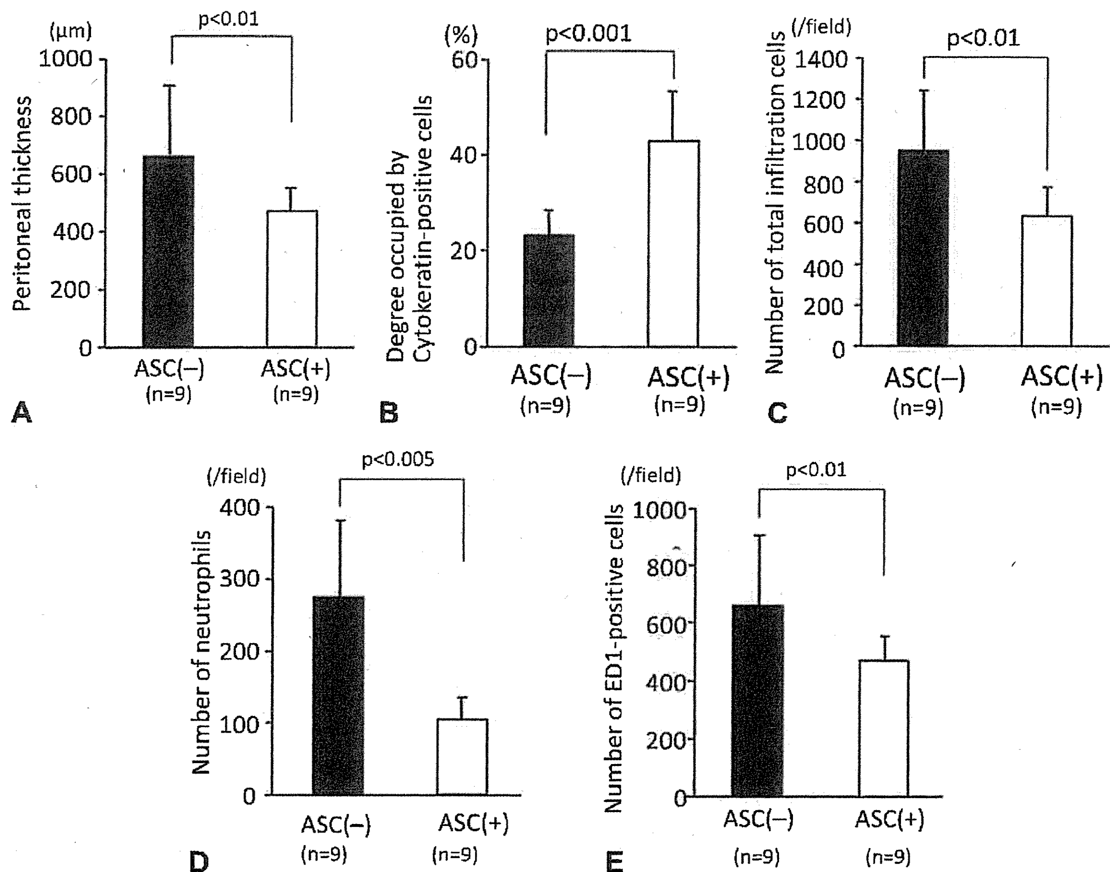

Figure 2. Peritoneal thickness, number of inflammatory cells and proportion of the peritoneal surface occupied by cytokeratin-positive cells in rats with Zy/scraping peritonitis and rats treated with rat ASCs. Peritoneal thickness (A), total number of infiltrating cells (C), number of neutrophils (D), and number of ED1-positive cells (E) were clearly suppressed by injection of rat ASCs (ASC(+)) compared with no rat ASC treatment (ASC(−)). In contrast, cytokeratin-positive mesothelial cells were observed more frequently in ASC(+) rats than in ASC(−) rats (B). Each value represents mean ± SD.

distinguish between mesothelial cells and CFSE-labeled rat ASCs in peritoneum, sections were incubated with anti-cytokeratin followed by tetramethylrhodamine isothiocyanate-labeled rabbit anti-mouse IgG (H+L) (Zymed, South San Francisco, CA, USA).

