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ORIGINAL ARTICLE

Functional interactions between pancreatic beta cells and (pre)adipocytes

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Abstract Type 2 diabetes is causally related to obesity and characterized by dysfunctional pancreatic beta cells. It is so far unclear whether direct interactions exist between adipocytes and beta cells and possibly raise any pathogenic relevance. In this study, we examined whether 9-day co-cultured 3T3-F442A (pre)adipocytes and primary rat pancreatic beta cells exert an influence on each other's function. In the presence of beta cells, 3T3-F442A cells became lipid-storing cells expressing markers of differentiated adipocytes and releasing adiponectin. This effect was attributed to the medium insulin levels (around 0.1 μM) and was associated with an elevated glucose consumption by the 3T3-F442A cells. The subsequent decrease in medium glucose concentration reduced the rate of insulin release by beta cells cultured at 10 mM glucose, and thus suppressed their degranulation during culture. These changes in beta cell function did not occur at 20 mM glucose and were reversible upon removal of the 3T3-F422A cells. They could not be reproduced by 3T3-F422A-conditioned medium containing varying adiponectin concentrations. These data indicate that insulin secreted by beta cells is sufficient to induce differentiation of preadipocytes without addition of exogenous adipogenic factors. Over 9 days culture, (pre)adipocytes did not directly and irreversibly affect beta cell functions.

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Introduction

Obesity, especially visceral obesity, is considered as one of the factors leading to insulin resistance and beta cell dysfunction. The mechanisms whereby adipose tissue could contribute to beta cell dysfunction are not well known, but they could involve adipocyte-secreted factors such as leptin, adiponectin, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), many of which have both central and peripheral effects.

Leptin, a protein produced by mature adipocytes, suppresses appetite, stimulates energy expenditure, inhibits insulin secretion and maintains insulin levels in the normal range. Although obese people have high leptin levels, they appear to be leptin resistant, and this probably leads to the exhaustion of beta cells [1]. Adiponectin expression and serum concentration are reduced in obese and insulin-resistant states [2]. Adiponectin is an insulin sensitizer and has protective effects on beta cells [3]. TNF-α and IL-6 are pro-inflammatory cytokines and their expression in human adipose tissue is positively correlated with body adiposity. TNF-α has been shown to inhibit insulin secretion and to induce beta cell apoptosis [1, 4]. In contrast, IL-6 has protective effects on beta cells. Therefore, the elevation of IL-6 production in obese and type 2 diabetic individuals may be involved in the beta cell compensation for insulin resistance in these conditions [1]. Although direct actions of some adipocyte-secreted factors on beta cell function have been reported [1, 3, 4], little is known about the combined effects of these factors.

In the present study, we investigate the influence of murine 3T3-F442A preadipocytes on the function of primary rat beta cells in vitro. The 3T3-F442A cell line is a frequently used preadipocyte cell line that was cloned from heterogenous Swiss 3T3 cells derived from dissociated near-term mouse embryos. Once differentiated in vitro, this cell line has many characteristics of adipocytes in vivo as the cells obtain a lipid-filled morphology and express adipocyte-secreted factors [5]. It was reported that insulin stimulates 3T3-F442A differentiation [6]. In addition, subcutaneous injection of 3T3-F442A preadipocytes into athymic mice gives rise to fat pads that are indistinguishable from normal adipose tissue [7, 8]. Therefore, the 3T3-F442A cell line appears to be a good model to study the influence of adipocytes and secreted factors on beta cell function.

Results

Effect of co-culture conditions on 3T3-F442A preadipocyte differentiation

3T3-F442A cells were co-cultured up to 9 days with primary rat beta cells with the aim to study the potential influence of preadipocyte-secreted factors on beta cell function.

We first evaluated the effect of beta cells on the differentiation of the 3T3-F442A cells, by evaluating the expression levels of different adipocyte markers in the co-cultured 3T3-F442A cells as compared to control cells. After 3 days, the concentration of insulin in the co-cultures increased to 319 ± 128 ng/ml at 180 mg/dl glucose and 598 ± 107 ng/ml at 360 mg/dl glucose (Table 1) and the mRNA levels for glyceraldehyde-3-phosphate dehydrogenase (GPDH), as well as glut-4, were increased in the co-cultured cells as compared to controls (Fig. 1a, right panels). After 9 days, insulin reached a concentration of 233 ± 168 ng/ml for the 180 mg/dl glucose condition and 823 ± 288 ng/ml at 360 mg/dl glucose (Table 1), and the co-cultured 3T3 cells expressed higher transcript levels encoding GPDH, glut-4, peroxisome proliferator activated receptor-γ (PPAR-γ) and adipocyte lipid-binding protein (the product of the aP2 gene) at both glucose concentrations (Fig. 1a, right panels). These changes in gene expression levels resemble the changes that were observed in 3T3-F442A cells that were induced to differentiate into adipocytes via addition of adipogenic hormones, as described under materials and methods (Fig. 1a, left panels), except, however, for preadipocyte factor-1 (pref-1), which was not suppressed under co-culture conditions.

