11. Krag DN, Anderson SJ, Julian TB, Brown AM, Harlow SP, Costantino JP, et al. Sentinel-lymph-node resection compared with conventional axillary-lymph-node dissection in clinically node-negative patients with breast cancer: overall survival findings from the NSABP B-32 randomised phase 3 trial. Lancet Oncol. 2010;11:927–33. doi:10.1016/S1470-2045(10)70207-2.
12. Giuliano AE, McCall L, Beitsch P, Whitworth PW, Blumencranz P, Leitch AM, et al. Locoregional recurrence after sentinel lymph node dissection with or without axillary dissection in patients with sentinel lymph node metastases: the American College of Surgeons Oncology Group Z0011 randomized trial. Ann Surg. 2010;252:426–32. doi:10.1097/SLA.0b013e3181f08f32 discussion 432–433.
13. Giuliano AE, Hunt KK, Ballman KV, Beitsch PD, Whitworth PW, Blumencranz PW, et al. Axillary dissection vs. no axillary dissection in women with invasive breast cancer and sentinel node metastasis: a randomized clinical trial. JAMA. 2011;305:569–75. doi:10.1001/jama.2011.90.
14. Galimberti V, Cole BF, Zurrida S, Viale G, Luini A, Veronesi P, et al. Axillary dissection versus no axillary dissection in patients with sentinel-node micrometastases (IBCSG 23-01): a phase 3 randomised controlled trial. Lancet Oncol. 2013;14:297–305. doi:10.1016/S1470-2045(13)70035-4.
15. Sanuki N, Takeda A, Amemiya A, Ofuchi T, Ono M, Ogata H, Yamagami R, Hatayama J, Eriguchi T, Kunieda E. Outcomes of clinically node-negative breast cancer without axillary dissection: can preserved axilla be safely treated with radiation after a positive sentinel node biopsy? Clin Breast Cancer. 2013;13(1):69–76. doi:10.1016/j.clbc.2012.09.005.
16. Goldhirsch A, Winer EP, Coates AS, Gelber RD, Piccart-Gebhart M, Thürlimann B, Senn HJ, Panel members. Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013. Ann Oncol. 2013;24(9):2206–23. doi:10.1093/annonc/mdt303.
17. Posther KE, McCall LM, Blumencranz PW, Burak WE Jr, Beitsch PD, Hansen NM, et al. Sentinel node skills verification and surgeon performance: data from a multicenter clinical trial for early-stage breast cancer. Ann Surg. 2005;242:593–9 discussion 599–602.
18. Krag DN, Anderson SJ, Julian TB, Brown AM, Harlow SP, Ashikaga T, et al. Technical outcomes of sentinel-lymph-node resection and conventional axillary-lymph-node dissection in patients with clinically node-negative breast cancer: results from the NSABP B-32 randomised phase III trial. Lancet Oncol. 2007;8(10):881–8.
19. Kim T, Giuliano AE, Lyman GH. Lymphatic mapping and sentinel lymph node biopsy in early-stage breast carcinoma: a meta-analysis. Cancer. 2006;106:4–16.
20. Wernicke AG, Goodman RL, Turner BC, Komarnicky LT, Curran WJ, Christos PJ, et al. A 10-year follow-up of treatment outcomes in patients with early stage breast cancer and clinically negative axillary nodes treated with tangential breast irradiation following sentinel lymph node dissection or axillary clearance. Breast Cancer Res Treat. 2011;125:893–902. doi:10.1007/s10549-010-1167-6.
21. Reznik J, Cicchetti MG, Degaspe B, Fitzgerald TJ. Analysis of axillary coverage during tangential radiation therapy to the breast. Int J Radiat Oncol Biol Phys. 2005;61:163–8.
22. Wong JS, Taghian AG, Bellon JR, Keshaviah A, Smith BL, Winer EP, et al. Tangential radiotherapy without axillary surgery in early-stage breast cancer: results of a prospective trial. Int J Radiat Oncol Biol Phys. 2008;72:866–70. doi:10.1016/j.ijrobp.2008.01.031.
23. Takeda A, Shigematsu N, Kondo M, Amemiya A, Kawaguchi O, Sato M, Kutsuki S, Toya K, Ishibashi R, Kawase T, Tsukamoto N, Kubo A. The modified tangential irradiation technique for breast cancer: how to cover the entire axillary region. Int J Radiat Oncol Biol Phys. 2000;46(4):815–22.
24. Takeda A, Shigematsu N, Ikeda T, Kawaguchi O, Kutsuki S, Ishibashi R, Kunieda E, Takeda T, Takemasa K, Ito H, Uno T, Jinno H, Kubo A. Evaluation of novel modified tangential irradiation technique for breast cancer patients using dose-volume histograms. Int J Radiat Oncol Biol Phys. 2004;58(4):1280–8.
25. Ohashi T, Takeda A, Shigematsu N, Fukada J, Sanuki N, Amemiya A, et al. Dose distribution analysis of axillary lymph nodes for three-dimensional conformal radiotherapy with a field-in-field technique for breast cancer. Int J Radiat Oncol Biol Phys. 2009;73:80–7. doi:10.1016/j.ijrobp.2008.04.003.
26. Fujimoto N, Amemiya A, Kondo M, Takeda A, Shigematsu N. Treatment of breast carcinoma in patients with clinically negative axillary lymph nodes using radiotherapy versus axillary dissection. Cancer. 2004;101:2155–63.
27. Zurrida S, Orecchia R, Galimberti V, Luini A, Giannetti I, Ballardini B, et al. Axillary radiotherapy instead of axillary dissection: a randomized trial. Italian Oncological Senology Group. Ann Surg Oncol. 2002;9:156–60.
28. Hoebers FJ, Borger JH, Hart AA, Peterse JL, Th EJ, Lebesque JV. Primary axillary radiotherapy as axillary treatment in breast-conserving therapy for patients with breast carcinoma and clinically negative axillary lymph nodes. Cancer. 2000;88:1633–42.
29. Cabanes PA, Salmon RJ, Vilcoq JR, Durand JC, Fourquet A, Gautier C, et al. Value of axillary dissection in addition to lumpectomy and radiotherapy in early breast cancer. Lancet. 1992;339:1245–8.
30. Louis-Sylvestre C, Clough K, Asselain B, Vilcoq JR, Salmon RJ, Campana F, et al. Axillary treatment in conservative management of operable breast cancer: dissection or radiotherapy? Results of a randomized study with 15 years of follow-up. J Clin Oncol. 2004;22:97–101.
31. Furukawa T, Kubota T, Tanino H, Oura S, Yuasa S, Murate H, et al. Chemosensitivity of breast cancer lymph node metastasis compared to the primary tumor from individual patients tested in the histoculture drug response assay. Anticancer Res. 2000;20(5C):3657–8.
32. Gentilini O, Veronesi U. Abandoning sentinel lymph node biopsy in early breast cancer? A new trial in progress at the European Institute of Oncology of Milan (SOUND: Sentinel node vs Observation after axillary UltraSouND). Breast. 2012;21(5):678–81. doi:10.1016/j.breast.2012.06.013.

