Complete lack of vitamin C intake generates pulmonary emphysema in senescence marker protein-30 knockout mice

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Vitamin C (VC) is a water-soluble, hexonic sugar acid that has two dissociable protons (40) and is abundant in fluids of the lung epithelial lining (50). VC scavenges free radicals such as superoxide (34), singlet oxygen (4), and hydroxyl radicals (2). Several reports indicate that VC has a beneficial effect on lung function but, when depleted during aging, may promote chronic obstructive pulmonary disease (COPD; Refs. 41, 44). On the other hand, VC also plays an essential role in collagen biosynthesis not only by promoting the activity of the prolyl hydroxylase, but also by increasing the mRNA of collagen (1 and III; Refs. 13, 35).

Senescence marker protein-30 (SMP30) is characterized by its ever-decreasing content in the liver, kidney, and lung with aging, a decrease that is androgen-independent (11). To clarify the physiological role of SMP30 in age-associated organ disorders, we used gene targeting to establish the SMP30 knockout (KO) mouse from C57BL/6 mice (17). Recently, we (24) reported that SMP30 has gluconolactonase (GNL) activity. Since GNL is a key enzyme in the VC biosynthetic pathway of mammals, mice deprived of GNL (SMP30 KO mice) lack the ability to synthesize VC (19). We (42) further discovered that oxidative stress is greater in the lungs of SMP30 KO mice than those of C57BL/6 mice since the protein carbonyls, a biomarker for oxidative stress, increased in the lungs of SMP30 KO mice when the mice were fed with a commercial chow.

Oxidative stress arises when the production of reactive oxygen species (ROS) overwhelms the antioxidant defenses (45). Glutathione peroxidase (GSH-Px), SOD, and catalase are major enzymatic antioxidants, whereas VC, vitamin E, and glutathione are the main nonenzymatic antioxidants. Oxidative stress has been suggested as a major cause of COPD (28, 51).

In contrast to humans, who cannot synthesize VC due to the lack of L-gulono-γ-lactone oxidase activity, most animals including mice and rats can synthesize VC, which prevents the development of scurvy even when their diet lacks VC. Accordingly, no information has been published about lung function in the complete absence of VC. In the present study, we utilized SMP30 KO mice deprived of VC and found that VC depletion caused pulmonary emphysema due to oxidative stress and a decrease of collagen synthesis by their third month of age.

MATERIALS AND METHODS

Animals. SMP30 KO mice were maintained and maintained as described previously (17). Wild-type (WT), C57BL/6, and SMP30 KO mice were fed with a commercial chow (CRF-1; Oriental Kobo, Tokyo, Japan) and had free access to water containing 1.5 g/l VC in 10 μM EDTA (pH 8.0) until they were weaned at the age of 30 days. We (12) have previously reported that this concentration of VC-containing water is sufficient to keep VC content within normal range in various organs of SMP30 KO mice. VC-containing water was prepared and exchanged twice a week. After weaning, each strain was fed with a VC-deficient chow (CL-2; CLEA Japan, Tokyo, Japan) and divided into the two groups, VC-supplemented [VC(+)] or VC-depleted [VC(−)]. The VC(+) group had free access to water con-
taining 1.5 g/l VC in 10 μM EDTA (pH 8.0), whereas the VC(−) group had free access to plain water without VC. Mice were maintained on a 12:12-h light-dark cycle in a controlled environment throughout the experiments. All experimental procedures using laboratory animals were approved by the Animal Care and Use Committee of the Tokyo Metropolitan Institute of Gerontology and Juntendo University School of Medicine.

Preparation and morphological evaluations of the lungs. Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg), and the lungs were removed and processed as described previously (23). The lungs were lavaged with 0.5 ml of phosphate-buffered saline through an intratracheal cannula four times. The bronchoalveolar lavage fluid (BALF) was pooled, and total cell counts and cell populations in each BALF specimen were determined. After BAL was performed, the right lungs were flash-frozen in liquid nitrogen and kept at −80°C until use. The left lungs were inflated and fixed by intratracheal instillation of 20% buffered formalin (pH 7.4) at a constant pressure of 25 cmH2O for 24 h.

