

FIGURE 1 – Proteomic analysis of OVISE CCC and OVSAHO SAC cell lines. Representative gel images of 3 independent experiments are shown. A 2D-DIGE gel image of OVISE CCC and OVSAHO SAC cell lines is shown in (a). Green spots correspond to proteins upregulated in OVISE cells (Cy3 labeled) compared with OVSAHO cells (Cy5 labeled). Red spots correspond to proteins upregulated in OVSAHO cells (Cy5 labeled) compared with OVISE cells (Cy3 labeled). Yellow spots correspond to proteins expressed at the same level in the OVISE and OVSAHO cell lines. A corresponding silver stain gel image is shown in (b).

Japan) in a hot water bath at 98°C for 40 min. In brief, quenching endogenous peroxidase with 3% H₂O₂ in methanol for 20 min. After treatment with BlockAce (Dainippon Sumitomo Pharmaceutical, Osaka, Japan) for 30 min at room temperature, the sections were incubated with a goat polyclonal anti-Anx A4 antibody at 1:100 dilution at 4°C overnight and subsequently incubated with a biotinylated anti-goat IgG antibody (Vector Laboratories Inc.) at room temperature for 1 hr. The antibody complex was detected by incubation with an avidin-biotin-peroxidase complex solution (Vector Laboratories Inc.) and visualized with 3,3'-diaminobenzidine tetrahydrochloride (MERCK, Darmstadt, Germany). Tissue sections were counter-stained with hematoxylin. Three gynecologic oncologists (A.K., T.M., Y.U.), blinded to the histological data, reviewed the stained sections. Cases with >90% of tumor cells staining positively with the anti-Anx A4 antibody were considered strongly positive (+++), cases with >50% but <90% Anx A4-positive cells medium positive (++), those with <50% positive cells weakly positive (+) and those with no or hardly any positive cells were considered negative.

Construction of Anx A4 expression vector

Total RNA from OVISE cells was purified with an RNA-Bee solution (Tel-Test Inc., Friendswood, TX) and cDNA was prepared with a SuperScriptTM III Reverse Transcriptase Kit (Invitrogen). To construct the Anx A4 expression vector, cDNA of human Anx A4 was amplified using KOD-plus (Toyobo Co. Ltd., Osaka, Japan) with the following primers: Anx A4 forward primer 5'-ttgacctagatcgtggcca-3' and Anx A4 reverse primer 5'-ttaaaccatctcctccacag-3'. The amplified cDNA was then inserted into pcDNA3.1/V5-His-TOPO vector (Invitrogen) and designated pcDNA3.1-Anx A4. The DNA sequence of Anx A4 cDNA inserted into the plasmid was confirmed using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, USA).

Generation of Anx A4 stable transfectant cells

To generate Anx A4 stable transfectant cells, the OVSAHO cell line was transfected with pcDNA3.1-Anx A4 using Lipofectamine 2000 (Invitrogen) according to the manufacturers' instructions, after which the cells were selected with 500 µg/ml of Geneticin (GIBCO, Invitrogen, Carlsbad, CA). We also transfected empty vector into the OVSAHO cell line using the same procedure described earlier to generate control cells. Stable clones were maintained in 250 µg/ml of Geneticin. Western blot analysis was performed to confirm the levels of Anx A4 expression in Anx A4 transfectant cells and empty vector control cells.

Measurement of IC₅₀ values after carboplatin treatment

Anx A4 transfected OVSAHO cells and empty vector control cells were seeded in 96-well plates (3,000 cells/well) (Costar; Corning Inc., Corning, NY) for 24 hr and then exposed to various concentrations (0-150 µM) of carboplatin for 72 hr. The cells were incubated with 10 µl of Cell Counting Kit-8 (Dojindo, Osaka, Japan) in 100 µl RPMI-1640 medium for 3 hr. Absorbance at 450 nm was measured with a microplate reader (Bio-Rad Model 680), and absorbance values were expressed as percentages relative to those for untreated controls, and the concentrations resulting in 50% inhibition of cell growth (IC₅₀ values) were calculated.

Measurement of intracellular platinum accumulation

Carboplatin accumulation in Anx A4 transfected cells and control cells was analyzed according to a previously established method¹⁷ with minor modifications. In brief, 1.5 × 10⁶ cells were seeded into a 60-mm tissue culture dish and incubated for 24 hr. The cells were then exposed to 2 mM carboplatin for 60 min at 37°C and washed twice with PBS either immediately or after 360 min of incubation in carboplatin-free RPMI 1640 medium supplemented with 10% FBS (HyClone Laboratories). After whole-cell extracts were prepared, the concentration of intracellular platinum was determined by using a polarized Zeeman atomic absorption spectrophotometer (model Z-8000; Hitachi, Ltd., Tokyo, Japan). The absolute concentration of platinum in each sample was determined from a calibration curve prepared with a platinum standard solution.

Statistical analysis

Student's *t* tests were used for statistical analyses. For the immunohistochemical analysis, a nonparametric analysis (the Kruskal-Wallis test) was used. A value of *p* < 0.05 was considered statistically significant.

Results

Anx A4 expression is elevated in CCC cell lines compared with SAC cell lines

The protein expression profiles of OVISE (CCC) and OVSAHO (SAC) cell lines were compared by means of 2D-DIGE analyses using fluorimimal dye-labeled protein extracts. The resulting gel images and corresponding silver-stained gels are shown in Figures 1a and 1b. Eight proteins highly expressed in OVISE cells and 6 proteins in OVSAHO cells were selected for identification by LC-MS/MS analysis. The results of these analyses (Table II) revealed

TABLE II - PROTEINS DIFFERENTIALLY EXPRESSED IN OVISE AND OVSAHO CELL LINES

Spot no.	Access. no.	Identified protein	M _w (Da)	pI	Coverage (%)
<i>Proteins upregulated in OVISE cells compared with OVSAHO cells</i>					
1	P09211	Glutathione S-transferase P	23,438	5.44	38
2	P09525	Annexin A4 (Annexin IV)	35,957	5.85	49
3	P04792	Heat-shock protein beta-1	22,826	5.98	39
4	Q13011	Delta3,5-delta2,4-dienoyl-CoA isomerase, mitochondrial precursor	36,136	8.16	28
5	P30040	Endoplasmic reticulum protein ERp29 precursor	29,032	6.77	15
6	O75874	Isocitrate dehydrogenase [NADP] cytoplasmic	46,915	6.53	39
7	P68104	Elongation factor 1-alpha 1	50,451	9.1	14
8	P68104	Elongation factor 1-alpha 1	50,451	9.1	19
<i>Proteins upregulated in OVSAHO cells compared with OVISE cells</i>					
1	Q07021	Complement component 1 Q subcomponent-binding protein, mitochondrial precursor	31,742	4.74	14
2	O75947	ATP synthase D chain, mitochondrial	18,405	5.22	41
3	P30084	Enoyl-CoA hydratase, mitochondrial precursor	31,823	8.34	27
4	P42126	3,2-trans-enoyl-CoA isomerase, mitochondrial precursor	33,080	8.8	10
5	P45880	Voltage-dependent anion-selective channel protein 2	38,639	6.32	25
6	P45880	Voltage-dependent anion-selective channel protein 2	38,639	6.32	25