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Single Center Experience of Cell-Free and Concentrated Ascites Reinfusion Therapy in Malignancy Related Ascites

Tetsuya Ito,[1] Norio Hanafusa,[2] Mieko Fukui,[1] Hiroko Yamamoto,[2] Yasuyuki Watanabe,[2] Eisei Noiri,[2] Satoru Iwase,[1] Kiyoshi Miyagawa,[3] Toshiro Fujita,[4] and Masaomi Nangaku[2]

Departments of [1]Palliative Medicine, [2]Hemodialysis and Apheresis, The University of Tokyo Hospital, [3]Laboratory of Molecular Radiology, Center for Disease Biology and Integrative Medicine, [4]Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo, Japan

Abstract: Cell-Free and Concentrated Ascites Reinfusion Therapy (CART) is expected to improve patients' symptoms related to ascites. Use of a patient's own proteins in ascites might reduce the risk of infection. However, several reports have described that reinfusion of concentrated ascites might elevate body temperature. The aim of this study is to examine the safety and efficacy of the CART system performed exclusively on patients with malignancies. In this retrospective cohort observational study, we examined 81 CART processes performed on 24 patients with malignancies. Data were collected from medical records and records during processing of ascites. We investigated the effectiveness and adverse events during the procedures. The amount of ascites processed was 2.6 ± 1.4 L on average. The concentration ratio was 9.31 ± 5.45 on average. We found an increase in the urine volume after the procedure, which was significantly related to the amount of reinfused protein. The body temperature increased by 0.44°C. Systolic blood pressure decreased by 4 mm Hg after paracentesis, but no significant difference was found between the pressure before paracentesis and after reinfusion. In platelet counts, no significant change was observed. After all, no clinically significant adverse event was confirmed during CART procedures. Results show that CART can be performed safely even on patients with malignancy-related ascites and that the procedure might improve diuresis. **Key Words:** Ascites, Cell-Free and Concentrated Ascites Reinfusion Therapy, Efficacy, Neoplasm, Plasmapheresis, Safety.

Cell-Free and Concentrated Ascites Reinfusion Therapy (CART) comprises three processes: (i) paracentesis; (ii) removal of cell components, including cancer cells, from ascites by filtration, and concentrating ascitic fluid; and (iii) reinfusion of fluid obtained through this process (1). During the second step of the process, the membrane separation technique is used, which resembles apheresis therapy. The first filter is virtually equivalent to a plasma-separating filter, which passes proteins and other molecules with fluid, but does not pass cells or micro-organisms. The second filter is virtually equal to a dialysis filter, ultrafiltrating isotonic salt and water from the filtered ascites fluid and concentrating the final product. Therefore, the volume is reduced such that the patient should not experience volume overload during reinfusion. CART is expected to improve patients' symptoms. Effective use of the patients' own proteins in ascites is expected to reduce the risk of infection by unknown pathogens. Moreover, it might be economically beneficial by avoiding the use of expensive donated blood products, although several reports indicate that a patient might experience elevated body temperature during the reinfusion process (2).

In this study, we examine the safety and efficacy of CART performed on patients with malignancies.

Received October 2012; revised January 2013.
Address correspondence and reprint requests to Dr Tetsuya Ito, Department of Palliative Medicine, The University of Tokyo Hospital, Tokyo 113-8655, Japan. Email: rachmaninov0531@aol.com

PATIENTS AND METHODS

Study design and population

We conducted this retrospective observational study to examine patients who were treated with CART at The University of Tokyo Hospital during June 2009–September 2010.

Procedures

Biologically clean ascites was obtained by paracentesis under local anesthesia. At least 1 L of ascites is necessary to conduct CART processing. Ascites was processed at the rate of 30–60 mL/min with the CART system (first filter AHF-MOW, second filter AHF-UNH; Asahi Kasei Medical, Tokyo, Japan) (Fig. 1A,B). We strove to process the ascites as slowly as possible because the processing speed has been regarded as correlated with elevation of body temperature after CART. The ascites were filtered using the first filter, which removes cancer cells and microbes. Then, the filtered ascites were concentrated using the second filter by gravity or by clamping the filter outflow. At an earlier stage of the observation period, coagulation occurred in the CART circuit. We started applying heparin to prevent fibrin formation in the ascites. Finally, in 67 of 81 sessions, 500 U of heparin per 1 L of ascites was added to the removed ascites. First cases showed slightly elevated body temperature, which can be a side-effect of CART. Therefore, we began to use steroids before reinfusion to reduce its risk. By the end of the observation period, in 56 of 81 sessions, 100 or 200 mg of hydrocortisone was administered to patients before reinfusion. The entire protocol for this study was approved by

