Metamorphosis of mesothelial cells with active horizontal motility in tissue culture

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Mesothelial cells, which have diverse roles in physiology and pathology, constitute the mesothelium along with connective tissue and the basement membrane; the mesothelium serves to shield the somatic cavities. After mesothelial injury, mesothelial cells undergo tissue recovery. However, the mechanism of mesothelial regeneration remains poorly understood. In this study, we used confocal time-lapse microscopy to demonstrate that transformed mesothelial cells (MeT5A) and mouse peritoneal mesothelial cells can randomly migrate between cells in cell culture and in vivo tissue culture, respectively. Moreover, peritoneal mesothelial cells changed their morphology from a flattened shape to a cuboidal one prior to the migration. Conversely, MDCKII epithelial cells forming tight cell–cell contacts with one another do not alter the arrangement of adjacent cells during movement. Our evidence complements the current hypotheses of mesothelial regeneration and suggests that certain types of differentiated mesothelial cells undergo morphological changes before initiating migration to repair injured sites.
Figure 1 | Mesothelial cells migrate through adjacent cells. (a) Confirmation of lack of interference of two types of cell tracking reagents (CellTracker Green CMFDA and CellTracker Red CMTPX). We used Hoechst 33342 to stain the cell nuclei. (b) MeT5A mesothelial cells changed places as indicated by the white and yellow arrowheads. MDCKII epithelial cells did not migrate but swayed back and forth. (c) and (d) Manual cell tracking of MeT5A mesothelial cells and MDCKII epithelial cells using time-lapse images that were acquired at 6-min intervals for 13 h. The initial and terminal places of tracked cells were connected using a line with gradation from white (initial) to yellow (terminal). Squares in the top panels are magnified in the bottom panels. Scale bars (a–d): 50 μm. (e) Cell tracking lines for one hundred cells in three independent experiments for each cell line. The xy coordinates indicate the location from the initial place of each cell. (f) Average velocities of each cell during time-lapse imaging. ***p<0.001.
studies. In the current study, we used a novel tissue culture method that was combined with a time-lapse confocal microscopy imaging system to provide supportive evidence for the migration and morphological change of mesothelial cells under conditions that resemble in vivo conditions.

**Results**

**Mesothelial cells migrate between cells.** First, we compared mesothelial cell behavior to epithelial cell behavior in vitro. We used a non-malignant human mesothelial cell line, MeT5A, which had been established by SV40 (simian virus 40) transformation. For comparison, we used the polarized non-transformed canine renal epithelial cell line, MDCKII. To observe cell motility, cultured cells were separately labeled with two types of cell tracking reagents (CellTracker Green CMFDA and CellTracker Red CMTPX). Separately labeled cells were later mixed together and co-cultured. These cells were seeded at a sub-confluent density to mimic in vivo conditions. The motility of green-labeled cells among the red-labeled cell population was tracked using time-lapse confocal microscopy. As shown in Fig. 1a, the admixed cells showed clear, distinct color labels with no cross-interference. Using confocal microscopy, we demonstrated that MeT5A cells were able to migrate in a horizontal manner by moving between the other surrounding cells. In contrast, the MDCKII cells remained in their original places and did not exhibit further migration other than slightly moving back and forth (Fig. 1b).

To determine the migratory distance of MeT5A cells, we tracked the cells for 13 h. MeT5A cells migrated in various directions to a distant site (Fig. 1c and d), whereas MDCKII cells slightly moved back and forth without changing their arrangement with neighboring cells. The quantitative data of cell motility are shown in Figs. 1e and f. We calculated the velocity of movement based on the location...
changes in tissue culture, we used a scanning electron microscopy. We measured the velocity of the mesothelial cells and confirmed that the cuboidal mesothelial cells exhibited migratory behaviors that were observed in the images that showed mesothelial cells when the observation began. However, cuboidal-morphology was clearly observed in the images that were captured approximately 11 h later. This result indicates that at least a portion of the mesothelial cells underwent a morphological change from a flattened to cuboidal shape. Although the altered cells were not facing the injured site, these cells horizontally migrated across the tissue by squeezing themselves through the gaps of surrounding cells. These ex vivo results are in accordance with those obtained using the cultured human mesothelial cell line, MeT5A. We found that the mesothelial cells migrated a greater distance compared to the epithelial cells. Therefore, our findings confirm the previous hypothesis that mesothelial cells undergo morphological changes and subsequent migration following injury.

**Discussion**

Mesothelial cells perform a plethora of diverse cellular functions. However, the normal physiology (e.g., motility, proliferation and differentiation) of these cells is not thoroughly understood. Although several previous studies have suggested that mesothelial cells undergo morphological transitions in response to inflammation or mesothelial injury, there has been a lack of direct evidence regarding this morphological change, especially in a setting that resembles in vivo conditions. This discrepancy might be due to the unavailability of an experimental approach that allows direct observation of the dynamics of mesothelial cell behavior.