The paraffin-embedded lung tissues were sectioned at 4-μm thickness and placed on glass slides. The lung specimens were stained with hematoxylin and eosin staining for morphological evaluation of the lungs, with Azan for the evaluation of collagen, and with Elastica-van Giesen (EVG) for the evaluation of elastic fibers. Air space size was assessed by using a computer-controlled small animal ventilator (flexiVent; SCIREQ, Montréal, Québec, Canada; Ref. 46).

Determination of total VC levels in the lungs. Lung tissues were homogenized in 50 mM phosphate buffer (pH 7.4) with a high-speed homogenizer (Polytron; Kinematica). The supernatant was obtained by centrifugation at 21,000 g for 15 min at 4°C and immediately frozen at −80°C until use. Total VC levels were determined by using an HPLC electrochemical detection method as described previously (12).

Determination of thiobarbituric acid reactive substances in the lungs. Lung tissues were homogenized in 50 mM phosphate buffer (pH 7.4) with a high-speed homogenizer (Polytron; Kinematica). The supernatant was obtained by centrifugation at 21,000 g for 15 min at 4°C and immediately placed at −80°C until use. ROS in the lungs was normalized by protein concentration. The assay mixture contained 50 mM potassium phosphate (pH 7.5), 1 mM EDTA (pH 7.5), 1 mM Na2S, 0.2 mM NADPH, 1 unit of GSSG reductase (Wako Pure Chemical, Osaka, Japan), 1 mM GSH, and tissue homogenate (approximately 0.025–0.1 mg of protein) in 700 μl of a total volume. After a 10-min preincubation at 25°C, the reaction was initiated by the addition of 2.0 mM H2O2 (100 μl), resulting in a final concentration of 0.25 mM H2O2. The decrease of the absorbance at 340 nm was measured, and the value was normalized by protein concentration.

Determination of thiobarbituric acid reactive substances in the lungs. Lung tissues were homogenized in 50 mM phosphate buffer (pH 7.4) with a high-speed homogenizer (Polytron; Kinematica). The supernatant was obtained by centrifugation at 21,000 g for 15 min at 4°C and immediately frozen at −80°C until use. The immunoprecipitation (IP) and Western blotting of sirtuin 1 (Sirt1) were performed according to Rajendrasozhan et al. (39). The supernatant of lung homogenate (100-μg protein) and 2 μl of rabbit polyclonal anti-SIRT1 antibody (Millipore, Temecula, CA) were included in a total volume of 400 μl containing 25 mM Tris-HCl (pH 7.2)-150 mM NaCl (IP buffer) and incubated for 2 h at 4°C. Immune complexes were obtained with the addition of 20 μl of protein A/G agarose beads (Pierce Biotechnology, Rockford, IL), incubation for 2 h at 4°C, and subsequent centrifugation at 4,000 g for 3 min at 4°C. The agarose beads were washed three times with IP buffer, suspended in 20 μl of 2× sample buffer containing 0.125 M Tris-HCl (pH 6.8)-4% SDS-50% glycerol-5% 2-mercaptoethanol-0.02% bromophenol blue, and then boiled for 5 min. After centrifugation at 21,000 g for 5 min at 4°C, the resultant supernatant was fractionated on SDS-polyacrylamide gel (10% acrylamide gel) and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was first blocked with 5% skim milk in 10 mM Tris-HCl (pH 7.5)-0.14 M NaCl-0.1% Tween 20 for 60 min at room temperature and incubated with rabbit polyclonal anti-SIRT1 (1:2,000 dilution; Abcam, Cambridge, MA) for 60 min at room temperature. After extensive washing, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin antibody (1:5,000 dilution; Bio-Rad Laboratories, Hercules, CA) for 60 min at room temperature. Antibody binding was detected using ECL Western Blotting Detection Reagents (Amersham Biosciences, Piscataway, NJ) according to the manufacturer’s instruction. Chemiluminescence signals were quantified with an LAS-3000 imaging system and Multi Gauge software (Fujifilm, Tokyo, Japan).

