Cellular Interaction Between Mouse Pancreatic α-Cell and β-Cell Lines: Possible Contact-Dependent Inhibition of Insulin Secretion

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The endocrine cells in the pancreatic islet have cellular communication between the heterotypic cells as well as the homotypic cells. The present study was conducted to elucidate the cellular interaction between pancreatic α cells and β cells utilizing differentiated mouse cell lines (i.e., αTC clone 6 and βTC cells). Co-culture of these two cell lines on a gyratory shaker generated numerous cellular aggregates of homogenous size within 48 h. Immunohistochemical staining for insulin and glucagon demonstrated that βTC cells were located in the central core of each aggregate, while αTC cells formed a mantle layer surrounding the βTC cells. This segregation was observed regardless of the ratios of the two cell types employed. Although glucagon at concentrations of 10^{-8} M or higher stimulated insulin secretion from βTC cells in both monolayer and aggregates, an increase in the ratio of αTC/βTC cells in aggregate cultures was accompanied by a decrease in secreted insulin and a rise in intracellular insulin content of the βTC component. The inhibitory effect of αTC cells on βTC insulin secretion was not limited to aggregate culture, since insulin secretion from βTC cells was also suppressed, and intracellular insulin content increased, by co-culture of αTC with βTC cells in monolayer. On the other hand, the secreted and intracellular insulin of βTC cells was not affected by αTC cells in a Transwell™ co-culture system in which direct cell-to-cell contacts were prevented by a semipermeable membrane that permitted chemical communication via medium metabolites. These data suggest that the insulin secretion from βTC cells may be inhibited possibly as a result of the contact with αTC cells. Exp Biol Med 228:1227–1233, 2003

Key words: cell-to-cell interaction; aggregate; pseudoislet; gyratory culture; pancreatic α-cell and β-cell lines

Mammalian pancreatic islets are composed of at least 4 different endocrine cell types. In rodents, these different cell types show particular intra-islet topography; β cells are located in the central core, while non-β cells surround β cells to form a mantle layer. It has been reported that when islets were dispersed into single cells and cultured for several days *in vitro*, they form islet-like cellular aggregates called "pseudoislets" (1, 2). This three-dimensional topography is implicated in the organized function of the islets.

The islet cells have cellular communications via gap junctions and show hormonal interactions via the vascular route and/or the paracrine route, both of which are implicated in the regulated secretion of islet hormones (3–5). In recent years, the cell adhesion molecules (CAMs) (6) and the gap junctional proteins have been extensively studied for analyzing the cellular communication between various types of cells including islet cells. Thus, one of the calcium-dependent CAM, E-cadherin (7), has been shown to be expressed in the mouse pancreatic β-cell line, MIN6 (8, 9). The expression of E-cadherin controls the connexin (Cx) 43-mediated gap junctional communication in mouse epidermal cells (10). Adequate levels of Cx43-mediated coupling is required for the proper insulin production and storage in rat insulinoma cell lines (11). Furthermore, the enhanced insulin secretory response in MIN6 pseudoislets concomitantly involved the intracellular Ca^{2+} response (8). The amount of expression of E-cadherin is correlated to glucose-induced insulin secretion in the MIN6 sublines (9). In addition to the cell-to-cell communication between β cells, pseudoislets formed from β and non-β cells have advantages in glucose-induced insulin secretion *in vitro* compared with those from β cells only (4, 5). However, there is a report in which the promoting effect of insulin secretion was not observed for the β cells in case of the contact with non-β cells (12).

The mouse β-cell line, βTC (13, 14), retains many characteristics of differentiated β cells. A mouse pancreatic

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α-cell line, αTC (15), produces both glucagon and insulin, and clonal lines producing only glucagon (αTC clone 6) were isolated from the original αTC (16). In this study, we examined the cellular interaction between these cell lines and assessed the cellular topographic relationships when cellular aggregates were formed by gyratory shaker (17). In addition, we assessed the changes of insulin secretion when βTC cells were maintained as mixed cellular aggregates or as mixed monolayer culture with αTC cells.

