

Insulin-like growth factor 2 enhances insulinogenic differentiation of human eyelid adipose stem cells *via* the insulin receptor

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Abstract

Objectives: Previously, we have isolated stem cells (HEAC) from human eyelid adipose tissue and functionally differentiated them into insulin-secreting cells. In the present study, we examined whether insulin family members might influence insulinogenic differentiation of HEAC.

Materials and methods: Following culture in differentiation media containing insulin family member or not, cells were examined for gene expression, protein expression and, particularly, insulin and C-peptide secretion, in response to high glucose challenge. Using antibodies against the specific receptor, target receptor mediating effect of the insulin family member was investigated.

Results: Insulin treatment during culture had little effect on either insulin or C-peptide secretion from HEAC, against high glucose challenge after culture. However, insulin-like growth factor (IGF) 1 treatment decreased both secretions, and interestingly, IGF2 greatly increased the secretions. HEAC treated with IGF2 had strong expression of *Pdx1*, *Isl1*, *Pax6* and *PC1/3* genes, and distinct staining after insulin and C-peptide antibodies, and dithizone. IGF2-enhanced insulinogenic differentiation was totally blocked by antibody against insulin receptor (IR), but not by anti-IGF1 receptor (IGF1R). Differentiated HEAC expressed both *IR* and *IGF1R* genes, whereas they expressed neither *IGF2* nor *IGF2R* genes.

Conclusions: From these results, it is suggested that IGF1 might inhibit insulinogenic differentiation of HEAC, whereas IGF2 enhances differentiation, and

that enhancement of IGF2 appeared to be mediated via IR.

Introduction

Insulin and insulin-like growth factor (IGF) family members have been studied as regulators of β -cell mass. Exogenous treatment by insulin at low nanomolar concentrations has been shown to reduce apoptosis of rat primary β cells induced by low cell density and low glucose (1) and of primary human and murine islet cells induced by serum withdrawal and low glucose (2,3). Insulin at high concentration has also been shown to increase proliferation of mouse primary islet cells (4). However, some studies have shown that high doses of insulin do not reduce apoptosis of primary human and mouse islet cells (2), but increase caspase-3-mediated cell death in a glucose-deprived apoptosis model of a mouse pancreatic β -cell line (5). IGF1 induces proliferation of rat INS-1 cells in a glucose-dependent manner (6,7) and also prevents cytokine-mediated apoptosis of islet cells from non-obese diabetic mice (8) and serum withdrawal-induced apoptosis of both human and rat primary islet cells (3). Several studies, however, have shown that liver-specific or pancreas-specific inactivation of *IGF1* gene causes pancreatic islets to enlarge, suggesting that IGF1 may serve an inhibitory function on islet growth *in vivo* (9,10). Treatment with IGF2 has been shown to protect against cytokine-mediated apoptosis of neonatal rat primary pancreatic islets *in vitro*, and the effect diminished when islets were incubated with neutralizing antibody against IGF2 (11). Overexpression of IGF2 during foetal development causes islet hyperplasia and low islet cell apoptosis (12). IGF2 supplementation increases the pool of β cells in pancreatic rudiments isolated from diabetic GK rats in which foetal production of IGF2 was impaired (13,14). Thus, IGF2 appears to protect islet cells from apoptosis and may serve as a survival factor. However, the above studies used β cells which already were determined or differentiated

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in vivo, and thus little is known about their roles during differentiation of stem/progenitor cells into β cells.

Various human cells can differentiate into insulin-secreting cells *in vitro*. Their transplantation *in vitro* has been shown to normalize or reduce blood glucose levels in animals of diabetic models (15–20). Results of some studies are promising for clinical application of insulin-secreting cells to treatment of human with type 1 diabetes, however, insulin-secreting cells to be used therapeutically need to secrete insulin in response to high glucose stimulation and to secrete high enough levels of it to regulate blood glucose level of the recipients. Researchers thus have been trying to develop various conditions for differentiation of various cell types into insulin-secreting cells. Many studies have used insulin or IGFs for insulinogenic differentiation of human embryonic stem cells (16,17,21,22), adult stem cells (18,23) or further types of cells (24,25); however, these studies have not clarified whether addition of insulin or IGFs promoted differentiation or not and thus, little is known concerning roles of these factors during differentiation of undetermined cells into insulin-secreting cells.