In functional terms, the differentiation of 3T3-F442A cells was reflected by their accumulation of lipid droplets positive in Oil Red O staining. After 3 days of co-culture, no morphological differences were observed as judged by phase contrast microscopy between control 3T3-F442A preadipocytes and co-cultured cells, and Oil Red O positive lipid droplets could not be detected (data not shown). However, after 9 days of co-culture, comparable amounts of Oil Red O positive lipid droplets accumulated in co-cultured preadipocytes, as well as in the hormone-induced cells, whereas no such accumulation was seen in control cells (Fig. 1b).

Effect of co-culture conditions on adipokine expression by 3T3-F442A cells

To further document the effect of the co-culture conditions on the differentiation of the 3T3-F442A preadipocytes, as compared to the hormone-induced cells, we examined the expression of adipokines that have been reported to affect beta cell function (leptin, adiponectin, TNF-α, and IL-6).

Co-cultured cells, as well as hormone-induced cells, expressed higher mRNA levels for adiponectin after 3 and

Table 1 Effect of 3T3-F442A cells on glucose and insulin concentration in culture media

Culture conditions	Glucose (mg/dl)		Insulin (ng/ml)		Insulin secretion (ng/K cells/24 h)			Insulin content (ng/K cells)		
	Day 3	Day 9	Day 3	Day 9	Day 3	Day 9	Day 11	Day 3	Day 9	Day 11
180 mg/dl glucose										
Beta cells	185 ± 15	164 ± 23	530 ± 66	497 ± 89	22 ± 2	23 ± 9	23 ± 5	17 ± 5	23 ± 4	30 ± 2
Beta + 3T3 cells	153 ± 13[b]	43 ± 26[c]	319 ± 128	233 ± 168	15 ± 6	3 ± 3[c]	13 ± 5	24 ± 4[2]	39 ± 9	34 ± 6
3T3 cells	167 ± 9	119 ± 4	/	/	/	/	/	/	/	/
360 mg/dl glucose										
Beta cells	364 ± 7	367 ± 12	631 ± 144	991 ± 179[3]	34 ± 6[3]	29 ± 9	37 ± 8	6 ± 1[3]	17 ± 1	14 ± 3
Beta + 3T3 cells	343 ± 9	271 ± 27[c]	598 ± 107	823 ± 288[3]	31 ± 5[3]	34 ± 11	46 ± 4[a]	6 ± 1[3]	7 ± 2[b2]	10 ± 4
3T3 cells	345 ± 10	326 ± 31	/	/	/	/	/	/	/	/

Data shown as means ± SEM, $n = 3$–6. Student's t test, [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$ co-cultured beta cells compared to beta cells alone; [1] $P < 0.05$, [2] $P < 0.01$, [3] $P < 0.001$ as compared to the 180 mg/dl glucose condition