Materials and Methods

Cells. βTC cells were derived from an insulinoma from a transgenic mouse of (B6D2)F1 segregating background and harboring rat preproinsulin II promoter and SV40 oncogene (13, 14). This cell line was not cloned since the secretion of glucagon was negligible (16). αTC cells were derived from a glucagonoma in a transgenic mouse of (B6D2)F1 segregating background and harboring rat preproglucagon promoter and SV40 oncogene (15, 18). This cell line was cloned by a limiting dilution method and established a glucagon-producing clonal cell line, αTC clone 6 cells, which, unlike the parental line, did not produce insulin (16). The expression of MHC class I and class II molecules on the surfaces of these cell lines were matched. Thus, both cell lines were heterozygous for MHC alleles derived from both parental strains used in the construction of the transgenic mice [C57BL/6J ($H\text{-}2^b$) and DBA/2J ($H\text{-}2^d$)] (16). Both lines were maintained in Dulbecco's Minimal Essential Medium (DMEM, 5.5 mM glucose; Nissui, Tokyo, Japan), and supplemented with 44 mM sodium bicarbonate, 15 mM HEPES, 100 mg/ml Kanamycin sulfate, and 10% heat-inactivated fetal bovine serum (Gibco Laboratories, NY).

Formation of Aggregates. Cells cultured in 25-cm^2 plastic culture flasks (Corning) were harvested and dissociated into single cells with 0.05% trypsin/0.5 mM EDTA (T-EDTA); 2 x 10^6 cells were inoculated into a 20-ml Erlenmeyer flask in 3 ml of DMEM medium with 10% fetal bovine serum. The flasks were sealed and shaken (70 rpm) on a gyratory shaker (Ikemoto, Tokyo, Japan) at 37°C. The 2 cell lines were mixed at various ratios: 1:4, 1:1, and 4:1.

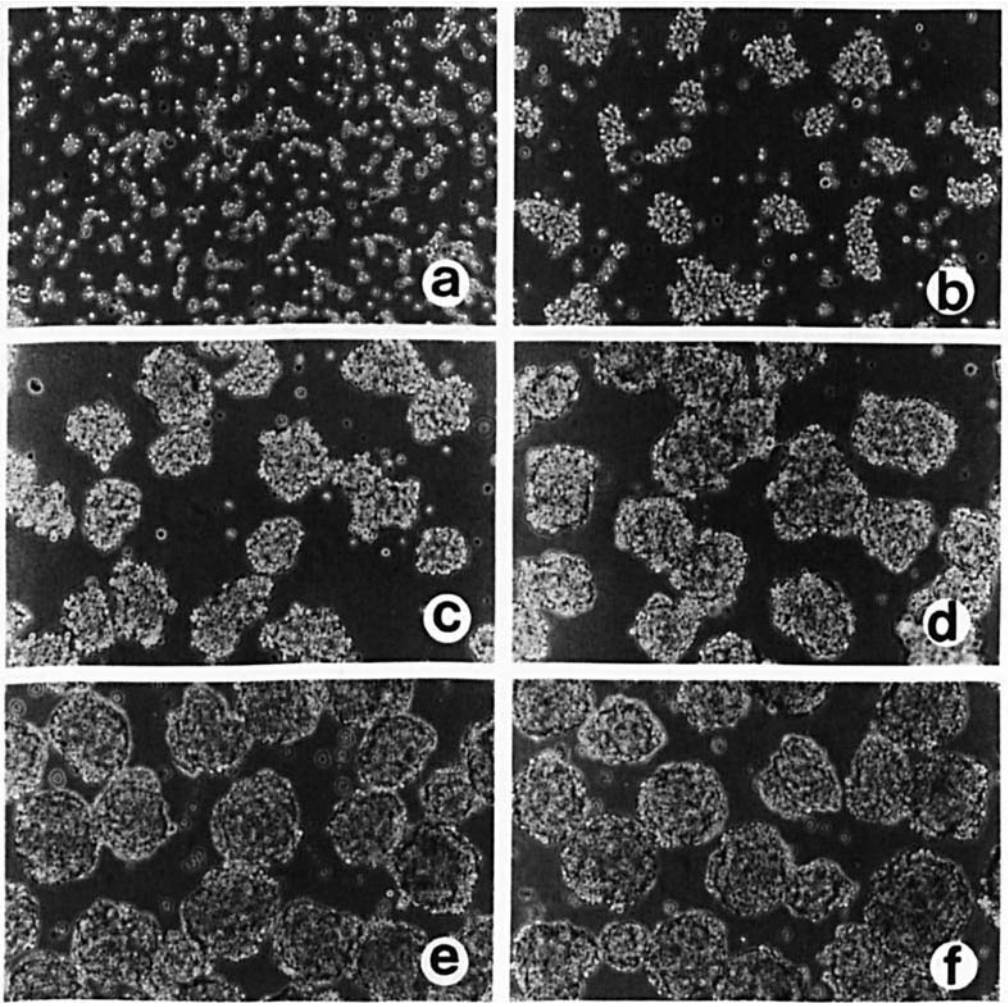

Figure 1. Time course of aggregate formation. αTC clone 6 cells and βTC cells were mixed at 1:1 ratio and cultured by 48 h on a gyratory shaker. Aggregates were observed by phase-contrast microscope at different time points; (a) 0 h, (b) 2 h, (c) 12 h, (d) 24 h, (e) 36 h, and (f) 48 h. Magnification ×62.

