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Original Article

Possibility of inhibiting arthritis and joint destruction by SSEA-3 positive cells derived from synovial tissue in rheumatoid arthritis

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ABSTRACT

Aim: Joint destruction progresses irreversibly once they occur in rheumatoid arthritis (RA), even with the recent development of anti-rheumatic drugs. Cells positive for stage-specific embryonic antigen-3 (SSEA-3), a marker of human embryonic stem cell, act as stem cells in the blood. The aim of this study is to investigate the effectiveness of SSEA-3 positive cells for the treatment for RA.

Methods: Synovial tissues were harvested at the time of joint surgery in RA patients. Cultured synovial cells were sorted by anti-SSEA-3 antibody using flow cytometry and were analyzed in *in vitro*. To investigate inhibitory effects on arthritis by SSEA-3 positive cells, collagen antibody-induced arthritis (CAIA) mice were used and transplanted with labeled cells intravenously.

Results: Presence of SSEA-3 positive cells was confirmed with approximately 1% in RA synovial cells. SSEA-3 positive cells were negative for CD34 and positive for CD44, CD90 and CD105. Multipotency of SSEA-3 positive cells was higher than that of SSEA-3 negative cells. Arthritis of the group transplanted with SSEA-3 positive cells in CAIA mice decreased over time.

Conclusions: SSEA-3 positive cells derived from RA synovial tissue might have the inhibitory effect on arthritis and would be one of cell source for new RA treatment.

1. Introduction

Rheumatoid arthritis (RA) is a refractory systemic autoimmune disease with synovial inflammation. Sustained synovial inflammation causes destruction of bone and cartilage and progresses into irreversible pathological conditions. Recently, biological agents targeting inflammatory cytokines have been shown to be effective for RA treatment, in which clinical remission is the treatment goal. However, treatment to restore joints that have been destroyed irreversibly is yet to be established. Therefore, a novel RA treatment that enables restoration of destroyed joints is needed.

Use of mesenchymal stem cells derived from bone marrow as a biological method for repairing articular cartilage defects have been investigated [1–5]. It is known that mesenchymal stem cells derived from bone marrow, fat tissue and synovial tissue contain multipotent cells that are capable of differentiating into various types of cells including chondrocytes, osteoblasts and adipocytes [6–12]. Mesenchymal stem cells are easily isolated from each tissue and proliferate rapidly in *in vitro*. According to previous researches as for mesenchymal stem cells, ethical dilemmas and risk of tumor formation, such as in ES cells and iPS cells, can also be avoided and therefore they are easy to use in clinical application. Furthermore, they possess strong immunosuppressive and anti-inflammatory effects [13]. For these reasons, we consider mesenchymal stem cells to be the most suitable cell type for arthritis and joint repair in RA.

Multilineage differentiating stress enduring (Muse) cells are pluripotent stem cells present in mesenchymal tissues reported by Dezawa et al., in 2010 [14], and are able to be isolated as

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stage-specific embryonic antigen-3 (SSEA-3) positive cells from mesenchymal cells. SSEA-3 positive cells act as stem cells in the blood and also possess immunosuppression effect [15–18].

The aim of this study is to investigate the possibility of inhibiting arthritis and joint destruction by SSEA-3 positive cells. It may lead to establishing a new treatment for RA.

2. Materials and methods

2.1. Preparation of synovial cell

Diagnosis of RA for all patients was based on the American College of Rheumatology (ACR) criteria in 1987 [19] or the ACR/European League Against Rheumatism (EULAR) classification criteria in 2010 [20]. Approval for this study was obtained from the Ethics of Human Experiments Committee at Hirosaki University Graduate School of Medicine, Hirosaki, Japan. Informed consent was obtained from all patients.

Synovial tissue was harvested from 13 patients with RA at the time of joint surgery in our hospital (Table 1). For comparison, synovial tissue from 13 patients with osteoarthritis (OA) was also collected. All 13 OA patients (11 female, 2 male) were performed total knee arthroplasty and the average age was 79.7 years old (range 75–85). For preparation of synovial cells, harvested synovial tissue was minced, digested with 3 mg/mL collagenase Type V (Wako Pure Chemical Industries: Osaka, Japan) for 3 h at 37 °C, washed with phosphate buffered saline (PBS) followed by centrifugation at 400 *g* for 5 min, was re-suspended in the αMEM (Sigma–Aldrich: Tokyo, Japan) containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific: Waltham, MA, USA) and antibiotics (100 units/mL penicillin G and 100 μg/mL streptomycin) (Thermo Fisher Scientific). Cells were then seeded in 100-mm culture dishes and cultured at 37 °C in a 5% CO_2 incubator. Medium was replaced twice a week and passaged at confluency.