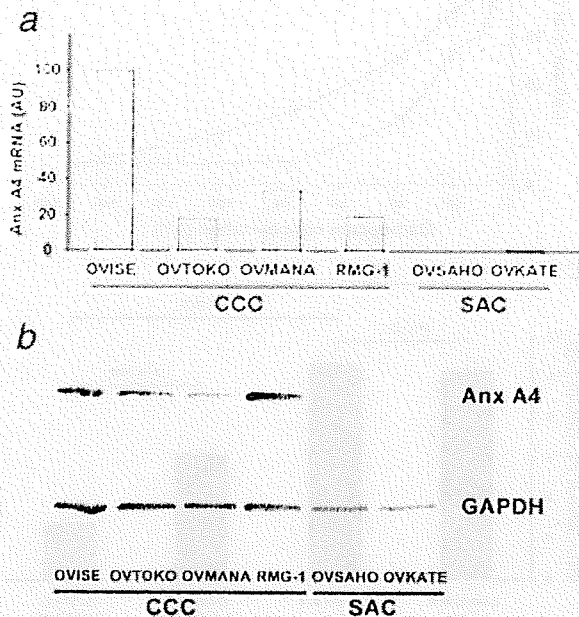


FIGURE 2 - Real-time RT-PCR and Western blot analysis of levels of Annexin A4 expression in ovarian cancer cell lines. Levels of Annexin A4 mRNA in ovarian CCC cell lines (OVISE, OVTOKO, OVMANA and RMG-1) and in ovarian SAC cell lines (OVSAHO and OVKATE) were determined by real-time RT-PCR (a). Levels of Annexin A4 protein in ovarian CCC cell lines (OVISE, OVTOKO, OVMANA and RMG-1) and in ovarian SAC cell lines (OVSAHO and OVKATE) were determined by Western blot analysis (b).

enhanced expression of the Annexin A4 protein in the OVISE cell line compared with the OVSAHO cell line.

The specific overexpression of Annexin A4 in ovarian CCC cell lines compared with that in SAC cell lines was further evaluated by real-time RT-PCR (Fig. 2a) and Western blot analysis (Fig. 2b). As shown in Figure 2a, expression of Annexin A4 (mRNA level) in OVISE, OVTOKO, OVMANA and RMG-1 (CCC) cell lines was enhanced compared with the OVSAHO and OVKATE (SAC) cell lines where Annexin A4 expression (mRNA level) was barely detectable. Western blot analysis (Fig. 2b) also demonstrated enhanced expression of Annexin A4 (protein level) in OVISE,

OVTOKO, OVMANA, RMG-1 (CCC) cell lines compared with the OVSAHO and OVKATE (SAC) cell lines.

Enhanced expression of Annexin A4 protein in tumors of ovarian CCC patients

Next, we determined whether levels of Annexin A4 protein are elevated in tumors of patients with ovarian CCC compared with other ovarian cancers. For this analysis, we performed an immunohistochemical study of Annexin A4 expression in tumor tissue samples from a large cohort of ovarian cancer patients (126 patients in total). In addition, we performed Western blot analysis using several frozen tumor tissue samples and compared the results with those of the immunohistochemical study. Representative immunohistochemical staining of Annexin A4 in tissue sections from patients with ovarian cancer revealed intense Annexin A4 staining in ovarian CCC compared with other histological types (Fig. 3a). Positive staining scores for Annexin A4 in tissue sections from patients with other types of ovarian cancers are shown in Figure 3b. We observed significantly stronger ($p < 0.01$) positive staining in tissue sections from patients with ovarian CCC compared with patients with ovarian endometrioid and serous adenocarcinoma. Of 43 CCC tissue sections, more than 30 were strongly positive for Annexin A4 (+++) compared with only 5 of the 62 SAC samples. Western blot analysis showed enhanced expression of Annexin A4 in CCC tumor samples that had demonstrated strong Annexin A4 immunohistochemical staining (+++) but barely detectable expression of Annexin A4 in SAC tumor samples that had demonstrated negative (-) Annexin A4 immunohistochemical staining (Fig. 3c).

Transfection of Annexin A4 cDNA into ovarian cancer cells enhances resistance to carboplatin treatment and modulates drug cellular efflux

Because Annexin A4 has been demonstrated to perform a functional role in chemoresistance in some cancer cell lines,¹⁴ we determined whether Annexin A4 can also confer chemoresistance to epithelial ovarian cancer cells. For this study, we generated Annexin A4 stably transfected OVSAHO cells. Figure 4a shows a Western blot analysis of Annexin A4 levels in OVSAHO parent cells, Annexin A4 stably transfected OVSAHO cells and empty vector transfected control cells. Figure 4b shows cell survival plots for control and OVSAHO/Annexin A4 cell lines after treatment with increasing concentrations of carboplatin (0–150 μ M). From this analysis, we determined the IC₅₀ carboplatin concentration values for the 2 cell lines. Higher (approximately double) IC₅₀ carboplatin concentration was observed in the OVSAHO/Annexin A4 (IC₅₀ = 42 μ M) cells compared with the empty vector control cells (IC₅₀ = 23 μ M). These results demonstrate that Annexin A4 can confer chemoresist-

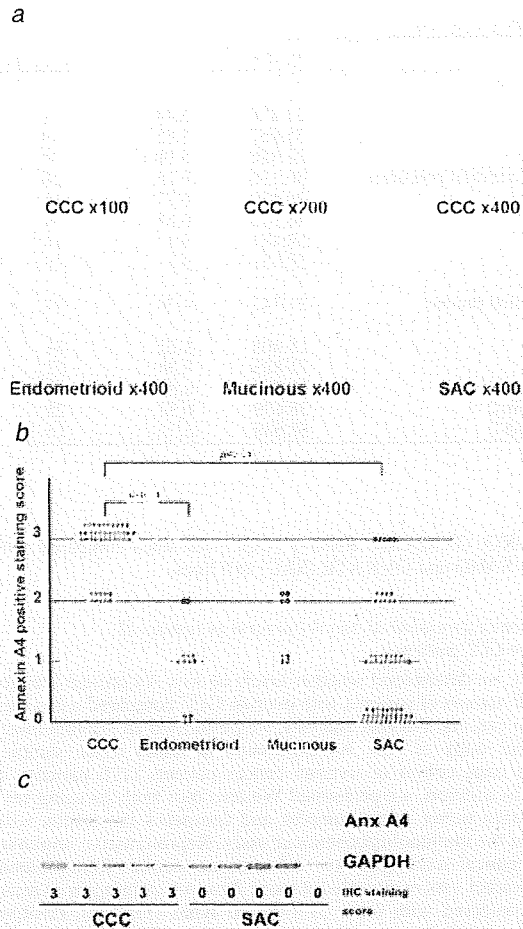


FIGURE 3 – Immunohistochemical analysis of Annexin A4 in ovarian cancer tumors. Levels of Annexin A4 protein in 126 epithelial ovarian cancer samples were determined by immunohistochemical analysis. Representative images of tissue sections from CCC ($n = 43$), endometrioid ($n = 8$), mucinous ($n = 13$) and serous adenocarcinoma ($n = 62$) ovarian cancer patients after immunohistochemical staining for Annexin A4 (a). Annexin A4-positive staining scores of tissue sections from ovarian cancer tumors (b). The p value between CCC and SAC is provided as determined by the nonparametric Kruskal–Wallis test. Western blot analysis using 5 CCC frozen tumor samples and 5 SAC frozen tumor samples (c).

