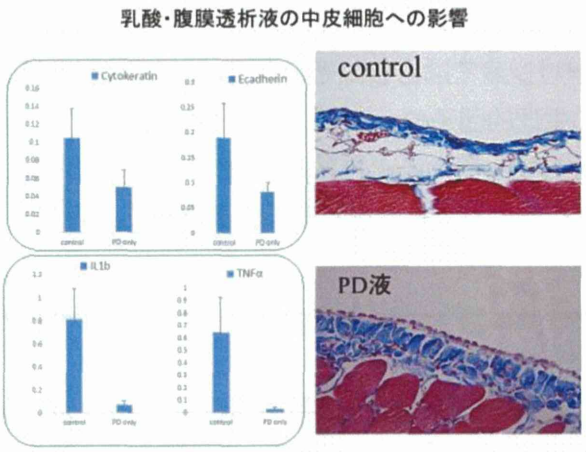
皮細胞を採取、フローサイトメトリーで形態観察、さらに細胞核酸抽出し各種遺伝子発現を検討する。

＊対象 PD 透析液

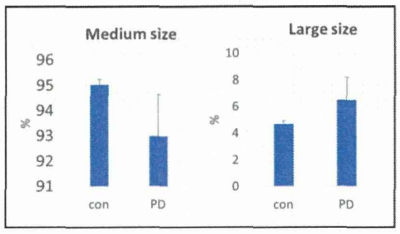
① 乳酸中性化液（2.5%グルコース(G)）
② 乳酸酸性液（4.25%G）
③ 乳酸・重炭酸中性化液（2.5%G）
④ 酸性イコデキストリン液
⑤ 中性化イコデキストリン液

C. 研究結果

下図：腹膜組織と中皮細胞 mRNA 発現
（コントロール群と乳酸中性液群の比較）



下図：中皮細胞サイズ：フローサイトメトリーによる解析
（コントロール群と乳酸中性液群の比較）



● 腹膜中皮細胞の細胞形態変化への影響
（各 6~9 匹の平均所見を示す）
コントロール群（無処理群）との比較：
乳酸中性化液：立方化＋＋,脱落＋
乳酸酸性液：立方体＋＋　扁平化＋　凝集＋
脱落＋＋
乳酸・重炭酸中性化液：立方化＋/－　脱落＋/－
酸性イコデキストリン液：立方化＋/－　脱落＋
中性化イコデキストリン液：立方化＋/－　脱落＋

● 回収細胞の形態フローサイトメトリー（乳酸中性液）
コントロールに比較して、乳酸中性化透析液群では、細胞面積が拡大している比率が増加する傾向が認められた。

● 回収細胞の mRNA 発現（乳酸中性液）
コントロールに比較して、乳酸中性液では、コラーゲンⅠ、フィブロネクチンの発現が増強、TGFβ、VEGF、Snail は著変なかった。一方、サイトケラチン、E-カドヘリン、インターロイキン 1、TNFα は有意に低下していた。

D. 考察

本研究は現行の PD 透析液の生体適合性を評価することを目的にしたものである。ラット腹腔内に１０日間透析液を投与、壁側腹膜を採取し中皮細胞の形態と各種因子の発現を検討した。形態変化として、無処理のコントロール群と比較し透析液投与群では中皮細胞の立方化が観察された。フローサイトメトリーの検討でも、乳酸透析液で細胞面積が増大する傾向が確認されたのは、この変化を反映するものと考えられた。しかし、この程度は透析液の種類によって異なり、乳酸・重炭酸透析液、イコデキストリン液では比較的軽度であった。一方、高濃度 G の乳酸酸性液では、中皮の立方化に加えて、細胞の凝集、脱落が確認され中皮障害が顕著であった。以上の結果から、乳酸、酸性、高濃度 G は中皮細胞障害に対する危険因子となることが考えられた。

現行の標準液である乳酸中性化透析液の群を対象に、中皮細胞の mRNA の発現を検討した。無処理群と比較して、サイトケラチン、E-カドヘリン発現が低下しており、上皮細胞の特性が変化している可能性が考えられた。しかし、Snail 発現に増加は認められなかったことから、この病態に

はEMTとは別の機序が関わる可能性が想定された。中皮細胞の機能として、局所免疫能がある。この指標としてサイトカインの発現を見た。透析液群ではインターロイキン１、TNFαは著明に低下しており、生理的機能の低下、障害が惹起されていること示唆された。

本邦においては、2004年以降、乳酸中性化透析液が標準的な透析液として使用されている。最近の検討で、乳酸中性化透析液の導入後、腹膜組織障害が抑制され、EPS発症頻度が低下していることが確認されている。しかしその一方で、PDの早期離脱に関しては改善傾向がなく、その理由としてPD関連腹膜炎が関与している(Nakayama M, et al. Perit Dial Int 2014;34:766-774.)。これらの報告は、腹膜硬化抑制に対する乳酸中性化透析液の有効性を示している。本検討でも、酸性液では中皮細胞障害は顕著であったのに対し、酸性液と乳酸中性化液とでは、中皮細胞障害は前者で著明だったことは、これを支持するものと考えられる。一方、PD関連腹膜炎が依然として大きな問題である事実は、現行透析液により腹膜の局所免疫能などの生理的機能が阻害されている可能性を示すものと考えられる。事実、本検討でも、乳酸中性化透析液で中皮細胞のサイトカインmRNA発現が低下していた。2015年から上市された乳酸・重炭酸透析液、中性イコデキストリン液がこれにどのような影響があるのか、検討する必要があると考えられる。

E. 結論

現行の標準PD液（乳酸中性化透析液）では中皮細胞障害性は抑制されているが、生理的機能の保全においては課題がある。

乳酸・重炭酸液や中性化イコデキストリン液がこれにどのような影響があるのかは重要な検討課題である。

F. 研究発表

1. 論文発表

なし

2. 学会発表

なし

G. 知的財産権の出願・登録状況

なし

厚生労働科学研究委託費
（障害者対策総合研究事業（障害者対策総合研究開発事業（身体・知的等障害分野））
「腎臓機能障害者に対する安全で効果的な腹膜透析法の開発等に関する研究」

研究成果の刊行物・別刷

研究成果の刊行に関する一覧表

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ORIGINAL ARTICLE

Efficacy and safety of fluoroscopic manipulation using the alpha-replacer for peritoneal catheter malposition

Yosuke Saka · Yasuhiko Ito · Yoshiyasu Iida · Shoichi Maruyama · Seiichi Matsuo

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Abstract
Background Catheter malposition is one of the reasons for outflow failure in peritoneal dialysis (PD) patients. Fluoroscopic manipulation is a non-surgical treatment option for catheter malposition. We retrospectively analyzed the efficacy and safety of fluoroscopic manipulation using an alpha-replacer guidewire.
Methods The alpha-replacer (JMS Co. Ltd., Tokyo, Japan) is a guidewire for the treatment of catheter malposition. We used the alpha-replacer in 23 PD cases at our hospital from January 2008 to December 2012. We evaluated body mass index, time interval between catheter placement and malposition, and interval between catheter exteriorization and malposition. Primary failure was defined as malposition at the time of catheter exteriorization, and secondary failure as malposition after functional PD therapy (correct position at time of exteriorization).
Results Successful catheter replacement rate using the alpha-replacer was 60.8 % (14 of 23 cases). This was similar to the rates in previous reports. Successful replacement was mostly observed in those with a long interval between catheter placement and malposition ($p = 0.048$), between catheter placement and exteriorization ($p = 0.047$) and with secondary failure ($p = 0.030$). In multivariate analysis, secondary failure cases had a higher rate of successful replacement than primary failure cases (odds ratio [OR] 7.33, $p = 0.038$). Serious complications, such as abdominal trauma or peritonitis, were not observed.
Conclusion Fluoroscopic manipulation using an alpha-replacer may be safe and effective for the management of peritoneal catheter malposition, particularly in patients who were under functional PD therapy until catheter malposition.

