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Influence of human adipose stem cells on prostate cancer cell growth

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ABSTRACT

In a novel regenerative cell-based treatment developed by us for the patients with stress urinary incontinence, autologous adipose-derived stem cells (ASCs) are injected into the periurethral region and the external urethral sphincter. Since the candidates for this treatment included prostate cancer patients after radical prostatectomy, we investigated the effects of ASCs on prostate cancer cell proliferation *in vitro* and *in vivo* to confirm the feasibility of our therapeutic approach. The LNCaP (human prostate cancer cell line) cells and ASCs were co-cultured, and prostate-specific antigen (PSA) concentration in their culture medium supernatant was measured at 48 and 96 h. The PSA concentration significantly decreased in the coculture medium supernatant as compared to the culture medium with LNCaP cells alone. On the contrary, PSA concentrations in the culture medium of LNCaP cells were not affected by supplementation with ASC culture supernatant. After subcutaneous transplantation of LNCaP cells, with or without ASCs, in immunodeficient mice, tumor growth was compared. The growth of LNCaP xenograft tumor in immunodeficient mice was significantly suppressed by ASC addition. These results indicated that ASCs inhibit prostate cancer cell growth, without no proliferative effect on prostate cancer cells.

Keywords: adipose stem cells, prostate cancer, cell growth

INTRODUCTION

Stem cell regenerative therapy using stem cells has been extensively investigated at an experimental level; however, exploring its clinical applications in a variety of fields is currently in progress. Embryonic stem (ES) cells, induced pluripotent stem (iPS) cells, cord blood stem cells, and adult stem cells can be applied for stem cell-based regenerative therapy. Mesenchymal stem cells are one of the multipotent adult stem cells that can proliferate into a variety of cell types in culture.[1-4] In clinical practice, various cell therapies have been utilized for damaged

tissue regeneration in several diseases, commonly using mesenchymal stem cells obtained from bone marrow. On the contrary, bone marrow as a tissue source has many limitations, such as the amount of bone marrow that can be collected from a donor is limited, the percentage of mesenchymal stem cells contained in bone marrow is not high, and it is necessary to expand culture to a required amount to be administered as regenerative medicine. Further, the time required for expansion culture is directly linked to the waiting time for transplantation, which imposes clinical limitations, including the response to changes in the patient's underlying disease environment. Adipose tissue contains multipotent stem cells that are similar to bone marrow-derived mesenchymal stem cells, and the number of stem cells in adipose tissue is comparatively more than that in the bone marrow. These adipose-derived stem cells can differentiate into bone, cartilage, nerve, blood vessels, and muscle. Unlike bone marrow cells, adipose tissue can be easily and safely harvested in large quantities with minimal morbidity, and a small tissue amount is required to obtain the therapeutic dose of cells.

We have developed a novel treatment strategy for stress urinary incontinence (SUI) resulting from external sphincter dysfunction by injecting autologous adipose-derived stem cells into the periurethral region.[5] This novel treatment strategy is minimally invasive, can be performed within 3 h in a single procedure, and does not require *ex vivo* culture of autologous stem cells. We have already reported the short- and long-term efficacy and safety of this regenerative treatment in a preceding clinical study.[5-7] Apart from this, an ongoing multicenter investigator-initiated clinical study, ADRESU,[8] is aimed at obtaining clinical data for demonstrating the efficacy and safety to obtain PMDA (Pharmaceutical and Medical Devices Agency) approval and a manufacturing and selling license. Since the subjects for this treatment comprise male patients who have undergone radical prostatectomy for prostate cancer, it is important to confirm that ASCs have no proliferative effect on the prostate cancer cells. The present study was conducted to investigate the effects of ASCs on prostate cancer cell proliferation.

MATERIALS AND METHODS

Tissue samples

Tissue samples from patients with prostate cancer were used after obtaining approval (approval number: 505-2) from the Ethics Committee of Nagoya University Medical School, Graduate School of Medicine, Japan guidelines and the study was performed according to the guidelines of The Declaration of Helsinki. The experimental study was also approved (approval number: 29330) by The Center for Animal Research and Education, the Nagoya University Graduate School of Medicine, Japan.

Prostate cancer cell lines

The LNCaP and PC3 cells were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). Both LNCaP and PC3 cells (4×10^4 cells per cell line) were cultured in RPMI 1640 (Sigma-Aldrich, Tokyo, Japan) and 1% penicillin/streptomycin (Life Technologies, Burlington, Ontario, Canada) with or without 10% fetal calf serum (FCS). The PSA concentration in the culture medium was measured with enzyme-linked immunosorbent assay (Abott, Wiesbaden, Germany).

