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Overexpression of Stat3C in Pulmonary Epithelium Protects against Hyperoxic Lung Injury

Xuemei Lian,*§ Yulin Qin,* Shaikh Abu Hossain,*¶ Li Yang,* Amanda White,* Huan Xu,**† J. Michael Shipley,¶ Tingyu Li,§ Robert M. Senior,¶ Hong Du,‡ and Cong Yan2*‡§

Acute lung injury is a side effect of therapy with a high concentration of inspired oxygen in patients. The molecular mechanism underlining this effect is poorly understood. In this study, we report that overexpression of Stat3C, a constitutive active form of STAT3 in respiratory epithelial cells of a doxycycline-controlled double-transgenic mouse system protects lung from inflammation and injury caused by hyperoxia. In this mouse line, >50% of transgenic mice survived exposure to 95% oxygen at day 7, compared with 0% survival of wild-type mice. Overexpression of STAT3C delays acute capillary leakage and neutrophil infiltration into the alveolar region. This protection is mediated at least partially through inhibition of hyperoxia-induced synthesis and release of matrix metalloproteinase (MMP)-9 and MMP-12 by neutrophils and alveolar resident cells. In some MMP-9−/− mice, prolonged survival was observed under hyperoxic condition. The finding supports a concept that activation of the Stat3 pathway plays a role to prevent hyperoxia-induced inflammation and injury in the lung. The Journal of Immunology, 2005, 174: 7250–7256.

Signal transducer and activator of transcription 3 is originally identified as the acute-phase response factor (1, 2). Stat3 is mainly activated by autocrine/paracrine growth factors and IL-6 family cytokines that share the common gp130 receptor subunit (3, 4). Upon activation by cytokines or growth factors, phosphorylation at Y705 by JAKs causes Stat3 dimerization to form heterodimer with Stat1, or to form homodimer with itself. Stat3 dimers translocate into the nucleus and activate downstream target genes. A Stat3C form has been designed to mimic the action of phosphorylated Stat3 (5). In this molecule, substitution of two cysteine residues within the C-terminal loop of the Src homology 2 domain of Stat3 produces a molecule that dimerizes spontaneously, binds to DNA, and activates gene transcription. The Stat3 signaling stimulates cell proliferation and prevents apoptosis (5). Overstimulation of the Stat3 pathway causes cellular transformation in mice (5). In contrast to other Stat-deficient mice that are born alive, targeted disruption of the mouse Stat3 gene leads to early embryonic lethality (6). Therefore, Stat3 is required for normal embryonic development and tissue formation. In the lung, Stat3 is expressed in alveolar type II epithelial cells and Clara cells along with IL-6Rs and JAKs (7, 8). Treatment of IL-6 or LPS activates Stat3 phosphorylation at Y705 in these cells in vivo (8, 9). Stat3 is required for maintaining the alveolar epithelial structure and function. Overexpression of a dominant-negative Stat3 to subvert the endogenous Stat3 activity in respiratory epithelial cells causes alveolar destruction (8). This pathophysiological phenotype is mediated partially through Stat3 regulation of surfactant protein B (SP-B) expression in the lung. In the absence of SP-B, neonatal lungs are collapsed during respiratory cycles that cause death after birth (10, 11). Stat3 stimulates SP-B gene expression in respiratory epithelial cells in a synergistic manner with retinoid acid receptors (7, 8). In an animal system, in which the Stat3 allele is selectively deleted in respiratory epithelial cells, surfactant proteins and lipids are decreased or absent in alveolar lavage material during hyperoxia. Pulmonary surfactant reduces surface tension forces and protects lung from collapse during respiratory cycles. Addition of SP-B to this system can improve animal survival during oxygen injury (12).