Determination of collagen I mRNA (Col1a2) in the lungs. Lung tissues were homogenized with a handy homogenizer in ISOGEN (Wako Pure Chemical), and total RNA was extracted according to the manufacturer’s protocol. RNA concentration was determined and confirmed as free from protein contamination by measuring absorbance at 260 and 280 nm. Then, cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol. The reactions were incubated at 42°C for 50 min and 70°C for 15 min and then cooled at 4°C in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). The cDNA was stored at −80°C until use. A real-time quantitative PCR (RT-QPCR) was performed in triplicate by using the qPCR SuperMix-UDG with ROX (Invitrogen) following the manufacturer’s protocol. The forward and reverse primers for collagen I (Col1a2; accession number: NM_00483883.1) were purchased from Applied Biosystems (TaqMan Gene Expression Assays). As an endogenous control gene, TaqMan Rodent GAPDH Control Reagent (Applied Biosystems) was used. The reactions were performed by using the RT-QPCR equipment (7500 Fast Real-Time PCR System; Applied Biosystems). For quantitative analysis of the mRNA level, a standard curve method was designed; an aliquot from each experimental sample was used to generate standard curves. The correlation coefficient of the standard curve was >0.999. The mRNA level of collagen I was evaluated as the value relative to that of GAPDH. The cycling condition was as follows: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 2 min, and 45 cycles at 95°C for 15 s and 60°C for 45 s.

Determination of protein concentration. The protein concentration was determined by BCA protein assay (Pierce Biotechnology) using bovine serum albumin as a standard.
Table 1. Body weights of WT and SMP30 KO mice (g)

<table>
<thead>
<tr>
<th></th>
<th>WT VC(−)</th>
<th>WT VC(+)</th>
<th>SMP30 KO VC(−)</th>
<th>SMP30 KO VC(+)</th>
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<tr>
<td>2 mo</td>
<td>24.8 ± 1.6</td>
<td>24.6 ± 0.6</td>
<td>24.7 ± 1.3</td>
<td>24.6 ± 1.3</td>
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<tr>
<td>3 mo</td>
<td>25.2 ± 1.6</td>
<td>24.5 ± 1.1</td>
<td>23.0 ± 2.0*</td>
<td>25.7 ± 0.8</td>
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</table>

Values are expressed as means ± SD of 5 animals. WT, wild type; VC, vitamin C; SMP30 KO, senescence marker protein-30 knockout. *P < 0.05 compared with 3-mo-old WT VC(−) mice.

Statistical analysis. Data are expressed as means ± SD. Statistical analysis was performed using one-way ANOVA followed by Tukey post hoc test for multigroup comparisons. All statistical analyses were performed using SPSS version 16.0 software (SPSS, Chicago, IL). P < 0.05 was considered statistically significant.

Results

Body weight change and BALF. To investigate the effect of VC on growth, we measured changes in body weights of our four experimental groups: VC(−) and VC(+) SMP30 KO mice and VC(−) and VC(+) WT mice (Table 1). After weaning at the age of 30 days, mice from both VC(−) groups were grown without dietary VC intake for 2 mo, whereas mice from both VC(+) groups obtained a sufficient amount of VC in their drinking water. At 2 mo of age, all mice provided with either plain water or VC-containing water for 1 mo gained similar amounts of weight; i.e., no significant difference was observed. However, by 3 mo of age, the mean body weight of SMP30 KO VC(−) group was significantly less, i.e., 2.2 ± 2.1 g lower, than that of the WT VC(−) group (Table 1).

