985). The mechanism underlying emphysema appears to be the decrease in the lung's elastic recoil properties caused by the destruction of alveolar elastin by elastase, an enzyme released by inflammatory cells (Kuhn et al. 1976; Foster et al. 1989). To a certain extent, elastin is protected by alpha-1-proteinase inhibitor, a blood plasma protein that inhibits the activity of elastase.

One hypothesis of how ozone could cause emphysema is that it increases the degradation of lung elastin by inactivating alpha-1-proteinase inhibitor (Johnson 1980; Pickrell et al. 1987). However, the results of recent experiments by Johnson and associates (1990) do not support this hypothesis. An alternative hypothesis that could account for enhanced degradation of lung connective tissue proposes that ozone alters the structure of connective tissue components, making them more susceptible to degradative enzymes. An

example of ozone-induced alterations in lung connective tissue components is provided by Dr. Radhakrishnamurthy (1994), an investigator in the NTP/HEI Collaborative Ozone Project. Dr. Radhakrishnamurthy reported that the glycosaminoglycan heparan sulfate, isolated from rat lung tissue exposed to 0.5 ppm or 1.0 ppm ozone, was chemically altered compared with that in normal lung tissue.

Investigators studying the content of lung elastin after acute and subchronic exposures of rodents to ozone have obtained conflicting results. Costa and colleagues (1983) observed that 12-week exposure to high (0.8 or 2.0 ppm) ozone levels increased elastin deposition in rat lungs. In the same study, however, elastin deposition decreased after exposure to 0.2 ppm ozone. Other investigators observed a decrease (Damji and Sherwin 1989) or no change (Dubick et al. 1981; Myers et al. 1984; Last et al. 1993) in lung elastin content after exposure periods of 1 to 11 weeks at ozone levels ranging from 0.25 to 1.5 ppm. The results of the NTP/HEI Collaborative Ozone Project suggest that a 20-month exposure to 1.0 ppm ozone caused an increased content of elastin in the proximal alveolar region of rat lungs (Chang et al. 1994). The conflicting results obtained by different investigators may be due to differences in species, duration of ozone exposure, or ozone concentrations.

METHODS TO DETERMINE OZONE'S EFFECT ON CONNECTIVE TISSUE COMPONENTS

Many studies of ozone's effects on lung collagen or elastin have used whole lung lobes. As discussed above, fibrotic changes may be localized to specific lung areas; therefore, the normal background level of collagen and elastin in lung lobes may obscure small but important focal changes. For example, in Part I of this series of Research Reports from the NTP/HEI Collaborative Ozone Project, Dr. Jerold Last and colleagues (1994) found no statistically significant ozone-induced effects in male rats. Statistically significant increases in collagen content and in the level of one specific crosslink were seen in female rats exposed to 1.0 ppm ozone when the data were expressed per lung lobe; however, these results are difficult to interpret, because the increases were not evident if the normalization parameters were changed.

The advantage of using methods that detect local changes in collagen content became apparent from Dr. Jack Harkema's histochemical analyses of lung tissue from ozone-exposed rats (included in Last et al. 1994). Using tissue from a small sample of animals, Dr. Harkema found that male and female rats exposed to 0.5 ppm or 1.0 ppm ozone had moderate to marked levels of stainable collagen in the centriacinar region, compared with controls. Although some control rats breathing clean air also showed small increases in collagen deposition, Dr. Harkema's results suggest that experimental approaches that pinpoint focal changes can lead to a more definitive picture of ozone's effect on the extracellular matrix of connective tissue than may be obtained by measuring the total content of a matrix constituent. Studies using electron microscopic morphometry have provided evidence that prolonged exposure to 0.5 ppm or 1.0 ppm ozone (Chang et al. 1994), or to a simulated urban pattern of ambient ozone (a background level of 0.06 ppm rising to a peak of 0.25 ppm and returning to 0.06 ppm) (Chang et al. 1992), caused an increased deposition of collagen that was localized to the proximal alveolar region of rat lungs.

A sensitive method used to assess the degree of active production of matrix proteins in localized areas is a technique called in situ hybridization, which detects the presence of specific messenger ribonucleic acid molecules (mRNAs) within cells. Because chronic lung injury is heterogeneous, the ability to assess local changes in connective tissue biosynthesis offers the advantage of discriminating individual cells that produce specific matrix molecule genes. Messenger RNAs are critical to the synthesis of a protein because they transmit the information encoded in the DNA to a cell's machinery for synthesizing protein.