2.2. Immunohistochemical staining

Immunohistochemical staining was performed to investigate the localization of SSEA-3 positive cells in RA synovial tissue. Synovial tissue harvested at the time of joint surgery was immediately fixed in 4% paraformaldehyde/PBS and embedded in paraffin as usual manner. Rat monoclonal antibody specific for human SSEA-3 (Merck Millipore, Darmstadt, Germany) was used as a primary antibody. Immunoreactivity was detected by incubation with a biotinylated anti-rat IgG antibody (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA), followed by streptavidin-biotin reaction (Vectastain ABC kit).

Table 1
Clinical data of patients with RA (n=13) for this study.

Pts	Age	M/F	Stage	Class	R/L	Surgery
1	73	M	IV	III	R	Total knee arthroplasty
2	45	F	I	II	L	Arthroscopic synovectomy
3	78	M	III	I	R	Total knee arthroplasty
4	82	F	IV	II	R	Total knee arthroplasty
5	77	M	IV	II	L	total knee arthroplasty
6	84	F	IV	II	R	Total knee arthroplasty
7	69	F	III	II	R	Total elbow arthroplasty
8	77	F	III	II	L	Total elbow arthroplasty
9	66	F	IV	II	R	2nd, 4th PIP arthrodesis
10	69	F	IV	III	L	1st IP arthrodesis
11	61	F	IV	II	R	Wrist arthroplasty
12	62	F	IV	II	R	1st IP arthrodesis
13	66	F	III	I	L	Total hip arthroplasty

Pts, patients; M/F, male or female; R/L, right or left; PIP, proximal interphalangeal joint; IP, interphalangeal joint.

2.3. Flow cytometry assay and surface epitopes

Synovial cells at second generation of subculture (passage 2) were used in the flow cytometry assay. Fluorescence was analyzed by FACScan (Becton Dickinson, Mountain View, CA, US) and compared to control. Antibody specific for human SSEA-3 from Biolegend (San Diego, CA, USA) and fluorescein isothiocyanate (FITC)-coupled anti-rat IgM antibody (Jackson Immunoresearch, MN, USA) were used as primary and secondary antibodies, respectively. Cells were characterized by Peridinin chlorophyll (PerCP)-coupled antibodies specific for human CD105 from R&D Systems (MN, USA), phycoerythrin (PE)-coupled antibodies specific for human CD34 from Becton Dickinson, Allophycocyanin (APC)-coupled antibodies specific for human CD90 from Becton Dickinson, and Brilliant Violet (BV421)-coupled antibodies specific for human CD44 from Becton Dickinson.

SSEA-3 positive cells were sorted by suspending 1×10^6 synovial cells at passage 2 in 100 μl FACS buffer containing 1 ml of EDTA, 5 ml of BSA and 44 ml of FluoroBrite DMEM (Thermo Fisher Scientific, Waltham, MA, USA). Cells were collected by using antibody specific for SSEA-3.

2.4. Multipotency of SSEA-3 positive cells

To investigate the multipotency of SSEA-3 positive cells in *in vitro*, osteogenic differentiation was performed using osteogenic medium (Lonza, Walkersville, MD, USA) consisting of dexamethasone, ascorbic acid, and β-glycerophosphate in a 6-well dish. *In vitro* adipogenic differentiation was also performed using adipogenic induction medium (Lonza) consisting of insulin, dexamethasone, indomethacin, and IBMX (3-isobutyl-methyl-xanthine) and adipogenic maintenance medium (Lonza) consisting of insulin in a 6-well dish. For *in vitro* chondrogenic differentiation, we utilized high-density three-dimensional micromass culture [21,22], in which cells were trypsinized and resuspended at a density of 1×10^5 cells/10 μl. Ten microliter droplets were seeded in culture dishes and allowed to form cell aggregates and substratum at 37 °C for two and a half hours. Chondrogenic medium (Lonza), consisting of ITS + premix (6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 μg/mL selenous acid, 5.33 μg/mL linoleic acid, and 1.25 mg/mL bovine serum albumin), pyruvate (1 mmol/L), ascorbate 2-phosphate (0.17 mmol/L), proline (0.35 mmol/L), dexamethasone (0.1 μmol/L) and recombinant human TGF-β3 (10 ng/mL) was then carefully added around the cell aggregates. This chondrogenic medium was replenished every three days.

2.5. Real-time PCR

Total RNA was prepared from each differentiated cultured cells using Qiagen RNeasy Mini Kit (QIAGEN, Hilden, Germany). Approximately 1 μg of total RNA was converted to cDNA, which was amplified by polymerase chain reaction (PCR) using ReverTra Ace qPCR RT Kit Master Mix (TOYOBO, Osaka, Japan). Real-time PCR was performed using an ABI prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). PCR primers were as follows: glyceraldehydes-3-phosphate-dehydrogenase (G3PDH) forward primer, 5′-TGCACCACCAACTGCTAGC-3′, G3PDH reverse primer, 5′-GGCATGGACTGTGGTCATGAG-3′;, sex determining region Y (SRY)-Box 9 (SOX9) forward primer, 5′-GAGCGAGGAGGACAAGTTCC-3′, SOX9 reverse primer, 5′-CCAGTCGTAGCCTTTGAGCA-3′; aggrecan (AGG) forward primer, 5′-TCGAGGACAGCGAGGCC-3′, AGG reverse primer, 5′-GAGATGTGCGATGTGGGAGCT-3′; alkaline phosphatase (ALP) forward primer, 5′-CCTCCTCGGAAGACAAC TCTG-3′, ALP reverse primer, 5′-GCAGTGAAGGGCTTCTTGTC-3′; bone morphogenetic protein 2 (BMP2) forward primer,