Morphological Examination. The aggregates were observed with a phase-contrast microscope at different time points, fixed with Bouin's solution for 1 h, dehydrated, and embedded in paraffin. Sections of 5-μm thickness were cut and used for light microscopic examination. They were stained immunohistochemically with mouse monoclonal antibody against human insulin (Nichirei, Tokyo, Japan), and rabbit antisera against pig glucagon (Dacopatts A/S, Denmark) as primary antibodies. Histofine SAB-PO Kit (Nichirei, Tokyo, Japan) for insulin antibody and Stravigen BSA-PO(R) Kit (Biogenex Laboratories, San Ramon, CA) for glucagon antibody were used for subsequent staining steps; non-immune sera as well as omission of primary antibodies were employed as specificity controls.

Functional Studies. In preliminary experiments, insulin secretion from monolayers of βTC cells was not significantly stimulated by glucose concentrations above 5.5 mM during 2 h of static incubation. We assessed the response of glucagon on insulin secretion from monolayer culture of βTC cells. βTC cells were inoculated in 24-well plastic culture plates (8 x 10^4/well) and cultured for 2 days to allow the cells to attach firmly; after 2 h of preincubation, the medium was substituted by the incubation media containing 0, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, or 10^{-5} M of glucagon (Novo, Denmark) in DMEM (5.5 mM glucose) supplemented with 0.2% BSA and 500 KIE Unit of aprotinin (Bayer, Germany). Incubation was carried out for 2 h and media were stored at −20°C for insulin assay. Intracellular insulin was also extracted overnight at 4°C from the monolayer of βTC cells with 1 ml/well of acid ethanol (1.5% HCl in 70% ethanol).

We assessed the effect of glucagon on insulin secretion from homotypic aggregates formed from βTC cells only. βTC cells were inoculated into 20-ml Erlenmeyer flasks (5×10^5 cells/flask) with 3 ml of medium and aggregates were formed. These aggregates were divided into 3 groups and inoculated into glass tubes for static incubation experiment. Incubation media containing 0, 10^{-8}, or 10^{-6} M of glucagon were employed.

Mixed cellular aggregates were formed from 50×10^4 cells/flask of βTC cells and various number of αTC clone 6 cells: 0, 25, 50, 75, or 100×10^4 cells/flask. These aggre-