Total cell counts in the BALF from the 2- and 3-mo-old mice indicated significant increases only in the SMP30 KO VC(−) group but not the VC(+) or WT mice at the corresponding ages (Table 2). Absolute counts of neutrophils, macrophages, and lymphocytes also significantly increased in the SMP30 KO VC(−) group at both 2 and 3 mo of age compared with those of the other three groups. However, no significant difference in cell populations in the BALF was observed among any of the four groups at either 2 or 3 mo of age.

Total VC levels in the lungs. We then measured VC content in the lungs of SMP30 KO mice to confirm that eliminating VC from their diet led to a complete absence of VC and that VC supplementation via ad libitum access to VC-containing water attained a relatively normal level of VC (Fig. 1). Total VC levels in the lungs from WT mice were almost stable (approximately 75–95 μg/g lung tissue) irrespective of the age at measurement or VC supplementation in drinking water. In contrast, total VC levels in lungs from the SMP30 KO VC(−) group decreased sharply to 3.2 ± 2.5 μg/g at 2 mo of age and were undetectable by their third month of life. The SMP30 KO VC(+) group, however, showed VC levels comparable with those of the WT group, 88.7 ± 5.2 μg/g lung tissue at 2 mo of age and 78.0 ± 13.3 μg/g lung tissue at 3 mo.

Effect of VC on the morphometric findings and respiratory mechanics. Lungs from the SMP30 KO VC(−) group showed significant air space enlargement compared with those in the WT group at 2- as well as 3 mo of age (Fig. 2). The SMP30 KO VC(−) mice had MLI increases of 42.5 and 38.3% at the 2- and 3-mo marks, respectively. Comparatively, MLI values for the SMP30 KO VC(+) vs. WT VC(−) mice were 85.2 ± 3.1 vs. 59.8 ± 1.8 μm at 2 mo of age and 87.0 ± 2.9 vs. 62.9 ± 2.5 μm at 3 mo of age (Fig. 2A). The SMP30 KO VC(−) animals also manifested significant destruction of the lung parenchyma, i.e., a DI of 12.0% (at 2 mo of age) and 11.8% (at 3 mo), compared with the WT VC(−) group at the corresponding ages (Fig. 2B). As published, a DI of >10% signifies major damage to lung parenchyma (47). Our results indicate that a complete lack of VC caused the pulmonary emphysema in SMP30 KO. In turn, VC supplementation prevented the increase of both MLI and DI in 2- and 3-mo-old SMP30 KO mice and maintained both indicators at levels comparable with

Table 2. Cell populations in bronchoalveolar lavage fluid from WT and SMP30 KO mice