ance in ovarian cancer cells. To investigate the molecular mechanisms of chemoresistance induced by Anx A4, we quantitated the intracellular platinum content after treatment of OVSAHO/Anx A4 and empty vector control cells with carboplatin. Figure 4c shows an analysis of intracellular platinum accumulation in OVSAHO/Anx A4 cells and empty vector control cells after carboplatin treatment with or without an additional incubation time (360 min) in carboplatin-free medium. Significantly ($p = 0.0025$) reduced levels of intracellular platinum accumulation were noted in OVSAHO/Anx A4 cells (OVSAHO/Anx A4 no. 40, 0 min) compared with empty vector control cells (Control no. 16, 0 min) when neither cell line underwent additional incubation in carboplatin-free medium. Control cells displayed no significant difference ($p = 0.178$) in intracellular platinum content between 0 min and 360 min of additional carboplatin-free incubation time (Control no. 16, 0 min vs. Control no. 16, 360 min), whereas

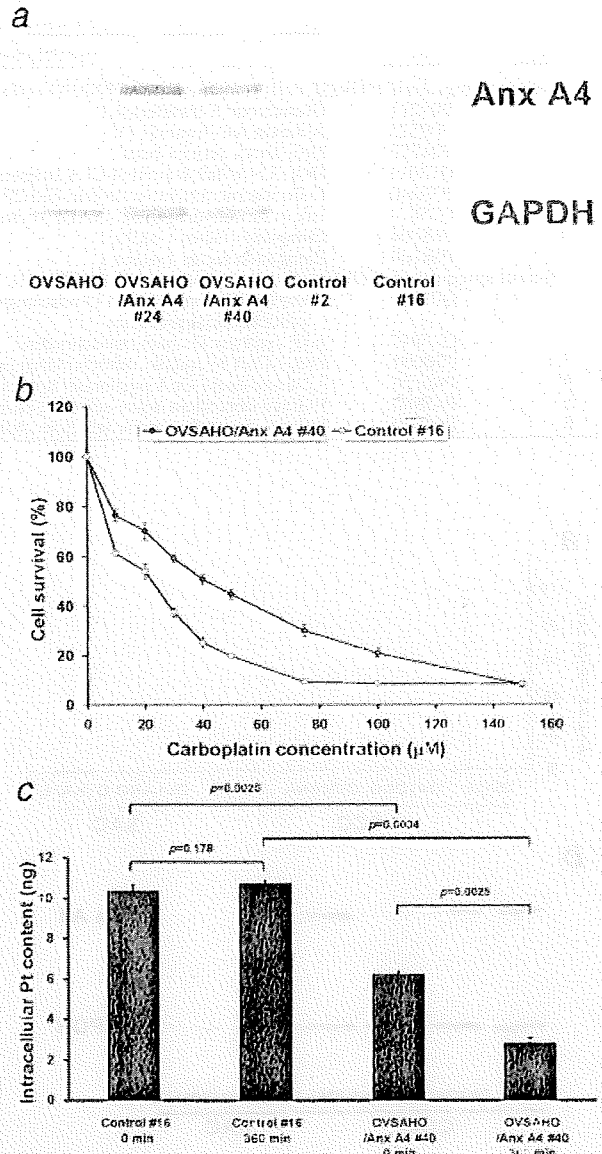


FIGURE 4 – Transfection of Annexin A4 cDNA into ovarian cancer cells confers resistance to carboplatin and decreases intracellular Pt accumulation. Cell survival (expressed as a percentage relative to control untreated cells) after 72 hr treatment of OVSAHO/Anx A4 and empty vector control cells with different concentrations of carboplatin (Figure 4a). The obtained IC50 values were 42 μM for OVSAHO/Anx A4 no. 40 and 23 μM for Control no. 16 (not shown in figure). Intracellular platinum content after treatment with 2 mM carboplatin for 60 min with or without after 360 min of incubation in carboplatin-free medium in OVSAHO/Anx A4 cells and control cells, as determined by atomic absorption spectrophotometry (Figure 4c).

OVSAHO/Anx A4 cells showed a significant decrease ($p = 0.0025$) in intracellular platinum content at 360 min as compared with at 0 min of additional carboplatin-free incubation time (OVSAHO/Anx A4 no. 40, 0 min vs. OVSAHO/Anx A4 no. 40, 360 min). Furthermore, OVSAHO/Anx A4 cells displayed significantly decreased ($p = 0.0004$) levels of intracellular platinum con-

tent compared with control cells after an additional 360 min of carboplatin-free incubation (Control no. 16, 360 min vs. OVSAHO/Anx A4 no. 40, 360 min), which suggests a role for Anx A4 in enhancing cellular platinum efflux.

Discussion

The use of carboplatin and paclitaxel for the treatment of ovarian cancers has significantly improved survival rates in patients with this disease.¹⁸ However, of the 4 major histological types of ovarian cancer, CCC of the ovary is characterized by strong chemoresistance.² Consequently, patients with this disease are associated with significantly lower 5-year survival rates than patients with other histological types of ovarian cancer.^{19,20} However, the molecular mechanisms of chemoresistance in this disease have remained poorly understood. Thus, the identification of proteins which are involved in chemoresistance in ovarian CCC is of major clinical importance because these proteins may constitute novel therapeutic targets in this disease.

In this study, we performed a 2D-DIGE proteomic analysis using ovarian cancer cell lines for the identification of a candidate protein associated with chemoresistance in ovarian CCC. We identified 8 proteins differentially upregulated in OVISE CCC cells compared with OVSAHO SAC cells (Table II). From among those 8 proteins, we focused on Anx A4, a calcium-dependent phospholipid-binding protein, which is localized proximal to the cell membrane and plays an important role in membrane fluidity or trafficking.¹⁰

We confirmed by means of both real-time RT-PCR (mRNA levels) and Western blot analysis (protein levels) that expression of Anx A4 was significantly enhanced in ovarian CCC cell lines compared with in non-CCC ovarian cancer cell lines (Figs. 2a and 2b). The findings of our analysis using ovarian cancer cell lines are in agreement with those of the proteomic study of Morita *et al.*,¹⁵ in which Anx A4 was identified as being differentially upregulated in ovarian CCC cell lines (OVISE and OVTKO) compared with an ovarian mucinous cancer cell line.

Previous studies have associated Anx A4 protein with chemoresistance. For example, in a study of Han *et al.*,¹⁴ Anx A4 was observed to be elevated in a paclitaxel-resistant human lung cancer cell line and transfection of Anx A4 cDNA into embryonic kidney 293T cells to confer resistance to paclitaxel. Because Anx A4 has been shown to be involved in modulating membrane permeability and membrane trafficking,¹⁰ it is conceivable that this involvement may result in modulation of both cellular drug influx and efflux after chemo-drug treatment. Taken together, these studies suggest that the strong chemoresistance characteristic of human ovarian CCC may be due to enhanced expression of Anx A4. However, it remained unclear whether levels of Anx A4 protein are significantly elevated in tumors of patients with ovarian CCC compared with other histological types.