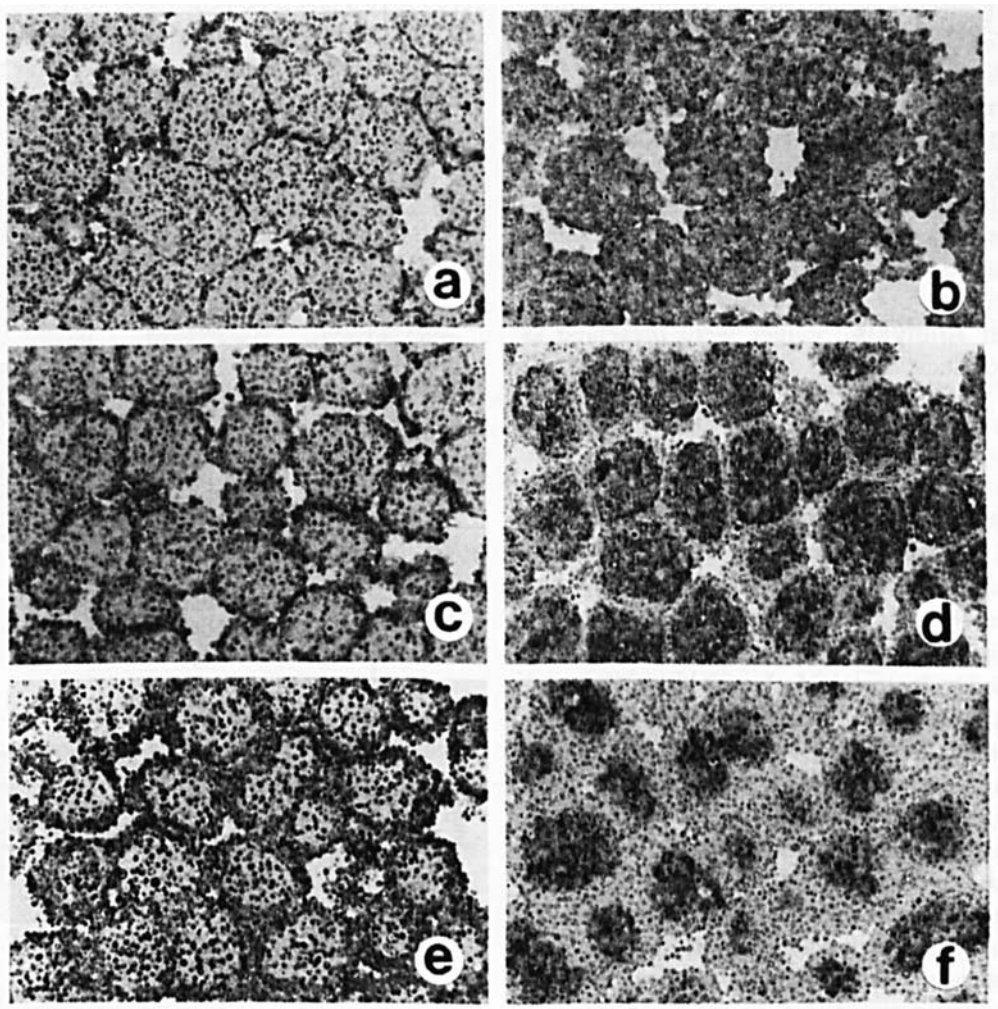

Figure 2. Immunohistochemical staining for glucagon (a, c, e) and insulin (b, d, f) of aggregates formed from αTC clone 6 and βTC cells at 3 different ratios of cell number. αTC:βTC = 1:4 (a, b), 1:1 (c, d), and 4:1 (e, f). βTC cells localize in the central core, while αTC cells form the mantle layer of each aggregates at all cell mixture ratios. Magnification ×68.

gates were incubated at 37°C for 2 h in the glass tubes. After the completion of static incubation, spent media were stored, and intracellular insulin was extracted as described previously.

To assess the architecture-dependent modulation of insulin secretion from βTC cells, we carried out the co-culture of 2 cell lines in 24-well plates as monolayers, and insulin secretion as well as intracellular insulin were assayed on culture day 2.

To rule out the possibility that αTC cells secrete soluble factors that influence the insulin secretion from βTC cells, we utilized the Transwell™ system (Costar, Cat. No. 3413, Cambridge, MA) in which the upper and lower compartments of the wells are separated by a microporous membrane (pore size 0.4 μm). Thus, 8×10^4 of βTC cells were inoculated into the lower compartment of the system, and then αTC clone 6 cells at various concentrations (0, 4, and 8×10^4) were inoculated into the upper compartment such that physical, but not chemical communication between these 2 cell lines was prevented. The media were stored frozen after 46 h of incubation and 2-h secretion study was carried out. Intracellular insulin was extracted as described previously. In these experiments, secreted and intracellular glucagon from αTC cells was also assayed to determine the effects on insulin secretion from βTC cells.

Insulin and Glucagon Radioimmunoassay. Insulin in the media and acid-ethanol extracts was assayed by radioimmunoassay (RIA) with porcine insulin as a standard and a polyethylene glycol method. RIA for glucagon was also carried out by a polyethylene glycol method with a C-terminal-specific antibody, OAL123 (Otsuka Assay, Tokushima, Japan).

Statistical Analysis. Values were presented as mean ± SE. Statistical analysis was performed with the Mann-Whitney *U* test.

Results

Formation of Aggregates. Numerous aggregates of homogenous size were formed from αTC clone 6 cells and βTC cells by 12 h, and these became compact and spherical by 36 h (Fig. 1). These pseudoislets were smaller in size than native mouse islets. Cellular composition of the aggregates studied by immunohistochemical staining for insulin and glucagon at several time points through 48 h of culture showed that βTC cells aggregated first, followed by attachment of αTC cells to the βTC cell aggregates (data not shown). Aggregates of various cell ratios were analyzed at 48 h of culture. βTC cells were located in the central core of each aggregate, while αTC cells comprised a mantle layer surrounding βTC cells regardless of the ratios of the 2 cell lines tested (Fig. 2). The relative sizes of "βTC-core" to "αTC-mantle" remained proportional to the ratios of each cell type in the mixture tested (Fig. 2). Insulin staining of the internal βTC core of cells appeared more intense in aggregates as the ratio of αTC/βTC cells increased.