Supplemental oxygen is commonly administered to patients with acute and chronic cardiopulmonary disorders to augment tissue oxygen delivery and enhance the alveolar and arterial oxygen levels. Unfortunately, prolonged administration of inspired oxygen at >50% concentration leads to a variety of forms of lung tissue damage, including acute lung injury and adult respiratory distress syndrome (13, 14). Animals exposed to hyperoxic conditions have been used as models to study acute lung injury and adult respiratory distress syndrome (13). There are three stages of morphologic changes in mouse models after exposure to a lethal dose of hyperoxia (e.g., 95–100% O2). During the first 24 to 72 h of exposure, most animals show no significant morphologic changes in the alveolar septa. This phase is soon followed by an inflammatory phase, which is characterized by an infiltration and accumulation of inflammatory cells (such as neutrophils) in the lung and the release of inflammatory mediators. This leads to remodeling and destruction of alveolar parenchyma that directly cause animal death (a destructive phase). The molecular mechanism involved in this whole process is poorly understood.

3 Abbreviations used in this paper: SP-B, surfactant protein B; ECM, extracellular matrix; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; TRE, Tet-responsive element; rTA, reverse tetracycline-responsive transactivator; WT, wild type; Balf, bronchoalveolar lavage fluid.

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In the mature lung, the interalveolar walls are supplied with capillaries. Epithelial cells and endothelial cells form a thin alveolar wall for easy penetration and exchange of oxygen and carbon dioxide. Extracellular matrix (ECM) is the major component in the endothelial-epithelial interstitial structure that is comprised of multiple structural proteins (such as collagens, elastin, gelatin, etc.). ECM can be modified by matrix metalloproteinases (MMPs) that are zinc-dependent matrix-degrading proteases. They degrade almost every component of the ECM and are required for normal lung development and remodeling after lung injury. However, aberrant or aberrant expression of MMPs can cause tissue damage (15). It is conceivable that MMPs play a major role in the alveolar destruction during hyperoxia. Under normal physiological conditions, the activities of MMPs are regulated at the level of transcription, posttranslational processing, and inhibition by endogenous inhibitors. Tissue inhibitors of metalloproteinases (TIMPs) are specific inhibitors of MMPs that participate in controlling the local activities of MMPs in tissues. Aberrant expression of TIMPs can also lead to tissue damage. Because inhibition of the endogenous Stat3 activity causes alveolar destruction and accelerates animal death during hyperoxia (8), it suggests that activation of the Stat3 pathway is required to protect against oxygen injury caused by hyperoxia. To test this hypothesis, an animal model has been established, in which a constitutively active form of Stat3C is overexpressed in respiratory epithelial cells under the control of a tissue-specific promoter by doxycycline treatment. Using this animal model system, we discovered that activation of the Stat3 pathway in respiratory epithelial cells protects against hyperoxia-induced capillary hemorrhage and neutrophil influx into the lung. In this animal model system, expression and enzymatic activities of MMP-9 and MMP-12 were highly induced during hyperoxia. Interestingly, these pathogenic increases were suppressed by Stat3C overexpression. As a result, the survival rate of transgenic mice was significantly improved during hyperoxia. The observation identifies a new mechanism by which the Stat3 signaling protects against the hyperoxia-induced lung inflammation and injury.

Materials and Methods

Animal care

All protocols involving the use of animals in this study have been approved by the Cincinnati Children’s Hospital Institution Animal Care and Usage Committee (IACUC) and follow guidelines established by the Panel on Euthanasia of the American Veterinary Medical Association. Protocols involving the use of rDNA or biohazardous materials have been reviewed by the Cincinnati Children’s Hospital Biosafety Committee and follow guidelines established by the National Institutes of Health. Animals were housed under IACUC-approved conditions in a secured animal facility at Cincinnati Children’s Hospital Medical Center, Cincinnati, OH). The CCSP-rtTA transgenic mice (16) was kindly provided by Drs. J. Tichelaar and J. Whitsett (Cincinnati Children’s Hospital Medical Center, Cincinnati, OH). The CCSP-rtTA transgenic mice were genotyped with an upstream primer corresponding to the CCSP promoter sequence (5’-ACTGCCCATTGGCCTTCAAGAAG-3’) and a downstream primer corresponding to the rtTA code region sequence (5’-AAATTCATGC CAGCTTTCCCC-3’). CCSP-rtTA(teto)-CMV-Stat3C double-transgenic mice were generated from cross-breeding of the CCSP-rtTA transgenic mice and teto-CMV-Stat3C transgenic mice.