5′-CAAACACAAACAGCGCAAACG-3′, BMP2 reverse primer, 5′-GCCACAATCCAGTCATTCCA-3′; peroxisome proliferator-activated receptor gamma (PPARγ) forward primer, 5′-TGAATGTGAAGCC-CATTGAA-3′, PPARγ reverse primer, 5′-CTGCAGTAGCTGCACGTGTT-3′; type II collagen alpha 1 chain (COL2A1) forward primer, 5′-CCGGGCAFAFFFCAATAGCAGGTT-3′, COL2A1 reverse primer, 5′-CATTGATGGGGAGGCGTGAG-3′. PCR was carried out under the following conditions; one cycle at 95 °C for 15 min, and 45 cycles at 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 1 min.

2.6. Intravenous transplantation of SSEA-3 positive cells into collagen antibody-induced arthritis (CAIA) mice

CAIA mice were established as the animal model for RA [23]. Induction of CAIA mice was performed on *scid*/*scid* mice 7 weeks old (CLEA Japan) in which they were injected with 1.5 mg of 5-clone cocktail (arthrogen-CIA arthrogenic monoclonal antibody (mAb), Chondrex, Redmond, WA) by intraperitoneal (IP) injection at Day 0. Fifty micrograms of lipopolysaccharide (LPS) (Chondrex) was injected by IP injection at Day 3. 3×10^4 SSEA-3 positive cells labeled with cell tracker green (CTG) (Thermo Fisher Scientific) were suspended in PBS, filtered, then intravenously injected via the tail vein after the injection of LPS at Day 3. SSEA-3 negative cells labeled with CTG were used in the same procedure as control. Mice were scored for clinical arthritis; Paws were assessed for signs of redness and swelling. Each paw was given a score of 0–4, giving a total maximum score of 16. (0, normal paw; 1, mild but definite redness and swelling in each one joint of the digit or wrist/ankle; 2, moderate redness and swelling in two joints of the wrist/ankle with digit involvement; 3, severe redness and swelling in whole paw; 4, maximum inflammation within the wrist/ankle with many digits involved) [24]. CAIA mice in both transplanted groups were euthanized on Day 5 and 28, embedded in paraffin, and fluorescent microscopy was used to investigate the localization of cells. We also examined immunohistochemical staining for human SSEA-3 (Merck Millipore, Darmstadt, Germany) in the same tissue section because there was a possibility of autofluorescence.

2.7. Statistical analysis

Student's *t*-test was used to assess significant differences. *P*-values < 0.05 was considered statistically significant.

3. Results

3.1. SSEA-3 positive cells in RA synovial tissue

Immunohistochemical staining for SSEA-3 showed a few positive cells in RA synovial tissue (Fig. 1a–c). These SSEA-3 positive cells were detected in the synovial sublining layer and not in the lining layer. Significant difference in average number of SSEA-3 positive cells between each 13 specimens of RA and OA synovial tissue on 10 fields selected randomly by Carl Zeiss Axio Imager was not observable (data not shown).

In FACS analysis, the ratio of SSEA-3 positive cells in cultured cells at passage 2 derived from RA synovial tissue and OA synovial tissue was similar. It was the values of approximately 1% in both of the cultured cells derived from RA synovial tissue and OA synovial tissue (Fig. 1d).

3.2. Immunophenotype and multipotency

SSEA-3 positive cells and SSEA-3 negative cells at passage 2 derived from RA synovial tissue and OA synovial tissue were similar in immunophenotype, respectively (Fig. 2). Cultured SSEA-3 positive cells derived from RA synovial tissue and OA synovial tissue strongly expressed CD44, CD90 and CD105 and lacked CD34. Cultured SSEA-3 negative cells derived from RA synovial tissue and

