Communication

Hyperoxic Exposure Alters Gene Expression in the Lung

INDUCTION OF THE TISSUE INHIBITOR OF METALLOPROTEINASES mRNA AND OTHER mRNAs*

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Exposure to high concentrations of oxygen can result in tissue damage, particularly in the lung. Lung pathology induced by hyperoxia includes changes in lung cell populations and morphology. Presumably, alterations in gene expression underlie some of these cellular changes. In order to better understand the molecular basis of these events, a cDNA library was constructed from the mRNA of the lungs of a hyperoxia-exposed rabbit and differentially screened for clones corresponding to hyperoxia-induced messages. This approach has led to the isolation of four clones, three of which are presented in this communication. One clone corresponds to a message whose steady state levels were induced 6-fold and encodes the tissue inhibitor of metalloproteinases, a protein that plays a key role in the regulation of connective tissue turnover in some cells and potentiates erythroid development in others.

Therapy with supraphysiological concentrations of oxygen (O2) is required in a number of clinical situations, but this use of O2 may be accompanied by tissue damage, particularly to the lung, which receives direct exposure (1). In virtually all animals in which lung injury from hyperoxic exposure has been studied, a specific pathology has been described (2, 3) which includes changes in alveolar membrane permeability causing edema and severe decreases in respiratory function (4). At the cellular level, the first visible signs of hyperoxic lung injury are a swelling of alveolar epithelial cells and an altered morphology of mitochondria and microsomes (5). Lung cell populations shift as a result of hypertrophy and hyperplasia of many cell types, and there is an inflammatory response (6). The initiating events in this process are probably mediated by highly reactive O2-derived molecules (1). Much attention has been paid to the induction of antioxidant defense mechanisms by hyperoxic lung injury (1, 4). Little is known, however, about the biochemical basis of the alterations in lung cell growth and morphology (7-9).

Studies in our laboratories (10) and others' (11) have characterized changes in pulmonary histology, mechanics, permeability, surfactant status, and antioxidant enzyme activity during and following a sublethal exposure of rabbits to high O2 concentrations. In order to better understand the molecular basis of the tissue response to hyperoxia, the studies described in this report utilized the hyperoxic rabbit model to take a novel approach to identifying the specific changes in gene expression that accompany hyperoxic lung injury. A number of genes were found to be induced by hyperoxia, one of which encodes a protein likely to play a key role in the remodeling of injured lung tissue, the tissue inhibitor of metalloproteinases.

EXPERIMENTAL PROCEDURES

Rabbits and RNA Isolation—Experiments were performed on New Zealand White rabbits (Hazelton) weighing approximately 2 kg. As previously described (14), rabbits were exposed to 100% O2 for 64 h in individual chambers and then returned to room air to recover for 20 h. Some rabbits were then re-exposed to 100% O2 for an additional 72 h and then killed. The lungs were perfused with saline through the heart before being removed for studies. Similarly-prepared lungs from untreated animals of similar age and weight were used as controls. Lung pieces were flash-frozen in liquid N2 and homogenized in guanidine isothiocyanate and RNA isolated by sedimentation through CsCl (12). Poly(A)+ mRNA was isolated from total RNA by affinity chromatography using oligo(dT)-cellulose (Pharmacia 77F) in batch.

Synthesis, Cloning, and Labeling of Complementary DNA—Complementary DNA (cDNA) was prepared using the cDNA synthesis kit of Pharmacia LKB Biotechnology Inc., according to their instructions and cloned into EcoRI cut Xgt10 DNA and packaged in vitro. Approximately 10⁶ recombinant phage were obtained (approximately 5 × 10⁶ plaque forming units/µg input poly(A)+ mRNA). Single-stranded cDNA was labeled with [3P]dATP to a specific activity of approximately 10⁶ cpm/µg by priming with random hexamer DNA and polymerization with Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories). Specifically, 0.5 µg of poly(A)+ RNA in 20 µl of H2O was mixed with 10 µl of 5 × transcriptase buffer (BRL) and 3 µl of 5 mg/ml random hexamer (dNdN, Pharmacia), heated at 68 °C for 10 min, and cooled on ice. Labeled cDNA was synthesized for 1 h at 37 °C in a 50-µl reaction containing the primed mRNA, 200 µM dGTP, 200 µM dCTP, 20 µM dATP, 20 µM dTTP, 50 µCi of [α-32P]dATP (Amersham Corp., 3000 Ci/mmol), 50 µCi of [α-32P]dCTP (Amersham Corp.; 3000 Ci/mmol), and 1 µl (20 units) of Moloney murine leukemia virus reverse transcriptase. The reaction was stopped by the addition of an equal volume of 0.6 N NaOH, 10 mM EDTA, and heated at 65 °C for 30 min to hydrolyze template. Unincorporated label was removed by passing the probe over a Sephadex G-50 spin column (Boehringer Mannheim).

