Antizyme is necessary for conversion of pancreatic tumor cells into glucagon-producing differentiated cells

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Abstract

Human pancreatic tumor cell lines – AsPC-1, PANC-1, MIA paca2, KP-1 and KP-59 cells – can be induced to differentiate into pancreatic hormone-producing cells by brief trypsin treatment and subsequent culture in a serum-free, chemically defined medium. During culture, AsPC-1 cells formed cell clusters resembling the pancreatic islets, expressed genes associated with the pancreatic development and produced glucagon but not insulin. When PANC-1, MIA paca2, KP-1 and KP-59 cells were treated and cultured the same way, they underwent similar morphological changes and produced insulin and glucagon. We used these systems to identify intracellular regulatory molecules involved in the conversion of pancreatic tumor cells into glucagon-producing cells. We found that the expression of antizyme 1 (AZ1), a negative regulator of ornithine decarboxylase, was increased and its localization was altered from the nucleus to the cytoplasm during AsPC-1 cell differentiation. Transient transfection of AsPC-1 cells with AZ1 siRNA resulted in inhibition of the morphological and functional cell differentiation as well as the specific suppression of AZ1 expression. By contrast, constitutive overexpression of AZ1 in AsPC-1 cells led to the enhancement of glucagon production. We also found that PANC-1 cells reduced the expression of glucagon mRNA when treated with AZ1 siRNA. These results suggested that AZ1 was necessary for the conversion of pancreatic tumor cells into glucagon-producing cells. Glucagon production in AsPC-1 cells was not affected by addition of putrescine, suggesting that the polyamines were not directly involved in the AZ1-mediated conversion of pancreatic tumor cells to differentiated state.

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Introduction

Alpha cell differentiation and glucagon gene expression are mediated by multiple transcriptional factors including Pax6 (St-Onge *et al.* 1997), ISL1 (Wang & Drucker 1995), Cdx-2/3 (Andersen *et al.* 1999, Ritz-Laser *et al.* 1999), Mafk (Artner *et al.* 2006), BRN-4 (Hussain *et al.* 2002), and Foxa-2 (Lee *et al.* 2005). Pax6 has been shown to interact with Cdx-2/3, c-Maf and Maf-B, and mediate the synergistic transactivation of the *glucagon* gene (Planque *et al.* 2001, Gosmain *et al.* 2007). POU3F4 and Foxa-2 have also been shown to activate *glucagon* gene expression and α -cell differentiation. Glucagon

is derived from proglucagon, an 18-kDa protein which contains glucagon and two related peptide sequences, glucagon-like peptides 1 and 2 (GLP1 and -2). The proglucagon molecule is processed by prohormone convertase 2 (PC2) in the pancreatic α -cells. Hormones processed by PC2 influence the processes that regulate α -cell differentiation and maturation (Vincent *et al.* 2003). At the present time, however, our understanding of the regulatory mechanism involved in α -cell differentiation is still incomplete, and thus requires further studies.

The polyamines including putrescine, spermidine and spermine play a crucial role in cell proliferation

and functional differentiation by influencing gene expression and protein synthesis (Pegg & McCann 1982, Tabor & Tabor 1984). Spermidine and spermine are present at high concentrations in the pancreatic islets (Hougaard *et al.* 1986). Both putrescine and spermidine are necessary for the maintenance of normal level of insulin biosynthesis in β -cells (Welsh & Sjöholm 1988). The polyamine synthesis is regulated negatively by antizyme 1 (AZ1) that binds to ornithine decarboxylase (ODC) and facilitates its degradation (Hayashi *et al.* 1996, Coffino 2001). AZ1 has a wide tissue distribution and has been implicated to play a role in various cellular functions through its influence on polyamine metabolism (Hayashi *et al.* 1996, Coffino 2001). The synthesis of AZ1 protein is mediated by a polyamine-dependent mechanism involving a programmed +1 frameshifting during translation of the *AZ1* mRNA (Matsufuji *et al.* 1995, Ivanov *et al.* 2000). Recent studies have shown that AZ1 also binds to several other proteins including cyclin D1, a cell cycle regulatory protein (Newman *et al.* 2004), Aurora-A, a mitosis regulatory protein (Lim & Gopalan 2007), and Smad1 (Gruendler *et al.* 2001, Lin *et al.* 2002, Xu *et al.* 2003). At present, however, the cellular function of AZ1 and its relationship with polyamine homeostasis in pancreatic islet cells is not known.