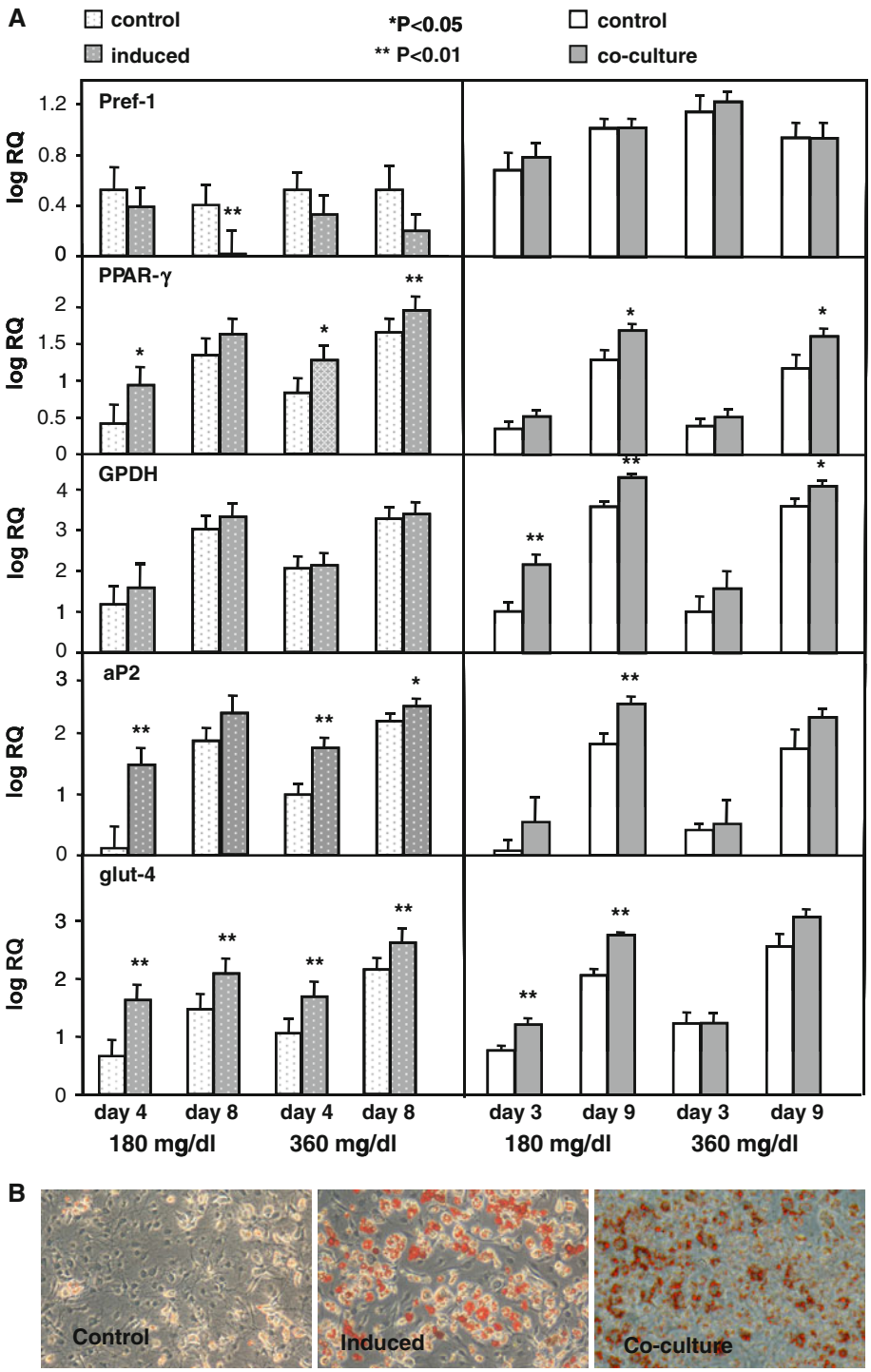

Fig. 1 Effect of co-culture conditions on 3T3-F442A preadipocyte differentiation. 3T3-F442A preadipocytes were co-cultured with primary rat beta cells for 3 up to 9 days in α-MEM medium with 180 or 360 mg/dl glucose (*right panels*); or were cultured for up to 8 days in the absence (control) or presence (induced) of adipogenic compounds (*left panels*). **a** Relative expression levels of pref-1, GPDH, PPAR-γ, aP2, and glut4 were determined by quantitative real-time PCR. All expression levels were normalized to β-actin and calibrated to the confluent 3T3-F442A cells at the start of co-culture. Data shown as means ± SEM, $n = 3$–6. Student's t test, * $P < 0.05$, ** $P < 0.01$. **b** The conversion of preadipocytes into mature lipid-filled adipocytes was characterized by Oil Red O staining of intracellular lipid droplets

9 days as compared to the control cells at both glucose concentrations, and the mRNA levels increased with culture time (Fig. 2a). Secretion of adiponectin was confirmed by measuring adiponectin accumulation in the 3T3-F442A-preconditioned media (Fig. 2b). Accumulation of adiponectin in the culture media was not influenced by the glucose concentration.

The mRNA levels for TNF-α and IL-6 were comparable in the co-cultured 3T3-F442A cells as well as in the control cells (Fig. 2a, right panels). However, lower expression levels for TNF-α and IL-6 were found when cells were induced (Fig. 2b, left panels). No representative levels of mRNA for leptin could be demonstrated.