Adipose stem cell lines

In addition to the commercially available ASCs, ASCs were cultured using subcutaneous adipose tissue obtained by liposuction from the abdomen of a patient enrolled in the preceded

clinical study.[6]

Commercially available ASCs, isolated from a 63-year-old male donor were purchased from Lonza (Lonza Inc., Walkersville, MD, USA) and maintained in MesenPRO RS™ medium (Thermo Fisher Scientific, Mass, USA) containing 2% FCS and 2 mM GlutaMAX™ (Thermo Fisher Scientific). The ASCs were characterized by flow cytometric analysis for CD13, CD29, CD44, CD73, CD90, CD105, and CD 166 (>90%) positivity, and CD14, CD31, CD34, and CD45 (<5%) positivity.

Adipose tissue obtained from a 70-year-old patient was washed with DMEM/F12 medium (Sigma-Aldrich) supplemented with penicillin and streptomycin (100 U/mL each) and was cut into 2 mm^3 pieces. Furthermore, they were digested in 2 mL Hank's balanced salt solution (Sigma-Aldrich) containing 1 mg/mL collagenase type I (Worthington Biochemical Corporation, Lakewood, NJ, USA) with reciprocal shaking for 1 h at 37°C. After removing the undigested tissues by passing the samples through a nylon mesh with a pore size of 100 μm, the stromal vascular fraction (SVF) was precipitated by centrifuging the filtrate at 1,200 rpm for 5 min at room temperature. The SVF was washed thrice by further centrifugation and suspended in DMEM/F12 medium; the nucleated cell number was counted by SVF staining with Turk's solution (Nacalai Tesque, Inc., Kyoto, Japan). The SVF cells ($2×10^5$) were seeded in human fibronectin (Sigma-Aldrich, Tokyo, Japan)-coated 25 cm^2 T-flasks (NUNC™) and cultured in 5 mL media at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After 24 h, the non-attaching cells, together with the medium, were removed and the culture was continued by feeding fresh medium every alternate day. All the cells used in this study were *Mycoplasma* negative, detected using the MycoAlert Mycoplasma Detection Kit, Lonza, Walkersville, MD.

PSA measurement in the media from LNCaP and PC3 cells with or without 10% FCS

LNCaP and PC3 cells were cultured for 48 h, with or without 10% FCS, and the PSA levels in the supernatant of each of the culture medium were measured.

Co-culture method of LNCaP cells and ASCs

LNCaP cells were co-cultured with ASCs in different ratios (LNCaP:ASCs 0:1, 1:0, 1:1, 2:0, and 2:1) ($1=1.8×10^6$ cells). First, LNCaP cells were plated in cell culture inserts with a Transwell 24-well plate (0.4 μm pore size) in complete RPMI 1640 medium (Thermo Fisher Scientific) and kept overnight. Further, the cells were transferred to a new Transwell 24-well plate in the presence of ASCs (commercial one or prepared one from the patient) for 48 and 96 h. Prostate-specific antigen concentration in the supernatant was measured at 48 and 96 h. Measured PSA levels were corrected with an equal LNCaP cell number, which was determined by a non-radioactive cell viability assay (Biomedica, Vienna, Austria).

Measurement of PSA concentrations in the supernatant of the culture medium of LNCaP cells supplemented with the supernatant of ASC culture medium

The LNCaP cells were cultured in RPMI 1640 medium with and without the addition of the supernatant of the culture medium, where ASCs were cultured for 48 h. Same experiments were performed with 10% fetal calf serum (FCS) and 20% knockout serum replacement (KSR). The PSA concentration in the supernatant of the LNCaP cell culture medium was measured after 48 h.