In the previous reports (Hardikar *et al.* 2003, Gershengorn *et al.* 2004), human pancreatic PANC-1 cells have been used as a model to study the process of pancreatic islet formation. Following brief trypsin treatment and culturing in a chemically defined serum-free medium (SFM), cells formed islet-like cell aggregates and expressed the functional markers of pancreatic islet cells including insulin and glucagon (Hardikar *et al.* 2003). In this study, we examined the functional potential of AsPC-1 cells, a human pancreatic adenocarcinoma cell line, and found that these cells can be converted to cells that produce glucagon but not insulin and form an islet-like structure by brief trypsin treatment and subsequent culture in SFM. We also studied the role of AZ1 in converting AsPC-1 cells into the differentiated state and found that the morphological and functional changes were blocked by depletion of AZ1 using siRNA, whereas overexpressing EGFP-AZ1 enhanced glucagon production. AZ1-mediated AsPC-1 cell differentiation was not affected by altering the cellular contents of putrescine and spermidine. Our results suggest that AZ1 plays a key role in converting AsPC-1 cells into glucagon-producing cells and its effect is distinct from the one on polyamine metabolism.

Materials and methods

Cell culture

Human pancreatic adenocarcinoma AsPC-1 cells (Dainippon Sumitomo Pharma, Osaka, Japan), PK-1 cells and PK-59 cells (Riken Cell Bank, Tsukuba, Japan) were maintained in 10% FBS-RPMI 1640. PANC-1 cells (Dainippon Sumitomo Pharma) and MIA paca2 cells (Riken Cell Bank) were maintained in 10% FBS-DMEM. Cells were plated at a density of 1×10^6 cells/well in six-well culture dishes. To induce differentiation, cells were washed with PBS three times and then treated for 30–60 s with 0.05% (v/v) trypsin/EDTA (GIBCO) at room temperature with care not to detach cells from culture plates. Cells were then cultured in serum-free DMEM/F10 medium containing 17.5 mM glucose and 1% BSA for the indicated time period without medium change at 37 °C in a humidified 5% CO₂ atmosphere.

Confocal microscopy

Cells were fixed with 4% (v/v) paraformaldehyde (WAKO, Osaka, Japan) for 30 min at room temperature and then permeabilized with 0.3% (v/v) tritonX-100. The cells were then treated with 3% (w/v) BSA in PBS to block the non-specific binding and were stained with anti-glucagon (Sigma) or anti-AZ1 antibodies (gift from Prof. S Matsufuji; Jikei.Med.Univ., Tokyo, Japan). Alexa 488 or Alexa 546-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR, USA) were used as the secondary mAb for staining. The stained cells were analyzed using a confocal laser microscope (Fluoview, FV1000, Olympus, Tokyo, Japan). Images were obtained using a 40 $\times$ oil-immersion objective lens (UPlan FL N 40 $\times$ /1.30). Glucagon production was estimated by measuring the intensity of fluorescent dye in at least 300 cells per culture dish using FV10-ASW software (Olympus).

Flow cytometry analysis

Cultured AsPC-1 cells were dissociated in PBS and single-cell suspensions of AsPC-1 cells were fixed in 4% paraformaldehyde for 10 min at 37 °C. Cells were washed twice with FACS buffer (1% (w/v) FBS, 0.09% (w/v) NaN₃ in PBS) and then incubated with anti-human glucagon mAb for 24 h at 4 °C. After washing twice with FACS buffer, cells were stained with Alexa-488-conjugated anti-mouse IgG for 30 min at room temperature. Cells were then passed through an iron mesh (0.55 μ m) and analyzed on FACSaria (BD Bioscience, San Diego, CA, USA) using the BD FACSDiva software.