Sensitive staining reactions, such as immunohistochemistry, can identify the presence of specific proteins; however, they cannot differentiate between preexisting protein and newly synthesized protein. In addition, because many proteins are rapidly secreted from cells, sometimes to areas far from their sites of synthesis, staining techniques cannot reliably identify their synthetic site. In contrast, identifying mRNAs that code for specific proteins provides important information concerning a cell's current function. Gene expression changes in cells due to a number of factors, and identification of mRNAs that code for specific proteins provides information about the expression of specific genes. By identifying the cell producing the mRNA, in situ hybridization provides information about the cells that are expressing specific genes. Immunohistochemical studies to detect the protein produced can be used in conjunction with in situ hybridization to define the sites of synthesis of specific proteins.

In situ hybridization requires researchers to synthesize RNA molecules whose structures are complementary to the mRNAs of interest. Because of their complementarity, these RNA molecules bind (hybridize) to mRNAs within cells and can be used as molecular probes for their presence. The degree of hybridization reflects the level of a specific mRNA and allows a better understanding of the synthetic capability of a specific cell type at a specific time.

Dr. Parks and Ms. Roby utilized in situ hybridization to determine if ozone inhalation affected the rat lung's content of mRNAs coding for procollagens I and III, tropoelastin,

and fibronectin. Because the content of a protein reflects a balance between synthesis and degradation, the investigators also examined lung tissue for the presence of the mRNA for collagenase, an enzyme that degrades collagen.

JUSTIFICATION FOR THE STUDY

The Health Effects Institute's primary objective for RFA 90-1 was to support biochemical, structural, and functional studies to determine whether prolonged inhalation of ozone causes changes in the respiratory system of rats that might be related to chronic lung disease in humans. Because these changes include alterations in connective tissue extracellular matrix proteins, an ad hoc review panel concluded that understanding the control of matrix protein synthesis was important for understanding the effects of prolonged ozone exposure. In situ hybridization was considered to be an excellent tool for these studies because of the anticipated focal nature of the ozone-induced changes and the ability of this technique to identify qualitatively the level of gene expression in the connective tissue matrix.

OBJECTIVES AND STUDY DESIGN

The overall objective of this study was to determine if prolonged ozone exposure influences the expression of genes that code for lung extracellular matrix proteins. To assess the possibility that continual ozone exposure is required for matrix gene expression, Dr. Parks and Ms. Roby examined the lungs of animals exposed to ozone for 20 months and killed immediately after exposure or after one week of recovery in clean air.

The specific aims were to:

1. Determine the presence of mRNAs that code for tropoelastin, α1(I) procollagen, α2(I) procollagen, α1(III) procollagen, α1(IV) procollagen, fibronectin, and matrix proteinases, including collagenase, in control rats and in rats exposed to ozone for 20 months.
2. Identify the cells that produce mRNAs.

Because of the generally negative results obtained in their initial experiments (discussed below), Dr. Parks and Ms. Roby added two more aims.

3. Determine the presence of mRNAs that code for tropoelastin and α1(I) procollagen in tissue from rats exposed to 0 or 1 ppm ozone for two months. (These experiments used tissue samples remaining from Dr. Parks' pilot study.)
4. Determine the presence of mRNAs that code for tropoelastin, α1(I) procollagen, α1(III) procollagen, and fibronectin in fetal lung tissue.

The investigators used in situ hybridization with specific, complementary, radioactively labeled RNA probes to determine the presence of the mRNAs that code for the matrix structural proteins. Identifying the mRNA for the degradative enzyme collagenase was included to evaluate whether changes in the content of connective tissue proteins could be affected by the level of protein turnover (the balance between synthesis and degradation). Autoradiography was used to determine the presence of hybridized complexes of the radioactive probes and mRNAs.

In his original application, Dr. Parks also proposed to perform immunohistochemical analyses. Tissues studied by in situ hybridization would be stained with antibodies specific for intracellular proteins such as α and γ actins to identify smooth muscle cells, and cytokeratin to identify fibroblasts; positive staining for actins and cytokeratin would identify myoepithelial cells. As sections were prepared for in situ hybridization, serial sections would be prepared and stored. After data were gathered by in situ hybridization, sections corresponding to those with a positive signal for matrix mRNAs would be analyzed by immunohistochemistry.

To determine whether ozone exposure may have mediated an early, transient effect on matrix gene expression, Dr. Parks reexamined the level of mRNAs that code for connective tissue extracellular matrix proteins in the anterior right lobe (rather than the accessory lobe used in the 20-month study). He used tissues from a set of rats that had been exposed to 0 or 1.0 ppm ozone for six hours per day, five days per week, for two months in his earlier pilot study (Parks 1992). Rats had been killed three days after ozone exposure ceased, and the lung tissue had been shipped to Dr. Parks' laboratory after being processed at Battelle Pacific Northwest Laboratories. These experiments were performed on tissue that had been fixed and embedded in paraffin, but not used in the earlier study (Parks 1992).