Keywords Peritoneal dialysis · Catheter malposition · Alpha-replacer

Introduction

In maintenance peritoneal dialysis (PD) patients, catheter malposition is one of the causes of outflow failure that requires urgent treatment, especially for cases accompanied by poor ultrafiltration [1, 2]. In general, surgical procedures, such as laparoscopic manipulation, are suggested or recommended for these patients due to the higher success rate [3–5]. However, surgical procedures are associated with the risk of several complications, such as abdominal peritonitis, hernia and dialysate leakage. Therefore, fluoroscopic manipulation is a useful non-surgical alternative, due to the lower risk of complications as compared to surgical procedures [6–11]. Various devices and techniques have previously been used for this, including fluoroscopic manipulation for peritoneal catheter malposition. Nonetheless, few reports have mentioned the differences in characteristics between successful and failed cases of catheter repositioning by fluoroscopic manipulation. We

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retrospectively analyzed the effectiveness and safety of fluoroscopic manipulation using an alpha-replacer, and determined the kind of cases that can be successfully treated by this technique.

Materials and methods

Alpha-replacer procedure

The alpha-replacer (JMS Co. Ltd., Tokyo, Japan) is a guidewire for treatment of catheter malposition. This wire is unique in being flexible if straightened, becoming harder when coiled [10]. We used the alpha-replacer in the same manner as previously described [9]. After checking the position of the tip of the PD catheter using a radio-contrast agent, an alpha-replacer is inserted aseptically (Fig. 1a, b). After further insertion of the alpha-replacer, the looped alpha-replacer is expanded, such that the tip of the PD catheter returns to the pelvis due to the elasticity of the wire (Fig. 1c). The alpha-replacer is then removed by pressing on the lower abdomen, while the tip of the PD catheter does not get displaced, remaining in the pelvic space (Fig. 1d).

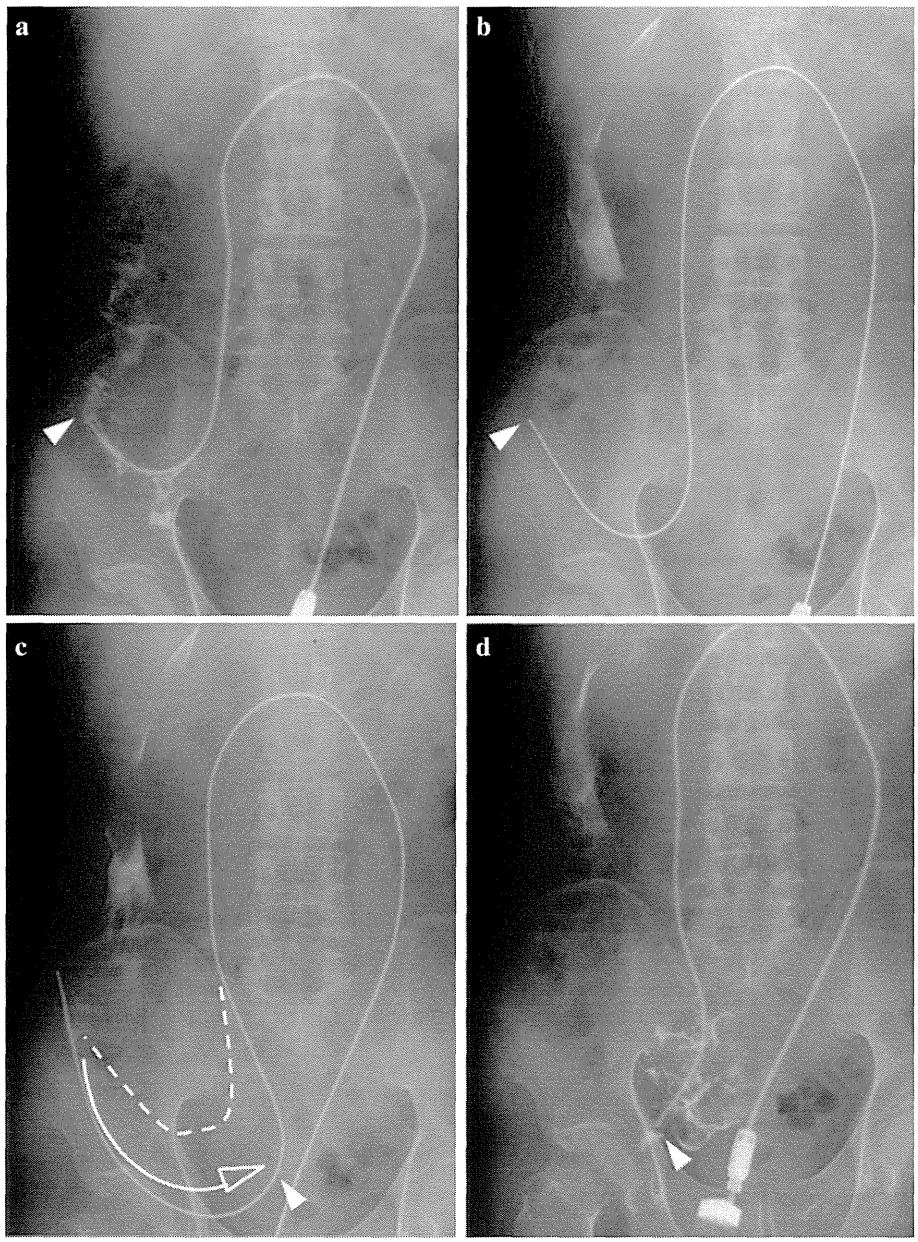

Fig. 1 Radiographic and schematic images of fluoroscopic manipulation using the alpha-replacer. **a** The tip of the displaced PD catheter is confirmed using a radio-contrast agent. **b** An alpha-replacer is inserted aseptically. **c** The looped alpha-replacer is expanded by its further insertion, resulting in the tip of the PD catheter returning to the pelvis due to the elasticity of the wire. The *dotted line* shows the displaced PD catheter. The *open arrow* shows the direction of movement of the PD catheter. **d** After removal of the alpha-replacer, the tip of the PD catheter remains in the pelvis. The *arrow head* shows the tip of the PD catheter

Patients

We reviewed 92 PD patients treated at our hospital between January 2008 and December 2012. Of these patients, we retrospectively analyzed 23 cases who received alpha-replacement treatment. The local ethical committee approved this research, and written informed consent was obtained from all patients before alpha-replacement treatment. We recorded age, sex, body mass index (BMI), dialysate volume of PD, history of previous abdominal surgery, and primary cause of renal failure as baseline data.