<table>
<thead>
<tr>
<th></th>
<th>Total Cell Count, ×10⁶</th>
<th>%Macrophages</th>
<th>%Lymphocytes</th>
<th>%Neutrophils</th>
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<tbody>
<tr>
<td>2 mo</td>
<td></td>
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<tr>
<td>WT VC(−)</td>
<td>0.7 ± 0.1</td>
<td>91.7 ± 1.2</td>
<td>7.5 ± 1.2</td>
<td>0.8 ± 0.6</td>
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<tr>
<td>WT VC(+)</td>
<td>0.9 ± 0.2</td>
<td>92.5 ± 1.9</td>
<td>7.0 ± 1.4</td>
<td>0.5 ± 0.5</td>
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<tr>
<td>SMP30 KO VC(−)</td>
<td>1.4 ± 0.3*</td>
<td>92.2 ± 1.7</td>
<td>7.3 ± 1.7</td>
<td>0.8 ± 0.6</td>
</tr>
<tr>
<td>SMP30 KO VC(+)</td>
<td>1.0 ± 0.3</td>
<td>92.0 ± 1.8</td>
<td>7.5 ± 1.8</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>3 mo</td>
<td></td>
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<tr>
<td>WT VC(−)</td>
<td>0.6 ± 0.1</td>
<td>92.6 ± 1.5</td>
<td>6.7 ± 2.0</td>
<td>0.7 ± 0.5</td>
</tr>
<tr>
<td>WT VC(+)</td>
<td>0.7 ± 0.2</td>
<td>91.7 ± 1.7</td>
<td>7.7 ± 1.2</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>SMP30 KO VC(−)</td>
<td>1.7 ± 0.5†</td>
<td>91.6 ± 1.5</td>
<td>7.3 ± 2.0</td>
<td>0.7 ± 0.5</td>
</tr>
<tr>
<td>SMP30 KO VC(+)</td>
<td>1.1 ± 0.2</td>
<td>93.0 ± 2.1</td>
<td>6.3 ± 2.2</td>
<td>0.8 ± 0.6</td>
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Values are expressed as means ± SD of 5 animals. *P < 0.01 compared with 2-mo-old WT VC(−) mice; †P < 0.01 compared with 3-mo-old WT VC(−) mice.
those of WT VC(+) mice at the corresponding ages (Fig. 2). In contrast, VC supplementation significantly increased the MLI in WT mice above that in 2- and 3-mo-old WT VC(-) mice. Although the DI also increased, it remained within the normal range (Fig. 2). The representative histological findings of the lungs from each group appear in Fig. 3. These results indicate that despite the protection VC provides to prevent the development of emphysema in SMP30 KO mice, VC may harm the lungs of WT mice. When we examined the mechanical properties of the lungs such as pressure-volume and lung compliance using a computer-controlled small animal ventilator, no significant difference was detected at 3 mo of age (data not shown). Similarly, another group reported that the computer-controlled small animal ventilator was not sensitive enough to detect the difference in lung mechanics between nonsmoking and smoking WT mice, although chronic smoke exposure developed emphysema in the WT mice (46). More sensitive methods might have detected the difference in lung mechanics between the VC-depleted SMP30 KO mice and the other groups under our experimental condition.

Effect of VC on oxidative stress. In SMP30 KO mice, ROS in the lungs from the VC(-) group increased well above that of the VC(+) group: VC(-), 2,625 ± 193; VC(+), 1,150 ± 63 arbitrary units (AU)-min⁻¹·mg⁻¹ protein at 2 mo of age and 2,786 ± 378 vs. 1,845 ± 353 AU-min⁻¹·mg⁻¹ protein, respectively, at 3 mo (Fig. 4). In WT mice, a significantly higher ROS...
was recorded in the lungs of the VC(+) mice than the VC(−) group only at 2 mo of age: 1.721 ± 0.505 vs. 1.142 ± 0.263 AU-min−1·mg−1 protein, respectively (Fig. 4). VC supplementation completely suppressed ROS generation in 2-mo-old SMP30 KO mice to a level like that of WT VC(−) group but not at the 3-mo increment.

To investigate the effect of VC depletion on oxidative injury in the lungs, we measured TBARS, one of the major biomarkers for oxidative stress. In SMP30 KO mice, TBARS in lungs from the VC(−) group significantly increased above that of the VC(+) group at both the 2- and 3-mo measurements: VC(−) vs. VC(+), 2.3 ± 0.2 vs. 1.6 ± 0.3 nmol/mg protein at 2 mo of age and 2.3 ± 0.2 vs. 1.8 ± 0.3 nmol/mg protein at 3 mo of age (Fig. 5). In WT mice, TBARS in the lungs from VC(+) mice exceeded those from VC(−) mice at both time periods: VC(−) vs. VC(+), 1.4 ± 0.2 vs. 1.8 ± 0.3 nmol/mg protein at 2 mo and 1.5 ± 0.2 vs. 1.9 ± 0.2 nmol/mg protein at 3 mo (Fig. 5). These results indicate that VC depletion in SMP30 KO mice increases ROS production and causes oxidative injury in the lungs and that VC partially protects the lungs from oxidative stress, although VC-supplemented SMP30 KO mice still showed higher ROS and TBARS in the lungs than WT VC(−) mice. In contrast, VC supplementation in WT mice increased TBARS in the lungs compared with those of the VC(−) group, suggesting that excess VC intake causes oxidative injury in the lungs (Fig. 5).