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

The investigators partially attained their objectives for specific aim 1 for the 20-month ozone exposure. They did not test for the presence of each mRNA at all three ozone levels because, as discussed below, they obtained negative results in the initial series of experiments at the highest ozone concentration, which suggested that lower concentrations would produce similar results. Because of these negative results, the investigators did not perform immuno-

histochemistry to identify the cells producing mRNAs (specific aim 2). In addition, the investigators reported that a suitable assay for α1(IV) procollagen was not available.

The investigators repeated their in situ hybridization experiments with tissue from rats exposed to ozone for two months (specific aim 3), and identified mRNAs in fetal lung tissue (specific aim 4).

ASSESSMENT OF METHODS AND STUDY DESIGN

The inhalation component of this project was conducted in compliance with the NTP Laboratory health and safety regulations, and with the Food and Drug Administration Good Laboratory Practice Regulations. Animal care and use were in accordance with the Public Health Service Policy on Humane Care and Use of Animals.

In situ hybridization is a highly sensitive method for detecting specific mRNAs. The investigators tested their probes to verify that they were specific for the mRNAs of interest, and used nonspecific probes as negative controls. (See the Investigators' Report for a technical discussion of these methods.) Because matrix gene expression could wane after ozone exposure ceased, several RNA probes were tested with tissue from (1) rats that were killed immediately after ozone exposure and (2) rats that breathed clean air for one week. The investigators assessed the degree of hybridization between their probes and the mRNAs by visually inspecting autoradiograms and comparing the abundance and location of silver grains in tissues from control and ozone-exposed lungs. By extending the time of autoradiographic exposure of their tissue slides to 30 days, the investigators considered that sufficient time had elapsed for any biologically significant reaction to be visualized. To strengthen the quality control of their in situ hybridization experiments, the investigators performed appropriate control experiments with nonspecific probes for the mRNAs of interest (see the Investigators' Report for a description of these analyses).

The investigators used two positive controls in this study. First, fetal lung tissue, which produces significant amounts of extracellular matrix components, was tested with RNA probes that also were used with lung tissue from the 20-month exposure. Second, because Dr. Parks' pilot study of lung tissue from rats exposed to ozone for two months provided positive results (Parks 1992), the investigators repeated these experiments and compared the effects of acute (2-month) and chronic (20-month) ozone exposure on the expression of matrix protein mRNAs. However, several differences in experimental design must be considered if the 2-month and 20-month exposure results are compared. First, different sets of F344/N rats were used for the two exposures. Second, Dr. Parks received the anterior right lung lobe for the 2-month study; the accessory lobe was used for mRNA analyses after the 20-month exposure. Third, because each exposure began with rats of the same age, those studied after 2 months were one and a half years younger than the animals studied after 20 months of exposure. Finally, tissues from only six rats were available to Dr. Parks after the 2-month exposure; in contrast, most experiments on rats exposed for 20 months were performed on tissue from 14 animals per exposure group.

STATISTICAL METHODS

The investigators did not quantify their results for two principal reasons. First, because the data for the major focus of this study, the 20-month ozone exposure period, were negative, there were no data to quantify. Second, the HEI Health Research Committee recognized that methods for quantifying in situ hybridization studies have not been adequately developed and recommended that Dr. Parks concentrate on localizing the effects, rather than on quantifying them.

RESULTS AND INTERPRETATION

Fetal Rat Lung Tissue

Fetal lungs synthesize large amounts of connective tissue during development; therefore, this tissue provided a positive control for testing the ability of probes to hybridize with connective tissue mRNAs. The investigators found a strong signal for tropoelastin mRNA in medial cells of developing blood vessels, a moderate signal in the stroma of airways, and a diffuse signal in alveolar stromal areas. Signals for α1(I) and α1(III) procollagen mRNAs were found in the same cells that were positive for tropoelastin mRNA.

Ozone Exposure for 20 Months

The investigators found weak autoradiographic signals for α1(I) procollagen and tropoelastin mRNAs in occasional cells in the interstitial areas of large airways and in conducting blood vessels; however, the location and strength of the signals did not differ between animals exposed to 1.0 ppm ozone and control animals. Probes for tropoelastin and α1(I) procollagen mRNAs were tested with lung tissue from animals killed immediately after ozone exposure ceased, and with tissue from rats that breathed clean air for one week after exposure, with similar negative results. No signal was obtained with probes for α1(III) procollagen, fibronectin, or collagenase in lung tissue from rats exposed to 1.0 ppm ozone compared with

control rats. Probes for these three mRNAs were tested only with tissue from rats killed immediately after exposure ceased (see Table 2 in the Investigators' Report for a summary of the in situ hybridization results).