In vitro co-culture assays with rat ASCs and rat mesothelial cells and WST-1 assay to confirm progression of mesothelial proliferation

First, rat mesothelial cells (4.5×10^5) from primary cultures were co-cultured directly with rat ASCs (0.45×10^5) or without rat ASCs on type I collagen-coated plastic dishes. At 48 h after co-culturing of both groups of cells, numbers of mesothelial cells were counted and compared. To adjust for the pure increase of mesothelial cell number, we incubated 4.5×10^5 rat ASCs in a separate dish to subtract the increase in ASCs (control rat ASCs) for 48 h. The increased number of mesothelial cells co-cultured with 0.45×10^5 rat ASCs was calculated using the following formula:

Number of co-cultured mesothelial cells (/dish) = (total cell count in a dish of co-cultured mesothelial cells and rat ASCs)−(number of control rat ASCs) $\times$ 0.45/4.5

To study whether some fluid-phase factors released from rat ASCs were related to proliferation of mesothelial cells, we used a Transwell system. Rat mesothelial cells were cultured with or without rat ASCs on 12-well Coster Transwell 0.4-μm membranes (Corning, Lowell, MA, USA). Rat mesothelial cells (2.0×10^5) were at the bottom of the well, and rat ASCs (2.0×10^5 or none) were on the inserter. After 48 h of culture, the number of mesothelial cells at the bottom of the well was counted.

In addition, Cell Proliferation Reagent WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate) (Roche Diagnostics,

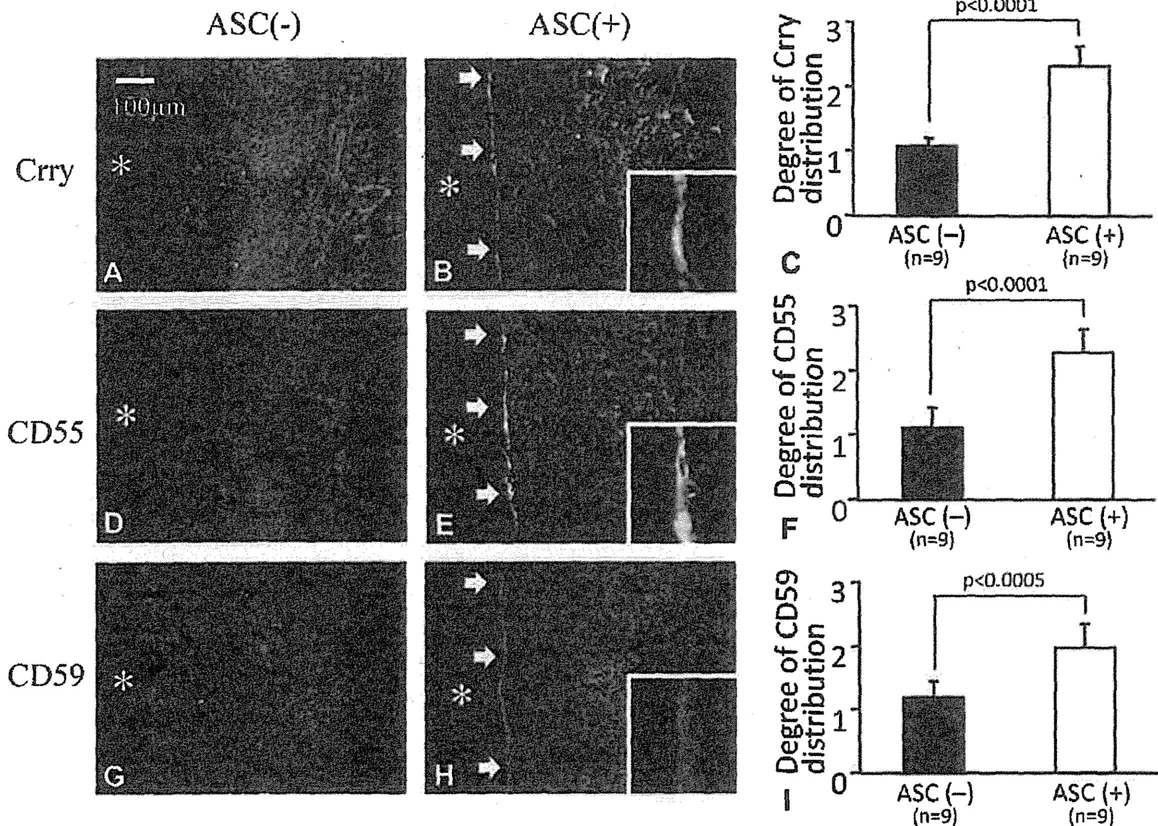

Figure 3. Changes in expression of membrane complement regulators Crry, CD55 and CD59 along peritoneal surfaces. Along the peritoneal surface of ASC(+) rats, distributions of CReg expression are clearly increased (B, E, H) compared with ASC(−) rats. Original magnifications are ×200, and scale bar is in upper left corner of (A). Insets (B, E, H) are ×4 magnifications of the originals. Arrows show expression of CRegs on peritoneal surface. *External face of the peritoneum. Degree of Crry (C), CD55 (F) and CD59 (I) distribution in peritoneum. Each value represents mean ± SD.