The mmp9-/- mice were made as previously described (17).

Total RNA isolation and RT-PCR

Adult CCSP-rtTA(teto)-CMV-Stat3C double-transgenic mice were treated with or without doxycycline-impregnated food (Harlan Teklar) for 2 mo. Animals were exposed with 95% oxygen or room air for 4.5 days. Total RNAs were isolated from lungs using the RNA purification kit (QiaGen). To detect Stat3C mRNA expression, an upstream primer (5’-TAC CATTGACCTGGGAGTTGCGCC-3’) and a downstream primer (5’-CCTGAAAACCTTTGCCC-3’) were used for RT-PCR. To detect SP-B mRNA expression, an upstream primer (5’-TGCTGTTGACCCCTAC GATAAGAAG-3’) and a downstream primer (5’-CATACTGGTTGTACT CGGTGTTCCC-3’) were used. RT-PCR was performed using the SuperScript One-Step RT-PCR kit (Invitrogen Life Technologies). Expression of GADPH mRNA was also examined using an upstream primer (5’-CAAGACCTGTTGGATGCCC-3’) and a downstream primer (5’-CTAGCCCACCCTTTGAGTGGC-3’) as an internal control. For MMP-9 mRNA RT-PCR assays, an upstream primer (5’-GATTCTTCCTGTAAC CCCAGGAAG-3’) and a downstream primer (5’-CAGTGGAAGGT GAGAGATCCCAAAG-3’) were used. For MMP-12 mRNA RT-PCR assays, an upstream primer (5’-CAAGACCAAACCCTTCCTCCAG-3’) and a downstream primer (5’-TGCTGTTGACCCCTACGATAAGAAG-3’) were used. The primers for TIMP1, TIMP2, TIMP3, and TIMP4 detection were purchased from SuperArray Bioscience.

Oxygen exposure

One-month-old wild-type (WT) and CCSP-rtTA(teto)-CMV-Stat3C double-transgenic mice were treated with doxycycline food for 2 mo (n = 20 per group). Animals were exposed to 95% oxygen in a 70 × 55 × 25-cm airtight plastic chamber (with free access to food and water) or room air. The oxygen concentration in the chamber was monitored with an oxygen analyzer (MSA), and was maintained with a constant flow of pressurized gas (3 l/min). Survival status of the mice was checked every 3–12 h. Differences in survival status between two groups were assessed using Kaplan-Meier Survival Analysis in StatView 4.5 software (Abacus Concepts). The same condition was used for mmp9-/- mice (3-mo-old study)

Bronchoalveolar lavage and cyrography analysis

After 4.5 days of 95% oxygen exposure, mice were anesthetized by i.p. injection with 0.1 ml of a 4:1:1 ketamine, acepromazine, and xylazine mixture, and exanguinated through the posterior abdominal aorta. The trachea was isolated and cannulated with a 22-gauge needle. Bronchoalveolar lavage fluids (BALF) were collected by lavaging the lung with three 1-ml aliquots of 0.9% sodium chloride. BALF were combined and centrifuged for 5 min at 1000 rpm and 4°C. The cell-free BALF samples were stored at −70°C for later analyses.

Equal volumes of the BALF (10 μl) from various samples were loaded on 10% Tricine gel with 0.1% gelatin (Invitrogen Life Technologies) for electrophoresis. To eliminate the SDS content, the gels were washed with 2.7% (w/v) Triton X-100 at room temperature for 30 min and incubated for 16 h in a buffer containing 50 mM Tris base, 40 mM HCl, 200 mM NaCl, 5 mM CaCl2, and 0.02% (w/v) Brij 35 for gelatinolytic enzymes to act. The digested gels were stained in Colloidal Blue Staining kit (Invitrogen Life Technologies) for 4 h to visualize the gelatin activity.