Screening the cDNA Library—The cDNA library was screened for hyperoxia-induced (H-I) clones by plaque lifts (24) onto cellulose nitrate disks (Millipore HATF). Approximately 3 × 10⁸ phage were grown on six 150-mm-diameter plates, each containing 5 × 10⁸ phage. Duplicate lifts were made from each plate and hybridized with labeled cDNA from either hyperoxia-induced or control lungs. Hybridization to radiolabeled cDNA from the hyperoxia-induced lungs was performed in the presence of (nonlabeled) total RNA from control rabbit lung at a concentration of 750 µg/ml hybridization buffer, to compete for hybridization to noninduced sequences. Both sets of hybridizations were performed in 6 × SSC (1 M NaCl, 0.1 M sodium citrate, 1 M Tris HCl, pH 7.5) for 16-18 h at 65 °C.

The abbreviations used are: H-I, hyperoxia-induced; nt, nucleotides; TIMP, tissue inhibitor of metalloproteinases.

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pH 7), 2 × Denhardt’s solution (0.04% bovine serum albumin, 0.04% polyvinylpyrrolidone-360, 0.04% Ficoll, 0.4 mM EDTA), 0.5% sodium dodecyl sulfate, at 68 °C for approximately 16 h with 10⁶ cpm of probe/ml hybridization buffer. Filters were washed once in 0.1 × SSC, 0.5% sodium dodecyl sulfate, at room temperature with gentle shaking, followed by five more washes in the same buffer, at 65 °C without shaking. Filters were blot-dried and autoradiographed with Kodak X-AR x-ray film, at −80 °C, using Quanta III (Du Pont) intensifying screens. Subsequent hybridizations were done without competitor RNA.

**Blot Hybridization and Analysis**—Southern blots were performed using the Pharmacia VacuGene, according to their instructions, and Northern and RNA dot blots were performed essentially as described by Thomas (14), except that charged nylon membranes (Magnagraph membranes that were stripped of probe following autoradiography, by boiling prior to rehybridization. Dot blots were prepared in triplicate from a single dilution of denatured RNA, for hybridization to three different probes. Serial 2-fold dilutions of each sample were blotted; results were quantitatively consistent throughout. After autoradiography, dots were cut out, and membrane pieces counted in a liquid scintillation counter to quantify hybridization.

**Subcloning and DNA Sequencing and Analysis**—Insert DNA was ligated to M13 mp 18 DNA followed by transfection of E. coli JM 13 (16). Clones with DNA inserted in opposite orientations were identified, and single-stranded phage DNA was isolated and used as templates for DNA sequencing using the dideoxy method. Sequencing reactions and template preparations were performed with kits from Bethesda Research Laboratories and United States Biochemical, according to the suppliers' instructions. Sequence data were analyzed using the University of Wisconsin Genetics Computer Group (UWGCG) software package, version 5.3.

**RESULTS**

**Isolation of H-I (Hyperoxia-induced) Clones**—A cDNA library was constructed from the lung of a rabbit which had been exposed to 100% O₂ for 64 h, recovered in room air for 200 h, and was re-exposed for another 72 h in 100% O₂ prior to killing. During the primary exposure and recovery period, the rabbit lungs underwent a well characterized pathology which includes altered pulmonary mechanics, pulmonary edema and atelectasis, increased alveolar permeability to macromolecules, increased alveolar phospholipid levels, and changes in the biophysical characteristics of surfactant (10, 11). After 200 h in room air, the animals recover from this injury, as measured by these same characteristics. These recovered rabbits have also developed a degree of O₂ tolerance; when re-exposed they survive about twice as long as control animals (17). Thus, a cDNA library from such animals may provide clones whose induction is linked to hyperoxic lung injury or hyperoxic tolerance. The same lung mRNA was also used as a template for probe for the isolation of H-I clones from the library. Unlabeled RNA of control rabbit lungs was added to the hybridization containing the H-I probe, to compete specifically for hybridization to abundant sequences common to both (induced and control) message populations. Clones were chosen which were of obviously greater intensity on autoradiograms of filters probed with the hyperoxic probe, relative to control probe.

