ONLINE SUPPLEMENTARY MATERIAL **R1**

DNA DAMAGE AS A MOLECULAR LINK IN THE PATHOGENESIS OF COPD IN SMOKERS

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METHODS

Immunofluorescence Staining

Deparaffinized tissue sections were autoclaved in a citrate buffer (10 mM, pH 6.0) for 30 minutes to expose the immunoreactive epitopes of antigens. After blocking the nonspecific binding sites with a serum-free protein blocking solution (Dako Japan, Tokyo) at room temperature for 10 minutes, the sections were incubated with primary antibodies at 4 ^{o}C overnight. The primary antibodies used were: rabbit monoclonal anti-phospho(Ser139)-histone H2AX (γH2AX) (clone 20E3, Cell Signaling Technology, Danvers, MA), mouse monoclonal anti-phospho(Ser139)-histone H2AX (γH2AX) (clone 3F2, Abcam, Tokyo, Japan), rabbit polyclonal anti-phospho(Ser25)-53BP1 (Abcam), rabbit polyclonal anti-phospho(Ser/Thr)-ataxia teleangiectasia mutated kinase (ATM)/ataxia teleangiectasia and Rad3-related kinase (ATR) substrate (Cell Signaling Technology), mouse monoclonal anti-p16^{INK4a} (p16) (clone F-12, Santa Cruz Biotechnology), rabbit polyclonal anti-cleaved(Asp175)-caspase-3 (Cell Signaling Technology), rabbit polyclonal anti-phospho(Ser536)-nuclear factor (NF)-κB (Abcam), mouse monoclonal anti-IL-6 (clone R-49L, Santa Cruz Biotechnology), mouse monoclonal anti-8-hydroxy-2-deoxyguanosine (8-OHdG) (clone N45.1, Japan Institute for the Control of Aging, Shizuoka, Japan), goat polyclonal anti-surfactant protein-C (SPC) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), goat polyclonal anti-aquaporin-5 (AQP5) (Santa Cruz Biotechnology), and mouse monoclonal anti-CD31 (clone JC70A, Dako Japan, Tokyo). The primary antibodies were reacted with secondary antibodies conjugated with Alexa Fluor 350, Alexa Fluor 488 or Alexa

Fluor 594 (Invitrogen, Carlsbad, CA), and, when necessary, the slides were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Sigma). The specificity of immunosignals was assessed by omitting the primary antibodies and preincubating the γH2AX antibody with a blocking peptide (Cell Signaling Technology).

Cell Culture and Irradiation

Normal human lung microvascular endothelial cells (fourth passage, Clonetics Co., Walkersville, MD) were plated onto an 8-well tissue culture plate and cultured in Microvascular Endothelial Cell Growth Medium®-2 (EGM®-2, Clonetics) supplemented with EGM®-2 SingleQuots® containing human recombinant epidermal growth factor, human fibroblast growth factor, vascular endothelial growth factor, vitamin C, hydrocortisone, human recombinant insulin-like growth factor, heparin, fetal bovine serum, gentamicin, and amphotericin. After reaching 50% confluence, cells were irradiated with a 10 Gy X-ray dose with an X-irradiator (Hitachi Medicotechnology, MBR-1520R-3, Hitachi, Japan) at a rate of 2.47 Gy/min, and 2 and 48 hours later, cells were fixed with 3% paraformaldehyde in PBS for 5 minutes. The cells were then permeabilized with 0.5% tritonX-100, blocked with a serum-free protein blocking solution (Dako Japan, Tokyo) for 10 minutes, and stained with appropriate primary antibodies and AlexaFluor-594-conjugated secondary antibodies (Invitrogen). The primary antibodies used were: anti-γH2AX (Cell Signaling Technology), anti-phospho(Ser25)-53BP1 (Abcam), anti-p16 (Santa Cruz Biotechnology), anti-cleaved(Asp175)-caspase-3 (Cell Signaling Technology), and anti-phospho(Ser536)-NF-κB (Abcam). Cell nuclei were stained with DAPI. The cell

culture supernatants were harvested 48 hours after irradiation, and the IL-6 concentration was measured by using an ELISA kit (Biosource International, Camarillo, CA).