Tissue processing, lung histology, and immunohistochemistry

Mice were anesthetized and lungs were inflation-fixed with 4% paraformaldehyde in PBS overnight at 4°C. Lungs were washed with PBS and dehydrated through a series of ethanol, followed by paraffin embedding. Five-micrometer tissue sections were placed onto slides for H&E staining and immunohistochemistry. In immunohistochemistry, MMP-9 Ab (1:500) (18) and MMP-12 Ab (1:200; Santa Cruz Biotechnology) were used. The signals were detected by biotinylated secondary Abs and Vectastain Elite ABC kit (Vector Laboratories). Positive immunohistochemical reactions were visualized by color development and counterstaining with hematoxylin. Sections were dehydrated through a series of ethanol, followed by paraffin embedding. The slides were heated at 56°C for 30 min and taken from various lung lobes and transferred by video camera to a computer screen using MetaMorph imaging software (Universal Imaging). A computer-generated, 121-point lattice grid was superimposed on each field,
and numbers of MMP-9-stained cells were determined by a point counting method. The statistical significance was assessed by ANOVA.

For immunofluorescence staining, Ly6G Ab (BD Biosciences) and MMP-9 Ab were used as primary Abs. A Cy2-conjugated donkey anti-rabbit IgG and a Cy3-conjugated donkey anti-rat IgG (Jackson ImmunoResearch) at a dilution of 1:200 were used as the secondary Abs. Slides were examined under a Nikon fluorescent microscope and analyzed for red (Cy3) and green (Cy2) fluorescence.

Results
Generation of CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice
A doxycycline-controlled double-transgenic mouse model was generated to specifically direct Stat3C expression in respiratory epithelial cells. In this system, a previously made CCSP-rtTA transgenic mouse line (16) was crossbred with a newly generated teto-CMV-Stat3C transgenic mouse line (Fig. 1). A Flag sequence was added at the C terminus of the Stat3C cDNA. Double-transgenic mice have been successfully obtained as detected by PCR using specific primers and mouse tail DNAs.

Expression of Stat3C and SP-B in the lung of CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice
Induction of Stat3C mRNA expression was achieved by doxycycline treatment for 2 mo as monitored by RT-PCR assay in CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice with 95% oxygen or room air treatment (Fig. 2). No Stat3C mRNA was detectable in double-transgenic mice without doxycycline treatment. In comparison, control GAPDH mRNA remained unchanged upon treatment. Wt and single-transgenic mice were also treated as controls with no Stat3C mRNA induction observed (data not shown). In addition, immunohistochemical staining of doxycycline-treated lung sections from CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice with Flag Ab showed expression of Stat3C-Flag fusion protein in alveolar type II epithelial cells, but not in untreated mice (data not shown). To test cellular response of Stat3C overexpression in activating downstream target genes in this system, SP-B mRNA expression was examined. The SP-B mRNA level was significantly augmented after doxycycline treatment in CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice with 95% oxygen or room air treatment (Fig. 2). This is in agreement with our previous finding that Stat3C activates SP-B gene expression (19).

Stat3C overexpression enhances animal survival under hyperoxic condition
To determine how constitutive activation of the Stat3 signaling pathway by Stat3C overexpression affects animal survival under oxygen toxicity, doxycycline-treated or -untreated CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic and Wt mice were exposed to 95% oxygen. Under this condition, the first mortality of doxycycline-treated or -untreated WT mice began at day 5. At day 7, all of the WT mice had succumbed (Fig. 3). Because both doxycycline-treated and -untreated WT animals showed identical survival curve, it seems that doxycycline treatment has no effect on animal survival during hyperoxia. The doxycycline-untreated CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice showed a similar survival curve like WT animals (data not shown). In comparison, induction of Stat3C overexpression by doxycycline treatment in CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice significantly delayed mortality under the same condition. The onset of death for doxycycline-treated double-transgenic mice began at day 6. At day 7, >50% of transgenic mice were still surviving 95% oxygen exposure, compared with 0% survival in WT mice. Some doxycycline-treated CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice were exposed to room air or 95% O2. Animal mortality was presented every 12 h. x-axis, Days exposed to 95% O2; y-axis, Survival percentage of animals. The solid line represents the survival rate of WT animals. The dashed line represents double-transgenic mice. In each group, n = 20. Log rank test showed statistical significance between survival rates of two groups (p < 0.05). WT, WT mice; Stat3C, CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice. The survival rates of doxycycline-untreated WT and double-transgenic mice were similar to doxycycline-treated WT animals (data not shown).