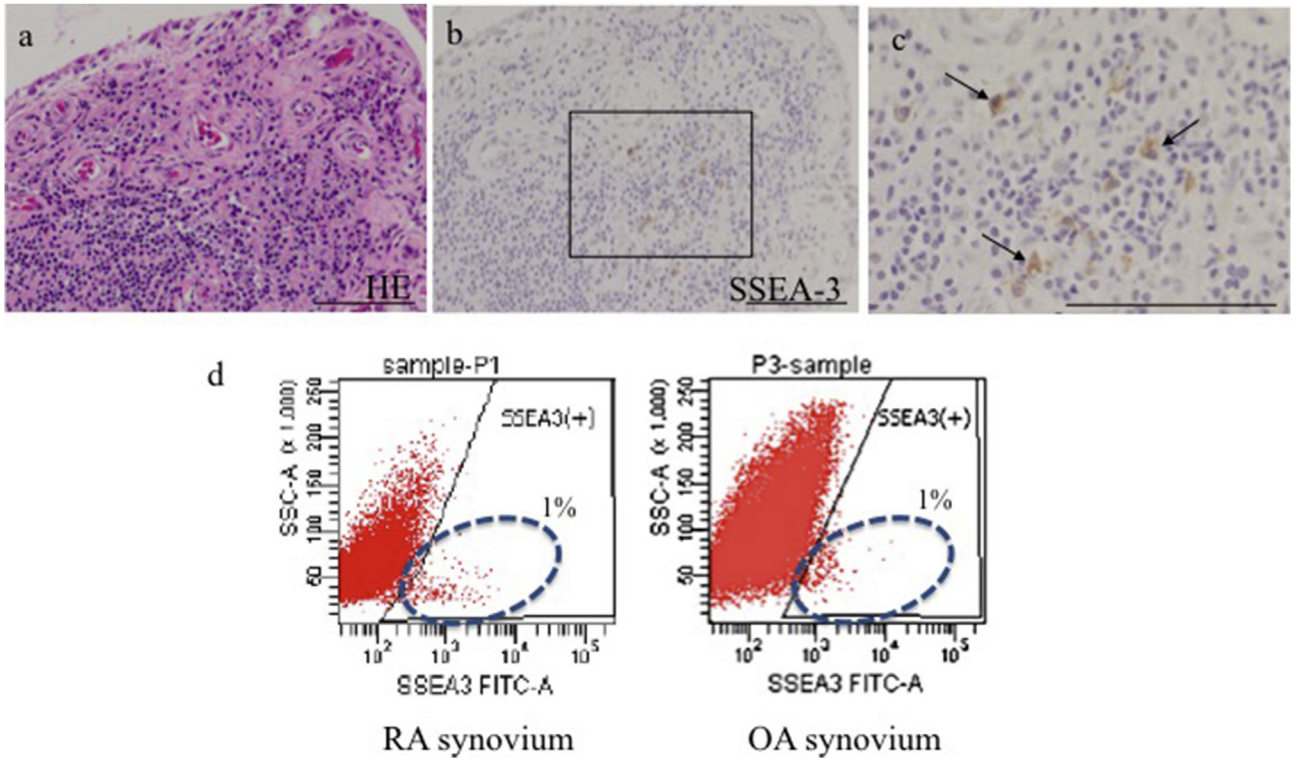

Fig. 1. (a) RA synovial tissue. Hematoxylin and eosin (HE) staining. (b) Immunohistochemical staining specific for SSEA-3 in RA synovial tissue. (c) Magnified feature of (b) Bar=100 μm. (d) Ratio of SSEA-3 positive cells in cultured cells at passage 2 derived from RA synovial tissue and OA synovial tissue.