Because the GSH-Px enzyme is widely recognized as a main antioxidant defender of the lungs, we measured this activity in all four groups (Fig. 6). However, no significant difference in GSH-Px activity was detected in any group at either 2 or 3 mo of age. Apparently, neither VC supplementation nor VC depletion influences GSH-Px activity in the lungs of SMP30 KO and WT mice.

Effect of VC on Sirt1. The silent information regulator 2 (Sir2) is a NAD+-dependent protein deacetylase that controls longevity in lower eukaryotes (49). Sirtuin (SIRT) is a nuclear protein and has the highest sequence similarity to Sir2 of any unit in the SIRT mammalian family (SIRT1–SIRT7; Refs. 3, 9). Lower levels of the anti-inflammatory and antiaging protein SIRT1 were found in the lungs of smokers and patients with COPD compared with those of nonsmokers (39), suggesting that SIRT1 plays a pivotal part in the interaction among closely related biological processes such as inflammation, oxidative stress, and aging.

Therefore, we determined the levels of Sirt1 in the lungs from our four experimental groups at 3 mo of age (Fig. 7). Amounts of Sirt1 in the lungs from the SMP30 KO VC(−) group decreased 21.1% compared with those of WT VC(−) group. However, Sirt1 levels were similar among SMP30 KO VC(+), WT VC(−), and WT VC(+) mice. Further investigation of the relationship between the morphometric findings and Sirt1 levels revealed a significant correlation between the MLI and Sirt1 content in the lungs (Fig. 7C; r = −0.68; P < 0.01).

Effect of VC on synthesis of collagen and elastic fibers. The SMP30 KO VC(−) group exhibited a decrease of collagen compared with SMP30 KO VC(+), WT VC(−), and WT VC(+) groups at 3 mo of age. No apparent difference in the collagen appeared to exist among SMP30 KO VC(+), WT VC(−), and WT VC(+) groups. Representative sections of Azan-stained lung tissue from each group are shown in Fig. 8. However, no significant difference in the amount of elastic fibers was observed in EVG-stained lung sections from any of the four groups at 3 mo of age (data not shown). To assess whether VC depletion affects collagen synthesis in the lungs, we measured collagen I mRNA in the lungs from our four experimental groups at 3 mo of age (Fig. 9). The collagen I mRNA of the SMP30 KO VC(−) group decreased 82.2% compared with that of the WT VC(−) group. VC supplementation partially restored the collagen I mRNA in SMP30 KO mice to the level of 52.2% compared with that of WT VC(−) group. No significant difference in collagen I mRNA was detected between WT VC(−) and VC(+) groups. These results indicate that VC and SMP30 play an important role in collagen synthesis.

DISCUSSION

Our results demonstrate that a complete lack of VC causes emphysema due to oxidative stress and a decrease of collagen...
with the decrease of hydroxyproline content in the skin, the dermis of VC-depleted SMP30 KO mice was thinner than that of WT mice.

A dose-response relationship seems to exist between VC intake and morphological changes of the murine lungs. We (32) reported previously that the SMP30 KO mouse is a novel animal model of senile lungs, since they develop air space enlargement prematurely without apparent parenchymal destruction. In that study, we fed CRF-1, an ordinary animal chow containing ~55 mg VC/kg, to SMP30 KO mice and recorded morphological changes of their lungs at the ages of 1, 3, and 6 mo. Although we did not measure VC levels of the lungs at that time, we expect that the total VC levels in the lungs were very low but sufficient to prevent the SMP30 KO mice from developing scurvy. This expectation is based on another of our studies documenting that the liver and kidneys contained only 6–8% of VC values of those from matched WT mice when the SMP30 KO group was fed CRF-1 for 80 days (24). Presumably, a reduction of VC in the lungs resulted in an enlargement of the air space without alveolar destruction, but complete lack of VC in the lungs generated pulmonary emphysema. Several epidemiologic studies indicate that dietary factors, particularly VC, may be involved in the etiology of COPD (31, 41, 44). A prospective study of dietary intake of antioxidant vitamins (vitamins A, C, and E) and decline of lung function performed in a general population consisting of 2,663 adults aged 18–70 living in the United Kingdom identified a significant correlation between declining lung function and intake of VC but not vitamins A or E (31). Siedlinski et al. (44) recently reported that an interaction between functional polymorphisms of glutamate-cysteine ligase, an endogenously acting antioxidant enzyme, smoking (packs/yr), and low VC intake all contributed to the oxidative burden and were associated with a loss of lung function.