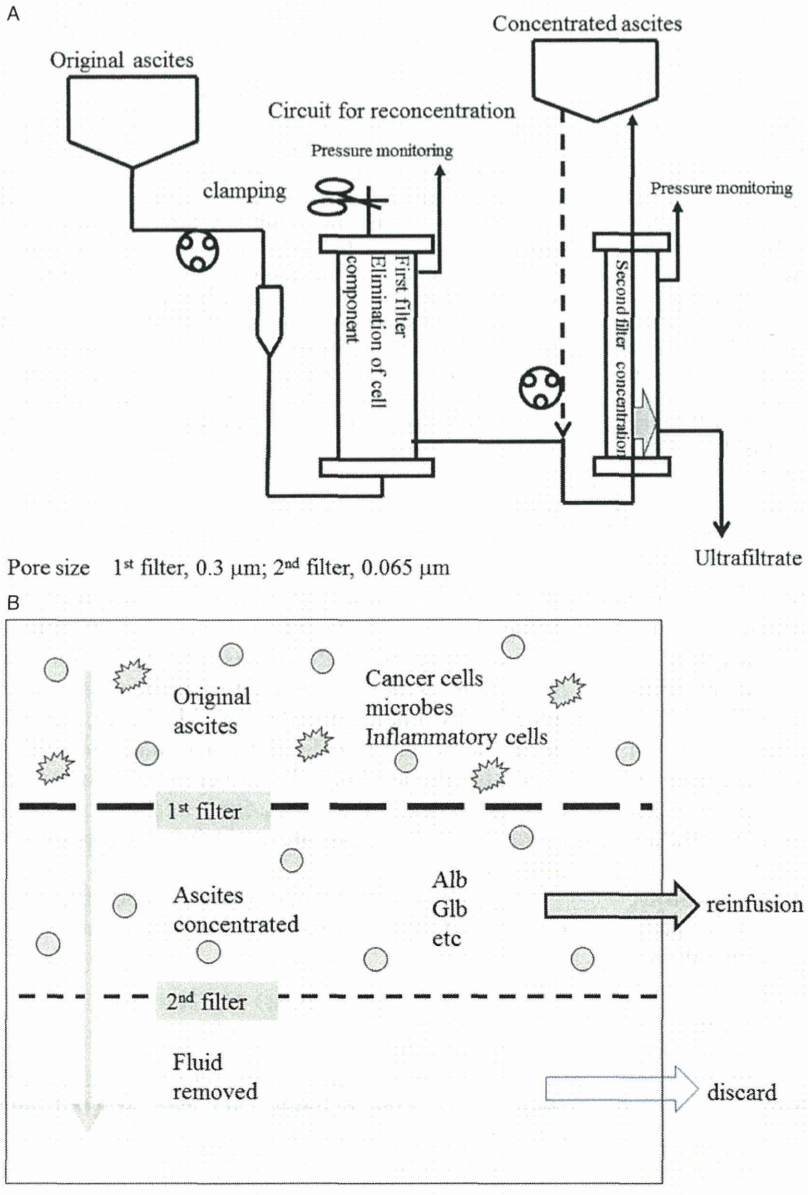

FIG. 1. Schematic illustration of Cell-Free and Concentrated Ascites Reinfusion Therapy (CART) circuit and its mechanism are shown. (A) Circuit for CART (B) Mechanism of CART. Biologically clean ascites was processed at the rate of 30–60 mL/min. The ascitic fluid was filtered by the first filter, removing cancer cells and microbes. Then, the filtered ascites was concentrated by the second filter to remove excess fluid. Finally, concentrated fluid, containing beneficial proteins such as albumin and globulin, was reinfused intravenously.

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the ethical committee of the Graduate School of Medicine and Faculty of Medicine, The University of Tokyo (approval number 2696).

Data collection

We collected data for patient age, sex, primary diagnosis, numbers of procedures, and volume and protein concentrations of unprocessed and processed ascites. We also checked changes in blood pressure, heart rate, body temperature, and platelet number during CART procedures, including the reinfusion phase. When any adverse event was observed, its severity was evaluated using CTCAE ver. 4.0; Common Terminology Criteria for Adverse Events ver. 4.0 (3). All data were collected from medical records to perform this retrospective study to clarify the safety and clinical problems associated with CART.

Statistical analysis

Paired *t*-tests were used for comparison of two groups. When we analyzed the relation between processing speed and body temperature, we divided the processing speed into two categories. The low-speed group was processed at a rate of 30 mL/h, the high-speed group was at either 50 or 60 mL/h. Spearman's correlation coefficient and multiple linear regression analysis were used to infer relations between continuous variables. All analyses were performed as two-tailed; *P*-values of less than 0.05 were regarded as statistically significant. JMP 8.0.2 (SAS Institute, NC, USA) was used for statistical analysis.

RESULTS

Patient backgrounds are presented in Table 1. During the study period, 24 patients were treated using CART. They were 58.7 years old on average. Diagnoses of the patients were mainly gastric cancer (21 of 24 patients). The number of procedures performed per patient was 3.64 ± 3.30 (mean ± SD; range 1–13). In all, 81 procedures were performed during the period. The average amount of reinfused protein was 63.1 g per session. The mean time necessary to finish the process was 102.4 ± 54.2 (mean ± SD; range 21.0–294.0) min.

Because CART is performed against diuretic-resistant ascites in many cases, it is important to clarify the correlation between the effects of protein reinfusion on clinical parameters. Regarding changes in daily urinary volume from Day 0 to Day 2, results revealed a significant increase in volume on Day 1 compared with Day 0 (787 mL on Day 0, 1309 mL on Day 1, $P = 0.009$). Subsequently, the volume decreased again on Day 2. No significant difference was found between Day 0 and Day 2 (854 mL on Day 2, $P = 0.396$). As Figure 2 shows, a significant correlation was found between the amount of protein reinfused during CART and change in urine volume from Day 0 to Day 1. The percentage of urine volume increased during the same period (change in volume 405.3 ± 788.1 mL correlation coefficient = 0.544, $P = 0.007$; percent increase of volume 75.7 ± 136.2% correlation coefficient = 0.499, $P = 0.015$).

TABLE 1. *Background of patients who were treated with Cell-Free and Concentrated Ascites Reinfusion Therapy (CART) at The University of Tokyo Hospital during June 2009–September 2010*

Age (years)	58.7 ± 12.5
Sex	Male, 13; Female, 11
Diagnosis	
Gastric cancer	21
Pancreatic cancer	1
Bile duct cancer	1
Myelofibrosis	1
Number of procedures	
Total	81
Per patient	3.64 ± 3.30 (1–13)
Ascitic volume (mL)	
Original	2635 ± 1360 (750–7300)
Processed	345 ± 185 (50–800)
Protein concentration (g/dL)	
Original	4.24 ± 1.12 (1.8–6.3)
Processed	19.17 ± 4.52 (11.0–30.0)
Ratio of concentration (original/processed volume)	×9.31 ± 5.45 (2.46–32.5)
Amount of protein reinfused (g)	63.12 ± 39.4 (5.5–163.8)

In Figure 3, systolic and diastolic blood pressure and heart rate during the CART procedures are shown. Regarding systolic blood pressure, a slight but significant decrease of 4 mm Hg was observed after paracentesis and before reinjection, compared with that before paracentesis. However, no significant change was found in diastolic blood pressure or the heart rate during CART.