In all patients, double-cuffed, swan-neck catheters with straight tips, JB-5A, (Hayashidera Co. Ltd., Kanazawa, Japan) were inserted under local anesthesia. None of the patients underwent preventive procedures for PD catheter malposition, such as the peritoneal wall anchor technique (PWAT) by which PD catheter was fixed to the peritoneal wall [12]. We defined catheter displacement as catheter tip migration out of the true pelvis, as detected by abdominal radiography. Six different nephrologists performed alpha-replacement treatment within 24 h after diagnosis of the catheter displacement. After alpha-replacement treatment, we confirmed outflow and catheter tip location. We did not use prophylactic periprocedural antibiotics for all patients because the treatment could be performed in a sterile manner.

We defined successful manipulation as cases in which relapse of outflow failure did not occur for at least 30 days after alpha-replacement treatment. To evaluate the differences between successful and failed cases, we analyzed the interval between catheter placement and malposition and that between catheter exteriorization and malposition. In addition, primary failure was defined as malposition at the time of catheter exteriorization, and secondary failure as malposition after functional PD therapy (correct position at the time of exteriorization) [11]. Complications, including peritonitis and abdominal injury, were defined as the occurrence of complications within 7 days after alpha-replacement treatment.

Statistical analyses

Unpaired t test, Mann–Whitney test and Chi-square test were used to compare normally distributed continuous variables, non-normally distributed variables and categorical variables, respectively, between successful and failed cases. Multivariate stepwise logistic regression analysis was used to evaluate parameters that correlated with successful outcomes of alpha-replacement treatment. Significant differences were defined as $p < 0.05$. We used IBM SPSS statistics version 21 (IBM Corporation, Armonk, NY, USA) for the analyses.

Results

Outcomes of alpha-replacement treatment

The total observation period was 241.4 patient-years. We conducted a retrospective analysis of 23 cases. The incidence rate of catheter malposition was 25.0 % (23/92) and 0.095/patient-year (23/241.4). It was similar to the rates in previous reports (16.8, 22.1 % and 0.073/patient-year, respectively) [11, 13, 14]. Of the 23 cases, 22 cases had outflow failure, and 1 case had both outflow and inflow failure. Mean patient age was 64 ± 14 years and 18 cases (78.2 %) were male. In 9 cases (39.1 %), diabetic nephropathy was the primary cause of renal failure. Two patients (1 male, 1 female) received alpha-replacement treatment twice. The success rate was 60.8 % (14 of 23 cases) (Table 1). Of the 9 failed cases, 5 cases, including 2 cases of omental wrapping, required surgical procedures. Of the 2 cases with omental wrapping, PD catheter re-implantation was performed in 1 case, while the other case underwent catheter repair by forefinger (CRF)[1] and PWAT. Three cases without omental wrapping, in whom the PD catheter was not trapped in abdominal organs, underwent PWAT for repositioning of the PD catheter. Four of the other cases managed to continue peritoneal dialysis therapy despite catheter malposition and outflow failure. The time required for alpha-replacement treatment was 15 min on average. No instances of serious complications, such as abdominal organ injury, bleeding or peritonitis, were observed.

Comparison between successful and failed cases

We divided the 23 cases into two groups depending on the response to the alpha-replacement procedure, as successful cases and failed cases. There were no significant differences between the two groups in terms of age, sex, BMI, dialysate volume of PD, history of previous abdominal surgery and prevalence of diabetes mellitus (Table 2). Success with the alpha-replacement procedure was mostly seen in patients with a long interval between catheter placement and malposition (successful vs. failed cases: 18.1 ± 18.5 months vs. 4.4 ± 7.1 months, $p = 0.048$) and a long interval between catheter exteriorization and malposition (successful vs. failed cases: 16.0 ± 18.8 months vs. 2.4 ± 4.1 months, $p = 0.047$). Secondary failure cases (14 cases), in whom catheters were located in the correct position at the time of exteriorization, had more successful

[1] CRF is a minimally invasive surgical procedure for the repair of displaced PD catheter. After a small cut in the peritoneum, the wrapped catheter is pulled out by the fore finger and released from wrapped tissues (mostly omentum).

Table 1 Baseline characteristics of peritoneal dialysis patients in whom alpha-replacement treatment was performed

Case no.	Age (years)	Sex	BMI	Dialysate volume (L/day)	Previous abdominal surgery	Primary cause of renal failure	Interval between catheter exteriorization and malposition (months)	Outcome
1	65	Male	17.4	11.0	None	CGN	23	Success
2	54	Male	27.3	9.0	None	DN	14	Success
3	67	Male	17.5	2.0	None	DN	0	Failure
4	75	Male	19.9	6.0	None	NS	0	Success
5	84	Female	18.8	5.0	None	Unknown	3	Success
6	84	Female	16.0	5.0	Omentectomy	Unknown	6	Failure
7	63	Male	20.2	6.0	None	CGN	17	Success
8	45	Male	23.6	5.2	None	NS	0	Success
9	74	Male	20.2	9.0	Umbilical hernia operation	DN	39	Success
10	60	Female	20.7	6.0	None	Unknown	63	Success
11	46	Male	21.5	7.7	None	NS	12	Failure
12	73	Male	25.0	7.5	None	DN	23	Success
13	54	Female	29.4	6.0	Appendectomy	CGN	2	Success
14	66	Male	29.5	4.5	Appendectomy	DN	0	Failure
15	27	Female	17.4	6.0	None	DN	0	Failure
16	81	Male	20.9	6.0	None	NS	33	Success
17	62	Male	21.9	11.5	None	Unknown	0	Failure
18	72	Male	18.3	11.5	None	NS	3	Failure
19	83	Male	18.4	4.5	None	NS	0	Success
20	61	Male	26.1	6.0	None	CGN	0	Success
21	57	Male	33.0	6.0	None	DN	0	Failure
22	68	Male	22.4	4.5	Appendectomy	DN	7	Success
23	56	Male	24.0	8.0	None	DN	1	Failure

Cases 5 and 8 and 6 and 11, respectively, were the same patients

Cases 11, 14, 15, 17 and 18 (cases 11 and 18: omental wrapping) required surgery

CGN Chronic glomerulonephritis, *DN* Diabetic nephropathy, *NS* Nephrosclerosis

outcomes than primary failure cases (9 cases) ($p = 0.030$) (Table 2). In multivariate stepwise logistic regression analysis, secondary failure cases were associated with a higher success rate (odds ratio [OR] 7.33; 95 % confidence interval [95 % CI] 1.11−48.26, $p = 0.038$) (Table 3).