Seven putative H-I clones were isolated and, in addition, one noninduced clone, CO II (see below), was isolated for use as a constitutively expressed control. Labeled insert cDNAs of purified clones were used to probe a Southern blot (not shown), indicating that the seven H-I clones fell into four hybridizing groups. Three clones (H-I 1, H-I 2, and H-I 3), corresponding to one member of each of the three hybridizing groups of two, were used in this study.

Northern blots of total lung RNA were performed in order to determine approximate sizes of the mRNAs corresponding to the H-I and control clones. Fig. 1 shows that the steady state levels of all three H-I messages increased in the lung after secondary hyperoxic exposure. In addition, CO II, which encodes the rabbit mitochondrial cytochrome oxidase, subunit II, and which did not change in intensity between the two sets of plaque lifts, also did not yield a significantly different signal when used to probe RNA of control and hyperoxic rabbit lung. H-I 1 hybridized to a single message, approximately 1000 nt long. H-I 2 hybridized as a broad smear ranging from 600 to 800 nt. H-I 3 hybridized to two messages. The message appearing to be more abundant is approximately 2000 nt; the apparently less abundant message is approximately 3200 nt. Both messages detected by H-I 3 are induced coordinately, as seen by autoradiographs of varying exposure, and on Northern blots of RNA from a variety of control and hyperoxic rabbits (not shown). The control message, CO II, is approximately 800 nt long.

**H-I 1 Is the Rabbit Tissue Inhibitor of Metalloproteinases**—In order to begin to understand how these induced genes may function in hyperoxic lung injury, it is necessary to determine the nature of the proteins they encode. The DNA sequence of one of them, H-I 1, and its derived amino acid sequence were therefore ascertained (Fig. 2). Although the H-I 1 clone is probably not full-length (H-I 1 mRNA is approximately 1000 nt; its transcript has not been mapped), it contains a long open reading frame between positions 16 and 733, encoding a 206-amino acid protein. A comparison of the DNA sequence of H-I 1 and all sequences in Genbank indicated that H-I 1 almost certainly encodes the rabbit tissue inhibitor of metalloproteinases (TIMP; 18) (see Fig. 3). TIMP is an inhibitor of collagenase, gelatinase, and proteoglycanase (all are metalloproteinases; 19, 20). TIMP is synthesized and secreted by fibroblasts of every type in which it has been studied (20–25) and also by erythroid precursor cells (26) and alveolar macrophages (27). TIMP has been identified as an autocrine growth factor for erythropoiesis and is also known as erythroid potentiating activity (28).

Fig. 3 shows the aligned sequences of the human, mouse,
position and the reading frame remains open until position 633. A putative

FIG. 2. The nucleotide and derived amino acid sequence of
cDNA clone H-I 1. The coding strand is shown above the dashed line. Translation is presumed to begin at the first ATG (position 16), and the reading frame remains open until position 653. A putative polyadenylation consensus sequence, AATAAA, is found starting at position 717. Data were obtained from both strands for about 75% of the sequence shown.

and rabbit TIMP proteins. The proteins are highly conserved; the overall sequence similarity between the human and rabbit proteins is 88%, between rabbit and mouse, 81%, and between mouse and humans, 84%.

The 3′-untranslated portions of each of the rabbit, human, and mouse TIMP cDNAs were translated from DNA sequences selected from the Genbank data base using the UWGCG programs Wordsearch, with default parameters set, and the derived amino acid sequences are shown in upper case.

FIG. 4. A comparison of the 3′-untranslated regions of rabbit, human, and mouse TIMP cDNAs. The nucleotide sequences of approximately 90 bases (coding strand) following the termination codons of the three TIMP cDNAs were compared using UWGCG programs Gap and Pretty, as described in legend to Fig. 3. Nucleotide sequences shown in upper case are identical in at least two genes.

suggests they may have a functional role. Others have noted sequence similarity in the 5′-untranslated region of the human and mouse TIMP cDNAs (21).