Although CART has been reported to increase body temperature (2), the average increase in body temperature was 0.44°C among the cases examined in this study (Table 2). The observed changes were less than or equal to grade 1 in the scale of CTCAE ver. 4.0. In 69.1% of the cases, 100 or 200 mg of hydrocortisone was administered. A significant difference was found between the increase in body temperatures of the steroid group participants and non-steroid group participants (steroid group 0.294 ± 0.473°C, non-steroid group 0.750 ± 0.689°C, $P = 0.001$). Correlation was also found between the increase in body temperature and processing rate of ascites (high-speed group 0.63 ± 0.09°C, low-speed group 0.21 ± 0.10°C $P = 0.003$). No significant

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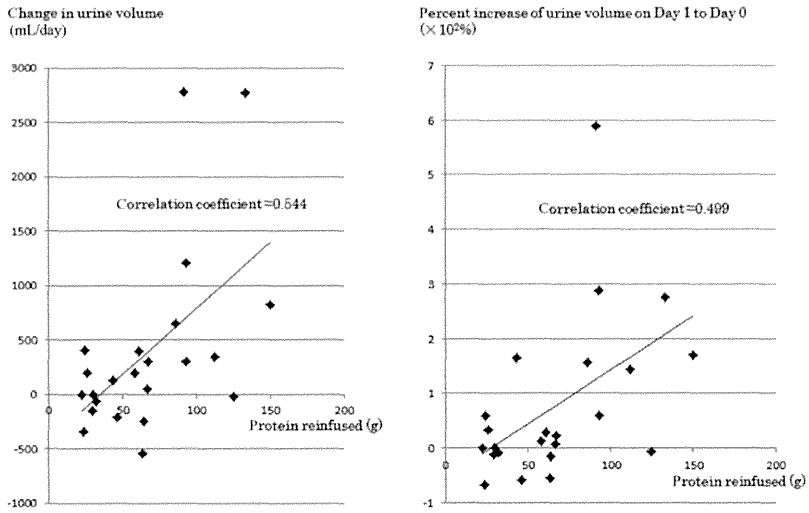

FIG. 2. Correlation between the amount of protein reinjected and increase in urine volume, and percent increase of urine volume on Day 1 from Day 0 is shown. A significant correlation was found between the amount of reinfused protein during the Cell-Free and Concentrated Ascites Reinfusion Therapy (CART) process and change in urine volume (difference between output volume from Day 0 and Day 1), and also between the percent increase of urine volume from Day 1 to Day 0 (change in volume $r^2 = 0.296$, $P = 0.007$ percent increase of volume from Day 1 to Day 0 $r^2 = 0.249$, $P = 0.015$).

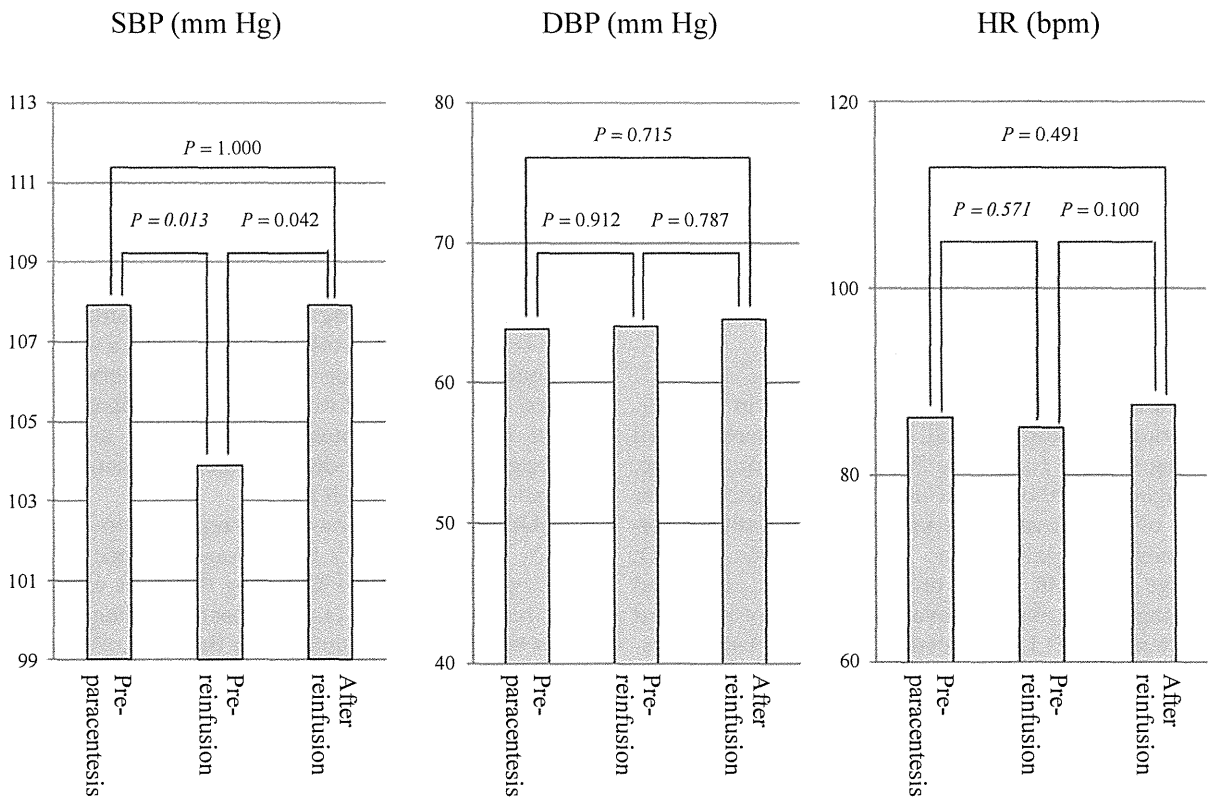

FIG. 3. Blood pressure and heart rate during Cell-Free and Concentrated Ascites Reinfusion Therapy (CART) procedure is shown. Systolic blood pressure was 107.9 ± 13.3 mm Hg before paracentesis, 103.9 ± 14.7 mm Hg before reinfusion, and 107.9 ± 15.8 mm Hg after reinfusion. A slight decrease of 4 mm Hg ($P = 0.013$) was observed after paracentesis and before reinfusion, with increase during the reinfusion process ($P = 0.042$). No significant change was found between systolic blood pressure (SBP) before paracentesis and after reinfusion. Diastolic blood pressure (DBP) was 63.9 ± 10.7 mm Hg before paracentesis, 64.1 ± 10.8 mm Hg before reinjection, and 64.6 ± 12.0 mm Hg after reinfusion. Heart rate (HR) was 86.1 ± 16.9 mm Hg before paracentesis, 85.1 ± 16.0 mm Hg before reinfusion, and 87.6 ± 14.6 mm Hg after reinfusion. No significant change was found in DBP or HR during the CART process.

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TABLE 2. *Body temperature before and at maximum after Cell-Free and Concentrated Ascites Reinfusion Therapy (CART)*

	Before CART	After CART
Mean ± SD (°C)	36.6 ± 0.384	37.0 ± 0.549
Body temperature (°C)	Number of patients	Number of patients
35.5–35.9	1 (1.3%)	0
36.0–36.4	27 (34.6%)	6 (8.0%)
36.5–36.9	37 (47.4%)	35 (46.7%)
37.0–37.4	11 (14.1%)	21 (28.0%)
37.5–37.9	2 (2.6%)	5 (6.7%)
38.0–38.4	0	6 (8.0%)
38.5–38.9	0	2 (2.7%)

association was found between the processing speed and the volume of ascites ($P = 0.373$). However, multiple linear regression analysis of body temperature elevation on steroid use and processing speed reveals that the change is affected by steroid use (adjusted difference 0.162°C $P = 0.037$), not by the processing speed. The maximum body temperature, shown in Table 2, reveals that the body temperature was less than 37°C in 54.7% of cases.