Discussion

Peritoneal catheter malposition is still a common problem in PD patients [14–16], although there are several management strategies available to deal with this problem [13, 17, 18]. Several previous reports have compared surgical procedures with fluoroscopic manipulation [3–5, 11]. In general, surgical procedures have a higher success rate (90−100 %) than fluoroscopic manipulation (44−78 %) [3–5, 7–9, 11]. On the other hand, the benefit of fluoroscopic manipulation is that it is minimally invasive. In this study as well, the success rate of alpha-replacement treatment was similar to the rate in previous reports [9, 10], which was lower than that of surgical procedures. In contrast, we were able to perform alpha-replacement treatment without complications such as abdominal organ injury or hernia that accompany surgical procedures. Alpha-replacement treatment contributes to minimizing labor costs due to the conciseness of the method. Furthermore, the alpha-replacement procedure can be carried out quickly because the operating room is not required. Although a simple comparison of medical cost between the two procedures is difficult, the cost of this treatment is expected to be lower than that of surgical procedures. In addition, fluoroscopic manipulation has several advantages, such as shortening of treatment time and better patient acceptance of the treatment. Our results demonstrate the merit of fluoroscopic manipulation, as is also described in other reports [6, 7, 11].

In addition to evaluation of the success rate of alpha-replacement treatment, we analyzed the differences between successful and failed cases. Of interest, this study showed that the success rate with alpha-replacement was

Table 2 Comparison between successful and failed catheter manipulation cases

Evaluated parameter	Successful ($n = 14$)	Failed ($n = 9$)	P value
Age	67.1 ± 11.8	59.7 ± 16.2	0.215
Male	$n = 11$	$n = 7$	0.964
Female	$n = 3$	$n = 2$	
BMI	22.3 ± 3.4	22.1 ± 5.8	0.922
Dialysate volume (L/day)	6.6 ± 1.9	6.9 ± 3.1	0.734
Previous abdominal surgery (%)	21.4	22.2	0.964
Primary cause of renal failure	DN: $n = 4$ Non-DN: $n = 10$	DN: $n = 5$ Non-DN: $n = 4$	0.203
Interval between catheter placement and malposition (months)	18.1 ± 18.5	4.4 ± 7.1	0.048
Interval between catheter exteriorization and malposition (months)	16.0 ± 18.8	2.4 ± 4.1	0.047
Primary or secondary failure	Primary: $n = 3$ secondary: $n = 11$	Primary: $n = 6$ secondary: $n = 3$	0.030

BMI Body mass index, *DN* Diabetic nephropathy

Table 3 Stepwise multiple logistic regression analysis predicting successful outcome of alpha-replacement treatment

Evaluated parameter	Odds ratio	95 % CI	P value
Primary vs. secondary failure	7.33	1.11–48.26	0.038

Both interval between catheter placement and malposition and interval between catheter exteriorization and malposition were excluded as evaluated parameters due to multicollinearity

higher in patients with catheter malposition after long-term PD therapy. In contrast, a lower success rate was observed in patients with catheter malposition during the PD initiation period. It is speculated that most cases of catheter malposition in long-term PD patients are easily corrected because PD catheters in these patients are appropriately implanted. On the other hand, technical or anatomical problems, such as omental wrapping, might be the main reasons for the lower success rate in patients during the PD initiation period, as previously described [11]. This indicates that alpha-replacement treatment could be a significantly effective method in patients who continue PD therapy for a certain period.

Miller et al. reported a higher success rate, especially in secondary failure cases (75.0 %) [11]. However, in their report, manipulated catheters were mostly located in the true pelvis, and not in the lower or upper abdomen. This indicates that these catheters were not displaced and did not require repositioning. In contrast, all catheters in our study were displaced to the lower or upper abdomen. The success rate of our study would probably be higher if cases similar to theirs were compared.

This study has several limitations. Firstly, it was a single center study with a small sample size. However, we demonstrated significant differences between successful and failed cases even with the limited number of samples. Secondly, alpha-replacement treatment was unable to correct catheter malposition in some cases, especially during the initiation period of PD. This could be due to technical problems accompanying the catheter insertion operation [19]. The International Society for Peritoneal Dialysis (ISPD) recommends that more than 80 % of PD catheters should be patent at 1 year [20]. The 1-year patency rate at our hospital was 83.7 % (77 of 92 cases), which is within the recommendation of the ISPD guideline. Further, preventive methods, such as PWAT, have also been reported in recent years. Although PWAT has not been performed as a preventive method at our hospital, such methods should be used in future cases presenting for PD catheter implantation. Thirdly, we used only one type of PD catheter. Therefore, it may be difficult to apply our study results to patients with other types of PD catheters. Lastly, several different doctors performed alpha-replacement treatment in the patients included in this study. As a result, the possibility of technical bias remains. Further analysis with a larger sample size may be warranted to resolve these limitations.

To summarize, fluoroscopic manipulation using the alpha-replacer is a safe and minimally invasive technique for PD catheter replacement that has a higher success rate in maintenance PD patients. For these reasons, alpha-replacement treatment can be chosen as the first-line therapy for catheter replacement, especially for maintenance PD patients.

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Conflict of interest None.

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Rat adipose tissue-derived stem cells attenuate peritoneal injuries in rat zymosan-induced peritonitis accompanied by complement activation

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Abstract

Background aims. In patients receiving peritoneal dialysis, fungal or yeast peritonitis has a poor prognosis. In rat peritoneum with mechanical scraping, severe peritonitis can be induced by zymosan, a component of yeast (Zy/scraping peritonitis). Administration of rat adipose tissue-derived stromal cells (ASCs) potentially can improve several tissue injuries. The present study investigated whether rat ASCs could improve peritoneal inflammation in Zy/scraping peritonitis. *Methods.* Rat ASCs were injected intraperitoneally on a daily basis in rats with Zy/scraping peritonitis. *Results.* Peritoneal inflammation accompanied by accumulation of inflammatory cells and complement deposition was suppressed by day 5 after injection of rat ASCs. The peritoneal mesothelial layer in Zy/scraping peritonitis with rat ASC treatment was restored compared with the peritoneal mesothelial layer without rat ASC treatment. Injected rat ASCs co-existed with mesothelial cells in the sub-peritoneal layer. *In vitro* assays showed increased cellular proliferation of rat mesothelial cells combined with rat ASCs by co-culture assays, confirming that fluid factors from rat ASCs might play some role in facilitating the recovery of rat mesothelial cells. Hepatocyte growth factor was released from rat ASCs, and administration of recombinant hepatocyte growth factor increased rat mesothelial cell proliferation. *Conclusions.* Because the peritoneal mesothelium shows strong expression of membrane complement regulators such as Crry, CD55 and CD59, restoration of the mesothelial cell layer by rat ASCs might prevent deposition of complement activation products and ameliorate peritoneal injuries. This study suggests the therapeutic possibilities of intraperitoneal rat ASC injection to suppress peritoneal inflammation by restoring the mesothelial layer and decreasing complement activation in fungal or yeast peritonitis.

Key Words: *adipose-derived stromal cells, complement, membrane complement regulators, peritoneal dialysis, peritonitis*

Introduction

Peritoneal dialysis (PD) is an important renal replacement therapy. There are >196,000 patients with end-stage renal disease currently receiving PD therapy around the world (1). However, many patients must be withdrawn from PD therapy for various reasons (2,3). Impairment of peritoneal function is one of the main reasons for withdrawal from PD therapy. Peritoneal impairment is caused by the use of non-biocompatible PD fluid such as acid-base PD fluid and long-term exposure of the peritoneum to PD fluid. Peritonitis is another important and serious complication leading to withdrawal from PD therapy (3,4). Both conditions are associated with peritoneal tissue injuries and may be related to the development of lethal encapsulating peritoneal sclerosis (5). Such problems must be resolved to improve the prognosis of PD therapy in the future. In particular, fungal or yeast peritonitis is a serious infection in patients on PD (6) because fungal infection is known to be associated with a poor prognosis of peritonitis and can lead to the development of encapsulating peritoneal sclerosis (7,8). We previously reported that zymosan, a component of the yeast cell wall, can induce severe peritoneal inflammation in association with complement activation (9). Zymosan activates the complement activation system through the alternative pathway (10). We also reported that dysregulation of the complement activation system could be one factor associated with the development and augmented severity of

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peritoneal injuries (9,11,12). Targeting the complement activation system might be one approach to control peritoneal injuries.