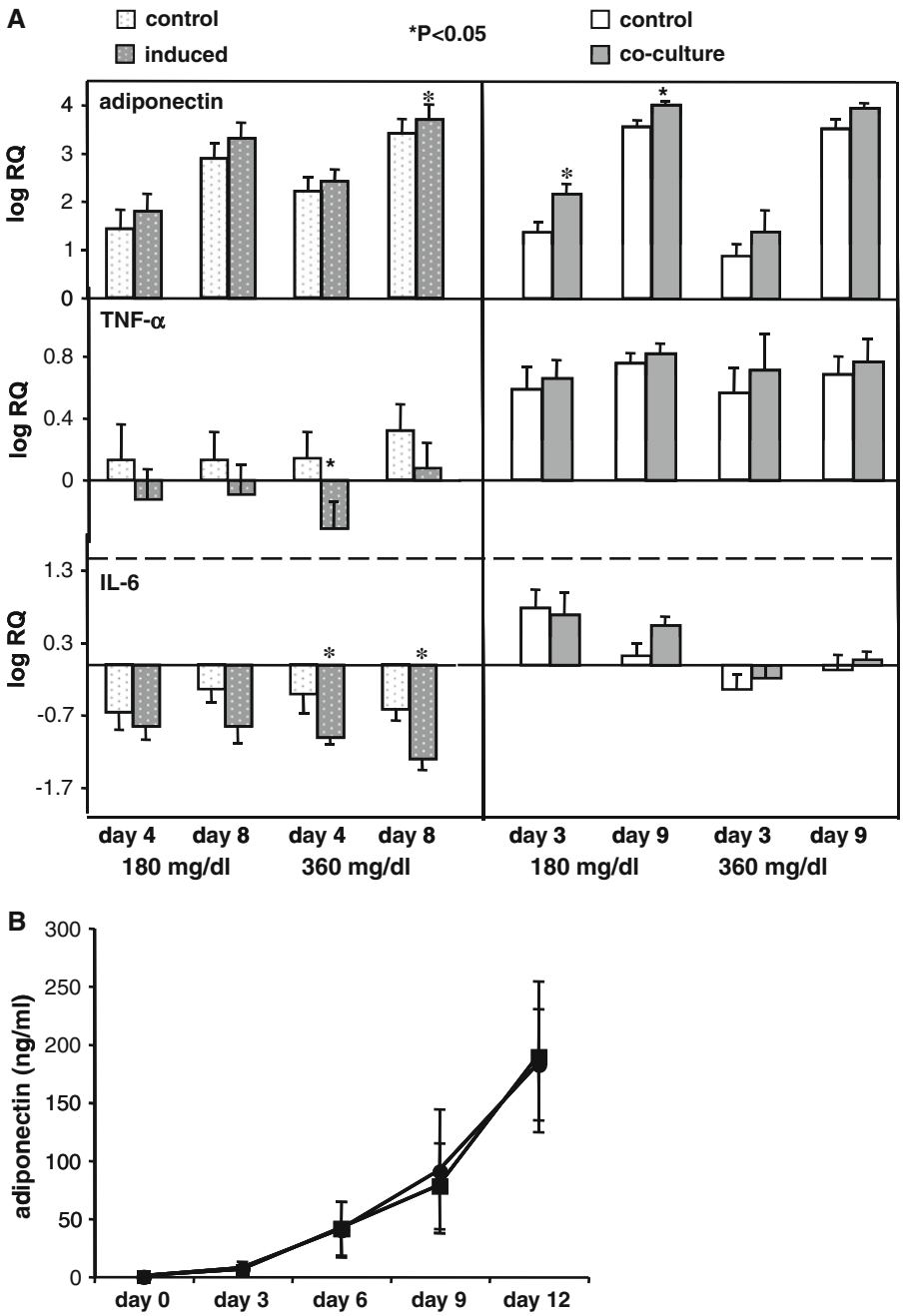

Fig. 2 Effect of co-culture conditions on adipokine expression by 3T3-F442A cells. 3T3-F442A preadipocytes were co-cultured with primary rat beta cells for 3 up to 9 days in α-MEM medium with 180 or 360 mg/dl glucose; or were cultured for up to 8 days in the absence or presence of adipogenic compounds. **a** Relative expression levels of adiponectin, TNF-α, and IL-6 were determined by quantitative real-time PCR. All expression levels were normalized to *β*-actin and calibrated to the confluent 3T3-F442A cells at the start of co-culture. Data shown as means ± SEM, $n = 3$–6. Student's t test, * $P < 0.5$. **b** Adiponectin concentration in 3T3-F442A (pre)adipocyte-conditioned media. 3T3-F442A preadipocytes were cultured for 3 up to 12 days in α-MEM medium with 180 mg/dl (*filled square*) or 360 mg/dl (*filled circle*) glucose. 3T3-F442A (pre)adipocyte-conditioned media and control media were collected after 3 to 12 days. Adiponectin concentration was measured via ELISA, data shown as means ± SEM, $n = 4$

Influence of co-culture conditions on beta cell function

In order to study the potential influence of 3T3-F442A cells on beta cell function, the cells were co-cultured for 3 up to 9 days in α-MEM medium at 180 and at 360 mg/dl glucose. After 9 days, beta cell containing inserts were removed and placed into fresh media for an additional culture period in the absence of 3T3 cells. Medium samples to measure insulin concentrations were taken at regular intervals as indicated (Table 1). Since 3T3-F442A cells are known to consume glucose during their differentiation into adipocyte-like cells, a process mediated by insulin [9], we also checked the glucose concentration in the media (Table 1).

3T3-F442A cells co-cultured with beta cells were found to consume significantly more glucose (1 mg/dl/24 h) than control 3T3-F442A cells (0.2 mg/dl/24 h) as soon as 40 h after starting the co-culture, reaching a rate of 3 mg/dl/24 h after 9 days (Fig. 3). In 3T3-F442A control cells, a sharp increase of their glucose consumption could only be measured after 5 days of culture, which reflects in the much slower differentiation of adipocyte-like cells in the absence of beta cells. The glucose consumption by the 3T3-F442A cells was not influenced by the initial glucose concentration.

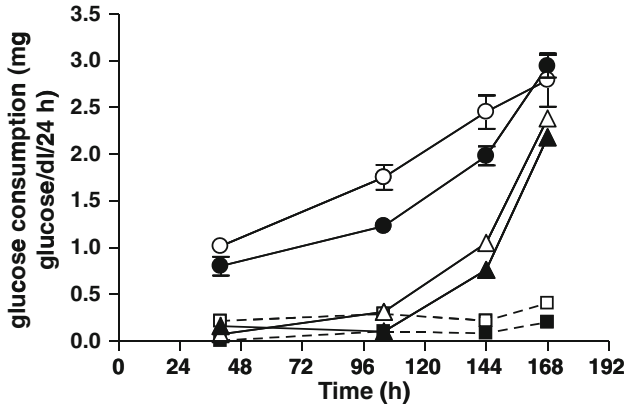

Fig. 3 Effect of co-culture conditions on glucose concentration in culture media. Glucose concentrations in the different culture media were measured as indicated after 40, 104, 144, and 168 h of co-culture: 3T3-F442A cells 180 mg/dl glucose (*open triangle*), 3T3-F442A cells 360 mg/dl glucose (*filled triangle*), beta cells 180 mg/dl glucose (*open square*), beta cells 360 mg/dl glucose (*filled square*), 3T3-F442A cells and beta cells 180 mg/dl glucose (*open circle*), 3T3-F442A cells and beta cells 360 mg/dl glucose (*filled circle*). Glucose consumption was calculated as the amount of glucose (mg/dl) used per hour. Data shown as means ± SEM, $n = 5–7$

Beta cells alone also had no effect on the glucose concentrations (Fig. 3).