FIGURE 1. Generation of doxycycline-controlled CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice. A, Construct maps for CCSP-rtTA transgenic mice and (teto)7-CMV-Stat3C transgenic mice. B, Genotyping of CCSP-rtTA transgenic (lane 1), (teto)7-CMV-Stat3C transgenic (lane 2), CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic (lane 3) and WT (lane 4) mice by PCR using mouse tail DNA.

FIGURE 2. Expression of Stat3C in CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice by doxycycline induction. Induction of Stat3C and SP-B mRNA expression in CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice (in duplicate) by doxycycline treatment with 95% O2 or room air by RT-PCR analysis. GAPDH mRNA expression was used as a control.

FIGURE 3. Survival of doxycycline-treated mice under hyperoxic condition. Doxycycline-treated adult WT and CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice were exposed to room air or 95% O2. Animal mortality was presented every 12 h. x-axis, Days exposed to 95% O2; y-axis, Survival percentage of animals. The solid line represents the survival rate of WT animals. The dashed line represents double-transgenic mice. In each group, n = 20. Log rank test showed statistical significance between survival rates of two groups (p < 0.05). WT, WT mice; Stat3C, CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice. The survival rates of doxycycline-untreated WT and double-transgenic mice were similar to doxycycline-treated WT animals (data not shown).
double-transgenic animals survived until day 9. Therefore, overexpression of Stat3C in respiratory epithelial cells enhances survival following exposure to hyperoxia.

Stat3C overexpression protects against pulmonary hemorrhage under hyperoxic condition

To further address the morphological changes caused by hyperoxia in the lung, histopathologic assessment was performed. Doxycycline-treated or -untreated WT and CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic animals were exposed to 95% oxygen for 4.5 days (right before the onset of mortality in WT animals). Lungs were inflated and paraffin embedded. After staining with H&E, tissue sections from WT and CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic animals were compared. In WT animals, alveolar hemorrhage was apparent after 95% oxygen exposure regardless of doxycycline treatment, indicating increased capillary permeability and damage of the endothelial-epithelial interstitial structure along the alveolar wall (Fig. 4). The doxycycline-untreated CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice showed a similar result (data not shown). However, induction of Stat3C overexpression after doxycycline treatment of CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice showed no obvious hemorrhage under the same hyperoxic condition (Fig. 4).

Stat3C overexpression reduces MMP-9 and MMP-12 mRNA levels under hyperoxic condition in the lung

Pulmonary hemorrhage during hyperoxia indicates alveolar structure damage, which may be caused by elevation of MMPs in the lung. To test this assumption, RT-PCR assay of MMP-9 and MMP-12 mRNAs was performed. Doxycycline-treated or -untreated WT or CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic animals were exposed to 95% oxygen for 4.5 days. Lungs were isolated and total RNAs were purified. Regardless of doxycycline treatment, MMP-9 and MMP-12 mRNA expression levels were significantly increased after 95% oxygen exposure in WT mice (Fig. 5). In doxycycline-untreated CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice, MMP-9 and MMP-12 mRNA expression levels were significantly increased after 95% oxygen exposure, which were similar to WT mice (Fig. 5). However, induction of Stat3C overexpression by doxycycline treatment of CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice significantly inhibited elevation of MMP-9 and MMP-12 mRNA levels in the lung (Fig. 5).

Stat3C overexpression protects against neutrophil infiltration under hyperoxic condition in the lung

Neutrophil infiltration is a manifestation of many acute inflammatory responses in the lung (20). Proteinases including MMP-9 (gelatinase B) are synthesized and stored in granules of neutrophils, and released upon inflammatory stimulation (21). Using polyclonal Ab against MMP-9 as a marker described previously (18), MMP-9 was observed (Fig. 7, upper three panels). Oxygen treatment significantly increased MMP-9- and Ly6G-positive staining cells in the lung (Fig. 7, middle three panels). The overlay of MMP-9- and Ly6G-positive cells was ~100% identical in the oxygen-treated WT lung (Fig. 7, middle right panel). This is a clear indication that the MMP-9-positive cells in oxygen-treated mice are neutrophils. In contrast, in doxycycline-treated CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice, both MMP-9- and Ly6G-positive cells were significantly reduced (Fig. 7, bottom panels).