Mannheim, Germany) was used to confirm enhancement of mesothelial cell proliferation according to information provided by the manufacturer. To confirm that fluid-phase factors from rat ASCs increased proliferation of mesothelial cells, rat mesothelial cells or rat ASCs were cultured in M199 with 0.5% FBS in 6-cm dishes for 48 h. Numbers of rat ASCs or rat mesothelial cells were prepared to approximately 2×10^6/dish after 48 h of incubation. Separately, rat mesothelial cells (1×10^4/well in 100 μL of M199 with 0.5% FBS) were incubated in 96-well multiple plates for 3 h. For each well in the 96-well multiple plates, the supernatant was changed to 100 μL of the supernatant from rat mesothelial cell culture or rat ASC culture dishes. After incubation for 22 h at 37°C, 10 μL of WST-1 solution was added to each well, and the cells were incubated for another 2 h. Each well of the 96-well multiple plate was measured at 450 nm in a micro-plate reader (Sunrise Rainbow RC; Tecan Trading, Mannēdorf, Switzerland). Each assay was repeated six times.

In vitro mesothelial cell wound-healing assay

To investigate paracrine effects of rat ASCs on recovery of the injured mesothelial cell layer, we used a modified *in vitro* wound-healing assay (31). Briefly, rat mesothelial cells were cultured on 35-mm collagen-coated dishes (Asahi Glass Co) as confluent monolayers. The monolayer was scraped in a line across the well with a 200-μL standard pipette tip. The wounded monolayer was washed three times with serum-free media to remove cell debris and incubated with 2 mL/dish of the supernatant of rat ASCs (ASC(+)) or with 2 mL/dish of the supernatant of mesothelial cells (control cells). For preparation of the supernatant, 4.5×10^5 rat ASCs and rat mesothelial cells were cultured in M199 with 10% FBS in a 6-cm dish for 48 h. Optical microscopy was performed using an IX70 inverted microscope (Olympus, Tokyo, Japan) under bright-field conditions. Optical images were captured using a model C-5060 digital camera (Olympus). The image

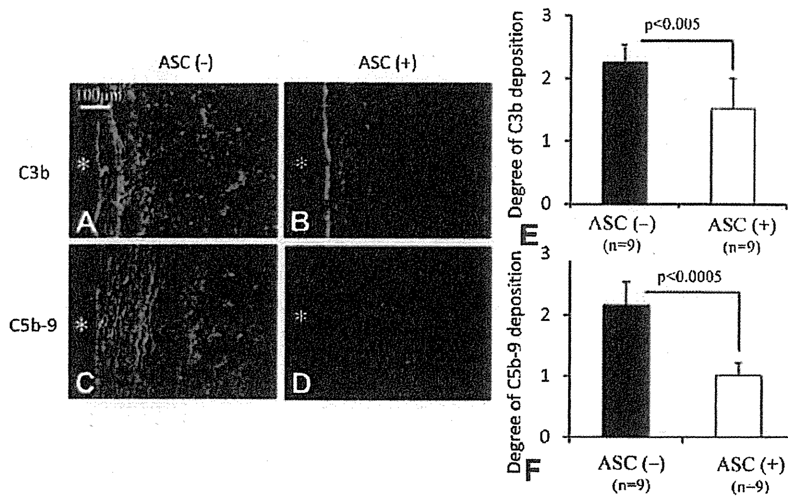

Figure 4. Depositions of C3b and C5b–9 along peritoneum in Zy/scraping peritonitis with (ASC(+)) or without (ASC(−)) treatment using rat ASCs. (A, B) C3b deposition in peritoneum. (C and D) C5b–9 deposition in peritoneum. In peritoneum treated with rat ASCs (B, D), depositions of C3b and C5b–9 are decreased compared with peritoneum without rat ASCs (A, C). Degree of C3b (E) and C5b–9 (F) deposition in peritoneum. Each value represents mean ± SD. Original magnification is ×400, and scale bar is shown in upper left corner in (A). *External face of peritoneum.

was subsequently analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA). The wound-healing effect was calculated as a percentage of the remaining cell-free area compared with the area of the initial wound.