Cell Culture and Bleomycin Treatment

Alveolar type II-like epithelial cell line A549 cells (ATCC #CCL-185) were plated onto an 8-well tissue culture plate and cultured in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% FCS under a humidified atmosphere of 5% CO_2 in air. After reaching 50% confluence, cells were treated with 50 μg/ml of bleomycin (Nippon Kayaku Co., Ltd., Tokyo, Japan) and 24 hours and 48 hours later, cells were fixed with 3% paraformaldehyde in PBS for 5 minutes. The cells were then permeabilized with 0.5% tritonX-100, blocked with a serum-free protein blocking solution (Dako Japan, Tokyo) for 10 minutes, and stained with appropriate primary antibodies and AlexaFluor-594-conjugated or AlexaFluor-488-conjugated secondary antibodies (Invitrogen). The primary antibodies used were: anti-γH2AX, anti-phospho(Ser25)-53BP1, anti-p21 (Abcam), anti-cleaved(Asp175)-caspase-3, and anti-phospho(Ser536)-NF-κB. Cell nuclei were stained with DAPI. The cell culture supernatants were harvested 48 hours after irradiation, and the IL-6 concentration was measured with an ELISA kit.

Guinea Pig Exposure to Cigarette Smoke

The animal protocol was approved by the Animal Care and Use Committee of Tokyo Women's Medical University. Male 4-week-old Hartley-strain guinea pigs, weighing 250 to 300 g were purchased from Japan SLC (Shizuoka, Japan) and housed in

polycarbonate cages fitted with high-efficiency particulate air filters. They were provided with access to food and water ad libitum. Commercial plain-tipped (non-filtered) cigarettes (Peace®; Japan Tobacco Inc., Tokyo, Japan) yielding 24 mg tar and 2.4 mg nicotine under a standard smoking regimen were used in this study. The guinea pigs were exposed with a vented nose-only chamber while awake. Each guinea pig was placed in an individual stall and exposed to the smoke of a cigarette by delivering it into the stall with an electric pump. The cigarette was lit and the smoke was delivered into the chamber for a period of 10 minutes and no smoke was delivered into the chamber for the next 20 minutes. The cycle was repeated until a total of 10 cigarettes had been smoked over a period of 5 hours. The controls were placed in the apparatus for the same period of time, but they were exposed to room air instead of cigarette smoke. Whenever an animal developed a bronchospastic reaction during cigarette-smoke exposure, the animal was removed from the chamber and intraperitoneally injected with adrenaline (0.2 ml, 1:10.000). The study was performed on a total of 16 animals in the same shipment lot after randomly assigning them to a control group (sham exposure, n = 8) and smoke-exposure group (n = 8). The initial design was to continue the exposures for 12 weeks, but we had to stop at the end of 10 weeks, because three animals in the smoke-exposure group died before 10 weeks as a result of bronchoconstriction during smoke exposure. These three animals were excluded from the final analysis.

Lung Processing, Morphometric Analysis, and Immunofluorescence Staining

The morphometric analysis of the lungs was performed according to the Thurlbeck

approach (E1). Each animal was weighed immediately before intraperitoneal injection of an overdose of pentobarbital. The lungs were inflated and fixed by intratracheal instillation of 10% formalin at a constant pressure of 25 cm H_2O. The left lung was embedded in paraffin and sagittally sectioned into 6 slices. Each slice was and cut into 3 μm sections, and these 6 sections were stained with hematoxylin and eosin and coded to prevent the observer from identifying the group from which it had been obtained. Twenty randomly selected fields were examined on each slide, and thus a total of 120 fields yielding 120 fields was examined per animal. All slides were analyzed at 200x magnification by using a computer program. Video images of each field were captured with an Olympus DP50 CCD camera and the video output of the camera was sent to an Olympus imaging microscope workstation (CUSL2G40) equipped with a computer running Microsoft Windows XP software. The video image of each field was projected onto the monitor of the computer on which 2 diagonal test lines were superimposed within an area measuring 12 cm x 14 cm. The mean linear intercept (Lm) was determined by counting alveolar wall intersections with the test lines on the video image and calculated by using the formula: Lm = 2LT/Iw, where Iw is the number of times the test line intersected with the alveolar wall and LT is the length of the test line.