Studies using embryonic or pluripotent stem cells are valuable for analyzing pathologies, restoring injuries involving hematopoietic cells, neurons, muscle or cardiovascular tissue and developing new therapeutic agents (13–17). As the stem cells of mesenchymal tissues, mesenchymal stromal cells have shown great potential to repair tissue injuries in various animal disease models as alternatives to conventional therapies (18,19). Adipose tissue-derived stromal cells (ASCs) have an added advantage in that the cells can be harvested more easily and less invasively than bone marrow-derived mesenchymal stromal cells (20) and reportedly display immunomodulatory properties (21,22).

Using a rat peritonitis model induced by administration of zymosan after scraping the peritoneum (Zy/scraping peritonitis) (9), we showed that the initial inflammation in severe peritoneal injury was related to complement activation and that peritoneal inflammation of this peritonitis was enhanced by complement activation, supporting the poor prognosis of fungal PD peritonitis. In the present study, we investigated a suppressive effect of rat ASCs in peritoneal injuries, targeting regulation of the complement activation system in the rat Zy/scraping peritonitis model.

Methods

Animals

Male Sprague-Dawley rats weighing approximately 250 g (Japan SLC, Hamamatsu, Japan) were used. All animal experiments described here were carried out in accordance with the Animal Experimentation Guide of Nagoya University School of Medicine.

Reagents and antibodies

Zymosan A was purchased from Sigma-Aldrich (St Louis, MO, USA). Dianeal NPD-4 1.5% (Baxter, Tokyo, Japan) was used as a 1.5% neutral PD fluid (pH approximately 6.4). To investigate the distribution of rat membrane complement regulators (CRegs), anti-rat Crry (monoclonal antibody [mAb] TLD-1C11) was purchased from Hycult Biotechnology (Uden, the Netherlands), and anti-rat CD55 (mAb RDIII7) and anti-rat CD59 (mAb 6D1), which were kindly donated by Prof B. P. Morgan (Cardiff University), were characterized as described (23–25). Fluorescein isothiocyanate (FITC)-labeled rabbit anti-mouse immunoglobulin G (IgG) was purchased from MP Biomedicals (Santa Ana, CA, USA). To observe C3b and C5b–9 (membrane attack complex) deposition, we used FITC-rabbit anti-rat C3 (MP Biomedicals) and mouse anti-rat C9 (clone 2A1; Hycult Biotechnology), respectively, followed by incubation with FITC-goat anti-mouse IgG (MP Biomedicals). Naphthol AS-D chloroacetate ($C_{20}H_{16}ClNO_3$), N,N-dimethylformamide and Fast Blue BB Salt hemi(zinc chloride) salt ($C_{17}H_{18}N_3O_3Cl \cdot 1/2\ ZnCl_2$) were purchased from Sigma-Aldrich for an esterase reaction to detect neutrophils. To observe ED1-positive cells, mouse anti-rat monocyte (clone ED1) was purchased from BMA Biomedicals (Augst, Switzerland). To recognize mesothelial cells along the peritoneal surface, we used monoclonal mouse anti-human cytokeratin, which was cross-reactive against rat (Dako, Glostrup, Denmark).

Preparation and characterizations of rat ASCs and primary cell cultures of rat mesothelial cells

Rat ASCs were obtained from inguinal adipose tissue from male Sprague-Dawley rats and cultured in low serum culture medium according to previous reports (26,27). Briefly, in the cell culture of rat ASCs, the basal medium was a 3:2 mixture of Dulbecco's modified Eagle's medium (Nissui Pharmaceutical, Tokyo, Japan) and MCDB 201 medium (Sigma-Aldrich), supplemented with 1 mg/mL linoleic acid-albumin (Sigma-Aldrich), ITS liquid media supplement (100×) as 1:100 (v:v) (Sigma-Aldrich), 0.1 mmol/L ascorbic acid phosphate ester magnesium salt (Wako Pure Chemical Industries, Osaka, Japan), 50 U/mL penicillin and 50 μg/mL streptomycin (Gibco Life Technologies, Grand Island, NY, USA). Cells were cultured in culture medium containing 2% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT, USA) and 10 ng/mL human fibroblast growth factor 2 (PeproTech, Rocky Hill, NJ, USA).

Primary culture of mesothelial cells was obtained according to our previous report (11). Briefly, dissected omentum from a Sprague-Dawley rat was digested in 10 mL of 0.25% trypsin with 1 mmol/L ethylenediamine tetraacetic acid (Gibco Life Technologies) for 30 min at 37°C, followed by incubation for 1 h in fresh 0.25% trypsin with 1 mmol/L ethylenediamine tetraacetic acid at 37°C. The residual omentum fragment was removed from the cell suspension. M199 medium (Invitrogen, Carlsbad, CA, USA) with 10% FBS and a mixture of 50 U/mL penicillin and 50 μg/mL streptomycin (Gibco Life Technologies) as 1:100 (v:v) was added into the cell suspension and centrifuged at 1000 rpm for 5 min. The cell pellet was re-suspended in 4 mL of M199 medium with 10% FBS, plated into type 1 collagen-coated 6-cm dishes (IWAKI; Asahi Glass Co., Tokyo, Japan) and incubated at 37°C for 4 days. Characterization

of the primary culture of rat mesothelial cells was performed according to our previous report (11).

In vivo experimental protocol

Zymosan-induced peritonitis was developed by five daily intraperitoneal injections of 5 mg of zymosan mixed with PD fluid in rats with mechanical scraping of the right-sided parietal peritoneum (Zy/scraping peritonitis) as described in our previous report (9), with a small modification to use 1.5% PD fluid instead of 4.25% PD fluid. Each intraperitoneal injection was administered to rats with 6×10^6 rat ASCs/rat suspended in 3 mL of phosphate-buffered saline (PBS) filled with 1.5% PD fluid (3 mL on days 0 and 1 and 8 mL on days 2–4), as described in our previous reports (26–28). Rat ASCs was injected 2 h after injection of zymosan on day 0 and at the same time as zymosan injection on days 1–4. Rats were sacrificed on day 5 to obtain tissues. The experimental protocol is summarized in supplementary Figure 1.

Separately, to investigate the effects of rat ASCs for treatment of peritoneal injuries, rat ASCs or vehicle were intraperitoneally injected on days 1, 2, 3 and 4 after the first injection of zymosan was administered intraperitoneally as for Zy/scraping peritonitis. The experimental protocol and number of rats in each group are shown in supplementary Figure 2A.