Measurement of hepatocyte growth factor levels in supernatants of rat ASC culture by enzyme-linked immunosorbent assay and acceleration of stimulation of rat mesothelial cell proliferation with recombinant hepatocyte growth factor

To measure levels of hepatocyte growth factor (HGF) in supernatant of rat ASCs, a rat HGF enzyme-linked immunosorbent assay kit was used according to the information from the manufacturer (Institute of Immunology, Tokyo, Japan). In addition, we investigated whether HGF could stimulate proliferation of rat mesothelial cells using recombinant rat HGF (Institute of Immunology). The rat ASCs as 1×10^4/100 μL/well were cultured in fresh medium with 0.5% FBS on a 96-well plate mixed with 0, 0.6 ng/mL, 1.25 ng/mL, 2.5 ng/mL, 5 ng/mL or 10 ng/mL of recombinant rat HGF. Each assay was repeated three times. After 24 h, the proliferation of mesothelial cells was assessed by WST-1 as described previously. The results are expressed relative to the optical density (OD) value without recombinant rat HGF (control). The OD values obtained from the WST-1 assay were used for calculation of relative OD value, and the calculation was performed as follows:

Relative OD value = (OD value with each concentration of recombinant rat HGF)/ (OD value without recombinant rat HGF)

Statistical analysis

All values are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using one-factor analysis of variance. When significant differences were identified, further analysis was performed using Scheffé *F* test between two groups. A significant difference between two groups was considered present for values of $P < 0.05$.

Results

Intraperitoneal administration of rat ASCs improved peritoneal tissue injuries in Zy/scraping peritonitis model under light microscopy

Although severe peritoneal thickness with many inflammatory cells was observed in Zy/scraping peritonitis (9), daily intraperitoneal injections of rat ASCs clearly improved the peritoneal injuries by decreasing peritoneal thickness in Zy/scraping peritonitis (Figures 1, 2A). In rats injected with rat ASCs (ASC(+)), number of infiltrating cells, neutrophils and counts of ED1-positive cells were significantly decreased (Figure 2C–E). On the peritoneal surface in rats treated with rat ASCs, recovery of mesothelial cells along the peritoneal surface detected by anti-cytokeratin was clearly increased in Zy/scraping peritonitis (Figures 1F, 2B). Many defects of

mesothelial cells stained by anti-cytokeratin remained along the peritoneal surface of Zy/scraping peritonitis rats without rat ASCs (Figure 1E).

Distribution of CRegs along peritoneal surface was increased by rat ASC injections, and deposition of complement-activated products such as C3b and C5b–9 in peritoneum was decreased by rat ASC treatments in Zy/scraping peritonitis model

Accompanying recovery of peritoneal mesothelium in rats injected with rat ASCs, the distribution of CRegs such as Crry, CD55 and CD59 was significantly increased along the peritoneal surface compared with rats without rat ASC treatment (arrows, Figure 3). Staining of CRegs was also observed to be weakly positive in accumulated inflammatory cells in peritoneum compared with the staining of CRegs on mesothelial cells. Depositions of C3b and C5b–9 on the peritoneum of rats treated with rat ASCs (ASC(+)) were significantly decreased compared with rats not treated with rat ASCs (ASC(−)) (Figure 4).

Distribution of rat ASCs injected in rats with Zy/scraping peritonitis

When CFSE-labeled rat ASCs were injected to observe the relationship between recovered mesothelial cells and rat ASCs in peritoneum, CFSE-labeled rat ASCs were observed close to mesothelial cells stained with anti-cytokeratin in peritoneum (Figure 5). However, CFSE-labeled rat ASCs were not merged with cytokeratin-positive mesothelial cells (Figure 5C), showing that rat ASCs co-localized with peritoneal cells close to the injured mesothelial cells.