Statistically significant decrease in platelet numbers was observed (pre-CART $30.79 \pm 16.83 \times 10^4/\mu L$, post-CART $28.14 \pm 14.58 \times 10^4/\mu L$, $P = 0.027$). However, we did not consider that such a decrease was clinically significant, which accounts for the lack of signs of disseminated intravascular coagulation (DIC).

DISCUSSION

Results show that the effects of CART include increased urine volume, mildly decreased blood pressure, and mild elevation in body temperature. These results, taken together, suggested that CART might be clinically useful with only limited adverse events.

In general, being at rest and given restricted sodium (2–5 g/day) and water (up to 1 L/day) intake, administration of diuretics combined with albumin (10–25 g/day) to maintain the serum albumin concentration of more than 2.5 g/dL are strategies that are often used to control ascites.

Cell-Free and Concentrated Ascites Reinfusion Therapy has been developed as a treatment against refractory ascites that do not respond to diuretics with albumin infusion. It is one method to recycle ascites, containing valuable proteins, following paracentesis (1). CART is expected to improve patients' symptoms, reduce the risk of infection, and obviate the use of potentially expensive donated blood products. Nevertheless, CART has been known to elicit elevated body temperature, especially during reinfusion.

Aside from CART, other strategies used against diuretic-resistant ascites include paracentesis with albumin infusion, peritoneovenous shunting, and transjugular intrahepatic portosystemic shunt (TIPS) (4–8). Among them, CART is applicable to the widest range of diagnoses, even for patients with malignancies or with poor performance status, but only if they have no severe jaundice or hemorrhagic ascites because bilirubin cannot be eliminated through the first filter and bilirubin can be reinfused through CART processing.

The mainstream treatment against malignant ascites is paracentesis. The therapy should be repeated at least once per 1–2 week period according to the rate of increase in ascitic fluid. Peritoneovenous shunting is also an available therapy alternative for refractory ascites. It reportedly lessens the frequency of paracentesis more effectively and enables control of symptoms caused by ascites for longer periods than other treatments do. However, its use demands that technical problems of indwelling catheters be overcome (6). Moreover, it entails high incidence of complications such as fever, sepsis, leakage of ascites, abnormality in the coagulation system, and pulmonary edema (6).

With TIPS, the ascitic volume can be reduced for a longer period than when using paracentesis alone, especially in patients with cirrhosis who have elevated portal vein pressure (7,8). Complications related to TIPS reportedly include shunt obstruction, hemolytic anemia, liver and heart failure, infection, hepatorenal syndrome, embolism, and increased occurrence of encephalopathy (7,8).

Results of our study show a significant increase in urinary volume at 1 day after CART. Correlation between increased urinary volume and the amount of protein reinfused might indicate favorable effects of protein reinfusion: increased plasma oncotic pressure and increased urinary volume. Bruno et al. reported effects of concentrated ascites filtration and reinfusion on cirrhotic patients with tense ascites (9). This strategy differs from CART in that cell components cannot be eliminated from the concentrate. That prior study found no increase in the urinary output of the participants at day one post-procedure. Bruno et al. also examined change in urinary volume after total paracentesis with intravenous albumin infusion of 4–6 g for each liter of ascites removed. Although the amount of reinfused protein was much less than that of our study, they observed a decrease in urinary output of the patients on day one after the procedure (9). These facts might reinforce the usefulness of CART in patients with diuretic-resistant ascites.

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Regarding CART, previous reports show that decreased blood pressure and elevation of body temperature are two main presumed adverse events. In general, hemodynamic change, neurohormone imbalance, and deterioration in renal function can occur during paracentesis. However, Stephenson et al. reported no significant or symptomatic hemodynamic change during paracentesis of up to 5 L with fluid replacement if needed. Blood pressure change in their study was not reported in detail (4). In our study, statistically significant decreased blood pressure was observed and confirmed in almost all cases after paracentesis, but we consider that such a decrease had no clinical significance as it did in Stephenson's report. Regarding body temperature, reinfusion of unfiltered and concentrated ascites reportedly caused fever higher than 38°C in 42.8% of patients, even though no bacterial contamination was found in reinfused ascites fluid (10). In this study, the observed changes were less than or equal to grade 1 in the scale of CTCAE ver. 4.0, although the change in body temperature is statistically significant and greater in the high-speed group. Steroids were administered before reinfusion in 70% of the cases, which might have prevented the elevation of body temperature. The reasons for the relation between body temperature and processing speed remain unclear. Moreover, the change becomes non-significant after adjustment for steroid use.

Miyamoto et al. reported that a peritoneovenous shunt causes clinical DIC in 13.3% of patients (11). Nevertheless, in this study, no sign of DIC was confirmed as long as we evaluated the laboratory data based on the platelet counts.

To conduct one CART procedure, the estimated expense was JPY 90 500 (62 400 for material costs and 28 100 for technical costs). Regarding the time course, we must expect at least half a day of processing to ensure safety, considering the time to remove ascites safely, to process the obtained fluid, and to reinfuse it. CART is an expensive and time-consuming method. Nevertheless, we believe in the method because the benefits described above outweigh these shortcomings.

CONCLUSION

Our study has some limitations. First, this study was a retrospective observational study performed at a single center with limited number of patients. Second, all participants had ascites with malignant disease origin. We did not examine cases performed on patients without malignancy. Third, this study had no control over those in which paracentesis alone was performed. Fourth, the effects on symptoms and quality of life of patients remain to be investigated. Nevertheless, this report is the first describing CART used for malignancy-related ascites and describing details of clinical benefits and side-effects.

In conclusion, this report describes that Cell-Free and Concentrated Ascites Reinfusion Therapy might be performed safely even on patients with malignancy-related ascites. The procedure possibly increases the urinary volume, presumably via colloid replacement.