For immunofluoresecence staining, the mid-sagittal paraffin-embedded tissue sections were deparaffinized, autoclaved, and stained with anti-γH2AX antibody as described above. The sections were counterstained with DAPI. We examined 10 randomly selected microscopic fields on each slide with an Olympus BX60 epifluorescence microscope equipped with a 100x objective, and visually counted the

numbers of γ□2AX foci per cell in alveolar wall cells.

REFERENCES

E1. Thurlbeck WM. Christie lecture: Emphysema then and now. *Can Respir J* 1994;1 : 121–139.

FIGURE LEGENDS

Figure S1. Correlations between the numbers of γH2AX foci and the numbers of phosphorylated 53BP1 foci per cell in type II cells (*A*) and between the numbers of γH2AX foci and numbers of phosphorylated ATM substrate foci per cell in type II cells (*B*). *Circles* = asymptomatic smokers; *triangles* = asymptomatic smokers; *squares* = COPD patients.

Figure S2. X-irradiation of lung microvascular cells induces DSBs, apoptosis, cell senescence, NFκB phosphorylation, and IL-6 production. Normal human lung microvascular cells were cultured and irradiated with a 10 Gy X-ray dose. Cells were fixed in 3% paraformaldehyde 2 hours later for immunostaining for γH2AX, phosphorylated 53BP1, and active caspase-3, or 48 hours later for immunostaining for p16 and phosphorylated NFκB. Cell culture supernatants were harvested 48 hours after irradiation, and IL-6 the concentration was measured by ELISA. (*A*) Representative images of immunofluorescence stained X-irradiated cells (*IR*) and unirradiated cells (*Cont*). Positive immunosignals are seen in red fluorescence. Cell nuclei were stained with DAPI. (*B*-*F*) Quantitative analyses of the results of immunofluorescence staining. $N = 4$ in each experiment. (*G*) Results of ELISA measurements of the amount of IL-6 secreted by X-ray-irradiated cells and unirradiated cells. $N = 8$ in each experiment.

Figure S3. Treatment of A549 cells with bleomycin induces DSBs, apoptosis, cell senescence, NFκB phosphorylation, and IL-6 production. A549 cells were treated with

50 μg/ml of bleomycin and fixed in 3% paraformaldehyde 24 hours later in preparation for immunostaining for γH2AX, phosphorylated 53BP1, and active caspase-3, or 48 hours later for immunostaining for p21 and phosphorylated NFκB. Cell culture supernatants were harvested after 48 hours, and the IL-6 concentration was measured by ELISA. (*A*) Representative images of immunofluorescence-stained bleomycin-treated cells (*Bleo*) and untreated cells (*Cont*). Positive immunosignals for γH2AX, phosphorylated 53BP1, active caspase-3, and phosphorylated NFκB are seen in the form of red fluorescence, and for p21 in the form of green fluorescence. Cell nuclei were stained with DAPI. (*B-F*) Quantitative analyses of the results of immunofluorescence staining. $N = 4$ in each experiment. (*G*) Results of ELISA measurements of the amount of IL-6 secreted by bleomycin-treated cells and untreated cells. $N = 8$ in each experiment.

Figure S4.

DSBs occurred in the lungs of guinea pigs with cigarette smoke-induced emphysema. (*A*) Representative photomicrographs of lung tissue sections stained with hematoxylin and eosin (*upper panels*) and stained with anti-γH2AX antibody (*lower panels*). *Inset* is an enlarged photomicrograph of γH2AX-positive cells. (*B*) Quantitative analyses of airspace size (Lm: mean linear intercepts) and the number of γH2AX foci in the alveolar wall cells. *CS* = lungs of guinea pigs exposed to cigarette smoke for 10 weeks ($n = 5$); *Air* = lungs of sham-exposed guinea pigs ($n = 8$). Data are expressed as means ± SEM.