In this study, we described a simple but novel technique that was established to observe the physiological changes in mesothelial cells. Using our cultured-tissue method, we provided direct evidence of the morphological transition in mesothelial cells without cell death (i.e., apoptosis). Using confocal microscopy imaging, we observed that the mesothelial cells within the mouse abdominal wall tissue morphologically changed from a flattened shape to a cuboidal one. Although the altered cells were not facing the injured site, these cells horizontally migrated across the tissue by squeezing themselves through the gaps of surrounding cells. These ex vivo results are in accordance with those obtained using the cultured human mesothelial cell line, MeT5A. We found that the mesothelial cells migrated a greater distance compared to the epithelial cells. Therefore, our findings confirm the previous hypothesis that mesothelial cells undergo morphological changes and subsequent migration following injury.
Figure 4 | Mesothelial cells were not apoptotic but slightly hypoxic after tissue culture. (a) Green fluorescence indicates apoptotic or digested-DNA. After 16 h of culture, muscle cells exhibit apoptosis (arrows), but mesothelial cells do not (dotted arrows). Before tissue culture, both muscle and mesothelial cells show no sign of apoptosis. Hoechst 33342 indicates nuclei. Mesothelial cells were distinguished from muscle cells for their flattened morphology and localization limited to the parietal surface of the abdominal wall. (b) Green fluorescence shows the abundance of pimonidazole-adducts, which reflects the extent of hypoxia (refer to text for details). After 16 h of tissue culture, mesothelial cells are slightly hypoxic (top left, arrows) compared with 2-h culture condition (bottom left, arrows). Scale bars (a and b): 100 μm. (c) Intensity of green fluorescence in panel (b) was measured with ImageJ. As-measured-intensity of fluorescence in the range 0 to 255 is shown in an arbitrary unit. *p<0.05.

We observed morphological transitions followed by migration in mesothelial cells, which were not situated immediately adjacent to the injured site.

Therefore, our evidence is consistent with the hypothesis that morphological changes in mesothelial cells are induced by putative soluble factors because mesothelial cells going through the conversion retain adjacent cells. However, what types of factors are involved is still elusive. Several previous studies have investigated the molecular mechanisms that regulate normal mesothelial cell behavior and have provided clues. Hott et al. have reported that thrombin enhances proliferation and migration of rat pleural mesothelial cells. Narsreen et al. have demonstrated that MCP-1, which is secreted by macrophages, induces proliferation and haptotaxis of human pleural mesothelial cells via the MCP-1/CCR2 signaling pathway. Therefore, thrombin- and MCP-1-mediated inflammation may contribute to mesothelial migration. A previous study has reported that mesothelial cells secrete IL-6 and IL-8 during inflammation and that these cytokines might be involved in a positive feedback loop that results in the activation of mesothelial cells. Necrotic factors also activate inflammatory responses in the mesothelial cells (i.e., activation of NF-κB and secretion of CXCL1 and interleukin-6). These factors represent potentially important candidates that may induce morphological changes and the subsequent migration of mesothelial cells after tissue damage. However, it is highly likely that other factors might be involved. Specifically, in the present study, hypoxia might also have contributed to the mesothelial changes. Therefore, further investigation is certainly necessary to characterize these factors. We expect that the identification of molecules that initiate or terminate the morphological and migratory changes will improve our understanding of mesothelial physiology and pathology.

In conclusion, we described a novel tissue culture approach for the observation of the dynamic behavior of mesothelial cells. This methodology was useful to evaluate mesothelial morphology and motility in conditions that resembled physiological settings compared to using an in vitro model. Using this approach, we provided supportive evidence that after injury, mesothelial cells undergo morphological changes and migrate to the injured area from a distant site. We hypothesize that these processes are induced by soluble factors that are released from damaged cells. Further investigation is required to identify the factors that comprise the key elements to this migratory event.

Methods

Materials. We obtained MeT5A cells from the American Type Culture Collection (Manassas, VA, USA). The MDCKII cells were a generous gift from Dr. Atsushi Enomoto (Nagoya University). C57BL/6 mice were purchased from Charles River Laboratories Japan, Inc. The Animal Experiment Committee of the Nagoya University Graduate School of Medicine approved the animal experiments. We purchased CellTracker Green CMFDA (Invitrogen), CellTracker Red CMTPX (Invitrogen), Hoechst 33342 (Dojindo Laboratories, Kumamoto, Japan), Hypoxyprobe Kit (Hypoxygen, Inc., Burlington, MA) and collagen (Cellmatrix Type I-C, Nitta Gelatin, Inc., Osaka, Japan).