Assessment of LNCaP tumor growth transplanted into nude mice with or without ASCs

The LNCaP cells ($2×10^6$) with 100 μL of Matrigel (Becton Dickinson Labware, Franklin Lakes, NJ) and 50 μL of serum-free RPMI 1640 were subcutaneously inoculated (with a 27-gauge needle) in the flank region of 6-week-old athymic male nude mice under anesthesia

(2% isoflurane inhalation). When mice bearing LNCaP tumors attained palpable tumor volume, they were castrated and randomly assigned in either 4×10^6 ASC (n=6) or PBS (n=6) subcutaneous injection with a 27-gauge needle around LNCaP tumors with 25 µL Matrigel and 25 µL RPMI 1640 (serum-free), under anesthesia by 2% isoflurane inhalation. Purchased ASCs were injected into growing LNCaP cells in 2:1 ratio. Tumor volume measurement was performed at 7, 14, and 28 d to calculate using the formula, length × width × depth × 0.5236.[9]

Statistical analysis

The data from the *in vitro* experiments are presented as mean±standard deviation (SD), and those from the *in vivo* experiments are presented as mean±standard error of mean (SEM). Statistical comparisons between the two groups were performed by Mann-Whitney U test. Multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test or two-way ANOVA with a repeated measurement test to determine the statistical significance (P-value of <0.05 was considered significant).

RESULTS

PSA measurement in the culture medium of LNCaP and PC3 cells

The PSA secretion was detected in the LNCaP-cultured medium, whereas it was not detected in the medium of PC3 cells, regardless of the presence of FCS (Fig. 1).

Measurement of PSA concentration in the supernatant of co-culture medium of LNCaP cells and ASCs

Figure 2 shows PSA level comparison in the supernatant of the LNCaP/ASCs co-culture medium in different ratios. In the experiment using commercial ASCs (Fig. 2a, b), the PSA remained undetected in the supernatant of the ASCs culture medium. However, the PSA level in the supernatant of the culture medium of LNCaP cells without ASCs increased depending on
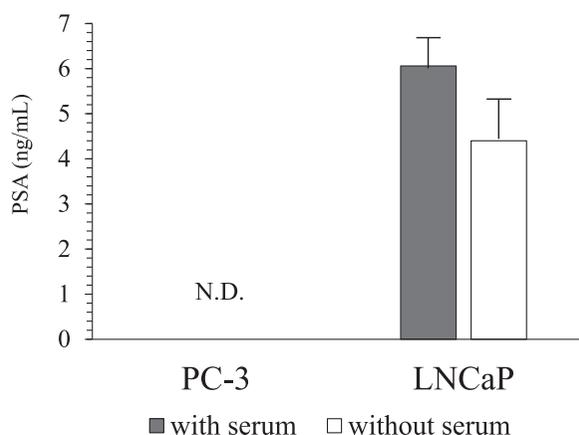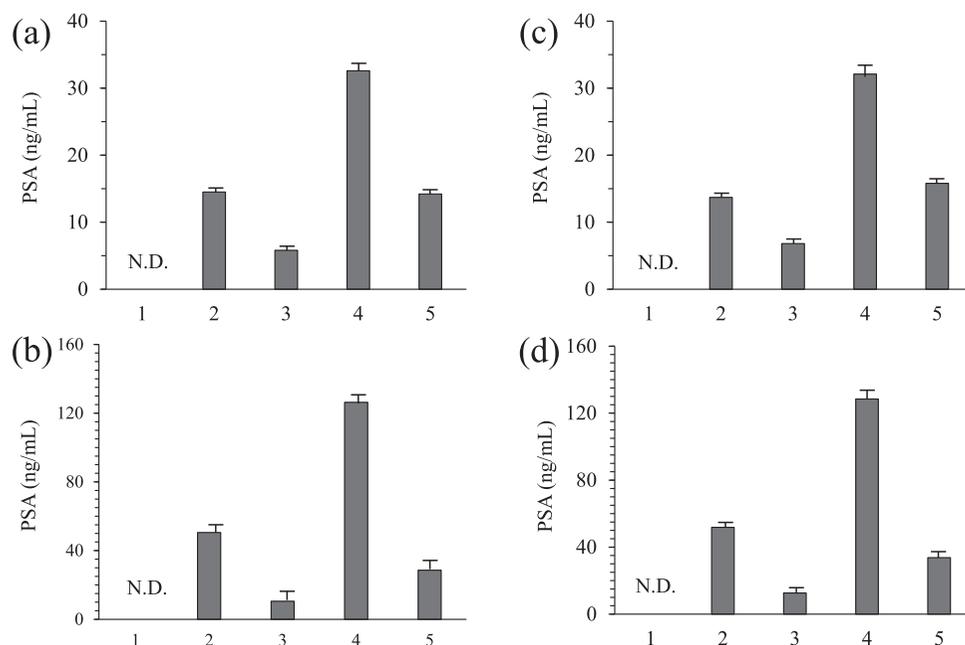

Fig. 1 PSA measurement in the culture media of LNCaP and PC3 cell with or without serum

Prostate-specific antigen was detected in the supernatant of LNCaP cell culture medium and was greater in the culture with FCS than without FCS. It was not detected in the supernatant of PC3 cell culture medium, both with and without FCS. The results are presented as mean±SD (n=4 dishes).

