Endothelial-mesenchymal transition

in bleomycin-induced pulmonary fibrosis

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Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and antibiotics (100U/ml penicillin, 100μg/ml streptomycin, and 0.25μg/ ml fungizone) were from Gibco-Invitrogen Corporation (Carlsbad, CA). Recombinant human EGF and recombinant human TGFβ were from R&D Systems (Minneapolis, MN). Phycoerythrin (PE)-conjugated rat anti-mouse CD34 antibody (Clone RAM34), streptavidin Cy-chrome (SAv-Cy-Chrome), biotin-conjugated rat anti-mouse CD31 (PECAM-1) antibody (Clone MEC13.3), purified rat ant-mouse CD144 (VE-cadherin) antibody (Clone 11D4.1) and BD cytofix/cytoperm kit were from BD Biosciences (San Diego, CA). Biotin-conjugated anti-rat IgG antibody and Vector M.O.M. Immunodetection Kit were from Vector Laboratories (Burlingame, CA). Biotin-conjugated rat anti-mouse Tie2 antibody (Clone Tek4) was from eBioscience (San Diego, CA). Biotin-conjugated rabbit anti-type I collagen (Col I) antibody was from Rockland (Gilbertsville, PA). FITC-conjugated mouse anti-α -smooth muscle actin (α-SMA) antibody (Clone 1A4), REDExtract-N-Amp Tissue PCR Kit, ITS (insulin, transferrin and selenium) liquid media supplement and 30% (W/W) H2O2 were from Sigma (St. Louis, MO). Histochoice MB was from Amresco (Solon, OH). Tyramide Signal Amplification

(TSA) Biotin System was from PerkinElmer (Boston, MA). Hoechst33342 was from Dojindo (Kumamoto, Japan). qPCR MasterMix Plus was from Eurogentec (Seraing, Belgium). SAv Alexa488 (SAv-488), SAv Alexa594 (SAv-594), SAv-594-conjugated anti rat antibody, SAv-594-conjugated anti rabbit antibody, superscript III reverse transcriptase and platinum Taq DNA polymerase were from Invitrogen Life Technologies (Carlsbad, CA). Collagenase type III and DNAse were from Worthington Biochemical Corp. (Lakewood, NJ). Costar Transwell was from Corning Costar Corporation (Cambridge, MA). Bleomycin (BLM) was kindly provided from Nippon Kayaku Co., Ltd (Tokyo, Japan).

Cells

The mouse microvascular endothelial cell line MS1 and activated Ras transduced MS1 (SVR) were maintained in DMEM supplemented with 2mmol/L L-glutamine, 100U/mL penicillin, 100μg/mL streptomycin, 0.25μg/mL fungizone, and 10% FCS (S1). For immunocytochemstry, some cells were cultured into an 8-well Lab-Tek Chamber Slide System (Nalge Nunc International, Naperville, IL).

Mice

Tie2-Cre transgenic (Tg) mice on C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME). CAG-CAT-LacZ Tg mice on C57BL/6 background were kindly provided by Dr. Jun-ichiro Miyazaki (Osaka University, Japan) (S2). To obtain Tie2-Cre/CAG-CAT-LacZ mice (CAG mice), Tie2-Cre Tg mice were bred with CAG-CAT-LacZ Tg mice. All animal studies have been reviewed and approved by the University Committee on Use and Care of Animals at Nagoya Graduate School of Medicine.

Determination of Cre-mediated transgene recombination

To analyze the Cre-mediated recombination, DNA was isolated from tail of Tie2-Cre/CAG-CAT-LacZ mice, using REDExtract-N-Amp Tissue PCR Kit according to the manufacturer's recommendations. Tail DNA was subjected to PCR analysis to make sure the deletion of the CAT gene and the expression of the LacZ gene by complete Cre-mediated recombination, using the set of primers used in the previous report (S2). The primers used for amplification of Cre and LacZ were designed on Cre gene and LacZ gene, respectively. The primers used for amplification of CAG were also designed on CAG promoter and the lacZ gene. Before the deletion, the sequence between the primer of sense and antisense for CAG is too long to amplify in the assay,

but can be amplified only after the CAT gene is removed (S2).

Mouse fibrosis model

Pulmonary fibrosis was induced by endotracheal BLM injection as before (S3).

Briefly, BLM was suspended in sterile saline at 3mg/ml. Tie2-Cre/CAG-CAT-LacZ mice were treated with 6μg/g body weight of BLM diluted in sterile saline or the same volume of sterile saline.

Generation of BM chimera mice.

BM chimeras were prepared as previously described (S3). Briefly, donor BM cells were collected from femurs and tibias of B6 mice and CAG mice by aspiration and flushing. Recipient B6 or CAG mice were exposed to 2 doses of 5Gy given 3hours apart. After irradiation, 4x106 BM cells from donor mice in a volume of 200μl sterile PBS were injected into recipient mice under anesthesia.