RT-PCR

Total RNA was isolated from cells using RNA-Bee RNA isolation reagent (Wako). Reverse transcription was performed according to the instructions provided with the Takara RNA PCR Kit (AMV version 3.0, Takara, Otsu, Japan). The PCR amplification employed 25–40 cycles with steps at 94 °C for 2 min, 60 °C for 30 s, and 72 °C for 1 min with TaKaRa EX Taq HS. The primers used were as follows: glucagon, forward 5'-aagtatctggactccaggcg-3' and reverse 5'-actggttaaaggtcccttcagc-3'; Pax6, forward 5'-caggaaggaggggagagaa-3' and reverse 5'-ttctttgcagcttcgcttc-3'; PC-2, forward 5'-gtccttgatgcaggtgccatg-3' and reverse 5'-actccttcagcacccttc-3'; Pdx-1, forward 5'-aagtcacgcgtggaaag-3' and reverse 5'-gccgtgagatgtactgtgaa-3'; ISL1, forward 5'-gcagcccaatgacaaaactaa-3' and reverse 5'-ccgtcgtgtctctctggact-3'; synaptophysin forward 5'-actatgggcagcaaggctac-3' and reverse 5'-gtcctcctgggcttcactg-3'; E-cadherin, forward 5'-cctcgacaccgattcaag-3' and reverse 5'-tcccaggcgtagaccaagaa-3'; β -actin, forward 5'-caaggccaaccgcga-3' and reverse 5'-gtccatcacgatgccagtgtga-3'; rat AZ1, forward 5'-gagcaactccaggctgacca-3' and reverse 5'-ttcttggttgagattgtgagacc-3'. After amplification, 10 μ l of each reaction mixture was analyzed by 2% agarose gel electrophoresis, and the products were then visualized by ethidium bromide staining.

Realtime PCR

cDNA was prepared from the total RNA of AsPC-1 cells with ExScript RT reagent kit (Takara) or PrimeScript RT reagent kit. The quantitative real-time PCR reactions were performed using SYBR Premix Ex Taq (Takara) in a LightCycler (Roche Diagnostics). The primer sequences used were the same as those in RT-PCR analysis. Samples were incubated for the initial denaturation at 95 °C for 10 min, followed by 40–50 cycles consisting of three steps at 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s. Cycle-to-cycle fluorescence emission readings were monitored at 72 °C at the end of each cycle and analyzed using LightCycler Software (Roche Diagnostics). All quantifications were normalized to β -actin.

Transfection and stably expression of EGFP-rat AZ1

An expression vector, pEGFP-rat AZ1, was a gift from Drs Noriyuki Murai and Senya Matsufuji (Jikei. Med.Univ., Tokyo, Japan). Rat AZ1 has an ~85% homology to human AZ1. It expresses a full-length AZ1 without the frameshift because of the deletion of

one thymine nucleotide (Murai *et al.* 2003). Transfection was carried out with 4.0 μ g plasmid DNA/well using Lipofectamine 2000. Transfected cells were subjected to antibiotic selection with 500 μ g/ml G418 (Wako) for 24 h. G418-resistant cells were detected two to three weeks. Rat AZ1/pEGFP-expressing cells were cultured with 2 μ M putrescine.

Small interfering RNA

The non-silencing control siRNA and AZ1 siRNA were purchased from Qiagen. Cells were transfected with the siRNA (150 nM) in the presence of Lipofectamine 2000 (Invitrogen). After 2 days, cells were induced to differentiate in SFM and further cultured in the presence of the siRNA. Various assays were performed 6 days after the first transfection of siRNA.

Western blot

Cell extracts were prepared by lysing cells for 30 min on ice in a lysis buffer containing 1% (v/v) NP-40, 0.1% (v/v) SDS, 0.5% (w/v) sodium deoxycholate and one tablet of Complete Mini (Roche Diagnostics). The extracts were centrifuged for 10 min at 14 000 *g* at 4 °C, and the supernatant was collected and boiled in SDS sample buffer for 5 min. The sample solution (100 μ g protein) was analyzed by SDS-PAGE (10% gel) and immunoblotted with the polyclonal anti-rat AZ1 antibody followed by a peroxidase-conjugated secondary antibody (Histofine, Nichirei, Japan). Immunoreactions were performed using the ECL Advance western blotting kit (GE Healthcare, Piscataway, NJ, USA) and visualized bands were analyzed on LAS-3000 (Fujifilm, Tokyo, Japan) using Multi Gage V3.0 software.