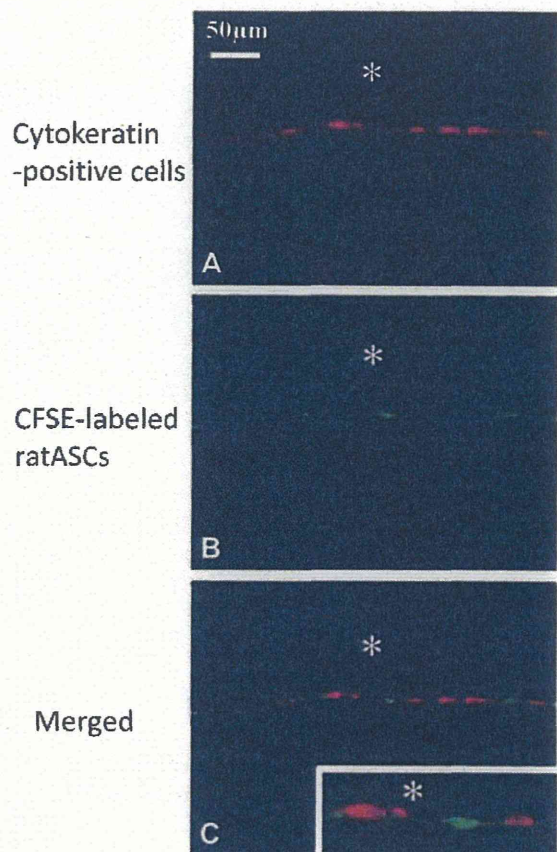

Figure 5. Recovery of mesothelium merged to distributions of rat ASCs in peritoneum. (A, B) Distributions of cytokeratin-positive mesothelial cells and of CFSE-labeled rat ASCs that were intraperitoneally injected. (C) Merged image for cytokeratin-positive cells (red color) and CFSE-labeled rat ASCs (green color). Inset (C) is a close-up view (×2 magnification of the original photo). *External face of the peritoneum. Original magnification ×400, and scale bar is shown in upper left corner in (A).

Proliferation of mesothelial cells from rat peritoneum was increased in two separated co-culture systems with rat ASCs

Under direct co-cultures of rat mesothelial cells mixed with rat ASCs, numbers of mesothelial cells were significantly increased compared with mesothelial cells alone (Figure 6A). In addition, when we used the Transwell cell-culture system to distinguish fluid-phase effects from effects of cell-cell communication, the number of mesothelial cells with rat ASCs was significantly increased compared with mesothelial cells without rat ASCs (Figure 6B). These observations suggest that some paracrine factors from rat ASCs increase mesothelial cells.

WST-1 assay supports increased proliferation of rat mesothelial cells incubated with rat ASC supernatant

We compared proliferation ability of rat mesothelial cells with supernatant from either cultured rat ASCs or rat mesothelial cells in the WST-1 assay to confirm paracrine effects such as fluid-phase factors released from rat ASCs. In the WST-1 assay, absorbance was significantly higher for mesothelial cells incubated with supernatant from rat ASC culture (supernatant of ASC in Figure 6C) than for mesothelial cells incubated with supernatant from mesothelial cell culture (supernatant of MC in Figure 6C). These results suggest that rat ASCs might have some paracrine effects facilitating proliferation of rat mesothelial cells.

Restoration of injured mesothelial cell monolayers was accelerated by supernatant from rat ASC culture

As an additional experiment, a wound-healing assay was performed to investigate the ability of mesothelial cells to proliferate as a paracrine manner. Under mesothelial cell wound-healing assay, closure of the

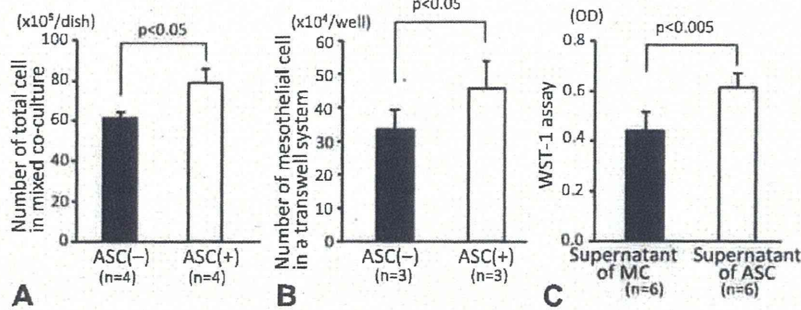

Figure 6. Rat ASCs enhance mesothelial proliferation under co-culture assays. (A) Total number of mesothelial cells mixed with rat ASCs as a co-culture assay. (B) Number of mesothelial cells in lower chamber separated from upper chamber without rat ASCs (ASC(−)) or with rat ASCs (ASC(+)) using the Transwell system. (C) Results of WST-1 cell proliferation assay. Incubation with supernatant from rat ASC culture (supernatant of ASC) shows higher OD values than for mesothelial cells incubated with supernatant from mesothelial cell culture (supernatant of MC).

wounded space of mesothelial monolayers was significantly accelerated by incubation with supernatant from rat ASCs culture (supernatant of ASC in Figure 7) compared with supernatant from mesothelial cell culture (supernatant of MC in Figure 7) at 6 h and 12 h.