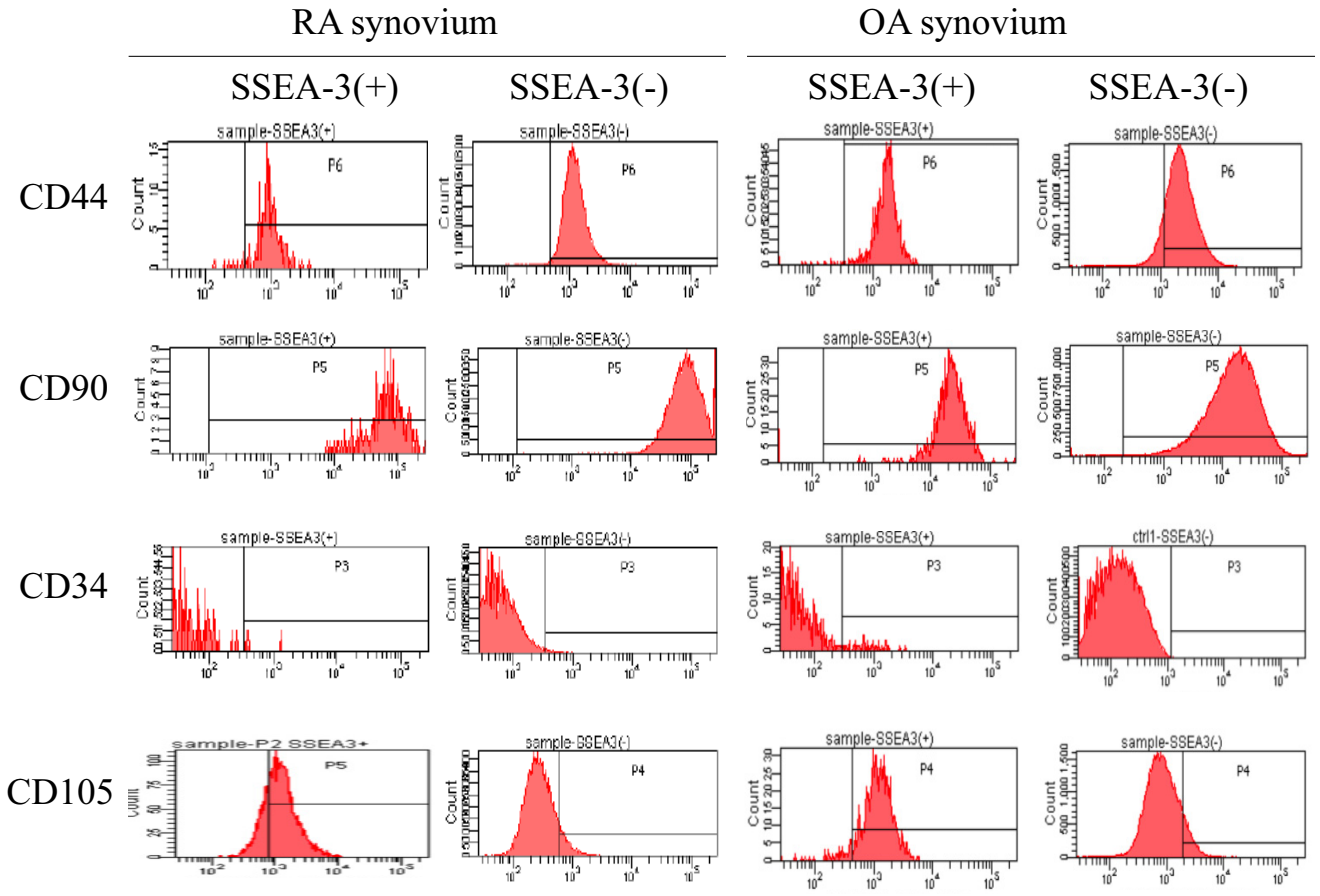

Fig. 2. Representative flow cytometry analysis data.

OA synovial tissue also strongly expressed CD44 and CD90 and lacked CD34, but they weakly expressed CD105.

Histological staining after differentiation induction culture proved multipotency of the cultured SSEA-3 positive cells. As shown in Fig. 3, alizarin red and alkaline phosphatase (ALP) staining showed differentiation into osteoblasts, oil red-O staining displayed differentiation into adipocytes, and toluidine blue (TB) staining presented deposition of extracellular proteoglycans and differentiation into chondrocytes. Both SSEA-3 positive cells and SSEA-3 negative cells derived from RA synovial tissue showed differentiation into osteoblasts, adipocytes and chondrocytes. However, many cells seemed to be stained in the differentiated tissues by SSEA-3 positive cells compared with them by SSEA-3 negative cells.

3.3. *Differentiation ability of SSEA-3 positive cells*

In all mRNA expression of ALP and bone morphogenetic protein 2 (BMP2) for osteogenic differentiation, peroxisome proliferator-activated receptor gamma (PPARγ) for adipogenic differentiation and type II collagen (COL2A1), sex determining region Y (SRY)-Box 9 (SOX9) and aggrecan (AGG) for chondrogenic differentiation, SSEA-3 positive cells showed higher gene expression level than SSEA-3 negative cells although there were individual differences (Fig. 4). These results indicate possibility of higher differentiation ability of SSEA-3 positive cells.

3.4. *Inhibitory effect on arthritis by SSEA-3 positive cells*

Fig. 5a displays the arthritis score of CAIA mice in the both transplanted groups after mAb injection. The group transplanted with SSEA-3 positive cells (n=3) was consisted of mice with intravenously transplanted SSEA-3 positive cells labeled with cell tracker green (CTG) seen in Fig. 5b, while the group transplanted with SSEA-3 negative cells (n=3) was consisted of mice with the transplanted SSEA-3 negative cells in the same procedure. Arthritis in the SSEA-3 negative cells group remained for 28 days, while arthritis score in the SSEA-3 positive cells group improved faster after peak inflammation (Fig. 5a). There was a significant improvement in arthritis in the SSEA-3 positive cells group. On day 5, CTG-labeled cells were detected in the synovial tissue (Fig. 5c and d) and were still present on day 28 (Fig. 5e and f) in the group transplanted with SSEA-3 positive cells. Fig. 5e shows representative joint in the group transplanted with SSEA-3 positive cells on day 28 and Fig. 5f shows SSEA-3 positive cells in synovial tissue in the immunohistochemical staining of Fig. 5e. Fig. 5g and h show the progression of joint destruction in the group transplanted with SSEA-3 negative cells on day 28. CTG-labeled cells were not detected in other healthy organs on day 28 by fluorescent microscopy (data not shown). These results indicate that SSEA-3 positive cells have the inhibitory effect on arthritis and systemic administration of them is safety.