Measurement of cellular polyamines

AsPC-1 cells were collected by centrifugation and washed with PBS. For extraction of polyamines, the cells were homogenized on ice in 100 μ l of 0.1 M HCl, and then 100 μ l of 0.5 M HClO₄ was added. The cell extracts were centrifuged (12 000 *g*, 10 min, 4 °C), and the supernatant was used for the measurement of putrescine, spermidine and spermine contents. The amount of putrescine, spermidine and spermine was quantitated by mass spectrometric analysis (Samejima *et al.* 2007).

Results

Pancreatic tumor cells can be induced to differentiate into glucagon-producing cells

Human pancreatic tumor cell lines, AsPC-1, MIA paca2, KP-1, KP-59 and PANC-1 cells, cultured in the growth medium exhibited no functional properties of pancreatic cells including the synthesis of pancreatic hormones, insulin and glucagon. However, when the cells were briefly treated with trypsin and then cultured in a chemically defined medium without serum (SFM), they all formed islet-like clusters and produced pancreatic hormones (Fig. 1). PANC-1, MIA paca2, KP-1 and KP-59 cells produced insulin and glucagon, whereas AsPC-1 cells produced glucagon but not insulin. Our results related to PANC-1 cells were in accord with those reported previously (Hardikar et al. 2003, Gershengorn et al. 2004).

As shown in Fig. 2, AsPC-1 cells cultured in SFM for 3 days stopped growing, formed islet-like cell clusters (the upper panel) and produced glucagon (the lower panel). After 6 days, the size of cluster and the extent of glucagon production were substantially increased. AsPC-1 cells that were not pre-treated with trypsin were not converted to differentiate when cultured in SFM. In addition, those cells that received trypsin treatment but cultured in the growth medium also did not form cell clusters and produced no glucagon. These results indicated that trypsin treatment and the subsequent culture in SFM were required for converting AsPC-1 cells into the differentiated state. The conversion of AsPC-1 cells to glucagon-producing cell clusters was a reversible process since these cells stopped glucagon synthesis and started to disaggregate

and grow when SFM was switched to the serum-containing growth medium.

The percentage of glucagon-producing AsPC-1 cells was determined by the intracellular immunofluorescent staining with the anti-glucagon mAb and flow cytometric analysis (Fig. 2B). Glucagon-producing cells comprised ~60% of the entire cultured cell population on day 6, whereas no glucagon-producing cells were detected on day 0. In addition, we found that differentiated AsPC-1 cells responded to the step-wise reduction of glucose concentration in the incubation medium by producing the increasing amount of glucagon. Moreover, glucagon production in differentiated AsPC-1 cells was suppressed by insulin treatment. Thus, AsPC-1 cells exhibited the functional properties of normal pancreatic alpha cells *in vivo* (Rall et al. 1973, Unger 1973).

Pancreatic α -cell differentiation is exquisitely regulated by various factors including transcription factor Pax6 (St-Onge et al. 1997), Pdx-1 (Wilson et al. 2003, Jensen 2004) and ISL1 (Wang & Drucker 1995) as well as PC2 (Vincent et al. 2003), while cadherin-mediated cell–cell adhesion is also critical in pancreatic islet differentiation (Dahl et al. 1996, Minami et al. 2008). Accordingly, we examined the expression of these genes during the AsPC-1 cell differentiation by RT-PCR analysis. As shown in Fig. 2C, mRNAs encoding Pax6, PC2 and E-cadherin were undetectable or very low on day 0 but increased markedly after 6 day culture in SFM. On the other hand, Pdx-1 and ISL1 mRNA were present at the beginning of culture and their levels did not change appreciably after 6 day culture in SFM. Synaptophysin mRNA, a neuroendocrine marker (Egawa et al. 1996, Lukinius et al. 2003),

Figure 1 Induction of pancreatic tumor cells to differentiated state. AsPC-1, MIA paca2, KP-1, KP-59 and PANC-1 cells were briefly treated with trypsin and then cultured in SFM for 6 days. Cells were fixed and stained with anti-pig insulin, anti-human glucagons, anti-guinea pig IgG-Alexa 488 and anti-mouse IgG-Alexa 546. The details were described in Materials and methods. Images were analyzed using a confocal laser microscope. Scale bar: 50 μ m.