Insulin and IGFs function primarily through insulin receptors (IR) including IR-A and IR-B, and IGF receptors including IGF1R and IGF2R, respectively (26). However, at high nanomolar concentrations, insulin activates the IGF1R, and IGFs can activate IR (27). In humans, adult pancreatic β cells express both mRNA forms of IR, IR-A and IR-B (28,29). Expression of IGF1R mRNA has been observed in intact tissues and cultured islets of foetal pancreas (30), and in adult pancreatic tissues (31). Expression of IGF2R mRNA and strong immunoreactivity to anti-IGF2R antibody have been observed in normal pancreatic tissues and in cytoplasm of islet cells, respectively (32).

In the present study, we have examined the effect of insulin and IGFs on differentiation of human eyelid adipose-derived stem cells (HEAC) into insulin-secreting cells. During insulinogenic differentiation culture, insulin treatment exhibited little effect and IGF1 had inhibitory effects; in contrast, IGF2 greatly enhanced differentiation. Interestingly, when cells were treated with IGF2 and anti-IR antibody together, their differentiation drastically decreased. These results suggest that IGF2 could enhance insulinogenic differentiation of HEAC through IR signalling.

Materials and methods

Reagents

All reagents used were purchased from Sigma (St Louis, MO, USA) unless specified otherwise. DMEM media and

type I collagenase were supplied by the Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was supplied by Hyclone (Logan, UT, USA) and Activin A, basic fibroblast growth factor (bFGF), IGF1 and IGF2 were supplied by Peprotech (Rocky Hill, NJ, USA). Mouse monoclonal antibodies to human proinsulin, insulin, IR and IGF1R were purchased from Abcam (Cambridge, MA, USA). Mouse monoclonal antibody to human C-peptide was supplied by Monosan (AM Uden, Netherland) and ELISA kits for human insulin and C-peptide were supplied by Mercodia (Winston Salem, NC, USA). Biotinylated goat anti-mouse IgG, horseradish peroxidase-conjugated streptavidin and 3,3'-diaminobenzidine tetrahydrochloride were purchased from DakoCytomation (Glostrup, Denmark). Human adult pancreatic mRNA (hPan) and human HepG2 mRNA were purchased from Ambion (Austin, TX, USA).

HEAC isolation and culture

Human eyelid adipose tissue was obtained from three subjects aged between 28 and 42 years (mean age: 36.2 years) undergoing cosmetic surgery, after providing informed consent. Fat tissues were surgically dissected from the subcutaneous zone and adipose-derived stem cells (HEAC) were obtained as previously described (20). Briefly, fat tissue was minced and treated with 0.075% type I collagenase in phosphate-buffered saline (PBS) for 30 min at 37 °C with gentle stirring. Following centrifugation at 1250 *g* for 10 min, cell pellets were isolated and washed twice in DMEM-low glucose type (5.5 mM; DMEM-LG). Cell suspensions were cultured in DMEM-LG supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 3.7 mg/ml sodium bicarbonate at 5% CO₂, 37 °C. After 2 weeks in culture, adherent cells were obtained and subjected to passage culturing; afterwards, medium was changed twice a week. All experiments were approved by Institutional Review Boards of Seoul Women's University.

Differentiation of HEAC into insulin-secreting cells *in vitro*

HEAC at passage (p) 3 to p5 were divided into five groups and plated on 48-well dishes (Nunc, Rochester, NY, USA) at 5×10^3 density cells/well and grown for 3 weeks. For control groups (C), cells were cultured in DMEM-high glucose type (25 mM; DMEM-HG) containing 10% FBS for 1 week, and then in DMEM-LG containing FBS for additional 2 weeks. For other experimental groups, cells were grown for 1 week in DMEM-HG containing 10% FBS, 4 nM activin A, 20 ng/ml bFGF, and 10 nM glucagon-like peptide (GLP)-1. During the subsequent

2 weeks, a group of cells (N group) was grown in DMEM-LG containing 10% FBS, 10 mM nicotinamide, 4 nM activin A, and 10 nM GLP-1 (NAG). Cells of other groups were grown in the same medium as N group, but supplemented with 50 ng/ml human insulin (Insulin group), IGF1 (IGF1 group) or IGF2 (IGF2 group).