Collection of lung samples from BLM-treated mice

At day28 post-BLM injection, mice were euthanized and both lungs were thoroughly perfused with saline to remove blood from the lung vascular beds as before (S4). To

collect freshly frozen lung samples for staining, lungs were lavaged twice with PBS and then removed from the thoracic cavity, and dissected free of extraneous tissue. Finally, the samples were frozen in OCT compound (Miles, Elkhart, IN). Serial cryostat sections (4-μm thick) were stained with H&E or with X-gal staining (see below).

Mouse lung fibroblast culture

Mouse lung fibroblasts were isolated from lung tissue by mincing and enzymatic digestion as previously described (S4). After filtration, released cells were centrifuged, washed and cultured in DMEM supplemented with 10% FCS, recombinant human PDGF (5ng/ml), recombinant human EGF (10ng/ml), ITS liquid media supplement (1:100) and antibiotics. The cells were maintained in culture and passaged as before (S4). Fibroblasts in this study were used after the second passage.

X-gal staining

For detection of β−galactosidase activity, cultured cells and lung tissues were fixed with 0.5% glutaraldehyde in PBS for 5minutes, then washed twice with PBS, and incubated in an X-gal solution, containing 8.4mM KCL, 84mM sodium phosphate buffer (pH 7.5), 1mM MgCl2, 3mM K4Fe(CN)6, 3mM K3Fe(CN)6, and 1mM X-gal

(5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) at 37°C overnight. Nuclei were stained with Hoechst33342 for cultured fibroblasts or hematoxylin for lung tissues, respectively. A minimum of 5 randomly selected high-power fields were examined per sample to count X-gal positive cells until at least a total cell count of 100 was reached. X-gal positive cells were counted and expressed as a percentage of total cells counted for each sample. A total of at least four samples were examined (S5).

Cell treatments and analysis of phenotype changes in vitro

To examine the combined effect of activated Ras and TGFβ on phenotype alterations in endothelial cells, MS1 and SVR were plated and treated with 10ng/ml TGFβ in medium without FCS for 24hours. They were then harvested for assessment of fibronectin and Col I mRNA by real time PCR, or endothelial-specific marker expression, including CD31, CD34, VE-cadherin, and Tie2, by FACS. For immunocytochemical determination of α-SMA, cultured cells in an 8-well Lab-Tek Chamber Slide were also treated with 10ng/ml TGFβ in medium without FCS for 24hours. To distinguish endothelial-MT *in vitro* from transient phenotype changes, defined as "scattering", these cells were cultured for another 24hours in medium with FCS after TGFβ removal and followed by phenotype analysis. After treatment with TGFβ, SVR were re-plated into the new culture dish to evaluate whether the cells can retain the phenotype changes.

In vitro analysis of phenotype alteration in endothelial cell line by FACS

After treatment, the harvested cells were stained with appropriate dilutions of biotin-conjugated rat anti-mouse CD31 antibody, PE-conjugated rat anti-mouse CD34 antibody, biotin-conjugated rat anti-mouse Tie2 antibody, purified rat ant-mouse VE-cadherin antibody, or the appropriate isotype-matched controls, and then detected by subsequent staining with SAv-Cy-chrome for CD 31 and Tie2, and SAv-488-conjugated anti rat antibody for VE-cadherin, respectively. Dead cells were excluded from flow cytometry analysis by appropriate gating (4) and total $2x10^4$ living cells were collected for each analysis on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). The results are presented as overlaid histograms and the relative mean fluorescence intensity (MFI). The relative MFI was calculated by dividing the MFI units of CD31, CD34, Tie2, or VE-cadherin staining by the MFI units of isotype control staining in each sample (S6).

Endothelial cell line transplantation in vivo and isolation of activated Ras transduced endothelial cells ex vivo

A million of MS1 or SVR cells were inoculated subcutaneously (s.c.) into the flank of

6-week-old female nude mice (S7). At day28 post-inoculation, the resulting nodule containing SVR cells was excised and *ex vivo* SVR cells from nodule were isolated by mincing and enzymatic digestion as previously described (S4). After filtration, released cells were centrifuged, washed and cultured in complete medium. To eliminate host-derived cells such as fibroblasts, cultured cells were incubated and selected with neomycin, since SVR cells have the neomycin resistance gene. Isolated *ex vivo* SVR cells were plated and treated with 10ng/ml TGFβ and then harvested for the analysis as above. To evaluate lung metastasis in treated mice, the lungs were resected at day28, fixed in 10% formalin, incorporated into paraffin blocks, sectioned at 4μm, and stained with hematoxylin and eosin (H&E).