Release of HGF was observed from rat ASCs, and proliferation of rat peritoneal mesothelial cells was increased by recombinant rat HGF

When we measured HGF levels in supernatant from rat ASCs, we confirmed the elevation of HGF levels dependent on the initial cell amount of rat ASCs (data not shown) as previously reported (26). To confirm whether HGF released from rat ASCs could directly accelerate proliferation of mesothelial cells in rat peritoneum, we stimulated rat mesothelial cells in medium mixed with various amounts of recombinant rat HGF. Proliferation of rat mesothelial cells increased with increasing amounts of recombinant rat HGF (Figure 8).

Effects of treatment of rat ASCs on day 1 after induction of Zy/scraping peritonitis

When administration of rat ASCs was started on day 1 after induction of Zy/scraping peritonitis (post-ASC(+)), peritoneal thickness was slightly, but not significantly, suppressed compared with lack of treatment of rat ASCs (ASC(−)) (see supplementary Figure 2B). Decreases of total number of infiltration cells and number of ED-1 positive cells were significantly observed in post-ASC(+) rats compared with control rats (ASC(−)) (see supplementary Figure 2C–E).

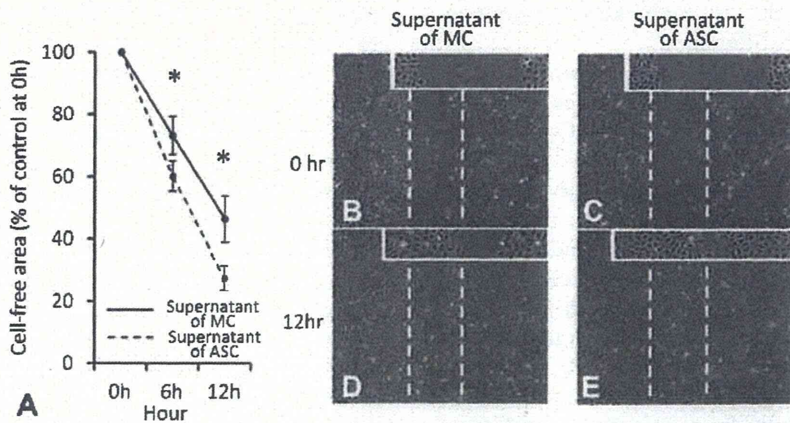

Figure 7. Restoration of injured mesothelial cell monolayers is accelerated by supernatant from rat ASCs in the wound-healing assay. Injured mesothelial cell monolayers were incubated with supernatant of rat ASCs (supernatant of ASC) or of mesothelial cells (supernatant of MC). Percentage of cell-free area at indicated time points compared with percentage of cell-free area at 0 h was determined (A). Each assay was performed in triplicate, and each value represents mean ± SD. Experiments were repeated three times, and results from a representative experiment are shown (*$P < 0.05$). Confluent mesothelial cell monolayers were injured with a pipette tip and treated with supernatant from supernatant of MC (B, D) or from supernatant of ASC (C, E) for the indicated time. Initial injuries are indicated by white dot lines. Cells growing into lines are considered to represent injury closure.

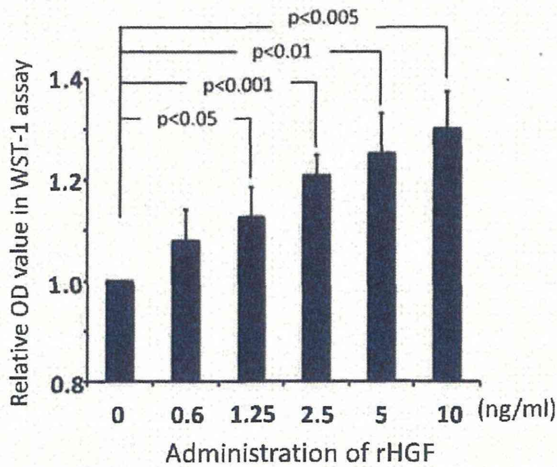

Figure 8. Administration of recombinant rat HGF (rHGF) increased proliferation of mesothelial cells. Each data point represents a relative OD value compared with conditional culture medium without recombinant rat HGF in the WST-1 assay.