On the other hand, we demonstrate here that VC supplementation affected lung morphology in WT mice. In those mice, VC supplementation caused a significant increase of MLI with increased ROS in the lungs compared with their WT counterparts deprived of VC. Although SMP30 was first identified in rats whose expression of this molecule decreased with aging (11), we later found a distinct temporal profile of lessening SMP30 expression in the liver, kidney, and lungs of mice. Their SMP30 mRNA level peaked at the age of 1–3 mo and decreased thereafter in the liver, was consistently low in the kidneys, but peaked at the age of 12 mo in the lungs (32). However, in the present study, VC levels in the lungs appeared to have peaked when the mice were only 2 or 3 mo old, since VC supplementation did not further increase the VC content in their lungs. VC is a water-soluble vitamin, and one can assume that excess VC would be easily excreted and, therefore, have no harmful effects on tissues. However, oxidative stress may develop because excess VC can generate ascorbate radicals and hydrogen peroxide as well as highly reactive hydroxyl radicals through the promotion of Fenton reactions (5, 7).

We found that Sirt1 expression decreased in the lungs under conditions in which VC was completely absent, and MLI correlated well with Sirt1 expression in the lungs of SMP30 KO mice at 3 mo of age. SIRT1 is known as both a type III histone deacetylase (HDAC) and a deacetylase for various transcription factors such as NF-kB, p53, and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1α; Refs. 14, 20). Moreover, SIRT1 is an important protein in the synthesis in the lungs of SMP30 KO mice. VC is not only a potent antioxidant, but also plays an essential role in collagen synthesis by promoting the activity of the prolyl hydroxylase and by increasing the mRNA of collagen (13, 33, 35, 38). The metabolism of extracellular matrix proteins of the lung is clearly important for the development of emphysema, since the breakdown of collagen by transgenic expression of collagenase in mice generated emphysema (6). In addition, oxidative stress appears to play a pivotal role in the development of COPD, as shown by increases in the markers of oxidative stress present systemically as well as in the lungs of patients with COPD (28). We (25) have recently reported that VC-depleted SMP30 KO mice specifically increased superoxide generation in a model of the living brain compared with that of WT mice, and the increase of ROS was prevented by administration of VC. We also reported that hydroxyproline content in the skin from VC-depleted SMP30 KO mice significantly decreased compared with that of WT mice, and the decrease of hydroxyproline was prevented by administration of VC (1). Consistent with the decrease of hydroxyproline content in the skin, the

Fig. 7. Sirtuin 1 (Sirt1) expression in the lungs and its correlation with MLI in the lungs of 3-mo-old mice. Sirt1 expression was determined by immunoprecipitation and Western blotting. A: representative results of Western blotting of Sirt1 in the lungs. B: relative levels of Sirt1 [% of WT VC(−) group] in the lungs. Values are presented as means ± SD of 5 animals. C: correlation between MLI and the levels of Sirt1 in the lungs from all 4 groups at 3 mo of age.
redox-sensitive NF-κB-mediated release of proinflammatory cytokines, apoptosis, and aging (39, 52), all of which are closely linked with the pathogenesis of COPD (15, 20). Supporting this interconnection, a deficiency of SIRT1 expression was reported in the lungs of smokers and patients with COPD compared with nonsmokers (39).