Functional Studies. As has been reported previously (16) and confirmed again in this study, αTC clone 6 cells neither contained nor secreted insulin. Moreover, glucagon secretion from βTC cells was negligible. As shown in Figure 3, insulin secretion from βTC cells in monolayer was responsive to glucagon at concentrations higher than 10^{-8} M; however, extractable insulin was not increased. Likewise, glucagon stimulated insulin secretion from homotypic aggregates consisting only of βTC cells at concentrations of 10^{-8} M or higher without increasing extractable intracellular insulin level (Fig. 4). Similar results were obtained when the same concentrations of glucagon used in the incubation study were included during the initial 48 h of the formation of aggregates, suggesting that glucagon in the medium during these periods does not influence the level of extractable insulin from aggregates. Insulin secretion from heterotypic aggregates formed from various ratios of αTC and βTC cells is shown in Figure 5. The amount of insulin secreted from mixed cellular aggregates decreased while the extractable insulin increased as the ratio of αTC/βTC cells increased. The concentration of glucagon in the medium after the 2-h incubation of heterotypic aggregates from αTC cells (100×10^4) and βTC cells (50×10^4) was 1.7×10^{-9} M. It is conceivable that this level of glucagon concentration could not stimulate the insulin secretion from βTC cells (Fig. 3). Therefore, the effect of glucagon secreted from αTC cells on the insulin secretion could be negligible in this

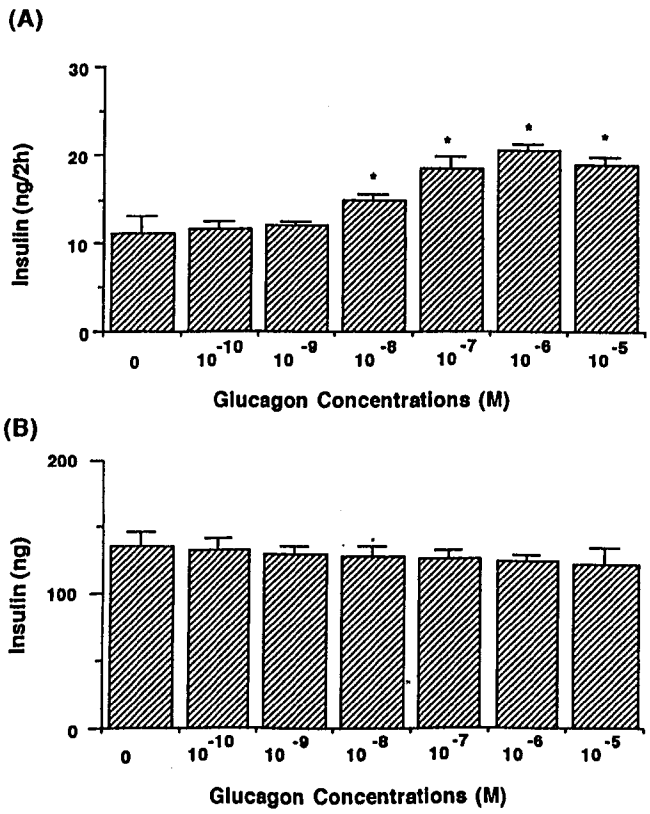

Figure 3. Effect of glucagon on secreted (A) and extracted (B) insulin from βTC cells in monolayer. Values are means ± SE ($n = 4$). *$P < 0.05$ vs control (glucagon 0 M).