Receptor blocking

To identify IGF2 target receptor molecule, cells were initially cultured for 1 week in DMEM-HG containing 10% FBS, 4 nM activin A, 20 ng/ml bFGF and 10 nM GLP-1. Then they were cultured for 2 weeks, for further differentiation in DMEM-LG containing 10% FBS, 10 mM nicotinamide, 4 nM activin A, 10 nM GLP-1 and 50 ng/ml IGF2. During the last 2 weeks in culture, 1 nM IR antibody, 1 µg/ml IGF-1R antibody or both antibodies were added to culture media. After 3 weeks differentiation culture, amounts of insulin and C-peptide released into culture media were measured using ELISA, as described below.

Measurement of insulin and C-peptide

To measure amounts of insulin and C-peptide released *in vitro*, cells after differentiation culture were incubated in DMEM-LG containing 0.5% bovine serum albumin (BSA) for 12 h. They were washed in PBS, and subsequently stimulated with DMEM containing 5.5, 15.0 or 25.0 mM glucose for 2 h at 37 °C. Amounts of insulin and C-peptide released into these stimulating media were measured using ultrasensitive human insulin and C-peptide ELISA kit according to the manufacturer's instructions.

RT-PCR

RNA was isolated using Tri-reagent according to the manufacturer's instructions. Purity of RNA was assessed by determining ratio of absorbance at 260 nm to that at 280 nm (>1.8) and RT-PCR was performed using a GeneAmp PCR system 2400 (Perkin Elmer, Boston, MS, USA). Subsequent PCR reactions were performed using cDNA, primer pairs (Table 1) and PCR mixture (Fermentas, St Leon-Rot, Germany) according to manufacturers' instructions. Amplified products were separated on 1.5% gel and visualized with ethidium bromide under a gel scanner (Ultima; Hoefer, Holliston, MA, USA). Transcript levels of genes were standardized to corresponding GAPDH level and relative mRNA levels were obtained.

Immunocytochemistry

After culture in Lab-Tek chamber slides (Nunc), HEAC were washed in PBS then permeabilized in 0.5% Triton

X-100 for 10 min. Endogenous peroxidase activity was inactivated using 3% hydrogen peroxide for 15 min at room temperature (RT). Slides were incubated in blocking solution, consisting of 2% BSA in PBS for 1 h at RT. Between each step, cells were washed in 0.1% BSA in PBS. Cells were incubated with primary antibodies overnight at 4 °C; the following primary antibodies against human proteins were used: mouse monoclonal antibodies to C-peptide (1:200), proinsulin (1:200) and insulin (1:200). After labelling with primary antibodies, cells were incubated in biotinylated goat anti-mouse IgG followed by horseradish peroxidase-conjugated streptavidin for 20 min, respectively, at RT. Immunoreactivity was visualized using 3,3'-diaminobenzidine tetrahydrochloride counterstained with Mayer's haematoxylin. Cells were photographed using bright-field microscope illumination (Axioskop2+; Carl Zeiss, Jena, Germany).

Dithizone staining

Cultured cells were incubated in 100 µg/ml dithizone (DTZ) solution at 37 °C for 15 min.

Statistical analysis

Data were expressed as mean ± SEM. Statistical significance was analysed by one-way ANOVA test using SPSS 18.0 (Somers, NY, USA). A *P*-value <0.05 or 0.01 was considered statistically significant.

Results

During maintenance culture up to p5 in DMEM containing FBS alone, morphology of HEAC remained unchanged. After culture in the five different differentiation culture conditions (Fig. 1A), amounts of insulin and C-peptide released by HEAC in response to 25.0 mM glucose was analysed using ELISA. As shown in Fig. 1B, HEAC of the control group demonstrated little (1.4 ± 0.7 pg/ml) insulin secretion, whereas those of N group secreted 113.9 ± 10.7 pg/ml. Addition of insulin to the N conditions, however, resulted in 288.3 ± 30.6 pg/ml insulin secretion. IGF1 addition instead of insulin somewhat lowered insulin secretion to 53.6 ± 20.2 pg/ml. Strikingly, IGF2 addition greatly increased insulin secretion to 950.1 ± 151.5 pg/ml. Amounts of C-peptide secreted from each HEAC group exhibited similar patterns, 14.8 ± 3.9 pg/ml by controls, 268.1 ± 60.3 pg/ml by N group, 167.0 ± 33.2 pg/ml by Insulin group, 55.4 ± 24.1 pg/ml by IGF1 group and 1257.5 ± 94.0 pg/ml by IGF2 group (Fig. 1C). These results demonstrated that IGF2 could strongly enhance differentiation of HEAC into