Stat3C overexpression decreases hyperoxia-induced MMP-9 enzymatic activity in the lung

To confirm that MMP-9-positive cells are indeed neutrophils, a colocalization of double immunofluorescent staining was performed using Abs against MMP-9 and Ly6G (a neutrophil-specific marker). In oxygen-untreated WT mice, no obvious MMP-9 or Ly6G staining was observed (Fig. 7, upper three panels). Oxygen treatment significantly increased MMP-9- and Ly6G-positive staining cells in the lung (Fig. 7, middle three panels). The overlay of MMP-9- and Ly6G-positive cells was ~100% identical in the oxygen-treated WT lung (Fig. 7, middle right panel). This is a clear indication that the MMP-9-positive cells in oxygen-treated mice are neutrophils. In contrast, in doxycycline-treated CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice, both MMP-9- and Ly6G-positive cells were significantly reduced (Fig. 7, bottom panels).

The process includes adhesion of MMP-9-positive cells on the endothelial surface of blood vessels, infiltration into the perivascular space region and the alveolar interstitial region (Fig. 6, A and B (pointed by arrows)). The staining of doxycycline-untreated CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice was similar to that of WT mice (data not shown). In comparison, MMP-9-positive cell influx was significantly reduced in doxycycline-treated CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice, in which Stat3C was overexpressed.

MMP-9 influx is associated with neutrophil infiltration

To confirm that MMP-9-positive cells are indeed neutrophils, a colocalization of double immunofluorescent staining was performed using Abs against MMP-9 and Ly6G (a neutrophil-specific marker). In oxygen-untreated WT mice, no obvious MMP-9 or Ly6G staining was observed (Fig. 7, upper three panels). Oxygen treatment significantly increased MMP-9- and Ly6G-positive staining cells in the lung (Fig. 7, middle three panels). The overlay of MMP-9- and Ly6G-positive cells was ~100% identical in the oxygen-treated WT lung (Fig. 7, middle right panel). This is a clear indication that the MMP-9-positive cells in oxygen-treated mice are neutrophils. In contrast, in doxycycline-treated CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice, both MMP-9- and Ly6G-positive cells were significantly reduced (Fig. 7, bottom panels).

Stat3C overexpression decreases hyperoxia-induced MMP-9 enzymatic activity in the lung

To determine whether neutrophil and MMP-9 influx results in elevation of MMP-9 enzymatic activity in the alveolar lumen, BALF were collected from doxycycline-treated WT and CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice, oxygen exposed or unexposed, for zymography analysis. As shown in Fig. 8, the MMP-9 activity was dramatically increased in WT animals after oxygen exposure. Doxycycline-untreated CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice showed a result similar to that of WT mice (data not shown). In comparison, induction of Stat3C overexpression blocked increase of the MMP-9 activity in
doxycycline-treated CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice. Therefore, elevation of the MMP-9 activity is partially responsible for damage of the endothelial-epithelial interstitial structure and capillary leakage in alveoli. This study suggests that activation of the Stat3 pathway blocks the MMP-9-damaging effect by delaying neutrophil influx into the alveolar region.

**MMP-9 deficiency enhances animal survival under the hyperoxic condition**

The above studies suggest that MMP-9 influx into the lung plays an important role in contributing alveolar structure damage and hemorrhage during hyperoxia. To confirm the pathogenic role of MMP-9 in hypoxic condition, oxygen injury study was performed using MMP-9 genetically ablated mice (mmp-9<sup>-/-</sup>) (17).

**Stat3C overexpression decreases hyperoxia-induced MMP-12 synthesis in resident cells around the alveolar wall**

MMP-12 is another important proteinase that degrades elastin and type IV collagen (15, 22). Previous studies showed that MMP-12 is primarily secreted by macrophages and plays a role in the pathogenesis of chronic lung injury, especially in smoking-related chronic obstructive pulmonary disease and emphysema (23, 24).