As a result of the increased glucose consumption by co-cultured 3T3-F442A cells, the medium glucose concentration dropped from 180 to 153 mg/dl on day 3 and further, to only 43 mg/dl after 9 days. This dramatic decrease, below beta cell stimulatory concentrations [10], was responsible for a decrease in the amount of insulin released over 24 h from day 3 onwards (Table 1). At the end of 9 days co-culture, beta cells released 2.9 ± 3 ng insulin/10^3 cells/24 h, as compared to 22.7 ± 9.4 ng insulin/10^3 cells/24 h by control cells; consequently, their cellular insulin content did not decrease as in control cells (39.1 ± 8.7 vs. 23 ± 3.6 ng/10^3 cells). These effects merely reflect the impact of the 3T3-F442A cells on the glucose concentration and thus do not indicate that they directly act on beta cell functions. They are also reversible upon removal of the 3T3-F442A cells as shown by the increase in insulin secretion and decrease in cellular insulin content when the inserts with beta cells were removed from the co-cultures and placed into fresh medium for an additional 48 h culture period (Table 1).

When the co-cultures were conducted at 360 mg/dl glucose instead of 180 mg/dl, the medium glucose concentrations were maintained at levels that maximally stimulated the beta cells (Table 1). Consequently, insulin release was not suppressed in co-cultured beta cells and neither was their cellular insulin content increased. These results strongly suggest that the presence of differentiating 3T3-F442A preadipocytes does not directly affect the secretory function of primary rat beta cells, at least not during a 9 days culture period.

We also examined the potential effect of 3T3-F442A cells on the stability of the secreted insulin. Control cells and co-cultured cells were incubated with I^{125}-insulin for 24 h after 2 or 8 days of culture. Twenty-four hours later, media, beta cells, and 3T3-F442A cells were harvested to measure I^{125}-breakdown by TCA precipitation as described, or to examine incorporation of the I^{125}-label in the cell fractions. As shown in Table 2, the degradation of I^{125}-insulin was not influenced by the presence of beta cells but increased two- to threefold in the presence of 3T3-F442A cells. No I^{125}-incorporation could be measured in the beta cell fraction, or in the 3T3-F442A preadipocyte fraction (results not shown).

Effect of 3T3-F442A (pre)adipocyte-secreted factors on beta cell function

In view of the data suggesting that (pre)adipocytes do not affect the function of co-cultured beta cells, we examined the effects of medium preconditioned by 3T3-F442A cells during 3–12 days of culture at 180 or 360 mg/dl glucose on beta cell function. Beta cells were exposed for 3 days to these preconditioned media. This approach allows an investigation of 3T3-cell released compounds at a fixed glucose concentration, which was not the case during co-culture. The glucose concentrations of the media did indeed remain stable over the 12 days of 3T3-F442A culture at 180 and at 360 mg/dl glucose (data not shown). Insulin secretion was measured

Table 2 Effect of 3T3-F442A cells on stability of insulin in culture media

Culture conditions			Insulin degradation %	
% FCS	Beta cells	3T3 cells	Day 3	Day 9
180 mg/dl glucose				
0	−	−	6 ± 2	2 ± 2
10	−	−	10 ± 6	6 ± 3
10	+	−	8 ± 4	4 ± 5
10	−	+	18 ± 6*	12 ± 2*
10	+	+	15 ± 3*	12 ± 2*
360 mg/dl glucose				
0	−	−	8 ± 9.2	9 ± 10
10	−	−	6 ± 2.8	7 ± 4.2
10	+	−	5 ± 4.2	6 ± 4.2
10	−	+	15 ± 5.6	17 ± 7.8
10	+	+	14 ± 6.4	18 ± 4.9

I^{125}-Insulin was added to the culture media after 2 or 8 days of co-culture and media were sampled 24 h later (days 3 and 9, respectively). Degradation of insulin was measured by TCA precipitation. Data shown as means ± SD, $n = 3$. Student's t test, * $P < 0.05$, as compared to medium without FCS, medium with 10% FCS in presence and absence of beta cells alone

Table 3 Effect of 3T3-F442A-conditioned media on insulin secretion and content of primary rat beta cells

Culture conditions	Adiponectin (ng/ml)		Insulin secretion (ng/K cells/24 h)		Insulin content (ng/K cells)	
	Ctrl	3T3	Ctrl	3T3	Ctrl	3T3
180 mg/dl glucose						
Day 3	/	5 ± 3	23 ± 5	20 ± 3	18 ± 4	19 ± 9
Day 9	/	70 ± 40	24 ± 5	22 ± 3	18 ± 9	30 ± 8
Day 12	/	189 ± 65	24 ± 8	23 ± 4	13 ± 3	17 ± 2
360 mg/dl glucose						
Day 3	/	7 ± 6	25 ± 5	19 ± 10	19 ± 4	21 ± 6
Day 9	/	86 ± 57	25 ± 5	18 ± 7	13 ± 4	18 ± 3
Day 12	/	182 ± 48	24 ± 6	23 ± 2	14 ± 3	19 ± 4

Data shown as means ± SD, $n = 3$–4

during the last 24 h. There were no significant differences in insulin secretion between beta cells cultured in control medium and in 3T3-conditioned media, and this for all media collected at different time points and containing adiponectin concentrations that increase with the duration of culture (Table 3). Adiponectin accumulated in the 3T3 medium but did not influence the insulin secretion. Similarly, the insulin content was not influenced by adiponectin or other (unknown) 3T3-F442A (pre)adipocyte-secreted factors (Table 3). In addition, we measured the insulin secretion and content of beta cells that were cultured in α-MEM medium to which recombinant adiponectin was added. Similar levels of insulin secretion and content were observed as for beta cells that were cultured in control α-MEM medium (data not shown).