In the study reported here, we, therefore, performed an immunohistochemical analysis of Anx A4 in tumor tissue samples from 126 patients with epithelial ovarian cancer to determine whether levels of Anx A4 protein are elevated in tumors of patients with ovarian CCC compared with other epithelial ovarian cancers. Because treatment with paclitaxel can enhance Anx A4 expression in cultured cells,¹⁴ all patients examined in this analysis had undergone preliminary diagnosis and had not received chemotherapy (including carboplatin or paclitaxel) before surgery. The results of this analysis revealed significantly ($p < 0.01$) strong positive staining (enhanced expression) of Anx A4 in tumor tissue samples from patients with ovarian CCC compared with endometrioid and serous adenocarcinoma, which are known to represent chemosensitive histological types (Fig. 3b). Western blot analysis using frozen tumor samples were compatible with results of the IHC study (Fig. 3c). Thus, our study was able to demonstrate the presence of enhanced expression of Anx A4 in tumors of patients with ovarian CCC. This finding is in agreement with that of our

proteomic analysis using ovarian cancer cell lines and indicates that Anx A4 may play a role in tumor resistance to cancer chemotherapy in patients with ovarian CCC.

To investigate a relationship between levels of expression of Anx A4 and patient prognosis, we reviewed clinical outcomes (recurrence, progression-free survival, etc.) of the 62 SAC patients including 5 patients with strong (+++)Anx A4 positive staining in IHC analysis. Among the 5 SAC patients with high levels of Anx A4 expression, 2 patients are well and alive with no recurrence, whereas the other 2 patients have recurred within 1 or 2 years after treatment and 1 patient was out of follow-up. We have compared the progression-free survival between these 5 patients and Anx A4 negative SAC patients and there was no statistically significant difference between the 2 groups. Because the number of Anx A4-positive SAC patients is small, further investigation will be necessary in a larger cohort of patients.

Although previous studies have demonstrated a role for Anx A4 in conferring chemoresistance to human cancer cell lines,¹⁴ a similar role in human epithelial ovarian cancer cells was not identified, and the specific mechanism of chemoresistance that Anx A4 confers was not previously determined. Therefore, we first studied cell survival after carboplatin treatment to confirm whether Anx A4 can enhance chemoresistance in epithelial ovarian cancer cells. We were unable to reduce Anx A4 protein levels in the OVISE CCC cell line, despite using various strategies (including siRNA), which may be due to the reported long half-life of Anx A4 protein (approximately 4 days).²¹ We then tested the effect of forced overexpression of Anx A4 in the OVSAHO non-CCC (SAC) ovarian cancer cell line in which Anx A4 is not endogenously expressed. We observed enhanced chemoresistance to carboplatin treatment in cells that stably expressed Anx A4 compared with empty vector control cells (Figs. 4a and 4b). Thus, our results demonstrate that Anx A4 protein plays a role in the enhancement of chemoresistance in epithelial ovarian cancer cells.

We next examined intracellular platinum accumulation in both Anx A4 expressing ovarian cancer cells (OVSAHO/Anx A4 cells) and empty vector control cells after carboplatin treatment (Fig. 4c). Our results of carboplatin treatment with no carboplatin-free incubation revealed significantly reduced levels of intracellular platinum content in OVSAHO/Anx A4 cells compared with control cells, which indicates that Anx A4 inhibits cellular platinum influx and/or promotes cellular platinum efflux. Comparison of the results for 0 and 360 min carboplatin-free incubation showed that OVSAHO/Anx A4 cells are more active in promoting cellular platinum efflux compared with control cells. Taken together, these results demonstrate that Anx A4 plays a part in the enhancement of cellular platinum efflux.

Our study has demonstrated for the first time elevated levels of Anx A4 protein in patients with ovarian CCC and an association between elevated Anx A4 levels and enhanced chemoresistance to carboplatin in human epithelial ovarian cancer cells. It has also found evidence for the first time that Anx A4 confers chemoresistance in part by enhancing drug efflux. Thus, it is conceivable that the observed strong resistance to cancer chemotherapy (including carboplatin) specific to ovarian CCC tumors, compared with that of other epithelial ovarian tumors, is mediated through the enhanced expression of Anx A4 in patients with this disease. Therefore, Anx A4 may constitute a novel therapeutic target for overcoming resistance to cancer chemotherapy in patients with ovarian CCC. In view of the reported half-life of Anx A4 protein, such a therapeutic strategy is likely to involve the inhibition of the function rather than of the expression of Anx A4 in patients with CCC.

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ORIGINAL ARTICLE

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Transdifferentiation of human adipose tissue-derived stromal cells into insulin-producing clusters

Abstract Type 1 diabetes mellitus is caused by autoimmune destruction of insulin-producing beta cells. The major obstacle to transplantation of insulin-producing cells to cure the disease is the limited source of these cells. To overcome this problem, we describe here a multistep protocol for generation of insulin-producing islet-like clusters from human adipose tissue-derived stromal cells (ADSCs). Analysis using reverse transcription polymerase chain reaction detected enhanced expression of various pancreatic genes during the differentiation of ADSCs. Immunofluorescence analysis revealed functional similarities between cells derived from ADSCs and pancreatic islet cells, i.e., the presence of insulin- and C-peptide-coexpressing cells in the clusters and glucagon expression on the cell surface. The glucose challenge tests revealed the production of insulin, and such production was regulated via physiological signaling pathways. Our insulin-producing cells derived from ADSCs could be potentially used for cell therapy of type 1 diabetes mellitus.

Key words Diabetes mellitus · Adipose tissue-derived stromal cells · Insulin · Islet-like cluster · Glucose response

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Introduction

Type 1 diabetes mellitus results from the destruction of insulin-producing beta cells by autoimmune responses. Although modern insulin regimens are improving outcomes,¹ the successes achieved over the past few decades by transplantation of whole pancreas and isolated islets suggests that diabetes can be cured by replenishment of deficient beta cells. Transplantation therapy was established by Shapiro et al., and many patients received islet transplantation and a large population of recipients did not need further insulin therapy.^{2,3} However, islets must be obtained from donors, but the resources are restricted and only a small proportion of patients can receive such transplantations. To overcome this problem, regenerative medicine for insulin-producing cells has been explored. Recently, it has been reported that insulin-producing cells could be differentiated from embryonic stem (ES) cells.⁴⁻⁹ However, the sources of ES cells are limited; ES cell-derived insulin-producing cells are not available worldwide. The limited supply of such ES cells has led to the generation of insulin-producing cells from somatic stem cells. If we can generate insulin-producing cells from adult somatic stem cells, autologous insulin-producing cells might be used for diabetic patients and inappropriate human leukocyte antigen (HLA) matching could be avoided.

This investigation was initiated to establish a method for generating insulin-producing islet-like clusters from adipose tissue-derived stromal cells (ADSCs), since the adipose tissue resection technique is easy and safe. Our strategy was to transdifferentiate ADSCs into islet-like clusters through multiple steps. The generated islet-like clusters secreted insulin in response to glucose stimulation and nonglucose secretagogues, and expressed various molecules that resembled those expressed by pancreatic beta cells, such as Isl-1, Pax4, Pax6, pancreatic duodenal homeobox 1 (Pdx1), prohormone convertase (PC) 1/3, PC2, Kir6.2, glucose transporter (Glut) 2, glucokinase (GK), and insulin. Here we report that ADSCs can be transdifferentiated to form islet-like clusters that produce insulin. It is hoped that these

islet-like clusters can be used clinically in the near future for patients with type 1 diabetes mellitus.