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REFERENCES

1. Inoue N, Yamazaki Z, Oda T et al. Treatment of intractable ascites by continuous reinfusion of the sterilized, cell-free and concentrated ascitic fluid. *Trans Am Soc Artif Intern Organs* 1977;23:699–702.
2. Hisa Y, Matsuoka T, Hisa T et al. Two cases of terminal ovarian cancer treated with Cell-free and Concentrated Ascites Reinfusion Therapy (CART). *Adv Obstet Gynecol* 2009;61: 226–9.
3. Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 (v4.03: Jun 14, 2010). 2009.
4. Stephenson J, Gilbert J. The development of clinical guidelines on paracentesis for ascites related to malignancy. *Palliat Med* 2002;16:213–18.
5. Luca A, Garcia-Pagan JC, Bosch J et al. Beneficial effects of intravenous albumin infusion on the hemodynamic and humoral changes after total paracentesis. *Hepatology* 1995;22: 753–8.
6. Greig PD, Langer B, Blendis LM et al. Complications after peritoneovenous shunting for ascites. *Am J Surg* 1980;139:125–31.
7. Lebrec D, Giuily N, Hadengue A et al. Transjugular intrahepatic portosystemic shunts: comparison with paracentesis in patients with cirrhosis and refractory ascites: a randomized trial. French Group of Clinicians and a Group of Biologists. *J Hepatol* 1996;25:135–44.
8. D'Amico G, Luca A, Morabito A et al. Uncovered transjugular intrahepatic portosystemic shunt for refractory ascites: a meta-analysis. *Gastroenterology* 2005;129:1282–93.
9. Bruno S, Borzio M, Romagnoni M et al. Comparison of spontaneous ascites filtration and reinfusion with total paracentesis with intravenous albumin infusion in cirrhotic patients with tense ascites. *BMJ* 1992;304:1655–8.
10. Zaak D, Paquet KJ, Kuhn R. Prospective study comparing human albumin vs. reinfusion of ultrafiltrate-ascitic fluid after total paracentesis in cirrhotic patients with tense ascites. *Z Gastroenterol* 2001;39:5–10.
11. Miyamoto K, Kusumoto N, Kawabata Y. The effectiveness of Denver peritoneovenous shunt for the treatment of refractory ascites. *Jpn J Gastroenterol Surg* 2006;39:422–7.

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STEM CELLS

TISSUE-SPECIFIC STEM CELLS

Stage-Specific Roles for Cxcr4 Signaling in Murine Hematopoietic Stem/Progenitor Cells in the Process of Bone Marrow Repopulation

CHEN-YI LAI,[a,b] SATOSHI YAMAZAKI,[a] MOTOHITO OKABE,[a] SACHIE SUZUKI,[a] YOSHIHIRO MAEYAMA,[c] YASUAKI IIMURA,[d] MASAFUMI ONODERA,[e] SHIGERU KAKUTA,[f,g] YOICHIRO IWAKURA,[f,h] MASANORI NOJIMA,[i] MAKOTO OTSU,[a,b] HIROMITSU NAKAUCHI[a]

Key Words. Chemokine receptors • Hematopoietic stem cells (stem and primitive progenitor cells) • Stem cell transplantation • Gene therapy (gene transfer to hematopoietic stem cells)

ABSTRACT

Hematopoietic cell transplantation has proven beneficial for various intractable diseases, but it remains unclear how hematopoietic stem/progenitor cells (HSPCs) home to the bone marrow (BM) microenvironment, initiate hematopoietic reconstitution, and maintain life-long hematopoiesis. The use of newly elucidated molecular determinants for overall HSPC engraftment should benefit patients. Here, we report that modification of C-X-C chemokine receptor type 4 (Cxcr4) signaling in murine HSPCs does not significantly affect initial homing/lodging events, but leads to alteration in subsequent BM repopulation kinetics, with observations confirmed by both gain- and loss-of-function approaches. By using C-terminal truncated Cxcr4 as a gain-of-function effector, we demonstrated that signal augmentation likely led to favorable in vivo repopulation of primitive cell populations in BM. These improved features were correlated with enhanced seeding efficiencies in stromal cell cocultures and altered ligand-mediated phosphorylation kinetics of extracellular signal-regulated kinases observed in Cxcr4 signal-augmented HSPCs in vitro. Unexpectedly, however, sustained signal enhancement even with wild-type Cxcr4 overexpression resulted in impaired peripheral blood (PB) reconstitution, most likely by preventing release of donor hematopoietic cells from the marrow environment. We thus conclude that timely regulation of Cxcr4/CXCR4 signaling is key in providing donor HSPCs with enhanced repopulation potential following transplantation, whilst preserving the ability to release HSPC progeny into PB for improved transplantation outcomes. STEM CELLS *2014;32:1929–1942*

INTRODUCTION

Hematopoietic stem cells (HSCs) have been used in transplantation to treat various intractable disorders [1–3]. Hematopoietic reconstitution is considered to be a process that can be divided into three major steps: (a) a homing/lodging step where HSCs transmigrate into the bone marrow (BM) cavity; (b) a BM repopulation step where HSCs replicate themselves while producing progenitor cells with multilineage differentiation potential; (c) a peripheral reconstitution step where mature cells are released from BM. Failure in any of these processes could impair transplantation outcomes [4].

HSC behavior is thought tightly regulated by certain extrinsic factors within the BM microenvironment (niche) [5]. HSC fate after transplantation may also be affected by similar external cues but much remains unclarified. We here focused on stromal cell-derived factor 1 (SDF-1)/CXCL12, abundant in BM [6]. The C-X-C chemokine receptor type 4 (CXCR4), a receptor for SDF-1/CXCL12, plays a role in hematopoiesis [7–9]. Knockout (KO) mouse studies have demonstrated the importance of the Sdf-1/Cxcr4 axis in HSCs in both fetal [10] and adult hematopoiesis, with a critical role in the latter identified for maintenance of HSC dormancy [7, 8, 11]. CXCR4 signaling has also been implicated in transplantation of adult hematopoietic stem and progenitor cells (HSPCs), as blockage of the SDF-1/CXCR4 axis in human HSPCs before transplantation compromised their engraftment/repopulation in Nonobese diabetic/Severe combined immunodeficiency (NOD/SCID) mice [12]. Similarly poor hematopoietic reconstitution was observed following transplantation of *Cxcr4*-KO mouse BM cells [7, 8]. In contrast, overexpression of CXCR4 in human HSPCs led to enhanced engraftment in mouse BM, suggesting that this modification might benefit transplanted patients [13, 14].