Discussion

Under normal physiological circumstances, the pancreatic beta cell maintains an insulin reserve that can meet varying demands for secreted insulin [11]. This balance is disturbed in patients with type 2 diabetes, in many induced or preceded by a state of obesity. Adipocytes can play a pathogenic role through different mechanisms. It is so far unknown, or at least uncertain, whether their secretory products can exert direct influences on beta cells. Previous studies have examined on the possible involvement of an elevated discharge of free fatty acids or of adipocyte-produced peptides or hormones by adding these compounds to isolated islets. In the present study, we assessed whether beta cell functions are affected during co-culture with a differentiating preadipocyte cell line and with its conditioned medium.

Effect of primary rat beta cells on 3T3-F442A preadipocytes

We first evaluated the effect of insulin released by the beta cells on the differentiation of the 3T3-F442A cells and compared the effects with cells that were induced with the adipogenic hormones [5]. Our results indicate that the amount of insulin secreted by the primary rat beta cells was sufficient to stimulate the differentiation of the 3T3-F442A cells in the co-cultures in the absence of other adipogenic hormones. This was illustrated by an elevated expression level of the adipogenic marker genes glut4, PPAR-γ, GPDH, and aP2 in the co-cultured cells, comparable to those in hormone-induced 3T3-F442A cells. In contrast, however, the mRNA levels for pref-1, a gene that suppresses adipocyte differentiation [12], was not downregulated under co-culture conditions whereas its expression was clearly suppressed in hormone-induced 3T3-F442A cells. This could eventually be explained by the absence of dexamethasone in the co-culture medium. Dexamethasone mediates an acute downregulation of pref-1 transcription and hence has a proadipogenic action on 3T3-F442A cells [12]. The failure of pref-1 mRNA levels to become downregulated did, however, not prevent the accumulation of Oil Red O positive lipid droplets in the co-cultured cells, while they were absent in control cells.

Differentiating 3T3-F442A cells were found to express adiponectin, TNF-α, and IL-6, and the expression of adiponectin was induced under co-culture conditions. The role of insulin in adiponectin expression and secretion, however, remains controversial. Insulin downregulates adiponectin gene expression in fully differentiated 3T3-L1 cells [13], whereas other studies have shown stimulatory effects of insulin on adiponectin secretion in 3T3-L1 adipocytes and in human primary adipocytes [14, 15]. In our co-culture system, beta cells upregulated the expression of adiponectin. Increased mRNA levels of adiponectin were also found in hormone-induced 3T3-F442A cells. In addition, 3T3-F442A cells secreted high levels of adiponectin in the culture medium, even in the absence of insulin. Therefore, expression and secretion of adiponectin may depend on the differentiation status of the 3T3-F442A cells and is not directly influenced by insulin.

The expression of the two major cytokines known to play a role in insulin resistance, TNF-α and IL-6, was not significantly altered by co-culture with beta cells. The lower expression of TNF-α in the induced cells is due to isobutylmethylxanthine that exerts its effects on differentiation by suppressing the production of endogenous TNF-α [16].

Effect of 3T3-F442A preadipocytes on the function of primary rat beta cells

Different strategies were used to examine the effect of 3T3-F442A (pre)adipocytes secreted factors on the function of primary rat beta cells.

In a first strategy, the beta cells were co-cultured with the 3T3-F442A cells. The function of beta cells was not altered in the presence of the 3T3-F442A preadipocytes during short-term (3 days) co-culture at glucose concentrations that were previously found to maintain best beta cell functions and survival [17, 18].

In contrast, during a longer co-culture period (9 days), the presence of preadipocytes resulted in tenfold lower rates of insulin secretion, thus maintaining higher cellular insulin reserves. This effect appeared indirectly caused through the increased glucose consumption by the differentiating 3T3-F442A cells. It is indeed known that differentiating preadipocytes take up glucose for intracellular lipid accumulation, a process that is mediated by insulin [9]. After 9 days of culture, the medium glucose concentration dropped from 180 to 43 mg/dl, despite medium change every alternate day. Therefore, the significant reduction in the insulin concentration may be related to lack of glucose-stimulated insulin secretion (GSIS), which is reflected in the high insulin content of the co-cultured beta cells. When this fall in medium glucose concentration was prevented by culturing the cells at 360 mg/dl, the co-cultured beta cells exhibited the same insulin release rates and cellular insulin content as the control cells. Our data are consistent with the study of Wang et al. [9], who measured the remaining glucose levels in the medium after stimulation of 3T3-L1 cells with insulin for 11 h. After stimulation, there was less than 9 mg/dl glucose remaining in the medium from cells incubated at low glucose concentration (102 mg/dl), whereas more than 306 mg/dl glucose remained in cells incubated at high glucose concentration (430 mg/dl). These results reflect the impact of the 3T3-F442A cells on the glucose concentration and thus do not indicate a direct effect on beta cell function.