Materials and methods

Adipose tissues from human subjects

Excess omental adipose tissues were resected from the gastro-omental artery during coronary artery bypass graft operation and excess subcutaneous adipose tissue was resected during mammary reconstruction procedures in ten subjects [four men and six women, 55 ± 5 years of age (mean \pm SEM); range 40–60 years] who had given informed consent. The protocol was approved by the Review Board for Human Research of Osaka University Graduate School of Medicine. All subjects fasted for at least 10 h before surgery. None was taking steroids or thiazolidinediones. A total of 1–10 g of abdominal subcutaneous (external to the fascia superficialis) and greater omental adipose tissue was obtained from each subject.

Isolation and culture of ADSCs

ADSCs were isolated as reported previously with minor modification.¹⁰ In brief, adipose tissue was minced and then digested in Hank's balanced salt solution (HBSS) containing 0.075% collagenase (Sigma-Aldrich, St. Louis, MO, USA) in a shaking water bath at 37°C for 1 h. Digests were filtered with a cell strainer (BD Biosciences, San Jose, CA, USA) and centrifuged at 800 g for 10 min. Red blood cells were excluded using density gradient centrifugation with Lymphoprep (density = 1.077) (Nycomed, Oslo, Norway). The cells were then plated using Dulbecco's modified Eagle's medium (DMEM) with 10% defined fetal bovine serum (FBS). After 24 h, the adherent cells were washed extensively, treated with ethylenediaminetetraacetic acid (EDTA), and the suspended cells were replated at a density 10 000 cells/cm² on human fibronectin-coated dishes in medium I: 60% DMEM low glucose, 40% MCDB201 (Sigma-Aldrich St. Louis, MO, USA), 10 µg/ml EGF (PeproTech, GmbH, Hamburg, Germany), 1 nM dexamethasone, 100 µM ascorbic acid, and 5% FBS. After three to five passages, the ADSCs were used in the experiments.

In vitro differentiation procedure

The cells were treated with trypsin-EDTA to dissociate them, and single cells were obtained and then suspended in medium II [80% knockout-DMEM (Invitrogen, Carlsbad, CA, USA), 20% defined FBS, 1 mM glutamine, and 1% nonessential amino acid (both from Invitrogen)] and applied to low-attachment culture dishes (Hydrocell; CellSeed, Tokyo, Japan.). Within one day, cells were self-aggregated into spheroids, named adipospheres. The resultant adipospheres (stage II) were cultured for 7 days with a change of medium every 3 days.

Although adipospheres resembled embryoid bodies, we traced the methods established by Segev et al.,⁹ in which insulin-producing cells were regenerated from embryoid bodies, to obtain insulin-producing cells from ADSCs. Seven-day-old adipospheres (which consisted, on average, of 1000 cells) were plated at a density of 300 adipospheres per well in six-well plastic culture plates and grown for another week in medium III: DMEM/F-12 1:1, 10 mg/l insulin, 6.7 mg/l transferrin, 5.5 mg/l selenium (ITS), and 1 mM glutamine (all from Invitrogen) with a supplement of 5 µg/ml human fibronectin (stage III). After 1 week in the ITS-fibronectin (ITSF) medium, the cells were dissociated and plated on 0.1% gelatin-coated plastic tissue-culture plates (BD Biosciences) at a concentration of 100 000/ml in medium IV: DMEM/F-12 1:1 with N2 supplement, B27 (with vitamin A) media (both from Invitrogen), 1 mM glutamine, and 10 ng/ml basic fibroblast growth factor (bFGF, Invitrogen). These cells expanded throughout the week; the media was changed every other day.

In the next step (stage V), the cells were cultured in medium V: DMEM free of glucose/F-12 1:1, supplemented with N2 and B27 (without vitamin A) media, 1 mM glutamine, 10 mM nicotinamide, and 10 nM exendin-4 (both from Sigma-Aldrich). After 7 days of culture in medium V, the cells were dissociated and applied onto low-attachment culture dishes (Hydrocell) to grow in suspension with medium V (stage VI).

Reverse transcription polymerase chain reaction

Total RNA was isolated from differentiated ADSCs using an RNeasy kit (QIAGEN, Hilden, Germany). After treatment with DNase, cDNA was synthesized from 500 ng total RNA using Superscript III reverse transcriptase RNase H minus (Invitrogen). The absence of DNA contamination in RNA samples was confirmed by the polymerase chain reaction (PCR) primers flanking an intron. Primers and the reaction conditions are described in Table 1. The PCR products were fractionated by 2% agarose gel electrophoresis.

Insulin secretion

The islet clusters obtained from the above procedure were rinsed three times in RPMI1640 (11879-020, Invitrogen) and preincubated for 1 h with the RPMI1640 containing 0.5% bovine serum albumin and 3.3 mM glucose. The masses of the clusters were expressed as islet equivalent (IEQ).¹¹ The clusters were then incubated for 2 h in RPMI1640 with 16.7 mM glucose and chased in RPMI1640 with 3.3 mM glucose with or without reagents such as theophylline (100 µM), isobutyl-methyl xanthine (IBMX) (100 µM), tolbutamide (10 µM), carbachol (100 µM), and nifedipine (50 µM) (all from Sigma-Aldrich). Then the conditioned media was analyzed for insulin and C-peptide levels. The insulin concentration was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Merco-

Table 1. Primers and reaction conditions for the polymerase chain reaction

Name	Sequence	Annealing (°C)	Size (bp)
Insulin			
F	AGGCTTCTTCTACACA	65	245
R	CAGGCTGCCTGCACCA		
Glucagon			
F	AGGCAGACCCACTCAGTGA	55	308
R	AACAATGGCGACCTCTTCTG		
Somatostatin			
F	TGCGCTGTCCATCGTCCT	55	258
R	GCCATAGCCGGGTTTGAGTT		
IAPP			
F	GAGAGAGCCACTGAAATTACTTGCC	60	471
R	CCTGACCTTATCGTGATCTGCC		
Nestin			
F	AGAGGGGAATTCCTGGAG	60	312
R	CTGAGGACCAGGACTCTCTA		
Is11			
F	GATTTCCCTATGTGTTGGTTGC	60	812
R	CTTCCACTGGGTTAGCCTGTAA		
Nkx6.1			
F	GTTCCCTCCTCCTCCTCCTC	60	381
R	AAGATCTGCTGTCCGAAAAAAG		
Pdx1			
F	GGATGAAGTCTACCAAAGCTCACGC	60	230
R	CCAGATCTTGATGTGTCTCTCGGTC		
Pax4			
F	GTGGGCAGTATCCTGATTCACT	60	308
R	TGTCACTCAGACACCTTTCTGG		
Pax6			
F	CCGAGAGTAGCGACTCCAG	60	239
R	CTTCCGGTCTGCCCGTTC		
GLUT2			
F	AGGACTTCTGTGGACCTTATGTG	60	231
R	GTTTCATGTCAAAAAGCAGGG		
Glucokinase			
F	AAGAAGGTGATGAGACGGATGC	60	230
R	CATCTGGTGTTGGTCTTCACG		
SUR1			
F	GTGCACA'CCACCACAGCACATGGCTTC	62	429
R	GTGTCTTGAAGAAGATGTATCTCCTCAC		
KIR6.2			
F	CGCTGGTGGACCTCAAGTGGC	60	497
R	CCTCGGGGCTGGTGGTCTTGCG		
PC1/3			
F	ITGGCTGAAAGAGAACGGGATAACATCT	60	457
R	ACTTCTTTGGTGATTGCTTTGGCGGTG		
PC2			
F	GCATCAAGCACAGACCTACACTCG	60	309
R	GAGACACAACCACCTTCATCCTTC		
E-cadherin			
F	AGAACAGCACGTACACAGCC	60	530
R	CCTCCGAAGAAACAGCAAGA		
GAPDH			
F	GTCAGTGGTGGACCTGACCT	60	394
R	AGGGGAGATTCACTGTGGTG		

The number of cycles was 35 in each case
Pax, Paired box gene

dia, Uppsala, Sweden), which detects human insulin with no cross-reactivity to proinsulin or C-peptide. C-peptide levels were also measured by an ELISA kit (Mercodia).