In this study, to determine the precise role of Cxcr4 signaling, highly purified murine HSCs and their progenies were used in both gain-of-function (overexpression) and loss-of-function (KO or desensitization) approaches. In

[a]Division of Stem Cell Therapy, [b]Stem Cell Bank, Center for Stem Cell Biology and Regenerative Medicine, [f]Laboratory of Molecular Pathogenesis, Center for Experimental Medicine and Systems, and [i]Division of Advanced Medicine Promotion, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan; [c]Upper GI Surgery Division, Department of Surgery, Hyogo College of Medicine, Kobe, Japan; [d]Kushiro City General Hospital, Kushiro, Japan; [e]Department of Genetics, National Research Institute of Child Health and Development, Tokyo, Japan; [g]Department of Biomedical Science, Graduate School of Agricultural and Life Sciences, University of Tokyo, Japan; [h]Division of Experimental Animal Immunology, Research Institute for Biomedical Sciences/Graduate School of Biological Science, Tokyo University of Science, Tokyo, Japan

Correspondence: Makoto Otsu, Ph.D., M.D., Stem Cell Bank and Division of Stem Cell Therapy, Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, 108-8639 Tokyo, Japan. Telephone: +81-3-6409-2342; Fax: +81-3-6409-2343; e-mail: motsu@ims.u-tokyo.ac.jp; or Hiromitsu Nakauchi, Ph.D., M.D., Division of Stem Cell Therapy, Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, 108-8639 Tokyo, Japan. Telephone: +81-3-5449-5330; Fax: +81-3-5449-5451; e-mail: nakauchi@ims.u-tokyo.ac.jp

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semiquantitative analysis of gain-of-function effects, we used not only wild-type (WT) Cxcr4-overexpressing cells but also cells expressing Cxcr4 with a specific C-terminal deletion (ΔC) homologous to that found in patients with the disorder termed warts, hypogammaglobulinemia, infections, and myelokathexis (WHIM) syndrome [15], for which a mouse model has recently been developed [16]. This truncation is regarded as a gain-of-function mutation, because it increases cell-surface stability of CXCR4 without impairing CXCR4 signaling capabilities [16–20]. By using this combinational approach, we assessed each of the multiple steps in donor cell reconstitution in mice that received Cxcr4-modified HSC/HSPCs and dissected stage-specific roles that Cxcr4 signaling plays in transplanted cells.

Materials and Methods

Mouse HSCs and HSPCs

C57BL/6 (B6)-Ly5.1 mice came from Sankyo Labo Service (Tokyo, http://www.sankyolabo.co.jp). B6-Ly5.2 and enhanced green fluorescent protein (EGFP)-transgenic (Tg) (CAG-EGFP) mice were from Japan SLC (Shizuoka, http://www.jslc.co.jp). Generation of *Cxcr4*-KO mice and of Kusabira Orange (KuO)-Tg mice is described in Supporting Information Methods [21–25]. Purified murine $CD34^{neg/low}$ c-Kit^+ $Sca\text{-}1^+$ Lineage marker-negative ($CD34^-$KSL) cells were used as the HSC source [26, 27]. Where indicated, KSL cells (referred to as HSPCs) were used. Considering the status of cells, that is, either fresh or postexpansion, we defined $CD34^-$KSL cells as fresh HSCs or cultured HS(P)Cs and defined KSL cells as fresh HSPCs or cultured HSPCs. Cell sorting was performed on a MoFlo cytometer (Beckman Coulter, Brea, CA, https://www.beckmancoulter.com) or a FACSAria II (BD Biosciences, San Diego, CA, http://www.bdbiosciences.com). The Institutional Animal Care and Use Committee, University of Tokyo, approved all animal experiments.

Retrovirus Transduction of HSC/HSPCs

The pGCDNsam-IRES-EGFP (DNsam-I/E) [25] was used as a control retroviral vector (Mock). Murine WT-*Cxcr4* cDNA was used to construct DNsam-WT-Cxcr4-I/E. We constructed DNsam-ΔC-Cxcr4-I/E, harboring mutant cDNA that mimics the type of mutation most frequent in WHIM syndrome [15, 18]. Production of VSV-G-retroviruses is described [25, 28, 29]. HSC/HSPCs were transduced with retrovirus as described [25]. Three days after transduction, cells expressing EGFP ($EGFP^+$ cells) were sorted and subjected to downstream assays (Supporting Information Fig. S1A).

Migration, Cell Proliferation, and Single-Cell Colony Assays in Liquid Culture

Chemotactic response toward SDF-1/CXCL12 was tested in $EGFP^+$ cultured HS(P)Cs [30]. Our single-cell colony assay in liquid medium is described [26, 31, 32]. Colonies were evaluated at days 11 and 14 for gain-of-function and loss-of-function studies, respectively (Supporting Information).

Assessment of Seeding Efficiency in Stromal Cell Coculture

The ability to repopulate an irradiated stromal cell layer was tested in sorted $EGFP^+$ HS(P)Cs or fresh HSCs using the in vitro coculture assay [33] that we developed from cobblestone-like area formation assays [34, 35] and is similar to a system recently shown to be useful to assess HSCs' clonogenic ability in stromal cocultures at a clonal level [36]. Test cells were sorted onto irradiated (50 Gy) C3H10T1/2 stromal cell layers pre-established in six-well plates containing Minimum Essential Medium Eagle Alpha in the presence of mouse stem cell factor (mSCF), mouse Thrombopoietin (mTPO), human Erythropoietin (hEPO), and mouse Interleukin-3 (mIL-3). Formed cobblestone-like areas were counted as described (Supporting Information).

HSPC Homing Assay

$EGFP^+$ cultured HSPCs (test cells) were cotransplanted into irradiated recipients with either fresh KSL or total BM cells from KuO-Tg mice (reference cells) numbered as shown. To desensitize HSPCs to SDF-1, fresh KuO-expressing (KuO^+) KSL cells preincubated with AMD3100 [37–39] (test cells) were cotransplanted into irradiated recipients with total BM cells from EGFP-Tg mice (reference cells). At the times indicated, both $EGFP^+$ and KuO^+ cells were counted in the same BM samples using flow cytometry analysis. Homing events were represented by the ratio of test cells to reference cells (schematic representation, see Supporting Information Figs. S1B, S2A, S3).

Early HSPC Repopulation Kinetics in Recipient BM

We estimated homing (16 and 24 hours) and subsequent repopulation (days 2–6) by infused test cells by counting colony-forming cells (CFCs) in recipient BM. Test cells were transplanted alone into lethally irradiated mice. Cells obtained at indicated times from recipient bones were subjected to colony-forming assays and scored [33] (Supporting Information).

Competitive Repopulation Assays

To assess both short-term (2–3 weeks) and long-term (>16 weeks) reconstitution ability in test cells, a competitive repopulation assay was used [26]. The detailed protocol is described in Supporting Information.

Flow Cytometry Analyses

To assess intracellular phosphorylation kinetics of signaling molecules, cultured $EGFP^+$ HS(P)Cs were cytokine-starved and restimulated with SDF-1/CXCL12. Cells were stained with anti-phospho-extracellular signal-regulated kinases 1/2 (Erk1/2), anti-phospho-Akt, or isotype-control rabbit mAbs (Cell Signaling Technology, Beverly, MA, http://www.cellsignal.com), followed by Alexa Fluor 647-anti-rabbit IgG (Invitrogen, Carlsbad, CA, http://www.invitrogen.com). Details are shown in Supporting Information together with other flow cytometry analyses.