A previous study demonstrated that 3T3-L1 adipocytes directly impair insulin secretion in MIN6 cells, a murine beta cell line. However, glucose levels during co-culture were not measured [19].

In a second strategy, we performed follow-up/recovery experiments to test whether the beta cells can regain their functional activity within a short period. Therefore, after the long-term co-culture, the inserts containing beta cells were removed from the co-cultures and placed into fresh medium for an additional 48 h. Both at 180 and 360 mg/dl glucose, insulin secretion and content were normalized, supporting the hypothesis/conclusion that differentiating 3T3-F442A cells have no direct effects on beta cell function.

In a third strategy, we examined the effects of adipocyte-secreted factors, or adipokines, on beta cell function. Therefore, we cultured beta cells in 3T3-F442A-preconditioned medium. There were no significant differences in insulin secretion and insulin content as compared to beta cells cultured in control medium, thus again indicating that factors secreted by 3T3-F442A (pre)adipocytes do not directly influence insulin secretion by beta cells. We especially focused on adiponectin because we observed a stimulatory effect of beta cells on adiponectin expression and there exist conflicting results on the effects of adiponectin on insulin secretory function. In normal islets, adiponectin had no significant effect on insulin secretion [20]. In contrast, in islets from mice rendered insulin-resistant by high-fat feeding, adiponectin shows a dual effect on insulin secretion: it inhibits insulin secretion at low glucose levels (50 mg/dl) but augments insulin secretion at high glucose levels (300 mg/dl). Another study showed that adiponectin stimulates insulin secretion both in vitro and in vivo at a glucose concentration of 100 mg/dl [21]. We showed that adiponectin is secreted by 3T3-F442A cells and accumulates in the preconditioned medium up to a concentration of ±0.2 μg/ml. In our culture system, adiponectin did not influence insulin secretion at 180 or 360 mg/dl glucose. Addition of recombinant adiponectin, up to a concentration of 12.5 μg/ml, also had no effect on insulin secretion and content of beta cells. The different effects of adiponectin on insulin secretion may be due to different experimental conditions. We use primary rat beta cells, while other studies describe the influence of adiponectin on pancreatic islets of murine [20, 21] or human [22] origin. In previous studies, only short-term effects (1 h incubation) of high levels (5–20 μg/ml) of recombinant adiponectin are examined, while we incubated the beta cells for 64 h in medium containing low levels of adipocyte-secreted adiponectin (0.2 μg/ml).

In this study, we examined the effects of 3T3-F442A (pre)adipocytes and of its secreted factors on primary pancreatic beta cell function, in a co-culture system. 3T3-F442A cells have the characteristics of mature adipocytes, as they differentiated and secreted adipokines under the influence of insulin, secreted by the beta cells. We demonstrate for the first time that the inhibitory effects on

insulin secretion, as seen in the co-culture experiments, are only due to the impact of the preadipocytes on the glucose concentration in the culture medium. This observation was confirmed in follow-up/recovery experiments and in 3T3-F442A-preconditioned medium experiments. We did not find any effect of adiponectin on insulin secretion in our culture system. The present study thus demonstrates that 3T3-F442A cells have no direct effect on the function of primary rat beta cells.

Materials and methods

Culture and differentiation of 3T3-F442A preadipocytes

3T3-F442A murine preadipocytes were grown in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Paisley, UK), with 10% iron-supplemented bovine calf serum (Hyclone, Logan, UT) and 5% penicillin/streptomycin, and passaged when pre-confluent. To stimulate adipocyte differentiation, cells were seeded at a density of 25,000 cells/cm^2 in DMEM medium and grown to confluency. Medium was then replaced by α-MEM medium (Invitrogen) containing 180 or 360 mg/dl glucose, 0.075 mg/ml penicillin, 0.1 mg/ml streptomycin, and 10% fetal calf serum (Hyclone) for 2 days and subsequently treated for another 2 days with α-MEM supplemented with 17 nM insulin, 2 nM triiodothyronine, 100 nM dexamethasone, and 100 μM isobutylmethylxanthine (induction medium). At day 4, cultures were switched to differentiation medium (α-MEM supplemented with 17 nM insulin and 2 nM triiodothyronine). 3T3-F442A cells cultured in α-MEM without addition of adipogenic hormones, served as control.

For collection of 3T3-conditioned α-MEM medium, a similar procedure was followed. 3T3-F442A preadipocytes were seeded at a density of 25,000 cells/cm^2 in DMEM medium and medium was replaced by α-MEM medium when cells reached confluency. Every 3 days, halve of the medium was collected and replaced by fresh α-MEM medium. Control medium was collected under the same culture conditions.

The degree of differentiation of the 3T3-F442A preadipocytes was monitored by Oil Red O staining of intracellular lipid droplets. Cells were washed with phosphate buffered saline (PBS) and fixed for 5 min with a 15% glutaraldehyde solution in PBS. Cells were washed with PBS, stained for 2 h with a 0.2% Oil Red O solution and washed and kept in tissue culture water. The Oil Red O stained lipid droplets were characterized by light microscopy (Zeis Axiovert 40 CFL microscope).