Figure 2 Induction of AsPC-1 cells to differentiated state. (A) Cell morphology and glucagons expression of AsPC-1 cells cultured in SFM. Immunostaining of AsPC-1 cells was carried out on days 0, 1, 3, and 6 in culture. Cells were fixed and stained with anti-human glucagons mAb and anti-mouse IgG-Alexa 546. Images were analyzed using confocal laser microscope. Scale bars: 100 μ m. (B) Analysis of glucagons-producing cell population. Cells were fixed and stained with monoclonal anti-glucagon and anti-mouse IgG-Alexa 488 on day 0 and day 6 in culture. Glucagon-containing cells were detected by flow cytometric analysis. (C) Gene expression in AsPC-1 cells. RT-PCR analysis was performed to examine the expression of gene encoding glucagons Pax6, PC2, PDX1, ISL1, synaptophysin, E-cadherin and β -actin in cultured cells on day 0 or on day 6.

was also present on day 0 and increased modestly (1.5-fold) on day 6. The above results demonstrated that AsPC-1 cells could be induced to express the morphological and functional properties of differentiated alpha cell. In the following studies, we used the islet-like cluster formation and glucagon production as the marker of pancreatic tumor cell differentiation.

AZ1 is up-regulated in pancreatic tumor cells during differentiation

To determine whether AZ1 was involved in AsPC-1 cell differentiation, we first examined the time course of AZ1 production in cells cultured in SFM. Western blot analysis showed that AZ1 was barely detectable on day 0 but its level began to increase on day 1 and increased further on day 3 and day 6 (Fig. 3A).

In addition, we found that the increase in *AZ1* mRNA was apparent in differentiated cells but not in undifferentiated cells (data not shown). The temporal patterns of AZ1 and glucagon production indicated that the increase of AZ1 preceded that of glucagon (Figs 2A and 3A).

We performed an immunocytochemical analysis to determine the intracellular localization of AZ1 in non-differentiated AsPC-1 cells on day 0 and differentiated cells on day 6 in culture. As shown in Fig. 3B, AZ1 was localized in the cytosol on day 6, whereas it was in the nucleus on day 0. The appearance of AZ1 in the cytosol was detected in some cells as early as day 1 in culture (data not shown). These results indicated that AsPC-1 cell differentiation involved the increase of AZ1 protein as well as the change in its localization from the nucleus to the cytosol.

Figure 3 The expression and intracellular localization of antizyme 1 in pancreatic tumor cells. (A) Western blot analysis of AZ1. Whole-cell extracts from AsPC-1 cells on days 0, 1, 3, and 6 in culture were analyzed by SDS-PAGE and immunoblotted with polyclonal anti-AZ1 and anti- β -actin antibodies, as described in the Materials and methods. (B) Intracellular localization of AZ1. Cells were stained with polyclonal anti-AZ1 and TO-PRO3 (nuclei staining) on day 0 (the upper panel) and day 6 in culture (the lower panel). Images were analyzed using a confocal laser microscope. Scale bars: 40 μ m (upper) and 100 μ m (lower).

Inhibition of AZ1 expression by AZ1 siRNA blocks pancreatic tumor cell conversion into glucagon-producing cells

In order to examine whether AZ1 was required for converting AsPC-1 cells into the differentiated state, we transfected AsPC-1 cells with control or AZ1 specific siRNA 2 days prior to inducing differentiation, and then cells were cultured in SFM in the presence of either control or AZ1 siRNAs for 4 days. The results of western blot analysis indicated that the level of AZ1 in cells treated with AZ1 siRNA was reduced $\sim 85\%$ when compared with that in cells treated with control siRNA (Fig. 4A). In addition, it was found that cells transfected with AZ1 siRNA formed islet-like cell clusters that were smaller and less organized (Fig. 4B). These cells also contained the lower level of *E-cadherin* mRNA when compared with cells transfected with control siRNA (Fig. 4D). In addition, the knock-down of AZ1 was associated with the reduction in the amount of *Pax6* and *glucagon* mRNAs that were decreased 85 and 70% respectively, relative to the corresponding amount in control cells (Fig. 4D). We also examined glucagon production in

AZ1-depleted cells by flow cytometric analysis. As shown in Fig. 4C, those cells expressed the substantially reduced level of glucagon. However, the ability of AZ1-depleted cells to produce glucagon was restored by expressing of EGFP-rat AZ1 in these cells.