4. Discussion

In this study, we found that SSEA-3 positive cells were present in synovial tissue even under pathological conditions such as RA. In previous studies, SSEA-3 positive cells have been detected in various organs such as pancreas, dermis, umbilical cord, fat, liver, trachea, bone marrow, spleen, and are contained at a proportion of

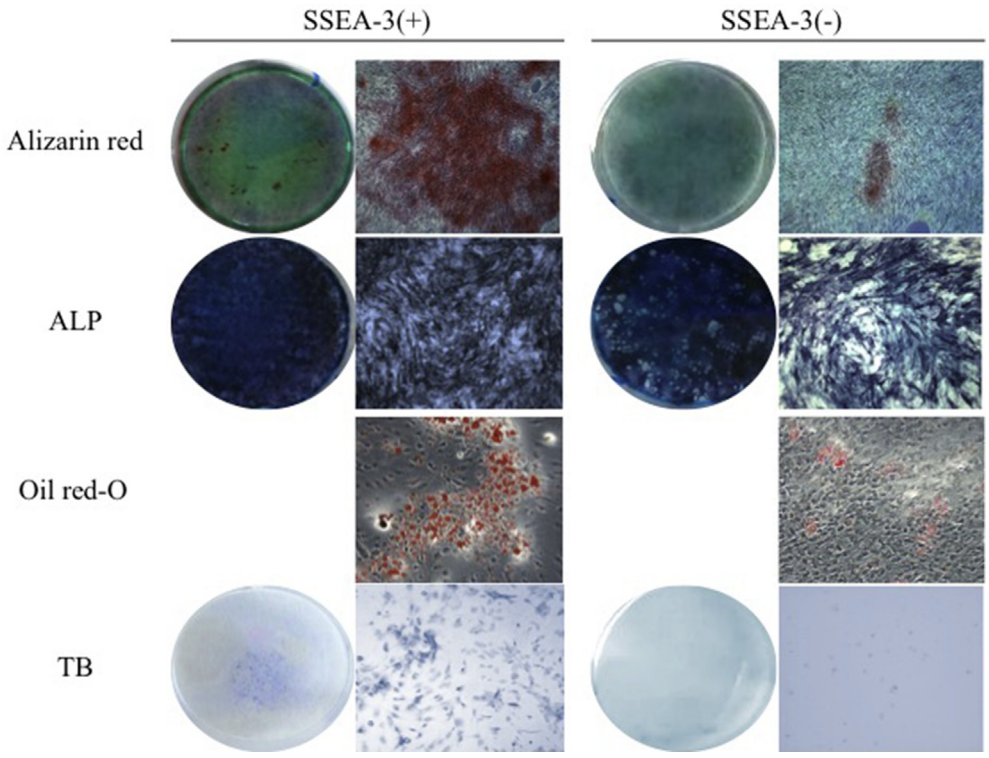

Fig. 3. Multipotency of SSEA-3 positive cells derived from RA synovial cells. Osteogenesis was shown by alizarin red and alkaline phosphatase (ALP) staining. Adipogenesis was shown by oil red-O staining and chondrogenesis was shown by toluidine blue (TB) staining.