Discussion

Rat ASCs have improved injuries from various tissues in more recent reports, including our own reports. Administration of rat ASCs clearly improved urethral resistance (32) and attenuated antibody-mediated rejection (26) and acute kidney injury induced by folic acid (28). In the present study, intraperitoneal injection of rat ASCs significantly suppressed peritoneal injuries in a rat Zy/scraping peritonitis model accompanied by decreased depositions of C3b and C5b–9 as complement activation products. Our results suggest that improvement of peritoneal injuries in rats with rat ASCs was at least partly attributable to suppression of complement activation in peritoneum.

We also investigated the reasons why complement activation and peritoneal injuries could be suppressed by administration of rat ASCs in rat Zy/scraping peritonitis. Although severe peritoneal inflammation developed with defects of the mesothelial cell layer in rats without rat ASC treatments, restoration of the mesothelial cell layer and decreased accumulation of inflammatory cells were observed in the peritoneum of rats treated with rat ASCs. Because mesothelial cells express abundant CRegs such as Crry, CD55 and CD59 (9), recovered mesothelial cells with CRegs in peritoneum might prevent the peritoneum from excessive complement activation in Zy/scraping peritonitis. We also observed that rat ASCs augmented proliferation of mesothelial cells in *in vitro* co-culture assays, and we observed that intraperitoneally injected rat ASCs distributed close to resident/recovered mesothelial cells, supporting the concept that injected rat ASCs facilitated the recovery of mesothelium. Results from our Transwell study suggested that paracrine effects of some factors released from rat ASCs could enhance the restoration of mesothelial cells. Concerning factors released to proliferate mesothelial cells as paracrine effects from rat ASCs, we focused on the roles of HGF for proliferation of mesothelial cells in *in vitro* assays because HGF is reportedly associated with mesothelial proliferation in peritoneal tissue repair (33,34). From our results, HGF released from rat ASCs might help to increase mesothelial cells, which have abundant CRegs. In addition, rat ASCs themselves might have the potential to prevent excessive complement activation locally because rat ASCs also express CRegs (data not shown). As other possibilities for effects of rat ASCs among mesenchymal stromal cells acting to protect against peritoneal injures, HGF released from mesenchymal stromal cells might decrease peritoneal tissue injures through anti-apoptotic effects (35). Another possibility involves factor H, a complement regulator, which is reportedly released from human mesenchymal stromal cells, suggesting that mesenchymal stromal cells themselves might have potential direct effects in protecting against complement activation (36).

Usage of ASCs in particular may prove advantageous because ASCs are easily and safely harvested from abundant adipose tissues compared with bone marrow-derived stromal cells. As mesenchymal stromal cells, ASCs may have multiple applications for tissue engineering and improvement of tissue injuries (37,38). As one aspect of the effects of ASCs, autocrine and paracrine effects of ASCs have been reported, such as regeneration of injured cardiac tissue using stem cell-derived cardiomyocyte sheets in a swine model of chronic myocardial infarction (39). Clinically, some trials of mesenchymal stromal cells have already succeeded in improving acute rejection of renal transplantation (40) and reducing infarction size in ischemic cardiomyopathy (41). In contrast, as unexpected problems, ASCs might present similar problems to the therapeutic use of mesenchymal stromal cells, such as infections, carcinogenesis, promotion of cancer metastasis or allergy caused by contaminating proteins such as growth factors (42–44). Although ASCs work to host with autocrine and paracrine effects, the present results suggest that paracrine effects of rat ASCs might play protective roles against peritoneal injuries.

In conclusion, our results suggest that rat ASCs facilitated recovery of mesothelial cells in the peritoneum, partly through suppression of complement activation, although how rat ASCs improve peritoneal injuries in Zy/scraping peritonitis remains unclear. In our study, administration of rat ASCs after

induction of Zy/scraping peritonitis limited the peritoneal injuries because the induced peritoneal injuries were severe. The present results suggest that administration of rat ASCs might provide a therapeutic approach to regulate the complement activation system in injured peritoneum under conditions of yeast-related peritonitis.

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Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.jcyt.2013.10.011.