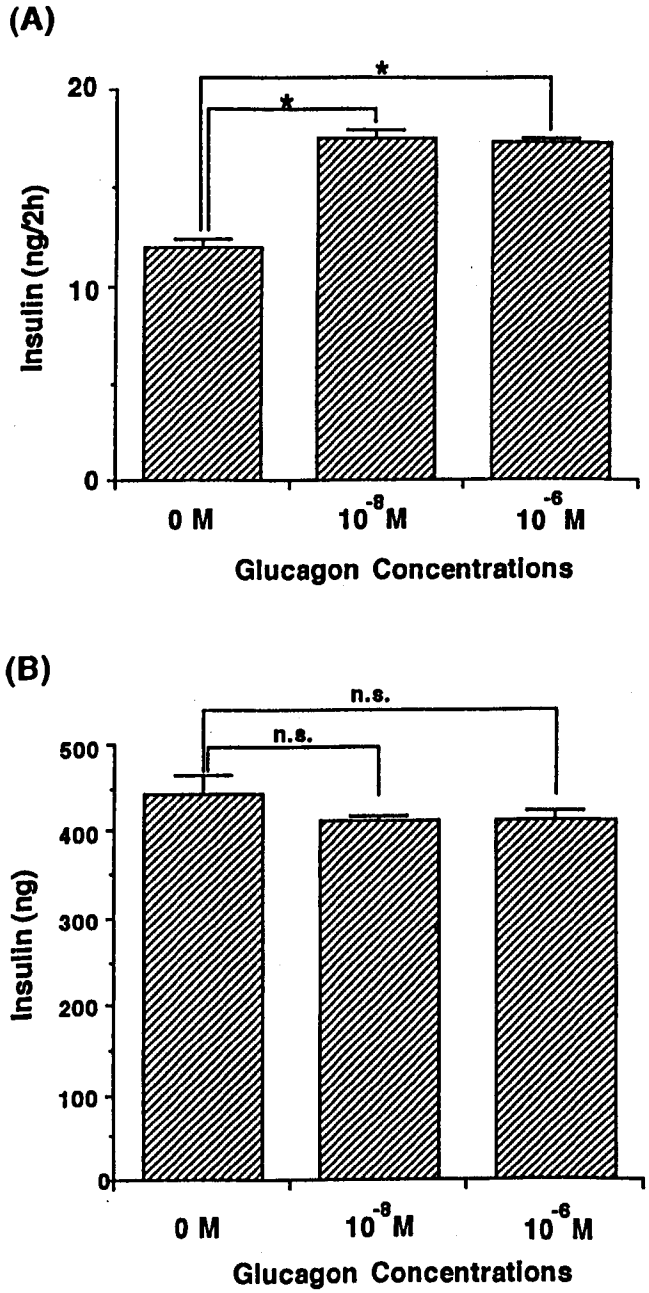

Figure 4. Effect of glucagon on secreted (A) and extracted (B) insulin from homotypic aggregates formed from βTC cells only. Values are means ± SE (n = 4). *P < 0.05. n.s.: not significant.

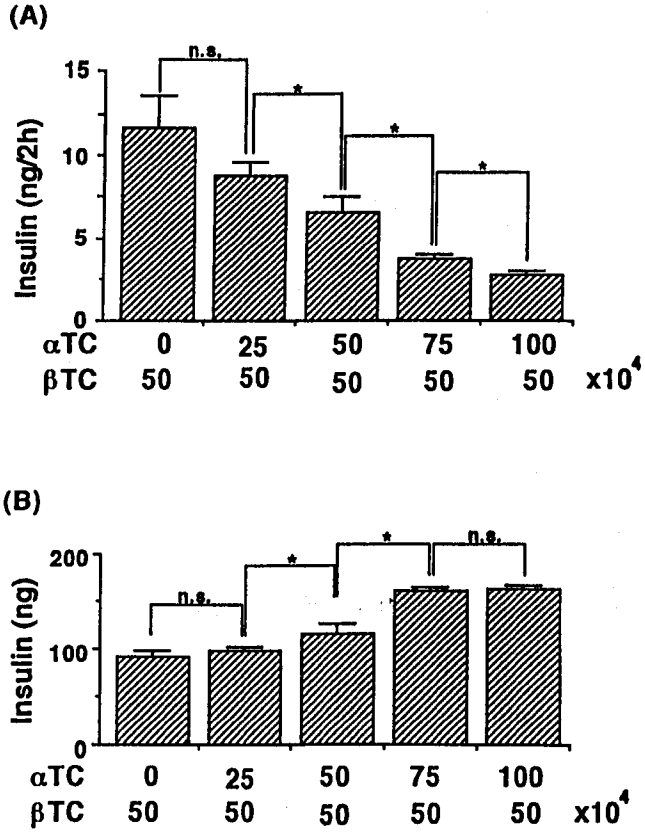

Figure 5. (A) Secreted and (B) extracted insulin from a heterotypic aggregates formed from αTC and βTC cells. Aggregates were formed from 5 × 10^5 cells/flask of βTC cells and various number of αTC clone 6 cells; 0, 25, 50, 75, or 100 × 10^4 cells/flask. Values are means ± SE (n = 4). *P < 0.05. n.s.: not significant.