Co-culture of 3T3-F442A cells with primary rat beta cells

It takes 8–12 days for 3T3-F442A preadipocytes to differentiate into adipocytes, as observed by Oil Red O stained lipid droplets and the expression of adipogenic markers. Only after entering this differentiation process, these cells can be expected to secrete significant levels of adipokines. The period of 9 days was chosen in this context and with the aim to examine the chronic influence of co-culture on both cell types. The follow-up period was 3 days. This period was chosen to be able to evaluate reversible changes (expected to normalize within 48 h) in phenotype over irreversible changes (typically maintained over a longer period). Beta cells can be kept in culture without major changes in vitality, cell numbers and phenotype for a period up to 16 days [23].

Unstimulated 3T3-F442A cells were plated as a monolayer in 24-well plates at a density of 20,000 cells/well (25,000 cells/cm^2) in DMEM with 10% iron-supplemented bovine calf serum to keep the cells undifferentiated [5]. 24 h after seeding, the medium was replaced by α-MEM medium containing 180 or 360 mg/dl glucose, 0.075 mg/ml penicillin, 0.1 mg/ml streptomycin, and 10% fetal calf serum, and freshly isolated beta cells, which are suspension cells, were added to the 3T3-F442A cultures using cell culture inserts (3 μm, Perkin Elmer, Germany) at a density of 12,500 cells/ml (the total volume in the well and insert), which is our standard density to evaluate effects on beta cell function and insulin production capacity [23]. The methods for rat islet isolation, dissociation and purification of islet beta cells have been previously described [24]. Procedures for animal experiments and sacrifice were approved by the local Ethical Committee for Animal Experimentation of the Vrije Universiteit Brussel. All manipulations were carried out in accordance with the European Community Council Directive (86/609/EEC). The purified beta cell preparation consisted of more than 90% insulin-positive cells [24–26] Unstimulated 3T3-F442A cells and rat beta cells were also cultured separately as controls. Beta cells cultured without 3T3-F442A cells in inserts with Ham F10 medium containing 180 mg/dl glucose, 0.5% BSA, 2 mM L-glutamine, 0.075 mg/ml penicillin, and 0.1 mg/ml streptomycin served as an internal standard.

Half of the medium was refreshed every 48 h and part of the medium was collected to determine the insulin release. Insulin concentrations were measured after 40 h, 64 h, and after 8, 9, and 11 days of culture. Beta cells were collected at start and after 64 h, 9 and 11 days to measure their cellular insulin content. Medium and cellular insulin concentrations were measured by radioimmunoassay as described prior [27] and glucose concentrations were

measured using Glucocard Memory strips (code DC 384 Menarini Diagnostics). Adiponectin concentrations were measured using an enzyme linked immunosorbent assay (R&D Systems, Minneapolis, MN). The beta cells were stained with Hoechst-Propidium iodide to evaluate their viability after 3 and 9 days of culture [28].

Insulin degradation assay

Insulin degradation assay was performed by adding 50 µl of radiolabelled ^{125}I-insulin to the cell and cell free culture medium on day 2 for short-term culture and on day 8 for long-term culture followed by 24 h incubation at 37°C. The percentage of intact ^{125}I-insulin in each sample was determined by 10% TCA precipitation as described prior [29].

Determination of gene expression levels

Total DNA-free RNA was extracted using the RNA Easy Qiagen kit (Qiagen, Valencia, CA), and the concentration was determined using the RiboGreen RNA quantification kit (Molecular Probes, Eugene, OR). The expression of the different adipocyte factors was determined by quantitative real-time PCR (7500 Fast Real-Time PCR System, Applied Biosystems) and normalized to the house-keeping gene β-actin. The following primers, probes, and gene assays (Applied Biosystems) were used: mPref1 fw: aaccatggcagtgcatctg; mPref1 rev: agcattcgtactggcctttc; mPref1 probe: aaatagacgttcgggcttgcacctc; mGPDH fw: ggtggcagaggcctttg; mGPDH rev: tgcccatttagcatctcctt; mGPDH probe: tcgaactggaaagtccattgagcagc; mPPAR-γ fw: ctgtcgg tttcagaagtgcct; mPPAR-γ rev: atctccgccaacagcttctc; mPP AR-γ probe: cccaaacctgatggcattgtgagaca; maP2 fw: ccttca aactgggcgtgg; maP2 rev: cgttttctctttattgtggtcgact; maP2 probe: atgctcttcaccttcctgtcgtctgcg; mLeptin fw: caaaaccctc atcaagaccattg; mLeptin rev: agtccaagccagtgaccctct; mLeptin probe: ttcacacacgcagtcggtatccgc; mAdiponectin: Mm00 456425_m1; mTNF-α: Mm00493736_m1; mIL-6: Mm004 46190_m1; mGlut4: Mm00436615_m1; β-actin: Mm0120 5647_g1. Expression levels of target genes were expressed versus a chosen calibrator (comparative $\Delta\Delta C_t$ method, Applied Biosystems).

Data analysis

Data are expressed as means ± SEM. Statistical significance for differences between groups is analyzed by non-parametric t-testing. Significance is set at $P < 0.05$.

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