FIG. 5. Steady state levels of TIMP mRNA increase 6-fold upon hyperoxic induction. 2 μg of poly(A) mRNA from control (−) and hyperoxic (+) lungs were dot-blotted (see “Experimental Procedures”) and hybridized with random hexamer-labeled probes for rabbit TIMP, and to two constitutively expressed, control probes: cytochrome oxidase subunit II (CO II) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Small differences in signal intensity between the induced and uninduced samples, seen in the dots hybridized to both control probes are probably due to small differences in the amount of RNA loaded. See “Experimental Procedures” and text for an explanation of quantitation.

DISCUSSION

The premise of these studies was that the cellular remodeling occurring in the lung in response to hyperoxic injury is partially due to changes in gene expression which result in synthesis of new proteins. Such proteins might be growth or chemotactic factors altering cell populations, proteolytic enzymes causing cell permeability changes and loss of the lung’s elasticity, or structural or regulatory proteins. It is reasonable to expect that the synthesis of some of these proteins is partially due to changes in gene expression which result in synthesis of new proteins. Such proteins might be growth or chemotactic factors altering cell populations, proteolytic enzymes causing cell permeability changes and loss of the lung’s elasticity, or structural or regulatory proteins. It is reasonable to expect that the synthesis of some of these
proteins is probably regulated by the steady state level of the messages encoding them. By differential screening of a cDNA library derived from hyperoxic lung, it should be possible to isolate clones of hyperoxia-induced (H-1) messages. Testing this hypothesis in hyperoxic rabbits led to the isolation of three H-1 cDNA clones.

Steady state levels of mRNAs detected by all three clones exhibited notable increases following a second hyperoxic exposure (Fig. 1); the extent of induction was different for each. The first of these clones to be studied in detail is H-1, which most probably encodes rabbit TIMP. TIMP is a M, 28,000 glycoprotein which inhibits collagenase, among other metalloproteinases, and is synthesized and secreted by a variety of cell types, including fibroblasts, macrophages, and erythroid colony forming blood cells (18–29). It is not yet known if the 6-fold increase in steady state levels of TIMP mRNA is correlated with commensurate changes in TIMP protein. This increase in TIMP message is, to our knowledge, the largest reported biochemical induction associated with hyperoxic lung injury. For example, antioxidant enzyme activities are rarely induced as much as 2-fold (30). Furthermore, other studies indicated that TIMP mRNA, and also H-1 and H-3 mRNAs, were induced in the lungs of hyperoxic rabbits whether they were exposed once or twice (not shown), although the kinetics of induction during primary O2 exposure, repeated, and secondary exposure have not yet been determined. In addition, animals which recovered in room air for 200 h had steady state levels of these messages which were comparable to unexposed animals (not shown). Because these studies were performed with RNA of whole lungs, it is not known in which lung cell type(s) these increases occurred, and, because these lungs were not lavaged prior to RNA isolation, it is not known to what extent the inductions were due to an influx of inflammatory cells. Furthermore, whether the increase in TIMP message abundance is the result of increased de novo transcription, slower message turnover, or both, is a subject of further investigation.

The 6-fold increase in TIMP message is based on normalization of hybridization to CO II. Fig. 4 shows that CO II is an appropriate control for these studies, because CO II mRNA levels are unchanged when compared to a constitutively expressed housekeeping gene encoding GAPDH. The abundance of both messages also remained unchanged when used to probe a variety of Northern and dot blots of injured and developing rabbit lungs.

The role of TIMP in the response to lung injury remains to be elucidated. One possibility is that induction of TIMP is necessary for remodeling extracellular matrix, including collagen. TIMP is known to play a key role in the regulation of connective tissue turnover (24). In this light, it is important to learn collagen synthesis and turn over is regulated in the O2-injured lung and if other proteases or antiproteases change their activities in this model. Another possibility is that TIMP functions as a growth factor in the injured lung. TIMP is identical to erythroid potentiating activity (18); erythroid potentiating activity stimulates the growth of erythroid progenitors in vitro (18, 19, 28). Erythroid potentiating activity also induces erythrocytosis in vivo (3). It is noteworthy that TIMP is one of a number of genes induced by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate, which also induces the protooncogenes c-myc, c-fos, c-sis, c-fms, and the growth factors platelet-derived growth factor interleukin-1 and T-cell growth factor (32). If TIMP induction by hyperoxic lung injury is mediated by the same mechanism utilized by 12-O-tetradecanoylphorbol-13-acetate, it would suggest that this set of growth regulatory genes may also be induced in the injured lung.

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