As previously reported (Hardikar et al. 2003) and confirmed in this study, PANC-1 cells also expressed glucagon mRNA when briefly treated by trypsin and cultured in SFM. However, when PANC-1 cells were treated with siRNA for AZ1, these cells reduced the expression of glucagon mRNA (Fig. 4E). These results suggested that AZ1 was necessary for converting pancreatic tumor cells into glucagon-producing cells.

Enhancement of glucagon production in differentiated pancreatic tumor cells by constitutive overexpression of AZ1

We next examined the effect of constitutive overexpression of AZ1 on AsPC-1 cell differentiation. AsPC-1 cells were transfected with expression vectors of EGFP-rat AZ1 or EGFP alone, and EGFP-positive cells were identified by flow cytometric analysis and fluorescence microscopy. The expression level of rat AZ1 in transfected cells was determined by RT-PCR. As shown in Fig. 5A, cells transfected with EGFP-rat AZ1 expressed rat AZ1 mRNA but cells transfected with EGFP did not. The effect of constitutive overexpression of AZ1 on AsPC-1 cell differentiation was assessed by examining glucagon expression during the 6 days culture in SFM. The RT-PCR analysis showed that the level of *glucagon* mRNA was dramatically elevated in cells transfected with EGFP-AZ1 when compared with that in EGFP transfected cells (Fig. 5B). In addition, we found by confocal microscopic analysis that glucagon production was markedly increased in EGFP-AZ1-transfected cells when compared with control cells (Fig. 5C). These results indicated that constitutive overexpression of AZ1 enhanced glucagon production in AsPC-1 cells.

Polyamine contents during pancreatic tumor cell differentiation

AZ1 is known to be a negative regulator of polyamine metabolism (Hayashi et al. 1996, Coffino 2001). It binds to ODC and enhances its degradation by proteasome, thereby inhibiting polyamine biosynthesis. We examined the polyamine contents in AsPC-1 cells during culture in SFM. As shown in Fig. 6A, the content of putrescine decreased progressively in differentiating AsPC-1 cells during 6 days culture. This change was consistent with the observed

Figure 4 The effect of antizyme 1 siRNA transfection on the differentiation of pancreatic tumor cells. AsPC-1 or PANC-1 cells were first transfected with AZ1 siRNA or control siRNA (150 μ m) for 2 days and then induced to differentiate in SFM culture medium in the presence of one of the two siRNAs for 4 days. (A) Western blot analysis of AZ1 protein was performed on AsPC-1 cells 6 days after the first transfection with AZ1 siRNA (Cont). Cell lysates were analyzed by SDS-PAGE and immunoblotted with polyclonal anti-AZ1 and anti- β -actin antibodies. (B) Morphological appearance of cells. AsPC-1 cells transfected with AZ1 siRNA (AZ1) and cells transfected with control siRNA (Cont.) were examined microscopically. Images were taken under the microscope (IX71, Olympus, subject lens $\times 10$). Scale bars 500 μ m. (C) Analysis of glucagon-producing cells 6 days after transfection with AZ1 siRNA (AZ1) or control siRNA (Cont.). Cells were fixed and stained with monoclonal anti-glucagon and anti-mouse IgG-Alexa 488. Intracellular glucagons polypeptides were detected by flow cytometric analysis. (D) Quantification of mRNAs encoding glucagons, Pax6 and E-cadherin in AZ1 siRNA (AZ1) or control siRNA (Cont.) transfected AsPC-1 cells by RT-PCR analysis. The expression level of each gene was normalized with that of β -actin. Data are given as mean $\pm$ s.d. ($n=3$) * $P<0.05$. (E) The effect of antizyme 1 reduction on PANC-1 cell differentiation. RT-PCR analysis of glucagons and AZ1 mRNA was performed on PANC-1 cells 6 days after the first transfection with AZ1 siRNA (AZ1) or control siRNA (Cont.).

increase in AZ1 in these cells (Fig. 3A). On the other hand, spermidine and spermine contents remained relatively constant.

To examine whether the decrease of putrescine was causally related to the conversion of AsPC-1 cells to the differentiated state, we assessed the effect of raising the intracellular putrescine content by adding exogenous putrescine into culture. As shown in Table 1, the addition of putrescine at the final concentration in the range of 1–10 mM increased its intracellular level in a concentration-dependent manner. However, these putrescine treatments caused no statistically significant difference in glucagon production. These results suggested that the change in putrescine concentration was not important for AsPC-1 cell differentiation.