Cell culture conditions. MeT-5A cells were maintained in Medium 199 (Invitrogen) that was supplemented with 1% antibiotic-antimycotic solution (Invitrogen), 10 ng/ml epidermal growth factor, 400 μM hydrocortisone, 870 μM insulin, 0.3% Trace Elements B (Mediatech, Manassas, VA, USA) and 10% FBS-Gold (PAA Laboratories, Ltd.). MDCKII cells were cultured in DMEM that was supplemented with 1% antibiotic-antimycotic solution and 10% FBS-Gold. All of the cells were maintained at 37°C with 5% CO₂.
Time-lapse imaging of sub-confluent cells in vitro. MoTSA and MDCKII cells were seeded at 30,000 cells/cm² per well of a 24-well plate. After 24 h, the cells were covered with 2 μM CellTracker Green CMFDA or CellTracker Red CMTPX according to the manufacturer’s protocol. After washing the cells with PBS, we added 0.3 ml of 0.125% trypsin-EDTA to detach the cells followed by 900 μl of culture medium in each well. We collected 1.2 ml of red-labeled cell suspension and 40 μl of green-labeled cell suspension, which were mixed together (cell number ratio: red:green = 30:1). We added 5 μl of culture medium to the mixture and centrifuged the cells. The cell pellet was resuspended in 150 μl of culture medium and was seeded onto a collagen-coated glass-based dish (0.5 cm²). After 24 h, images of the cells were captured every 6 h for 13 h or longer using a Nikon A1Ri confocal microscope (Nikon, Tokyo, Japan). To confirm the lack of interference from each cell-labeling solution (CellTracker Green CMFDA and CMTPX), we stained the cells with Hoechst 33342 and took high-resolution images after time-lapse imaging.

Manual tracking of cells. We analyzed the images using ImageJ software (http://rsb.info.nih.gov/ij). We used the LOCI Bio-Format plug-in (http://rsbweb.nih.gov/ij/plugins/bio-formats/) to process the acquired images and the Manual Tracking plug-in (http://rsbweb.nih.gov/ij/plugins/track/track.html) to track cell migration.

Tissue culture of the murine abdominal wall. Four- to ten-week-old C57BL/6 mice (Charles River Japan) were used. Their anterior abdominal walls were excised, washed with warm HBSS at least three times and incubated in culture medium (50% Opti-MEM, 25% FBS-Gold, 24% HBSS% and 1% antibiotic-antimycotic solution). The tissue was labeled using 5 or 10 μM CellTracker Green CMFDA according to the manufacturer’s protocol. After labeling, the tissue was subjected to time-lapse imaging using a Nikon A1Ri confocal microscope. We took images of peritoneal mesothelial cells every 20 min for 10 h or longer. The motility of thirty or more cells was tracked from each sample by ImageJ.

Scanning electron microscopy. Immediately following dissection or after 1 day of incubation, the murine abdominal walls were fixed in phosphate buffer containing 2% glutaraldehyde for 30 min. The samples were washed and fixed in 0.1% osmium tetroxide for 30 min. The tissue was dehydrated and coated with osmium tetroxide at a thickness of 10 nm. We observed the samples using S-8005 (Hitachi, Tokyo, Japan) scanning electron microscope.

TUNEL assay. Immediately following dissection or after 16 h of incubation, the murine abdominal walls were fixed in 10% neutral phosphate-buffered formalin. The tissues were subsequently sliced into sections, attached to slide glasses and deparaffinized. The specimens were washed with distilled water and subjected to antigen retrieval by pressure digestion for 10 min at 37°C. The specimens were washed with distilled water again and were incubated with TdT (Terminal deoxynucleotidyl transferase) for 50 min at 37°C. TdT reaction solution was prepared by the addition of TdT solution (Wako Pure Chemical Industries, Osaka, Japan) and fluorescein-12-UTP (Roche Applied Science, Indianapolis, IN) to TdT reaction buffer (pH 7.2, 30 mM Tris-HCl, 140 mM cacodylic acid, 1 mM CoCl2, 140 mM NaOH). The specimens were later washed with PBS and incubated with blocking solution (N101, NOF Corporation, Tokyo, Japan) for 30 min at room temperature. Followed by PBS washing, anti-fluorescein antibody conjugated with Alexa 488 and Hoechst 33432 were added to each slide. After 2 h incubation at 37°C, the slides were washed again with PBS and subjected to inverted confocal microscopy. To make positive controls, we added a slide for 30 min at 37°C with DNASe I solution (Invitrogen) before TdT reaction and incubated a slide without TdT enzyme during TdT reaction solution, respectively.

Pimonidazole-based evaluation of hypoxia. Murine anterior abdominal walls were prepared as described above. Hypoxyprobe (200 μM) was added to each tissue immediately after time culture started or after 14 h incubation. After 2 h of incubation with hypoxyprobe, tissues were fixed and subjected to immunohistochemistry using anti-pimonidazole monoclonal antibody at a concentration of 1.4 μg/ml. The tissues were observed using BZ8000 (Keyence, Japan).

Statistics. All experiments were performed three or more times. The unpaired Student’s t-test was used to calculate each statistic. The statistics was calculated using Prism 5 (GraphPad Software Inc., San Diego, CA, USA).

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**Author contributions**

H.N. conceived the project, performed the experiments and wrote the paper. C.S.H. edited the manuscript and performed animal experiments. A.E., T.K., T.N., J.L. and S.A. gave conceptual advice. Y.O. and S.F. helped animal experiments. S.T. supervised the project and edited the manuscript.

**Additional information**

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

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