experiment. To assess the possibility that the decline of insulin secretion was related to the acquisition of three-dimensional architecture described above, we co-cultured the 2 cell lines in monolayer in 24-well plates. In this experiment, insulin secretion again decreased as the number of αTC cells increased (Fig. 6). The extractable insulin was increased as the number of αTC cells increased, except for the co-cultures consisting of βTC cells (8 × 10^4) and αTC cells (16 × 10^4) (Fig. 6). To rule out the possibility that some soluble factors released from αTC cells could suppress insulin secretion from βTC cells, we utilized the Transwell™ system in which direct cell-to-cell contact was prevented but chemical communication via diffusion through semipermeable membranes was permitted. As shown in Figure 7, the insulin secretion and the extractable insulin from βTC cells in the lower compartment of the Transwell™ system were not altered through 46 h by co-culture of αTC cells in the upper compartment. In this experiment, 8 × 10^4 αTC cells in the upper compartment released glucagon at concentrations of 0.7 × 10^{-9} M at 2 h and 2.5 × 10^{-9} M at 46 h into the medium of lower compartment. These levels of glucagon concentrations again could be negligible in terms of the stimulation of insulin from βTC cells (Fig. 3). Moreover, these glucagon concentrations, especially the latter, exceeded that of the medium when the significant reduction of insulin secretion was observed in the incubation of heterotypic aggregates. If αTC cells in the upper compartment secrete soluble factors that suppress insulin secretion from βTC cells, and if the concentration of glucagon could be a marker for the diffusion of these factors through microporous membrane, ample concentrations of these factors could exist in the medium of the lower compartment. Since the concentration of insulin was not depressed in the Transwell™ system, the secretion of these soluble factors detectable by this system was ruled out.

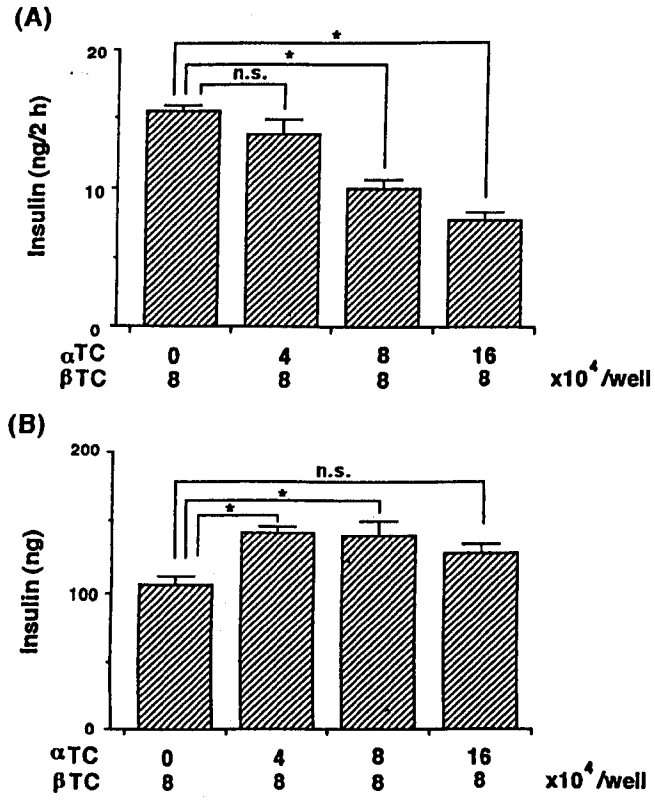

Figure 6. (A) Secreted and (B) extracted insulin from βTC cells co-cultured in monolayer with αTC cells in 24-well plates. The data are representative of 3 different experiments that showed the similar tendency of the insulin secretion and extraction. Values are means ± SE (n = 4). *P < 0.05. n.s.: not significant.