Immunohistochemistry

Stage VI clusters were fixed for 24 h in 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized

using 0.5% Triton X-100 in PBS, and then incubated overnight with the primary antibody guinea-pig anti-insulin (1:100, Dako, Glostrup, Denmark) with either rabbit anti-C-peptide (1:100, Linco, St. Charles, MO, USA), rabbit anti-glucagon (1:100, Dako), or rabbit anti-somatostatin (1:200, Dako). After rinsing, secondary antibodies were added to the samples, which were then incubated for another 1 h at room temperature. Finally, the cells were rinsed three times, sliced into 10- μ m slices, and mounted

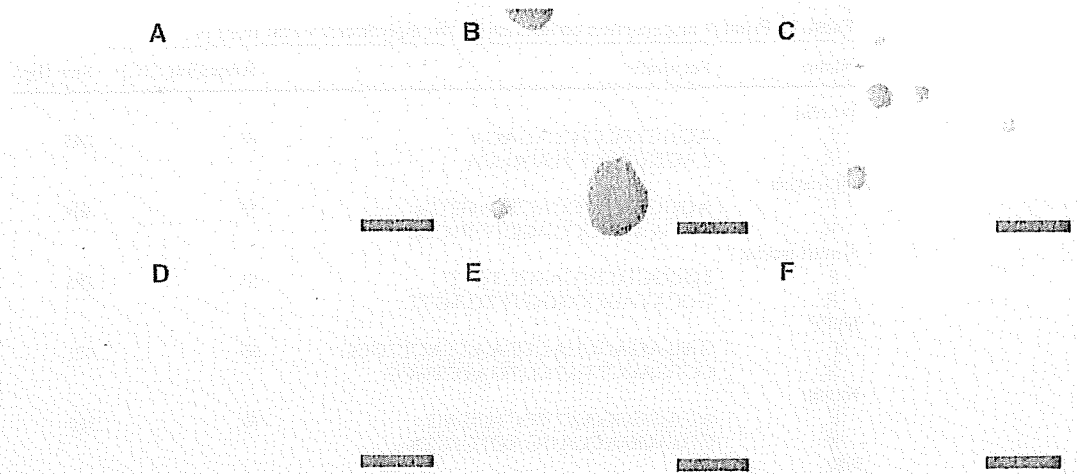


Fig. 1A–F. General outline of the differentiation protocol, which consists of six stages. **A** Stage I: growth of adipose tissue-derived stromal cells (*bar* 500 μm). **B** Stage II: formation of adipospheres in suspension (*bar* 500 μm). **C** Stage III: plating adipospheres in medium I for 1 week (*bar* 500 μm). **D** Stage IV: dissociating the cells and plating them in

medium II on gelatin-coated dishes for 1 week (*bar* 500 μm). **E** Stage V: change to medium III and culture for 1 week (*bar* 250 μm). **F** Stage VI: dissociation of the cells and growing them in suspension in low-attachment dishes with medium III for 3 days to form clusters (*bar* 250 μm)

with Permaflur (Thermo Fisher Scientific, Waltham, MA, USA). The slides were examined using a fluorescence microscope (Fluoview FV1000, Olympus, Tokyo, Japan).

Results

Multistep procedure for differentiation of ADSCs into insulin-producing islet-like clusters

To promote the differentiation of human ADSCs into insulin-producing cells, we applied the procedure outlined in Fig. 1. In the first stage, ADSCs were expanded and passaged three to five times. In the next stage, ADSCs were cultured in suspension. Within 48 h of incubation, these cells started forming cell aggregates, named adipospheres, which grew bigger with time, forming spheroids. In the third stage, the adipospheres were plated and expanded in insulin-transferrin-selenium-fibronectin (ITSF) medium. In the fourth stage, the expanded cells in stage III were collected individually and cultured in N2 and B27 supplemented with basic fibroblast growth factor (bFGF). The fourth stage cells could be split and passaged. In the fifth stage, these cells were cultured in a medium lacking bFGF and vitamin A, and containing nicotinamide and exendin-4. In the last stage (stage VI), the cells obtained in stage V were cultured in suspension. Within 3 days of incubation, the cells started forming cell islet-like aggregates.

Dynamics of pancreatic gene expression during differentiation of ADSCs into insulin-producing clusters

Analysis with reverse transcription polymerase chain reaction (RT-PCR) demonstrated enhanced expression of pancreatic genes in the differentiated insulin-producing islet-like

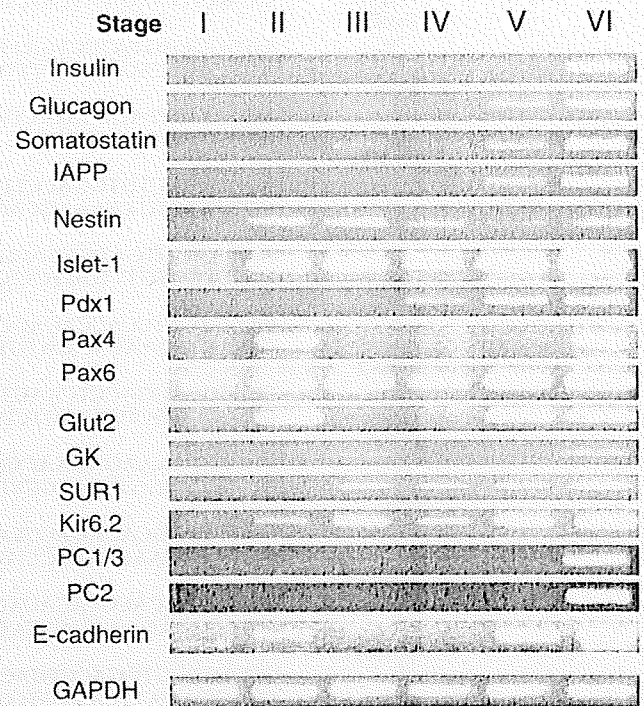


Fig. 2. Reverse transcription polymerase chain reaction (RT-PCR) analysis of pancreatic gene expression at the six differentiation stages. Total RNA isolated from the cells of each differentiation step was subjected to RT-PCR analysis with primers for the indicated genes. *IAPP*, islet amyloid polypeptide; *Pdx1*, pancreatic duodenal homeobox 1; *Glut2*, glucose transporter 2; *GK*, glucokinase; *PC*, prohormone convertase; *GAPDH*, Glyceraldehyde 3 phosphate dehydrogenase

clusters of ADSCs (Fig. 2). Insulin was expressed in stage VI, while other pancreatic hormones, such as glucagon, somatostatin and islet amyloid polypeptide (IAPP), were observed in stages V and VI. Nestin appeared in stage II