Immunocytochemistry for cultured fibroblasts and endothelial cells lines

Unless otherwise specified, immunostaining procedures were done as previously described with TSA biotin system (S8). Immunostaining procedures for slides of cultured fibroblasts were performed after X-gal staining. All slides were air-dried and fixed in Histochoice MB solution for 20minutes. Endogenous peroxidase activity was inactivated by 0.3% H2O2 in methyl alcohol for 30minutes, and after washing the slides were additionally immersed in 0.2% Triton X-100 in PBS for 15minutes for immunostaining of α-SMA and/or Col I. After blocking nonspecific protein binding

with the blocking buffer (supplied in TSA system kit), the slides were incubated with the appropriate dilutions of primary antibodies. When mouse monoclonal antibody was used, the M.O.M. immunodetection kit was used according to the manufacturer's recommendation. As negative controls, each primary antibody was substituted with the appropriate species and isotype-matched immunoglobulins. For CD31 or Tie2 staining, the slides were incubated for one hour with biotin-conjugated rat anti-mouse CD31 antibody or biotin-conjugated rat anti-mouse Tie-2 antibody. For immunostaining of α-SMA and/or Col I, the slides were incubated overnight at 4°C with appropriate dilutions of biotin-conjugated rabbit anti-Col I antibody and/or FITC-conjugated mouse anti-α-SMA antibody. All slides were sequentially incubated with streptavidin-peroxidase complex (HRP-SAv) and biotinyl tyramide, with washes in-between each incubation. Finally, SAv-488 or SAv-594 was utilized to visualize antibody binding. For cultured fibroblasts, nuclear staining was performed with Hoechst33342. The sections were mounted with Immu-mount (Shandon, Pittsburg, PA).

PCR analysis for expression of targeted genes

Total RNA was isolated from treated cells as previously described (S4). Semiquantitative PCR analysis was done as previously described (S9). RNA (500ng)

from each sample was reverse transcribed with oligo-dT20 primer and Superscript III. Real time PCR was performed using a TaqMan ABI 7300 Sequence Detection System (PE Applied Biosystems, Foster City, CA). After initial denaturation at 95°C for 10minutes, cDNA was amplified with qPCR MasterMix Plus as follows: 40cycles of a two-step PCR program at 95°C for 15seconds and 60°C for 60seconds. Fibronectin, Col I, Snail, and Twist mRNA were detected, using mixture of oligonucleotide primers and probe from TaqMan Gene Expression Assays (Assay IM; Mm00692642_m1 for fibronectin, Mm01302050_g1 for Col I, Mm00441533_g1 for Snail, and Mm00442036_m1 for Twist, respectively) (Applied Biosystems, Foster City, CA). The mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA signal using TaqMan rodent GAPDH control reagents from Applied Biosystems (S10). In preliminary experiments, we confirmed that the PCR products, which were performed using the target gene-specific primers, were not contaminated with fragments amplified from genomic DNA by comparing the products generated with and without reverse transcriptase. RT-PCR analysis for mRNA of TGFβR I and II, and GAPDH was performed with platinum Taq. Primer sequences used for TGFβR I (GenBank accession no. NM_009370) and II (GenBank accession no. NM_009371 for variant1 and NM_029575 for variant2), and GAPDH were as follows: TGFβR I (552bp

product), sense: (5'-TGGTCTTTGCTTTGTCTC-3'), antisense (5'-ACAGCAACTTCTTCTCCC-3'); TGFβR II (variant1; 381bp product, variant2; 303bp product, respectively), sense: (5'- GCTGCATATCGTCCTGTG -3'), antisense (5'- AGAGTGAAGCCGTGGTAG -3'); GAPDH (308bp product), sense: (5'-GCAGTGGCAAAGTGGAGATT -3'), antisense (5'-GCAGAAGGGGCGGAGATGAT -3') (S11).

Statistical analysis

The results were analyzed using the Mann-Whitney test for comparison between any two groups, and by non-parametric equivalents of analysis of variance (ANOVA) for multiple comparisons. $P<0.05$ was considered to indicate statistical significance.

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Supplemental Figure Legends

Figure S1 *Immunohistochemical evaluation for the lungs of saline-treated CAG mice and BLM-treated CAG mice*

The lungs from saline-treated mice and BLM-treated mice were analyzed by fluorescence microscopy (**A**-**F**). The lungs from saline-treated mice were stained with both isotype-macthed control IgG for Col I (**A**) and anti CD31 antibody (green) (inset in **A**). Nuclear staining was performed by Hoechst33342 (blue). The lungs from saline-treated mice were stained with both anti Col I antibody (red) (**B**) and anti CD31 antibody (green) (**C**). Merged image for CD31 and Col I was shown in (**D**). The lungs from BLM-treated mice were stained with both anti Col I antibody (red) (**E**) and anti CD31 antibody (green) (**F**). Magnification was 200X for (**A**-**F**). A representative example of at least three independent experiments is show. All scale bars indicate 50μm.

Figure S2 *Characterization of lung fibroblasts derived from saline-treated CAG mice*

Combined staining with X-gal and sequential immucytochemistry for Col I (red) and α-SMA (green) were performed for SLF derived from CAG mice (**A**; X-gal staining and **B**; immunocytochemistry for Col I and α-SMA, respectively). Arrowheads indicate

X-gal (+)/Col I (+)/α-SMA (-) fibroblasts. A representative example of at least three independent experiments is shown. All images were photographed at 200X magnification.

Figure S3 *Altered expression of endothelial-specific markers on endothelial cells by combined treatment with activated Ras and TGFβ*

Tie2 in (**A**) and VE-cadherin in (**B**) on endothelial MS1 and SVR treated with vehicle or TGFβ at 10ng/ml for 24hours were shown as overlaid histogram by FACS.