Our study clearly has limitations in terms of examining the genuine effect of VC depletion on pulmonary morphology. First, we evaluated the lungs of SMP30 KO mice at 2 and 3 mo of age when the lungs are still developing and maturing. Accordingly, we cannot adequately explain whether emphysema arises from developmental or destructive problems or both. Since SMP30 KO mice receive VC in mother’s milk until weaning (~1 mo of age), examination of the lung morphology during this developmental period is not helpful for the present study. Second, SMP30 has multiple functions aside from synthesizing VC. We (10) and other groups (43) reported that SMP30 regulates the homeostasis of intracellular Ca^{2+}. Furthermore, SMP30 regulates the intracellular free Ca^{2+} concentration by enhancing plasma membrane Ca^{2+} pumping activity (10). Therefore, possible dysregulation of intracellular Ca^{2+} homeostasis due to the genetic disruption of SMP30 may have contributed to the development of emphysema in the present study. When disrupted, α-Klotho, a regulator of calcium metabolism in the plasma membrane, is associated with a premature aging-like phenotype including emphysema (16, 26). Additionally, the lack of α-Klotho induces hyperfunction of renal tubular 1α-hydroxylase resulting in an abnormal increase of serum Ca^{2+}, which activates the calcium-dependent matrix proteases such as calpain and induces emphysema (29). We anticipate a need to examine the possible role of intracellular calcium homeostasis as well as oxidative stress in the development of aging-like phenotypes in SMP30 KO mice. We have noted further that SMP30 is localized in both the nuclei and cytoplasm of cultured murine hepatocytes and that a domain consisting of 51 amino acid residues has a 60 – 66% similarity to bacterial and yeast RNA polymerases (18). The diverse subcellular localization of SMP30 may support its many functions in intracellular signaling processes such as activation of Akt on apoptosis (30) or suppression of nuclear DNA and RNA synthesis (21, 22). These divergent properties of SMP30 seem to explain why VC supplementation did not completely restore lung morphology or levels of collagen I mRNA and ROS in SMP30 VC(−) mice despite the comparable VC level attained in their lungs. To address the precise role of VC in fully developed, mature lungs rather than during the course of development, we must prepare VC-free SMP30 KO mice for observation at more advanced ages. However, from the viewpoint of the multifunctional properties of SMP30, the older SMP30 KO mice grow, the more their lungs would be impacted by the other malfunctions of SMP30 in addition to the VC deficiency. Third, we did not examine any markers or evidence of alveolar cell apoptosis and inflammation. Although we focused on oxidative stress in this study, we may have oversimplified the overall multifactorial nature of lung destruction, since it is now well-recognized that lung destruction occurs due to the mutual interaction among oxidative stress, alveolar cell apoptosis, protease/antiprotease imbalance.

Fig. 8. Azan staining in the lungs of VC(−) and VC(+) groups of WT (A and B) and SMP30 KO (C and D) mice at 3 mo of age. Collagen is stained blue, and nuclei are stained red.

Fig. 9. Relative levels of collagen I (Col Ia2) mRNA [% of WT VC(−) group] in the lungs from VC(+) and VC(−) groups of WT and SMP30 KO mice at 3 mo of age. Values are presented as means ± SD of 5 animals.
ance, and inflammation (28). The possibility certainly exists that alveolar cell apoptosis occurred due to oxidative stress induced by VC depletion and contributed to the development of emphysema in our SMP30 KO VC(−) group. Similarly, the increase in absolute counts of inflammatory cells in the BALF of our SMP30 KO VC(−) group may denote the expression of inflammation in their lungs. Finally, we did not dissect the roles of altered oxidative stress and collagen synthesis or the interaction between them that was caused by a complete lack of VC. Experiments in which alternative anti-oxidants are administered instead of VC should resolve this issue.

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