and was highly expressed in stages II, III, and VI. Further examination of the transcriptional factors related with beta cell development showed expression of islet-1 in all stages except stage I, and expression of Pdx1 in stage IV with further increase with differentiation. Expression of Pax4 appeared in stages II and VI, and that of Pax6 was noted in all stages of differentiation. Next, we examined messages of *Glut2*, *GK*, the K_{ATP} -channel genes *Kir6.2* and *SURI*, *PC1/3*, and *PC2*. *Glut2* appeared in stage II, disappeared, and then reappeared in stages V and VI. *GK* was detected in stage III and the final stage. *Kir6.2* was expressed in stages II to VI, and *SURI* was expressed in stages III to VI. *PC1/3* and *PC2* were observed in stage VI. Thus, the generated insulin-producing clusters could process proinsulin into insulin and generate C-peptide. *E-cadherin* was observed in stages V and VI, and enhanced pancreatic beta cell cluster formation into islets. These results indicate that the adipocyte-derived differentiated cells can transcribe, process, and secrete insulin in response to glucose.

Islet-like clusters release insulin in a similar way to pancreatic islets

The generated clusters were examined for their insulin-secretion potential (Fig. 3A). The glucose challenge tests showed that the generated clusters secreted insulin in the conditioned media at concentrations of $50.9 \pm 1.9 \mu\text{U}/1000 \text{ IEQ}$ (islet equivalents) (mean \pm SEM) at high glucose challenge (16.7 mM) and $26.1 \pm 1.3 \mu\text{U}/1000 \text{ IEQ}$ at low glucose challenge (3.3 mM) (Fig. 3A). Next, we examined the effects of 10 μM IBMX on glucose insulin response and C-peptide secretion. IBMX increased insulin release from adipocyte-derived differentiated cells 3.6-fold to $185.0 \pm 17.0 \mu\text{U}/1000 \text{ IEQ}$ and C-peptide release increased 2.3-fold from 1.6 ± 0.1 to $4.4 \pm 0.5 \text{ ng}/1000 \text{ IEQ}$ (Fig. 3A). To determine whether these cells regulate insulin release via physiological signaling pathways, we examined the effects of several agonists and antagonists on insulin secretion (Fig. 3B). In the presence of high glucose levels, theophylline (100 μM) induced a 3.6-fold increase in insulin secretion; tolbutamide (10 μM), an inhibitor of the K_{ATP} -channel, a 4.2-fold increase; and carbachol (100 μM), an agonist of muscarinic cholinergic receptors, a 1.6-fold increase. Nifedipine (50 μM), a blocker of Ca^{2+} channels present in beta cells, did not alter insulin secretion in response to low ($25.7 \pm 0.6 \mu\text{U}/1000 \text{ IEQ}$) or high glucose levels ($29.3 \pm 0.6 \mu\text{U}/1000 \text{ IEQ}$). These results indicate that glucose induced the pancreatic machinery in the differentiated cells to release insulin.

Generated islet-like clusters express insulin, glucagon, somatostatin, and C-peptide in a similar way to pancreatic islets

Stage VI clusters were subjected to immunofluorescence analysis for the expression of insulin, C-peptide, glucagon, and somatostatin (Fig. 4). Insulin-positive cells were the major population of cells among the generated islet-like

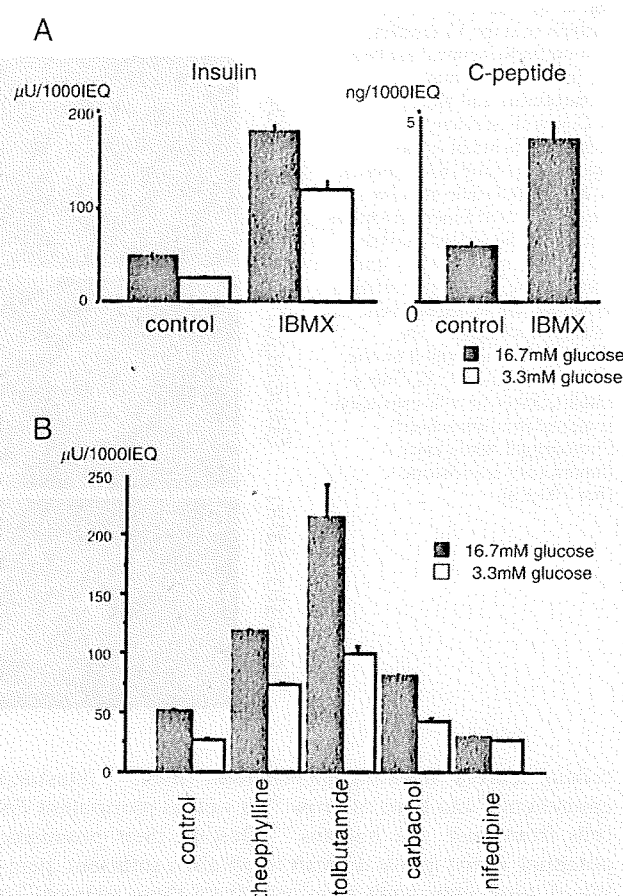


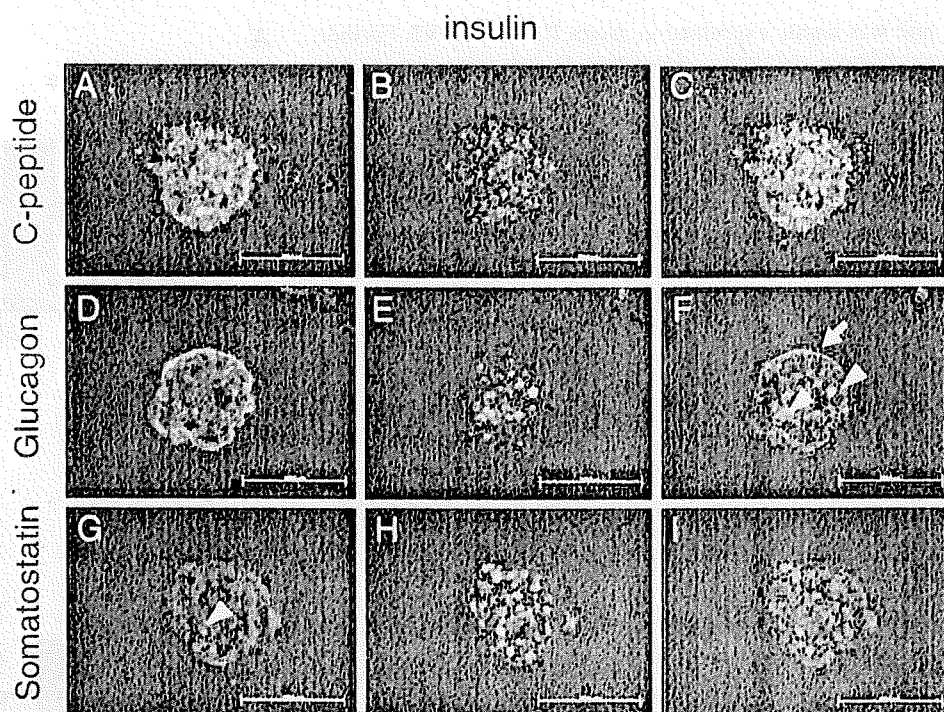
Fig. 3A,B. Regenerated insulin-producing cluster derived from adipose tissue-derived stromal cells. **A** Glucose-induced insulin and C-peptide release in response to low (3.3 mM) and high (16.7 mM) glucose levels. **B** Effects of various agonists and antagonists on insulin secretion. Data represent the effects of theophylline, isobutyl-methyl xanthine (IBMX), tolbutamide, carbachol, and nifedipine. All data represent the mean \pm SEM of experiments performed in triplicate.