Statistical Analysis

All data are expressed as means ± SD. Comparisons between two groups were subjected to Student's unpaired *t* test using *Prism 4* software (GraphPad La Jolla, CA, http://www.graphpad.com), and comparisons of more than two groups were performed by Dunnett's multiple comparison (vs. control) unless otherwise annotated. *p* for trend was tested using polynomial contrast in general linear models. A level of $p < .05$ was considered significant.

Results

Stepwise Gain-of-Function Effects of Exogenous Cxcr4 on Transduced HS(P)Cs, with Greater Response for the ΔC-Type Receptor

We constructed a system for gain-of-function studies as detailed in Supporting Information. With this system, we

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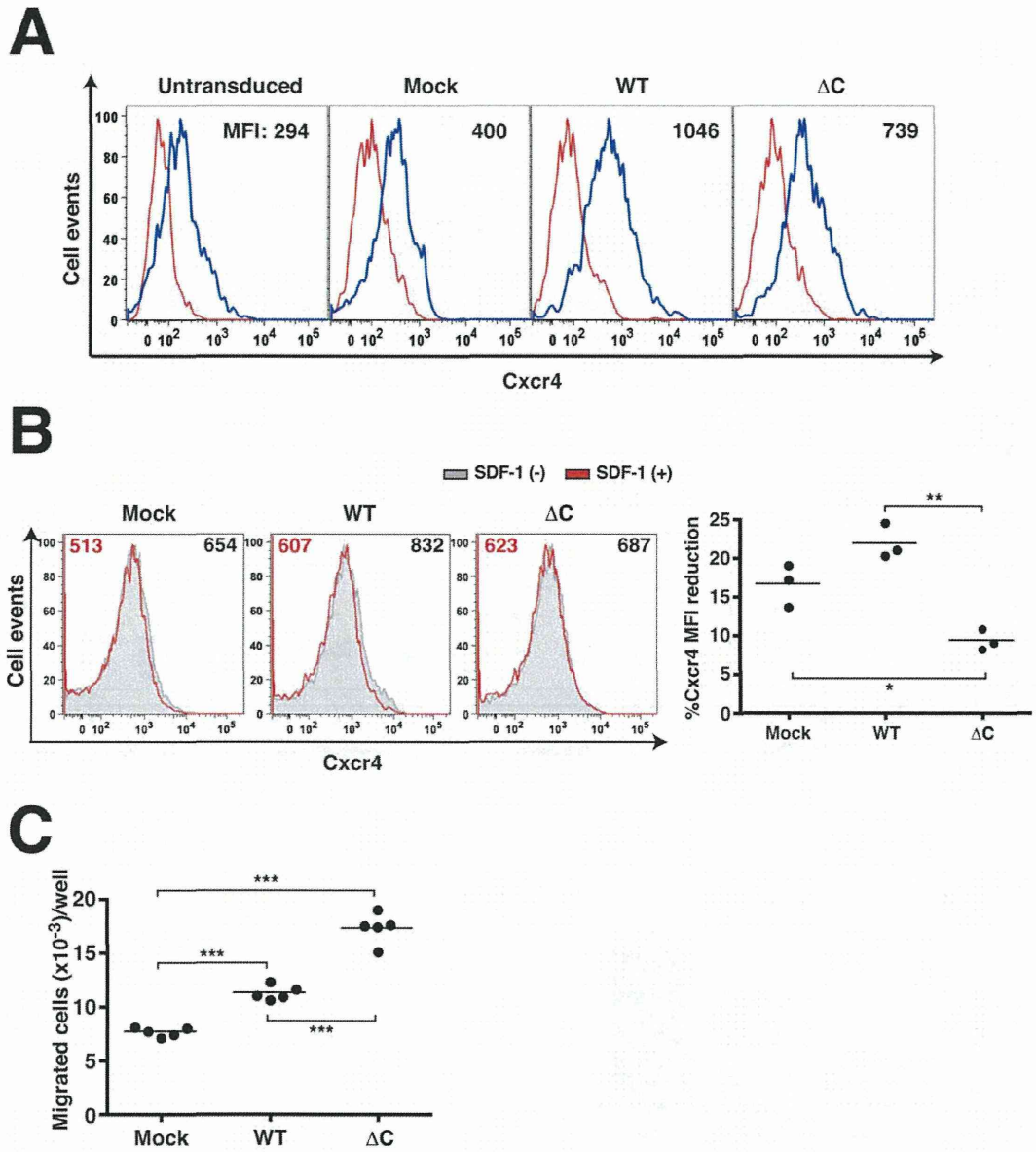

Figure 1. Rationale for gain-of-function experiments to assess role of C-X-C chemokine receptor type 4 (Cxcr4) signaling in murine hematopoietic stem/progenitor cells (HSPCs). **(A):** Assessment of cell surface Cxcr4 expression in HS(P)Cs after 1 week of cultivation following transduction and enhanced green fluorescent protein positive ($EGFP^+$) cell sorting. Cxcr4 intensity (blue histograms) represents endogenous Cxcr4 expression by untransduced cells (Untransduced) and Mock-transduced cells (Mock), or the sum of both endogenous and exogenous Cxcr4 expression by transduced cells (WT, ΔC). MFI values are shown. Red histogram: isotype control. **(B):** Receptor internalization. Cultured and sorted HS(P)Cs were left unstimulated or were stimulated with SDF-1 (500 ng/ml) for 30 minutes, after which surface expression of Cxcr4 was compared by flow cytometry. Gray/Black: Unstimulated; Red: Stimulated with SDF-1. Estimated MFI values are shown in the corresponding colors. Percent reduction of Cxcr4 expression was calculated as described (Materials and Methods section). Shown are representative data from three independent experiments. Mean values are indicated as bars ($n = 3$, *, $p < .05$; **, $p < .01$). **(C):** Transwell migration assay. Test HS(P)Cs (3×10^4 cells) were added to individual upper chambers of transwell plates in which lower chambers contained SDF-1 (50 ng/ml) as an attractant. After incubation for 90 minutes at 37°C, migrated cells were counted. Shown are representative data from two independent experiments with five replicates per group. ***, $p < .001$. The differences between groups were tested by Tukey-Kramer multiple comparison. Abbreviations: ΔC, C-terminal deletion; MFI, mean fluorescence intensity; SDF-1, stromal cell-derived factor 1; WT, wild type.

demonstrated overexpression of Cxcr4 receptors, either in wild-type configuration or in their C-terminus truncated form, in transduced HS(P)Cs (Fig. 1A; Supporting Information Fig. S4A, S4B) and in the murine hematopoietic cell line 32D (Supporting Information Fig. S4C). Defective internalization of ΔC-Cxcr4 in response to SDF-1/CXCL12, a feature predictable

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