Discussion

Previously pancreatic tumor-derived hormone-producing cells were employed as a model to study the pancreatic cell differentiation (Borden *et al.* 1993). For

example, PANC-1 cells were shown to undergo differentiation into hormone-producing cells after stable expression of Pdx-1 and exposure to GLP1 (Hui *et al.* 2001) or brief trypsin treatment followed by cultivation in SFM (Hardikar *et al.* 2003, Gershengorn *et al.* 2004). Studies on these islet cell-model systems provided valuable information for understanding the mechanism of pancreatic cell differentiation. For example, it was shown that PANC-1 cells produced FGF2 that acted as a paracrine chemo-attractant to stimulate clustering of precursor cells, an early step leading to the formation of islet-like cell aggregate (Hardikar *et al.* 2003). In the present study, we found that other pancreatic tumor cell lines, AsPC-1, MIA paca2, KP-1 and KP-59 cells, all formed islet-like clusters and produced pancreatic hormones following brief trypsin treatment and the subsequent culture in SFM (Fig. 1). We found that AsPC-1 cells were converted to form an islet-like cluster and produce glucagon following brief trypsin treatment and the

Figure 5 The effect of antizyme 1 overexpression on the differentiation of pancreatic tumor cells. (A) The expression level of AZ1 mRNA in EGFP-AZ1 cells. AZ1 and β -actin expression were analyzed by RT-PCR using total RNA from AsPC-1 cells stably transfected with pEGFP or pEGFP-AZ1 constructs. (B) The expression level of glucagons mRNA in EGFP (control) or EGFP-AZ1 overexpressing AsPC-1 cells. Total RNA from differentiated AsPC-1 cells were analyzed for glucagons mRNA expression by RT-PCR. The expression level of glucagons was normalized relative to that of β -actin. Data are given as mean $\pm$ s.d. ($n=3$) * $P<0.05$. (C) Analysis of glucagon-producing cells in EGFP and EGFP-AZ1 overexpressing cells after 6 day culture in SFM. EGFP of EGFP-AZ1 overexpressing AsPC-1 cells were fixed and glucagon polypeptides were detected by immunofluorescence staining using anti-human glucagons mAb and anti-mouse IgG-Alexa 546. Images were analyzed using a confocal laser microscope.

subsequent culture in SFM (Figs 1 and 2A). However, production of insulin was undetectable in AsPC-1 cells. The reason for their inability to produce insulin is not known. In addition, we found that AsPC-1 cells synthesized the increasing amount of glucagon in response to the decreasing glucose concentrations in the culture medium. Moreover, glucagon production by AsPC-1 cells was suppressed by insulin treatment. Earlier studies showed that insulin inhibits transcription of *glucagon* gene and glucagon synthesis in pancreatic alpha cells *in vivo* (Maruyama et al. 1984, Philippe 1989). Thus, findings indicated that AsPC-1

cells exhibited several functional and morphological properties of normal pancreatic alpha cells (Rall et al. 1973, Unger 1973).

Our present study as well as the previous work (Hardikar et al. 2003, Gershengorn et al. 2004) indicated that trypsin treatment was the prerequisite for conversion of pancreatic tumor cells into the differentiated state. Recently, the effect of trypsin was shown to be mediated by a protease activated cell-surface receptor, PAR, that enhances cell aggregation (Wei et al. 2006). Since AsPC-1 cells strongly expressed PAR (Ohta et al. 2003), it was possible that the induction of AsPC-1 cell differentiation was triggered by the activation of PAR. After trypsin treatment, AsPC-1 cells cultured in SFM formed an

Figure 6 The change in the polyamine contents and the effect of putrescine during the differentiation of pancreatic tumor cells. Polyamine contents in cultured AsPC-1 cells. Cells were cultured for 0, 1, 3, and 6 days in SFM and the level of putrescine, spermidine and spermine contents was determined by mass spectrometric analysis as described in the Materials and methods ($n=3$) * $P<0.001$.