1: ASCs x 1, 2: LNCaP x 1, 3: LNCaP+ASCs (1:1), 4: LNCaP x 2, 5: LNCaP+ASCs (2:1)

Fig. 2 PSA concentration measurement in the co-culture medium of LNCaP cells and ASCs
In the experiment using commercial ASCs, PSA production by LNCaP was low in the supernatant of co-culture medium of LNCaP cells and ASCs at 48 h (a) and 96 h (b). Similar results were obtained at 48 h (a) and 96 h (b) in the experiments where ASCs, prepared from a patient, was used. The results are presented as mean±SD (n=4 dishes).

the cell number at 48 and 96 h. On the contrary, in the supernatant of the coculture medium, including both LNCaP cells and ASCs (1:1 or 2:1), PSA concentrations significantly decreased as compared to those in the supernatant of the LNCaP cell culture medium. Similar results were obtained when ASCs obtained from the patient enrolled in the ADRESU trial were used (Fig. 2c, d).

Measurement of PSA concentrations in the medium of LNCaP cells supplemented with supernatant from ASC cultures
No significant change was observed in the PSA concentration in the supernatant of LNCaP cell culture medium by addition of the supernatant of ASC culture medium in both the conditions (with FCS, or KSR) (Fig. 3).

Assessment of LNCaP tumor growth transplanted into a nude mouse with/without ASCs
The mean tumor volume was similar in all the groups before each treatment. Tumor growth was significantly inhibited by ASC addition at 28 d after LNCaP cell transplantation. The mean tumor volume of LNCaP in the presence of ASCs was significantly lower than that with LNCaP alone (Fig. 4).
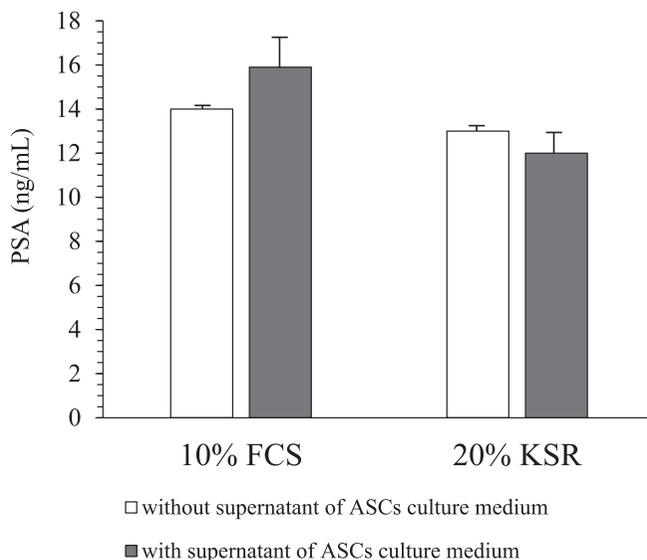
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Fig. 3 asurement in the supernatant of LNCaP cells culture medium supplemented
with the supernatant of ASC culture medium

Supplementation of the supernatant of ASC culture medium into LNCaP culture did not affect PSA production both in the presence of 10% FCS and 20% KSR. The results are presented as mean±SD (n=4 dishes).
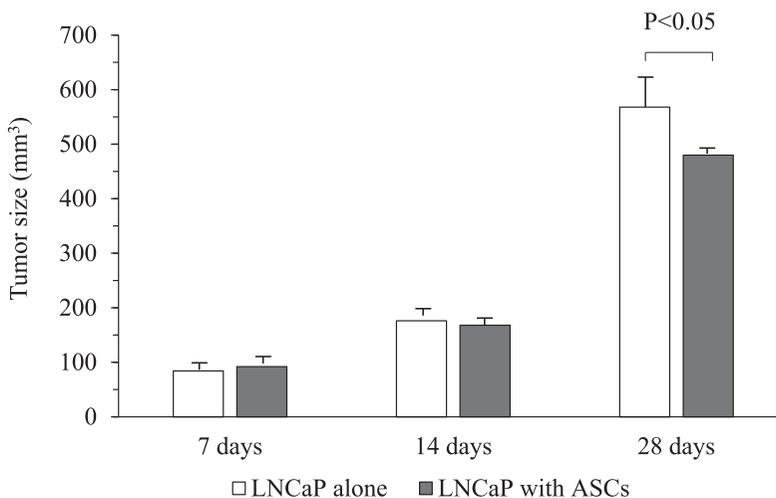

Fig. 4 Growth of the LNCaP tumor transplanted into a nude mouse with or without ASCs

The tumor growth was significantly suppressed at 28 d after ASC supplementation to LNCaP cells as compared to LNCaP alone. The results are presented as mean±SEM (n=6 mice).