Using polyclonal Ab against MMP-12, MMP-12 expression was significantly induced in the resident cells along the alveolar wall in doxycycline-treated or -untreated WT animals after 4.5 days of 95% oxygen exposure on immunohistochemical staining (Fig. 10). The doxycycline-untreated CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice showed a similar result (data not shown). In contrast, Stat3C overexpression blocked MMP-12 expression in doxycycline-treated CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic animals in the same condition, indicating that activation of the Stat3 pathway suppresses the regional MMP de novo synthesis in resident cells along the alveolar wall.

**Stat3C overexpression has no protective effect on TIMP1 mRNA down-regulation under hyperoxic condition in the lung**

TIMPs are specific inhibitors for MMPs in tissues. One possible mechanism to explain alveolar structure damage and pulmonary hemorrhage during hyperoxia is down-regulation of TIMPs. To test this possibility, doxycycline-treated or -untreated CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic animals were exposed to hyperoxia. The WT and mmp-9<sup>-/-</sup> mice were exposed to 95% oxygen. In comparison to WT mice, some mmp-9<sup>-/-</sup> mice survived longer under the same condition (Fig. 9). Therefore, overexpression of MMP-9 is partially responsible for pathogenic destruction during hyperoxic condition. In the absence of MMP-9, the damage effect for the alveolar structure was reduced.

![FIGURE 6. MMP-9 expression in the lungs of WT and CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice during hyperoxia. A, Neutrophil staining with MMP-9 Ab in the perivascular space and the alveolar regions (black arrows) in doxycycline-treated WT and CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice with exposure to room air or 95% O<sub>2</sub>. Double-transgenic mice without oxygen treatment showed no difference from WT mice (data not shown). Original magnification, ×400. B, Counts of MMP-9 positively stained cells. The same size areas were selected from various lung sections. In each group, multisections from three lungs (n = 3) were studied. ANOVA showed significant differences among different animal groups (p < 0.05). WT, WT mice. Stat3C, CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice. BV, Blood vessel.](image)

![FIGURE 7. MMP-9 expression in the WT lung is neutrophil associated during hyperoxia. Immunofluorescence staining of MMP-9 (green) and Ly6G (red) in WT and doxycycline-treated CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice with exposure to room air or 95% O<sub>2</sub>. The overlay of two Ab staining is represented as yellow. Double-transgenic mice without oxygen treatment showed no difference from WT mice (data not shown). Arrows point to representative stained cells. Original magnification, ×200.](image)

![FIGURE 8. The MMP-9 level in BALF of WT and CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice during hyperoxia. BALF were obtained from doxycycline-treated WT and CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice with exposure to 95% O<sub>2</sub> or room air for 4.5 days. Equal amounts of BALF samples (in duplicate) were separated on gelatin gel for zymographic analysis. WT, WT mice; Stat3C, CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice.](image)
95% oxygen for 4.5 days. Total RNAs were isolated from whole lungs and evaluated by RT-PCR. Among four TIMPs, the TIMP1 mRNA expression level was significantly decreased after 95% oxygen exposure (Fig. 11). The TIMP3 mRNA expression level remained the same. There was no detectable TIMP2 or TIMP4 expression in the lung regardless of oxygen treatment. The WT animals showed a similar result (data not shown). This result suggests that down-regulation of TIMP1 plays a role in alveolar structure damage during hyperoxia. However, unlike MMP-9 or MMP-12, induction of Stat3C overexpression by doxycycline treatment of CCSP-rtTA/(teto)7-CMV-Stat3C double-transgenic mice showed no protection against down-regulation of TIMP1 mRNA expression in the lung (Fig. 11).

Discussion

Oxygen-induced lung injury is a complex process, which is affected by multiple factors. The mechanism underlying this process is not very clear. Because pulmonary edema is a major histopathologic finding in mice treated with 95% oxygen (Fig. 4), the interstitial structure of endothelial-epithelial cells must be damaged during hyperoxia. As reported previously, hyperoxia decreases pulmonary surfactant (12). This does not directly lead to ECM degradation and pulmonary hemorrhage. Other mechanisms must be involved in the process.