Table 1 The effect of putrescine on glucagon production. AsPC-1 cells were cultured for 6 days in serum-free medium (SFM) in the presence of 0, 1, 3 and 10 mM putrescine. Cells were fixed with 4% paraformaldehyde, and then stained with anti-glucagon mAb and anti-mouse IgG-Alexa 546. Glucagon production was measured by FV10-ASW software (means $\pm$ s.d.; $n=3$ dishes) as described in the Materials and methods. The level of putrescine content was determined by mass spectrometric analysis (means $\pm$ s.d.; $n=3$ dishes)

Concentration of putrescine in culture	Intracellular putrescine (nmol/mg protein)	Glucagon production (unit/300 cells)
0	0.8 $\pm$ 0.4	1681 $\pm$ 447
1	3.3 $\pm$ 0.9	2064 $\pm$ 418
3	10.0 $\pm$ 0.3	1545 $\pm$ 185
10	25.5 $\pm$ 0.6	1196 $\pm$ 179

islet-like cluster and increased the expression of *E-cadherin* (Fig. 2D) and β -catenin mRNA (data not shown) that were involved in cell–cell adhesion. Inhibition of the functional activities of E-cadherin and catenins blocked the formation of pancreatic β -cells (Dahl *et al.* 1996, Minami *et al.* 2008). In this study, we found that single AsPC-1 cell that did not form the cluster produced no glucagon (data not shown), whereas glucagon production was prominent among cluster-forming cells. Thus, it appears that the functional and morphological differentiations of AsPC-1 cells are coupled.

We found that the cultivation of AsPC-1 cells in SFM is another prerequisite for converting cells to the differentiated state. Under these conditions cells stopped growing and remained in the G1 phase of cell cycle, which may provide the environment suitable for cell differentiation to take place. In addition, serum starvation may alter the pattern of gene expression that may favor the induction of differentiated properties. Serum starvation culture was used to prepare nuclei for transplantation to make cloned animals (Campbell *et al.* 1996, Wilmot *et al.* 1997).

The model of glucagon-producing α -cell differentiation was relatively scarce. In this study, we used AsPC-1 cells as a model to study the regulation of glucagon-producing α -cell differentiation. Several lines of evidence indicated that AZ1 was required for AsPC-1 cell differentiation. Thus, depletion of AZ1 expression using an AZ1-specific siRNA inhibited the formation of cell clusters and expression of genes such as *glucagon*, *Pax6* and *E-cadherin* in AsPC-1 cells (Fig. 4D). We also showed that the number of glucagon-producing cells and glucagon production were increased by overexpression of AZ1. Furthermore, the data shown in Fig. 3A indicated that the

levels of AZ1 both mRNA and protein were increased during AsPC-1 cell differentiation, suggesting that the increase of AZ1 protein occurred, at least in part, through transcriptional regulation. The expression of glucagon mRNA in PANC-1 cells was also inhibited by treatment of AZ1 siRNA (Fig. 4E). These results indicated that transcriptional up-regulation of AZ1 is the key event in conversion of pancreatic tumor cells into the differentiated state.

The previous report showed that AZ1 is primarily localized in the nucleus where it transports ODC from the cytosol for degradation by nuclear proteasome (Gritli-Linde *et al.* 2001, Murai *et al.* 2003, Schipper *et al.* 2004). By contrast, we found that AZ1 was mainly localized in the cytosol during conversion of AsPC-1 cells to the differentiated state (Fig. 3B). These findings suggested that the site of AZ1 action is limited in the cytoplasm wherein it may interact with some proteins involved in regulation of α -cell differentiation. Future work is required to identify the target molecules of AZ1 to promote AsPC-1 cell differentiation. It was of interest to note that sex-lethal and cyclin B acting downstream of the Hedgehog signal were the target of *Drosophila* gutfeeling, the homologue of AZ1 (Vied *et al.* 2003).

Earlier studies demonstrated that AZ1 serves as a negative regulator of polyamine biosynthesis by binding to ODC and facilitating its degradation (Hayashi *et al.* 1996, Coffino 2001). Our data in Fig. 6 showed that the intracellular putrescine content was decreased during AsPC-1 cell differentiation when the AZ1 level increased. However, AsPC-1 cell differentiation was not affected by raising the intracellular level of putrescine by exogenously adding putrescine (Table 1). These results indicated that the decrease in putrescine was not important for the AZ1-mediated conversion of AsPC-1 cells, and suggested that the effect of AZ1 was separate from the one involved in inhibition of polyamine biosynthesis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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