Histologic analysis

The parietal peritoneum was cut into strips (approximately 5 × 20 mm for a strip; four strips were obtained from the right-sided parietal peritoneum with mechanical scraping). Two strips of the parietal peritoneum were randomly obtained and fixed in 10% buffered formalin and embedded in paraffin. Sections 4.5 μm thick were stained with hematoxylin-eosin for histologic analysis. The other two strips were used for immunohistochemistry analysis. To estimate peritoneal thickness as a marker of tissue damage to the parietal peritoneum, we randomly observed 20 fields/rat at ×100 magnification under light microscopy, using the mean value for that rat as the peritoneal thickness. To estimate total accumulated cells, the total number of infiltrated cells was counted in 20 fields at ×200 magnification under light microscopy. The total number of infiltrated cells was calculated as follows:

(Total count of infiltrated cells in sequential peritoneal areas under × 200 magnification)/20

Immunohistochemistry analysis

To investigate infiltrations of neutrophils and ED1-positive cells, paraffin-embedded sections were de-waxed in xylene, re-hydrated and washed in PBS. To detect neutrophils, we used the Fast Blue Salt method as an esterase reaction according to a previous report (29). Briefly, de-paraffinized sections were incubated in chloroacetate solution (5 mg naphthol AS-D in 1 mL of N,N-dimethylformamide mixed with 25 mg fast blue BB salt in 40 mL of PBS) overnight at 4°C in the dark. After rinsing with distilled water, slides were stained with Nuclear Fast Red counterstain (Vector Laboratories, Burlingame, CA, USA) to counterstain nuclei. To detect infiltrated ED1-positive cells, de-paraffinized sections were pre-treated with 0.3% H_2O_2 to block endogenous peroxidase and incubated in 10% normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in PBS to block nonspecific binding, followed by treatment with 10 mmol/L citrate buffer (pH 6.0) for antigen retrieval at 98°C for 30 min. De-paraffinized sections were also incubated with mAb ED1, followed by addition of conjugated goat anti-mouse IgG and horseradish peroxidase-labeled polymer (Histofine Simple Stain; Nichirei, Tokyo, Japan) as a secondary reagent. Development was performed using deaminobenzine tetrahydrochloride development reagent (Histofine Simple Stain). The number of neutrophils or ED1-positive cells was calculated using the following formula:

Number of neutrophils or ED1-positive cells = (total count of neutrophils or ED1-positive cells in 20 sequential peritoneal areas under × 200 magnification)/20

From snap-frozen peritoneal strips, sections 4.5 μm thick were prepared with a cryostat and fixed in acetone according to our previous report (9). To investigate recovery of the mesothelial cell layer along the injured peritoneum, frozen sections were first stained with anti-cytokeratin, followed by conjugated polyclonal goat anti-rabbit IgG antibody absorbed with untreated rat serum (1:1/v:v) and horseradish peroxidase-labeled polymer (Histofine Simple Stain) as a secondary reagent. Enzyme activity was finally detected using 3-amino-9-ethyl-carbozole (Dako). Recovery of the mesothelial cell layer in the injured peritoneum was estimated according to our previous report (30). Briefly, the length of cytokeratin-positive mesothelial cell layer in relation to the total length of the peritoneal surface was calculated under ×100 magnification as the proportion occupied by cytokeratin-positive cells. To investigate C3b deposition as a complement activation product, FITC-labeled anti-rat C3 was incubated on frozen sections. For detection of C5b–9 as another activation product, frozen sections were incubated with mouse anti-rat C9 followed by incubation with FITC-labeled goat anti-mouse IgG

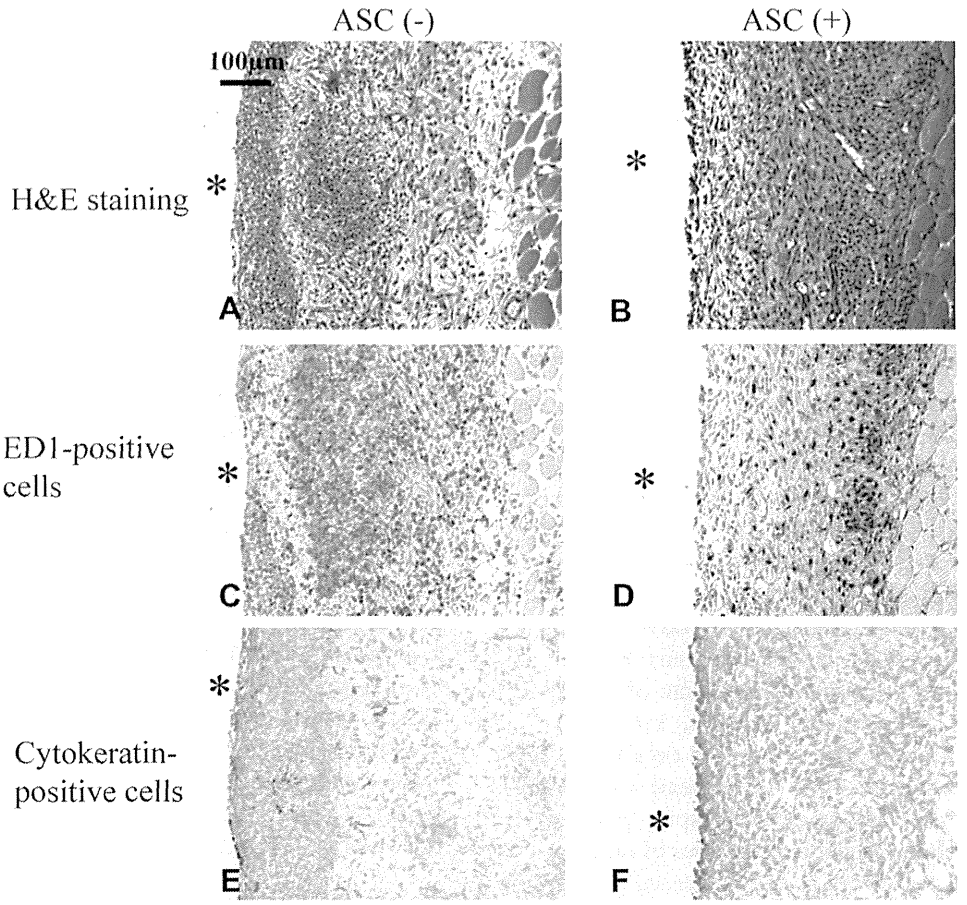

Figure 1. Microscopic findings in peritoneum treated with (ASC(+)) or without (ASC(−)) rat ASCs in Zy/scraping peritoneal peritonitis. (A, C, E) Micrographs show peritoneum without injection of rat ASCs in Zy/scraping peritonitis (ASC(−)). (B, D, F) Micrographs show rat ASC-injected rats (ASC(+)). Micrographs with hematoxylin-eosin (H&E) staining (A, B), ED1-positive cells (C,D) and distribution of cytokeratin in mesothelial cells (E, F). The thickness of peritoneum and accumulation of infiltrative cells are clearly decreased in rats treated with rat ASCs (ASC(+)) (B). Accumulation of ED1-positive cells is also reduced by rat ASCs (D). Cytokeratin-positive cells are apparent along the recovered peritoneal surface after injury in Zy/scraping peritonitis (F). *External face of the peritoneum. Scale bar is in upper left corner in (A).

absorbed with untreated rat serum (1:1/v:v). For C3b or C9 staining, we used normal rabbit serum or mouse serum as negative controls for antibodies. To score deposition of C3b or C5b–9 under ×200 magnification, a semi-quantitative scale was used: 0, negative result or only trace staining; 1, positive staining area <10% of total surface area; 2, positive staining area 10–30%; 3, positive staining area >30%. The mean value for degree of C3 or C5b–9 deposition from 20 fields was used. To examine the distribution of CRegs Crry, CD55 and CD59, mAbs (TLD-1C11, RDIII-7 and 6D1) were first incubated with the tissue sections, followed by FITC-labeled anti-mouse IgG. Distribution of the CRegs was also semi-quantified with the following scale: 0, negative; 1, positive staining area<20% of total surface area; 2, positive staining area 20% to <40%; 3, positive staining area 40% to <60%; 4, positive staining area 60% to <80%; 5, positive staining area >80%. The mean value for degree of CRegs expression from 20 fields was used.