MMPs are the major enzymes that degrade ECM proteins in the lung. It is reasonable to speculate that the damage of the alveolar interstitial structure results from the elevated levels of MMPs in oxygen-treated mice. Indeed, the RT-PCR and zymography studies showed dramatic elevation of the MMP-9 and MMP-12 levels in the WT lung before the onset of animal mortality following hyperoxia exposure (Fig. 5). MMP-9 can be synthesized by multiple cell types in the lung (21). The increase of the MMP-9 activity may come from either the migrating circulatory cells (e.g., neutrophils), or from alveolar resident cells. To clarify this issue, immunohistochemical and immunofluorescent staining was performed. The results indicated that MMP-9 staining is tightly associated with invading neutrophils from the circulatory system (Figs. 6 and 7). This occurs in both the alveolar region and the blood vessel region in the lung. It seems that hyperoxia triggers neutrophil adhesion to endothelial cells and infiltration into the lung. The molecular mechanism for this phenomenon remains to be elucidated. No MMP-9 expression was detected in alveolar resident cells. In comparison, newly synthesized MMP-12 was detected in resident cells along the alveolar walls after hyperoxia exposure (Fig. 10). MMP-12 is generally regarded as macrophage elastase and is secreted by macrophages. There was no obvious macrophage increase in the alveolar region during hyperoxia. In addition to MMP elevation, expression of an MMP inhibitor TIMP1 was decreased after hyperoxia exposure (Fig. 11). Taken together, hyperoxia enhances MMP activities in alveoli either by inducing MMP expression or by inhibiting TIMP expression. As a consequence, these enzymes directly cause ECM destruction, leading to capillary permeability and subsequent pulmonary hemorrhage.

Because oxygen injury is initiated by inflammation, molecules that play important roles in mediating inflammatory responses may participate in the process of oxygen injury. Transgenic mouse model systems have been proven to be very useful in identifying signaling molecules and transcription factors in various lung disease processes. In this study, a doxycycline-controlled Stat3C double-transgenic mouse system has been created. In this system, the Stat3 signaling pathway is constitutively activated by overexpression of Stat3C. The studies showed that activation of the Stat3 signaling pathway in respiratory epithelial cells exhibits the protective effect against oxygen injury that is associated with increased capillary permeability, neutrophil invasion, and MMP synthesis in the lung. However, this pathway does not protect against down-regulation of TIMP1 expression caused by hyperoxia.

Based on the observation made in this report, a model has been proposed to explain the molecular mechanism by which Stat3C protects against lung oxygen injury. High oxygen concentration induces expression of MMPs (e.g., MMP-12) in lung epithelial cells, endothelial cells, and bronchoalveolar macrophages. These
gene products cause ECM degradation. In addition, high oxygen concentration induces expression of signaling molecules that bind to the cognate plasma membrane receptors and results in the increased expression of adhesion molecules in endothelial cells to facilitate migration and adhesion of neutrophils to the vascular endothelium. Attached neutrophils migrate through the capillary wall into the lung. The activated neutrophils in the lung release proteinases (e.g., MMP-9) to exacerbate capillary permeability and lung injury. When Stat3C is overexpressed in alveolar type II epithelial cells, it activates a set of genes. Through paracrine and autocrine mechanisms, these gene products are released from alveolar type II epithelial cells to inhibit influx of neutrophils and production of MMP-9 and MMP-12 in the lung. Therefore, alveolar type II epithelial cells play a critical role in damaging and repairing of lung oxygen injury.

Other mechanisms may also be involved in the protection against oxygen-induced lung injury. SP-B is a critical component for the surfactant structure and is required for epithelial cell repairing of lung oxygen injury. When Stat3C is overexpressed in alveolar type II epithelial cells, it activates a set of genes. Through paracrine and autocrine mechanisms, these gene products are released from alveolar type II epithelial cells to inhibit influx of neutrophils and production of MMP-9 and MMP-12 in the lung. Therefore, alveolar type II epithelial cells play a critical role in damaging and repairing of lung oxygen injury.

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References