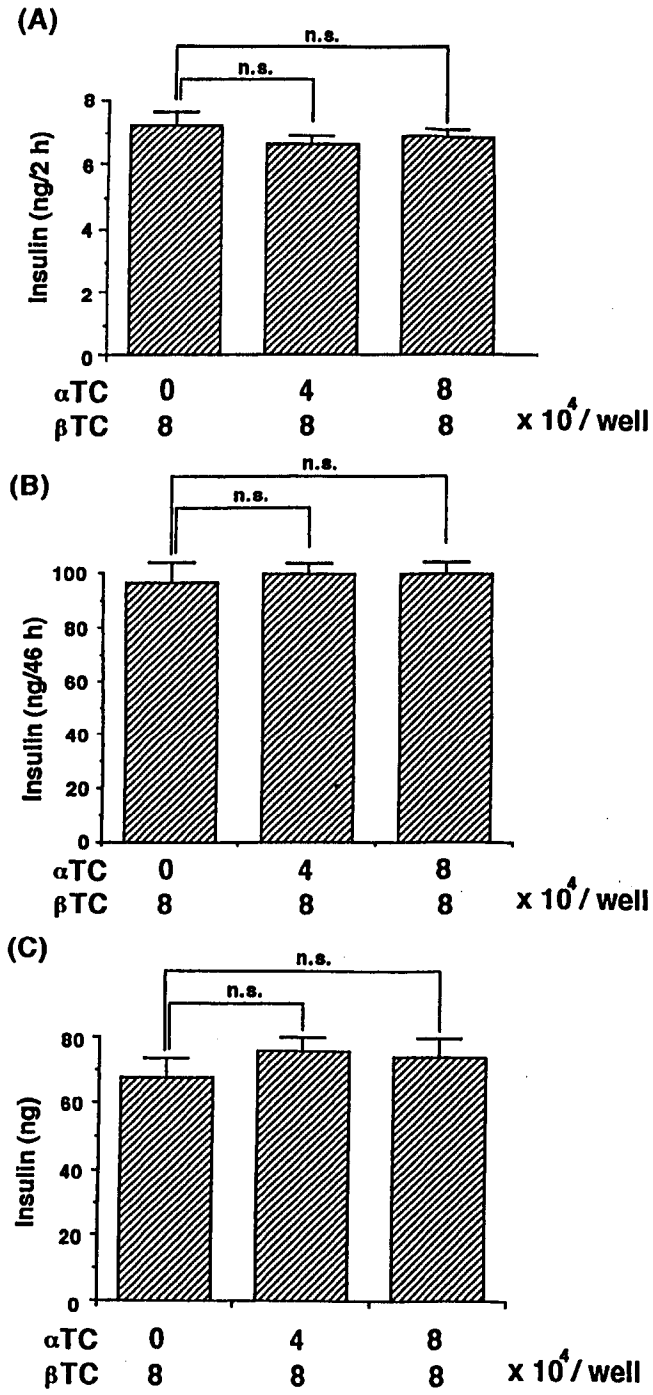

Figure 7. Secreted (A: 2 h; B: 46 h) and extracted (C) insulin from βTC cells co-cultured with αTC cells in the Transwell™ system described in the text. Values are means ± SE (n = 4). *P < 0.05. n.s.: not significant.

Discussion

The present study demonstrated that αTC clone 6 and βTC cells retain cell surface properties discriminating each other, although they are originated from the pancreatic endocrine organ of the same (B6D2)F1 segregating background of the mice. In addition, the heterotypic aggregates formed from these cell lines showed similar three-dimensional architecture to native islets *in vivo*. The normal rat islet cells have been reported in a static culture in the collagen matrix to reassociate spontaneously and form aggregates with similar anatomical topography to native islets (2, 19). However, utilizing gyratory shaker culture as was the case of the present study, Shizuru *et al.* (20) indicated a non-native reorientation of normal rat islet cells to produce aggregates of inverted cellular composition. The discrepancy of these results may stem from the methods used (21), or from the changes of cell-surface properties associated with the cellular transformation (22).

In our system, since αTC cells secrete glucagon, which stimulates insulin secretion from βTC cells, we assumed at first that αTC cells might coordinate βTC cells to stimulate insulin secretion. However, the insulin secretion from aggregates was decreased and the insulin extracted from aggregates increased as the elevation of αTC/βTC ratio (Fig. 5). The morphological study clearly showed that βTC aggregates were enveloped by the thicker outer mantle layer as the number of αTC cells increased (Fig. 2). Therefore, the αTC mantle might act as a barrier to the outward diffusion of insulin into the surrounding medium. However, the reduction of insulin secretion was also observed in the co-culture experiments in monolayer (Fig. 6), in which both chemical communication and physical contacts, but not the particular three-dimensional architecture, were pre-

served. Furthermore, the fact that some soluble factors produced by αTC cells suppress insulin secretion from βTC cells was not evident from the experiments utilizing Transwell™ system (Fig. 7). Therefore, these data indicated that the insulin secretion from βTC cells may be inhibited as a result of the close contact with αTC cells. It has been reported that the cellular contact between β cells as aggregates inhibits the basal insulin release (3, 23, 24); however, it has not been reported that the insulin secretion from β cells are inhibited via the contact with α cells. The results of our study, nevertheless, did not completely rule out the possibility of the soluble factor, which is effective exclusively as a paracrine fashion. These factors might not be detected as a soluble factor in the Transwell™ system. The other explanation of the suppressed insulin secretion is that the cell-to-cell contact may influence the replication rate or the apoptosis of βTC cells, and may modulate the insulin release and stores.

In conclusion, we have demonstrated that aggregates formed from α- and β-cell lines retain typical cellular composition similar to native islets, and also the inhibition of insulin secretion from the β-cell line via the possible contact with the α-cell line. The precise mechanism remains to be elucidated; however, these islet cell lines provide the useful model system in which the cellular interaction between the rare cell types and the pancreatic islet cells, could efficiently be examined.

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