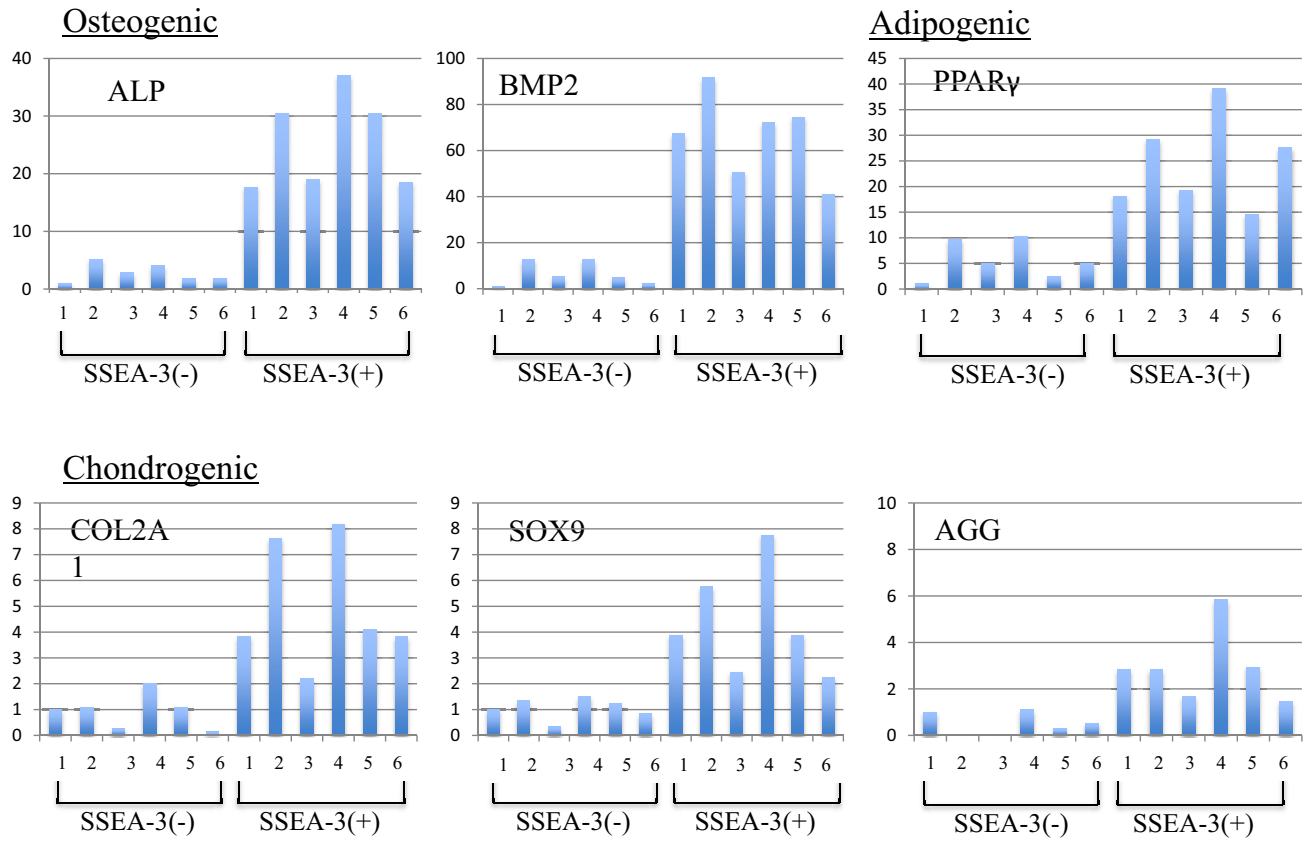

Fig. 4. Real-time polymerase chain reaction (PCR) analysis. Results are reported as the mean of three independent experiments and the messenger RNA (mRNA) value of glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) was set as an internal control. Osteogenesis was shown by the expression of ALP mRNA and BMP-2 mRNA, adipogenesis was shown by the expression of PPARγ mRNA and chondrogenesis was shown by the expression of COL2A1 mRNA, SOX9 and AGG. Each data represents an average of three times. Note that the x-axis numbers represent patients in Table 1. The mRNA values of y-axis were arbitrary set.