Detecting distribution of 5-carboxy-fluorescein diacetate N-succinimidyl ester-labeled rat ASCs injected in rat Zy/scraping peritonitis

5-Carboxy-fluorescein diacetate N-succinimidyl ester (CFSE) was labeled on rat ASCs using a Cell Trace CFSE Cell Proliferation Kit (Molecular Probes, Eugene, OR, USA) in accordance with the instructions provided by the manufacturer. To observe the distribution of injected rat ASCs, 6×10^6 CFSE-labeled rat ASCs were injected on days 0 and 1 in the Zy/scraping peritonitis model. On day 3 after starting the experiment, rats were killed to harvest the peritoneum. Frozen sections from rats injected with CFSE-labeled rat ASCs were cut into 5-μm-thick sections and fixed with acetone for 10 min. First, to enhance the fluorescence signal, CFSE-labeled rat ASC tissues were incubated with anti-fluorescein/Oregon Green, goat IgG fraction (Molecular Probes) followed by FITC-labeled anti-goat IgG (Sigma-Aldrich). Second, to

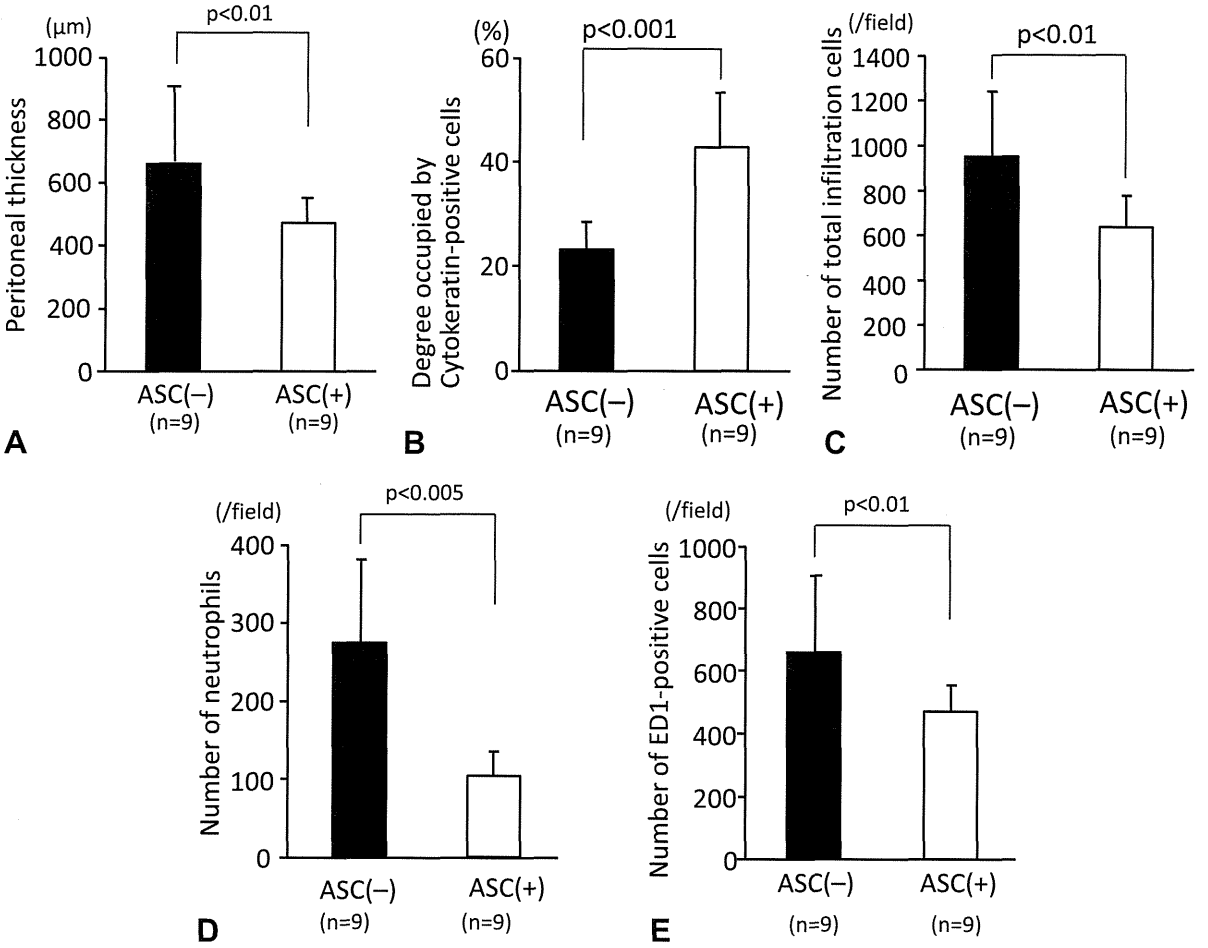

Figure 2. Peritoneal thickness, number of inflammatory cells and proportion of the peritoneal surface occupied by cytokeratin-positive cells in rats with Zy/scraping peritonitis and rats treated with rat ASCs. Peritoneal thickness (A), total number of infiltrating cells (C), number of neutrophils (D), 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) × 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,