clusters. As shown in Fig. 4F, insulin was localized inside the cells (arrowheads) while glucagon was identified on the cell surface (arrows). Only a small number of cells expressed somatostatin (Fig. 4G; arrowhead). These expression patterns of pancreatic hormones in the generated clusters were similar to those identified in pancreatic islets.¹⁰ Almost all insulin-expressing cells coexpressed C-peptide, indicating the production of insulin (Fig. 4C). A substantial number of cells coexpressed insulin and glucagon (Fig. 4F), and insulin and somatostatin (Fig. 4I), in the same way as immature pancreatic endocrine cells described in the development of the pancreas.¹⁰ These results indicate that the generated islet-like clusters derived from ADSCs resemble pancreatic islets.

Discussion

Recent studies have demonstrated the feasibility of generating insulin-producing cells from progenitor cells of various

Fig. 4. Immunocytochemical analysis of stage VI clusters. Immunohistochemical analysis for insulin, C-peptide, somatostatin, and glucagon. **A** C-peptide staining, **D** somatostatin staining, **G** glucagon staining (all in green), **B**, **E**, and **H** insulin staining (red). **C**, **F**, and **I** merged images of insulin and second marker (C-peptide, somatostatin, and glucagon, respectively) (orange). **F** Insulin was localized inside the cells (arrowheads), while glucagon was identified on the cell surface (arrow). **G** Only a small number of cells expressed somatostatin (arrowhead). Photographs taken using a fluorescence microscope (bar 100 μ m)



cellular sources, including the pancreas,¹² liver,^{13,14} intestinal epithelium,¹⁵ and bone marrow,¹⁶ as well as pluripotent ES cells from mice and humans.⁴⁻⁹ Despite their promising potential, it may prove difficult to obtain a sufficient quantity of autologous adult stem cells from these sources. To overcome these limitations, we explored the possibility of using human adipose tissues as a source for transdifferentiation into insulin-producing cells. Adipose tissue is a safe and abundant source of large amounts of somatic stem cells. In the present study, we generated functional insulin-producing cells from human ADSCs using a multistep in vitro differentiation procedure. These studies provide direct evidence that human ADSCs could be programmed in vitro to become functional insulin-producing cells.

To differentiate human ADSCs into insulin-producing cells, we used a six-step transdifferentiation procedure. To design the protocol, we supposed that noncommitted cells were expanded in the first step, multipotent cells were selected in the second step, endodermal-committed cells were selected in the third step, the committed cells were expanded in the fourth step, these cells were committed into pancreatic cells in the fifth step, and in the sixth step these cells were expected to mature into insulin-producing cells.

In detail, in the first step, human ADSCs were grown to expand their numbers and their undifferentiated properties were maintained with selenium. Selenium is used in serum-free cultivation to protect mesenchymal stem cells against damage due to cultivation and to maintain their properties as stem cells.¹⁷ The second step was performed to select and enrich the undifferentiated cells. By using floating culture in stage II, undifferentiated cells formed spheroids, named adipospheres. Adipospheres expressed islet-1, nestin, and

Pax4, as shown in Fig. 2. Previous studies indicated that cells positive for islet-1, nestin, and/or Pax4 are pancreatic endocrine progenitor cells.¹⁸⁻²⁰ Mesenchymal stem cells were grown under floating conditions where they generated spheroids. Multipotent stem cells in the form of spheroids are also known as neurospheres, and have been described as differentiating into neuronal lineages.²⁰ In the third step, we were able to expand the nestin-positive cells and increase their numbers under insulin-transferrin-selenite-fibronectin (ITSF) serum-free conditions. Because of the similarities between the development of beta cells and that of neuroepithelial cells,²¹ a similar transient expression of nestin was reported to occur in human insulin-producing beta cell precursors.^{18,19} In the fourth step, the cells were transferred to a medium containing N2, B27, and bFGF. N2 and B27 were first designated as supplements for serum-free media for culture of neural cells primarily for protection against oxidants. Just as for neural cells, beta cells are sensitive to reactive oxygen species.²² bFGF and vitamin A have growth promoting effects on pancreatic epithelial cells reported as precursors of beta cells.^{6,23-25} At the end of the differentiation procedure, bFGF and vitamin A were withdrawn and nicotinamide and exendin-4 were added to promote maturation of precursor cells into insulin-producing cells. Nicotinamide increases the rate of proinsulin biosynthesis, and the resultant increase in insulin production and content is due to formation of new beta cells through differentiation.^{26,27} Exendin-4 has been reported to stimulate both the differentiation of beta cells from ductal progenitor cells and proliferation of beta cells when given to rats.^{28,29} In the final step, the cells were cultivated under floating conditions to enhance the maturation of progenitor cells into beta cells.^{6,30} Taken together, almost all the steps of the procedure seem

to promote the transdifferentiation of human ADSCs into insulin-producing clusters.

Recent studies illustrated that when cultured *in vitro*, bone marrow-derived mesenchymal stem cells obtained from mice and rats could be differentiated into insulin-producing cells.^{31,32} In addition, Sun et al. demonstrated that human bone marrow-derived stem cells can differentiate into insulin-producing cells under appropriate conditions *in vitro*.³³ In the differentiating conditions, the bone marrow-derived mesenchymal stem cells were cultured with bFGF, betacellulin, activin A, and nicotinamide under high glucose conditions. In our procedures, ADSCs were cultured with bFGF, exendin-4, and nicotinamide under low glucose conditions. Timper et al. supposed that ADSCs could express insulin message after 3 days of cultivation and differentiate into pancreatic beta cells.³⁴ Unfortunately, they could not show that the insulin-producing cells processed according to their method could secrete insulin in response to glucose challenge, supposing that their cells did not fully differentiate into pancreatic beta cells but into their precursors or progenitors. In our method, the cells were cultured under floating conditions in the final step in differentiation. To fully differentiate into insulin-producing cells, cell-cluster formation might be critical, as suggested by Segev et al.⁹

In the clinical context, our present study demonstrated the potential for cell-based therapy of diabetes involving the generation of autologous insulin-producing cells *in vitro* from ADSCs. Although autoimmunity against autologous regenerated islets could be a concern, these *in vitro*-generated insulin-producing cells could, in theory, provide a potentially unlimited source of islet-like cells without the limitation of immune rejection based on alloimmunity. However, because there are multifactorial influences in the transdifferentiation of ADSCs into competent insulin-producing cells, there are many questions left unanswered, and unresolved issues remain. In addition, the insulin-producing capacity of the ADSC-derived islet-like clusters was similar to that of ES cell-derived islet-like clusters but was less than that of isolated islets by a factor 10 to 100.^{9,11} Obviously, further research is required to address these important questions, yet we believe the results demonstrated in this study provide direct evidence supporting the notion that transdifferentiation of ADSCs to insulin-producing cells may represent a viable therapeutic option for type 1 diabetes.

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