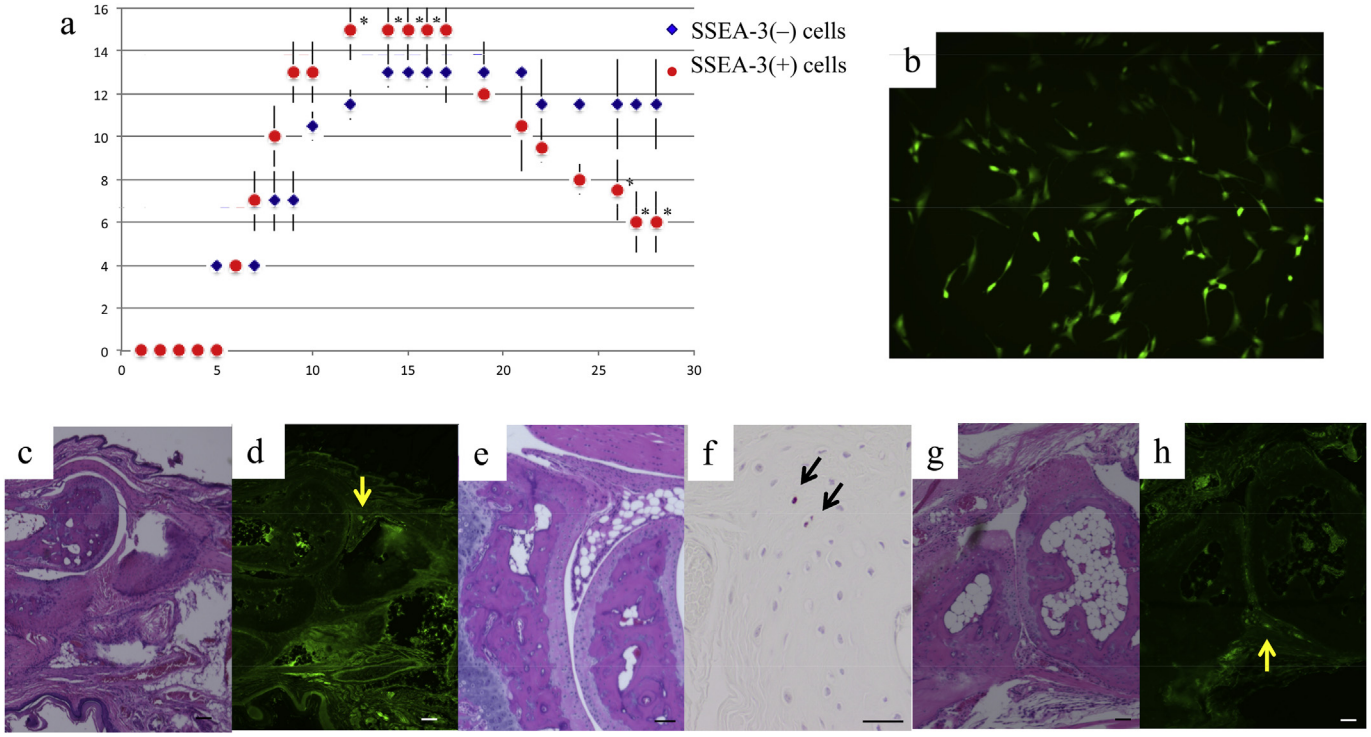

Fig. 5. (a) Arthritis score of CAIA mice after mAb injection. SSEA-3 (−) cells group consisted of mice transplanted with SSEA-3 negative cells. SSEA-3 (+) cells group consisted of mice transplanted with SSEA-3 positive cells. Both groups contained three mice. Each score shows average values of three mice and *means $p < 0.05$. The X-axis means the day from Day 0 when the monoclonal antibody cocktail was administered. (b) Cell tracker green (CTG)-labeled SSEA-3 positive cells (10X). (c, d and e, f) Histology of joint of CAIA mouse after CTG-labeled SSEA-3 positive cells injections on day 5 and 28, respectively. Arrows shows CTG-labeled cells and positive cells for SSEA-3 in immunohistochemical staining Bar=100 μm. (g and h) Representative histology of joint of CAIA mouse after CTG-labeled SSEA-3 negative cells injections on day 28 Bar=100 μm.

several % in cultured mesenchymal stem cells [14], 4–9% in human adipose tissue [25], and 1–2% in human skin fibroblasts [14]. We used synovial tissue because they could be harvested certainly during surgery. Also, it is known that human synovial mesenchymal stem cells have a higher capacity for proliferation and greater chondrogenic potential than those from other cell sources, such as bone marrow from previous studies [26–28]. Actually, although synovial tissue were collected from RA patients with various disease stages and surgical sites, SSEA-3 positive cells were detected with values of approximately 0.5–1% in all cases by FACS analysis. The proportion in synovial tissue in our study seemed to be comparable to previous reports. We used RA derived cells in further experiment because the therapeutic goal of our study was to improve arthritis in RA by using autologous cells. There are some reports that fibroblast-like synovial cells (FLSs) in RA synovial tissue have many similarities with mesenchymal stem cells (MSCs) in their morphology and differentiation potential and MSCs in RA synovial tissue are not working well [29,30]. The difference between RA and OA as a source of SSEA-3 positive cells is yet unknown, but we could show that SSEA-3 positive cells in RA synovial tissue were present at the same level as OA.

Collected SSEA-3 positive cells had higher gene expression level and differentiation ability in *in vitro* compared with SSEA-3 negative cells that were occupying most of mesenchymal stem cells. Wakao S., et al., reported that Muse and non-Muse cells had differentiation ability of osteocytes, chondrocytes, and adipocytes, while differentiation ability in non-Muse cells was lower rate [18]. We think that SSEA-3 positive cells in this study had a similar nature as Muse cells, considering also the results that SSEA-3 positive cells strongly expressed CD105 in FACS analysis. SSEA-3 positive cells can be systemically administered by intravenous administration like Muse cells and can also differentiate into osteoblasts, adipocytes and chondrocyte. These suggests the possibility of repairing degenerative cartilage and destroyed joints in RA.

In CAIA mice experiment, SSEA-3 positive cells systemically administered had inhibitory effect on arthritis. In the transplanted group consisting of mice transplanted with SSEA-3 positive cells, arthritis score quickly decreased after the onset of arthritis compared with SSEA-3 negative cells group. In this study, we did not measure the blood level of various inflammatory cytokines in CAIA mice. However, there were some previous studies on immunosuppressive effect of mesenchymal stem cells. Ozawa K., et al., reported that mesenchymal stem cells could suppress T cell proliferation, especially Th17 and have immunosuppressive ability [31]. Other researchers reported that mesenchymal stem cells derived from human umbilical cord tissue (UC-MSC) suppressed various inflammatory effects of FLSs and T cells of RA and therefore UC-MSC can be useful for treating RA [32,33]. In our study, we believe that the reason for suppressing arthritis in CAIA mouse is due to immunosuppressive effect and therapeutic effect by SSEA-3 positive cells in *in vivo*.

There are some problems in our study. First, each number of CAIA mice in the both transplanted groups was very small (n=3). We think that it is necessary to increase the number of mice in order to accurately evaluate arthritis. Second, there is a need for further long-term follow-up to investigate sustained effect of suppressing arthritis and effect of joint repair. Third, blood level of inflammatory cytokines should be measured to prove improvement of arthritis in *in vivo*. Although there are such problems, our study suggests the possibility of inhibiting arthritis and joint destruction by SSEA-3 positive cells derived from synovial tissue in RA. Further study of SSEA-3 positive cells for clinical application in humans will be lead to future development as a new treatment in RA.

5. Conclusion

We demonstrated that SSEA-3 positive cells derived from RA synovial tissue might have the inhibitory effect on arthritis and joint destruction. SSEA-3 positive cells would be one of cell source for new RA treatment.

Conflict of interest

All authors declare no conflicts of interest associated with this manuscript.

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