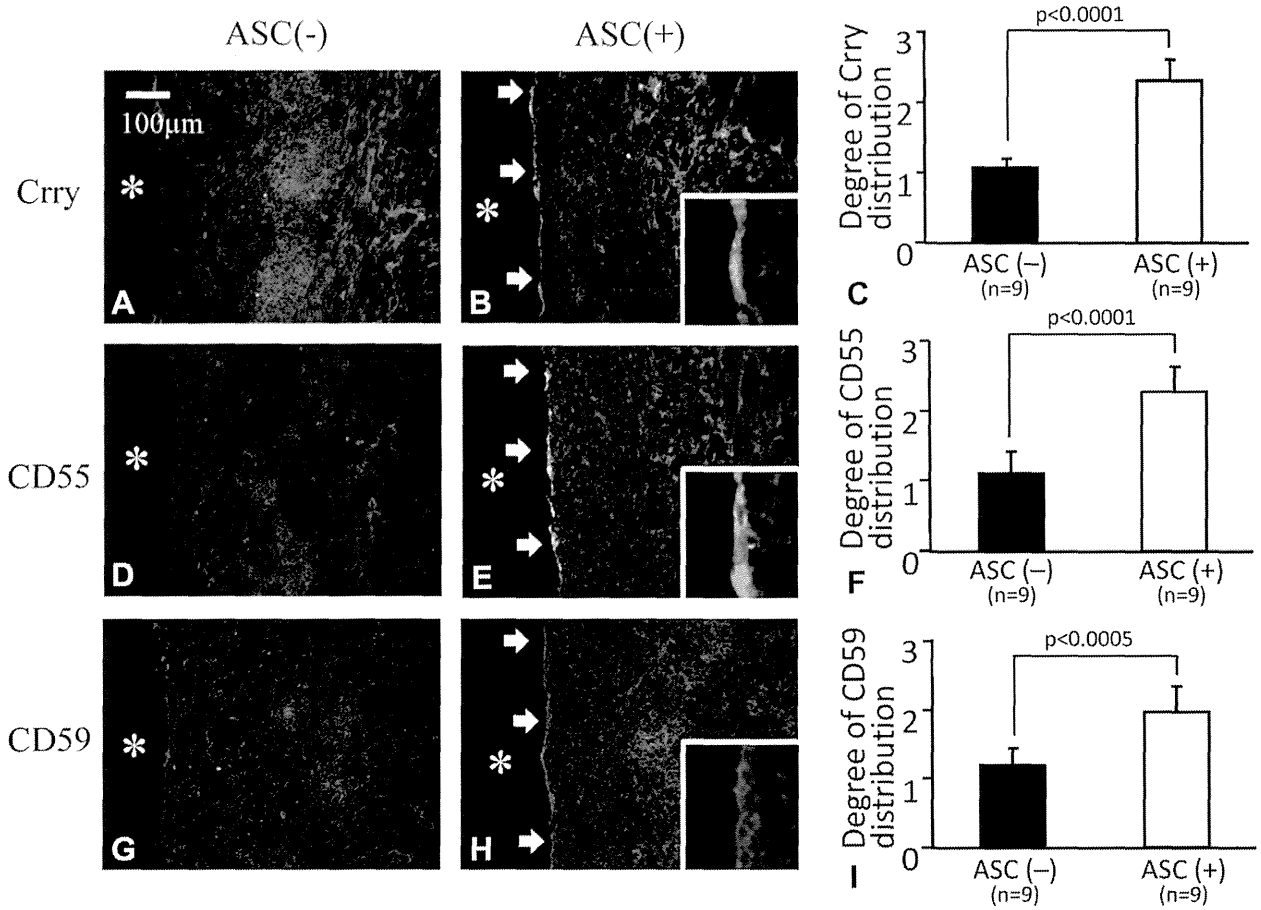

Figure 3. Changes in expression of membrane complement regulators Crry, CD55 and CD59 along peritoneal surfaces. Along the peritoneal surface of ASC(+) rats, distributions of CReg expression are clearly increased (B, E, H) compared with ASC(−) rats. Original magnifications are ×200, and scale bar is 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, Mannëdorf, 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

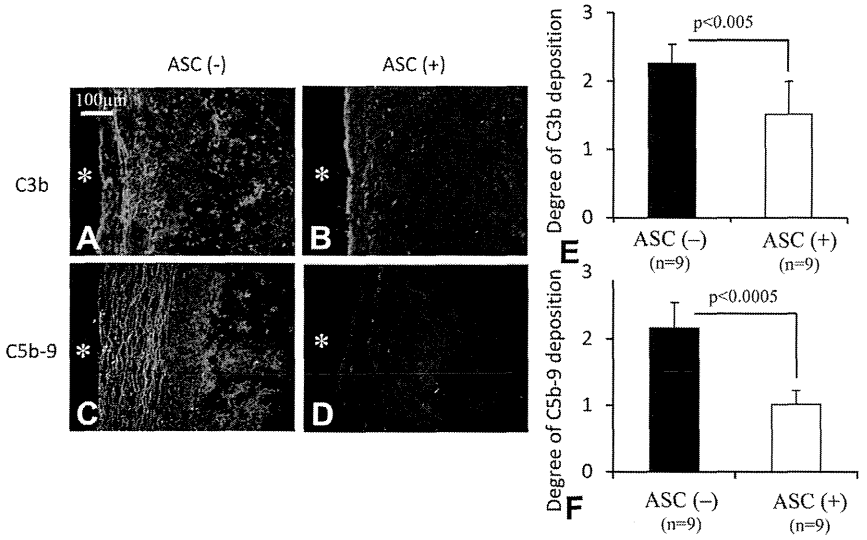

Figure 4. Depositions of C3b and C5b–9 along peritoneum in Zy/scraping peritonitis with (ASC(+)) or without (ASC(–)) treatment using rat ASCs. (A, B) C3b deposition in peritoneum. (C and D) C5b–9 deposition in peritoneum. In peritoneum treated with rat ASCs (B, D), depositions of C3b and C5b–9 are decreased 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.

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

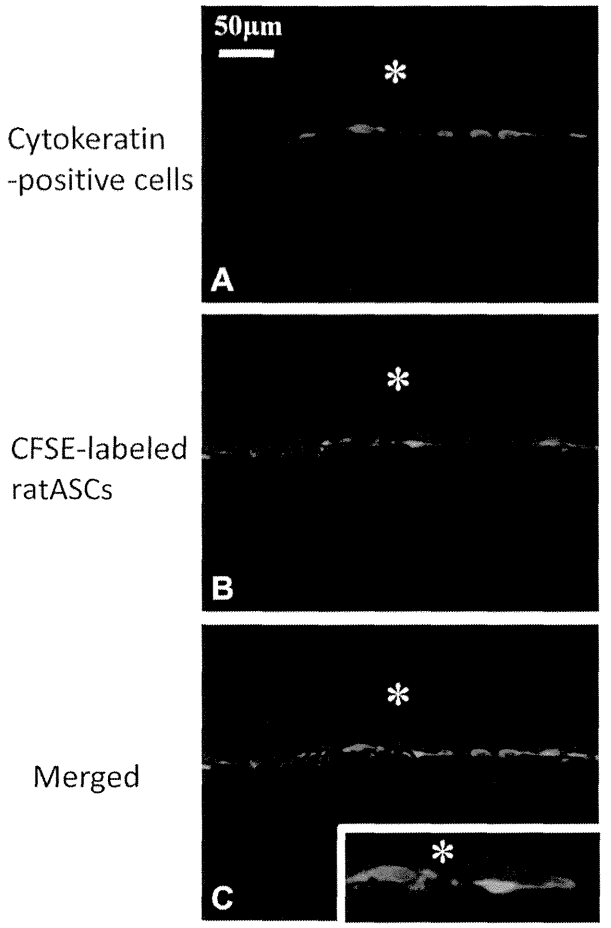

Figure 5. Recovery of mesothelium merged to distributions of rat ASCs in peritoneum. (A, B) Distributions of cytokeratin-positive mesothelial cells and of CFSE-labeled rat ASCs that were intraperitoneally injected. (C) Merged image for cytokeratin-positive cells (red color) and CFSE-labeled rat ASCs (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).

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