Because little response was seen in lung tissue exposed to the highest ozone level (1.0 ppm ozone) compared with controls, tissue samples from rats exposed to lower ozone levels (0.12 and 0.50 ppm ozone) were not studied in detail. However, to determine if exposure to lower ozone levels caused elevated levels of mRNAs, the investigators tested one probe (for tropoelastin mRNA) with lung tissue from rats exposed to the two lower levels of ozone and killed immediately after exposure ceased. The results of these experiments also proved negative.

Because weak signals for tropoelastin and α1(I) procollagen mRNAs were detected in occasional cells in the interstitium of large airways and in conducting blood vessels, degradation of mRNA in tissue samples was not considered to be an explanation for the lack of a stronger signal after 20 months. These findings also suggest the possibility that a continued, low-level production of these matrix proteins occurred over an extended time.

Ozone Exposure for Two Months

The investigators reexamined lung tissue from their earlier pilot study of rats exposed to 0 or 1.0 ppm ozone for two months. Only a small number of animals were available for these experiments; however, positive results were obtained with tissue from four of six animals. Individual cells in many alveoli of ozone-exposed lungs were strongly positive for tropoelastin and α1(I) procollagen mRNAs. No signals were detected in alveolar cells from control rat lungs. Comparing these results with those from the 20-month study suggests that ozone exposure may have caused an early response that disappeared between 2 and 20 months. However, it cannot be excluded that differences in experimental design (which were not under the investigators' control) may account, in part, for the different outcomes observed at 2 months and at 20 months of exposure.

IMPLICATIONS FOR FUTURE RESEARCH

Two major issues have important implications for future research on the effects of ozone on lung connective tissue. The first concerns the observation that matrix genes may be expressed transiently after ozone exposure. If this observation is confirmed by future studies, the difference in levels of gene expression seen after 2- and 20-month exposures to 1.0 ppm ozone suggest that the lung possesses mechanisms for decreasing matrix gene expression despite continued exposure to ozone levels that cause a biological response. In this case, future studies could be directed toward better defining the time at which genes expression is decreased by studying more experimental timepoints during prolonged ozone exposure. The experimental design mandated by the NTP protocol did not allow the investigators to measure mRNA levels at a series of time points during the 20-month exposure. However, future studies measuring mRNA levels at earlier time periods are needed to address the temporal nature of the response.

Future research also should seek to determine the cellular and molecular mechanisms for the increased expression of connective tissue genes seen in lung tissue from rats exposed to ozone for two months, including identifying possible mediators that influence matrix synthesis and their cells of origin. Because the low level of matrix mRNAs seen after 20 months did not differ between control and ozone-exposed lung tissue, a second major observation from this study is that the deposition of collagen and elastin in the proximal alveolar region of rats exposed to ozone for prolonged periods (Chang et al. 1992, 1994) may be caused by mechanisms other than a continued high level of matrix gene expression. For example, alternative mechanisms could involve increased efficiency of information transfer from mRNAs to other elements of the protein synthetic mechanism, and nongenetic changes such as perturbations in the activity of enzymes that degrade matrix components. The latter mechanism would stabilize newly synthesized matrix because of decreased turnover. Thus, if researchers obtain definitive evidence for ozone-induced increases in connective tissue deposition, investigations of collagenase activity could help determine whether decreased degradation of matrix components plays a role.

CONCLUSIONS

The most important conclusion drawn from the 20-month exposure study is that the expression of genes that code for lung tropoelastin and α1(I) procollagen is not increased by ozone exposure. The similarity between the low levels of tropoelastin and α1(I) procollagen mRNAs in control rats and in rats exposed to 1.0 ppm ozone suggests that a continuous basal synthesis of these proteins occurs that is not related to ozone exposure.

However, the results of a smaller pilot study of rats exposed to 1.0 ppm ozone for 2 months indicate that at this time point the expression of genes coding for tropoelastin and α1(I) procollagen was higher than in control animals. The difference in gene expression between the 2-month and 20-month exposures implies that the lung may possess mechanisms for decreasing

matrix gene activity during prolonged exposure to ozone. However, because the two studies were not conducted concurrently and differed in experimental design, these intriguing results must be interpreted with caution and require further experimental validation.

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