DISCUSSION

The regenerative treatment developed for SUI includes injecting the adipose-derived regenerative cells, including ASCs, into the periurethral area.[5-7] The main cause of SUI in male patients

is iatrogenic sphincter impairment following radical prostatectomy for prostate cancer; ASCs are injected into the periurethral area at the prostate removal site. It is important to confirm that ASCs do not have a proliferative effect on prostate cancer cells for the successful application of this regenerative treatment in post-radical prostatectomy patients. The present study suggests that ASCs do not show a proliferative effect on the prostate cancer cells, rather they might inhibit prostate cancer cell proliferation.

In the present study, the effect of ASCs on prostate cancer cell proliferation was indirectly assessed by measuring the PSA produced by the prostate cancer cells. However, enhanced PSA production represents one of the most important characteristics of prostate cancer growth; therefore, the inhibition of PSA production by ASC co-culture confirmed that ASCs suppressed the prostate cancer cell biological activity. The result of LNCaP cells implantation into nude mice also supported the inhibitory effect of ASCs on prostate cancer cell proliferation. The effect of ASCs on cancer cell proliferation is debatable. *In vitro* experiments have reported ASCs to enhance breast cancer cell[10,11] and colon cancer cell[12] proliferation due to changes in the microenvironment, such as cytokine secretion and angiogenesis. On the contrary, *in vivo* and *in vitro* experiments by Cousin et al[13] demonstrated that ASCs strongly inhibited cell proliferation by inducing cell death via altering the cell cycle progression in pancreatic cancer. Although a report had suggested that ASCs promoted cancer cell proliferation,[14] Takahara et al[15] recently provided evidence suggesting that ASCs directly inhibit the proliferation of androgen-responsive and androgen-nonresponsive prostate cancer cells. The LNCaP and PC3 cells co-cultured with ASCs demonstrated that ASCs induced cancer cell apoptosis by activating the caspase 3/7 signaling pathway. They also demonstrated *in vivo* that local transplantation of ASCs delayed tumor growth, derived from both LNCaP and PC3 xenografts in immunodeficient mice. Furthermore, they reported that ASCs inhibited prostate cancer cell growth by inducing cell apoptosis along with reduced Bcl-xL activity by miR-145, including exosomes released from ASCs.

As for the mechanism of the ASCs inhibitory effect on prostate cancer cell proliferation, it is not necessary that ASC-secreted substances would cause an anti-proliferative effect since, in the present study, the addition of ASC culture medium supernatant to LNCaP culture medium did not affect PSA production in the cancer cells. Instead, the ASCs would have a direct anti-proliferative effect on the prostate cancer cells. On the contrary, Takahara et al[16] suggested that on the basis of DNA microassay with ASCs that candidate molecules play a role in proliferation/apoptosis regulation; in particular, TGF-β1 as a key secretory factor for the paracrine effect of ASCs. Thus, further studies are necessary to elucidate the mechanism of the anti-proliferative effect of ASCs on prostate cancer cells.

Limitation of the present study was that cell number was not examined as a direct measure of prostate cancer cell proliferation in the co-culture experiment. Since PSA production reflects the viability of the LNCaP cells, decrease of PSA production by LNCaP cell in co-culture with ASCs suggests the suppression of LNCaP cell viability by ASCs. On the other hand, the *in vivo* experiment showing the ASC-mediated decrease of LNCaP xenograft tumor growth in immunodeficient mice indirectly proves the inhibitory effect of ASCs on LNCaP cell proliferation.

The present study suggested that ASCs do not promote, but rather inhibit prostate cancer proliferation, although the mechanism remains unelucidated. The result confirms that the regenerative treatment developed for SUI is safe to perform on male patients who have undergone radical prostatectomy for prostate cancer. In addition, the anti-proliferative effect of ASCs is an important finding suggesting the possibility of applying ASCs in exploring a new strategy of cancer treatment.

SOURCES OF FUNDING

CONFLICT OF INTEREST/DISCLOSURES

None.

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