Table 1. Primers used for the RT-PCR analysis and expected size of PCR products

Gene	Primer sequence	Size (bp)	Annealing temp. (°C)	Reference
GAPDH	5'-aca act ttg gta tcg tgg aa-3' 5'-aaa ttc gtt gtc ata cca gg-3'	456	53	NM 002046
Insulin	5'-aac caa cac ctg tgc ggc tc-3' 5'-aag ggc ttt att cca tct etc tcg-3'	320	59	J00265
NeuroG3	5'-cgt gaa etc ctt gaa ctg agc ag-3' 5'-tgg cac tcc tgg gac gag ttt c-3'	211	61	AF 234829
pdx1	5'-ccc atg gat gaa gtc tac g-3' 5'-gtc etc etc ctt ttt cca c-3'	262	54	NM 000209
Glucokinase	5'-gaa tac ccc cca gag acc ttt tc-3' 5'-ggg ttc ttc ctg agc cag cg-3'	182	61	M90299
Isl1	5'-agcatcaatgctcctcaactcc-3' 5'-tgttggcaaggcaatgacc-3'	493	57	NM 002202
Nkx6.1	5'-acacgagaccacttttccg-3' 5'-tgctggactgtgcttctcaac-3'	336	59	NM 006168
Glut 2	5'-agc ttt gca gtt ggt gga at-3' 5'-aat aag aat gcc cgt gac ga-3'	300	57	NM 000340
NeuroD1	5'-cag aac cag gac atg ccc-3' 5'-atc aaa gga agg gct ggt g-3'	216	60	NM 002500
PC1/3	5'-ttg gct gaa aga gaa cgg gat aca tct-3' 5'-act tct ttg gtg att gct ttg gcg gtg-3'	457	60	NM 000493.3
PC2	5'-gca tca agc aca gac cta cac tcg-3' 5'-gag aca caa cca ccc ttc atc ctt c-3'	309	63	NM 003594.2
GLUT1	5'-ctc act gct caa gaa gac atg g-3' 5'-ctg ggt aac agg gat caa aca g-3'	366	60	NM 006516.1
Nkx 2.2	5'-ttc tac gac agc agc gac aac c-3' 5'-cgt cac etc cat acc ttt etc g-3'	393	56	NM 002509.2
IGF I	5'-cac tgt cac tgc taa att ca-3' 5'-tga aat aaa agc ccc tgt gtc t-3'	386	55	NM 00618.3
IGF II	5'-ctg gag acg tac tgt gct ac-3' 5'-ggg gtt taa agc caa tcg att-3'	547	55	NM 000612.4
IR	5'-cat caa ggg tga ggc aga ga-3' 5'-ttg gcc tca tct tgg ggt tg-3'	710	59	NM 000208.1
IGF IR	5'-tgg agt gct gta tgc etc tg-3' 5'-tga tga cca gtg ttg gct gg-3'	329	59	NM 000875.3
IGF IIR	5'-tca aca tct gtg gaa gtg tgg a-3' 5'-gaa tag aga agt gtc cgg atc gga gtc-3'	430	59	NM 000876.2

insulin-secreting cells and that IGF1 might be an inhibitor of insulinogenic differentiation. Insulin did not appear to affect differentiation.

To examine whether secretion of insulin and C-peptide from differentiated HEAC might occur in a glucose-dependent manner, cells incubated in presence of 5.5, 15 or 25 mM glucose concentration for 2 h then cultured in medium, were analysed. HEAC of the control group secreted 7.5 ± 3.4 , 15.3 ± 3.0 and 11.4 ± 2.9 pg/ml insulin in response to the above glucose concentrations, respectively. However, cells of the N group secreted 15.3 ± 3.0 , 52.2 ± 2.8 and 127.3 ± 7.8 pg/ml insulin; cells of IGF2 group secreted 25.7 ± 2.6 , 113.9 ± 1.0 and 687.8 ± 58.1 pg/ml insulin, respectively (Fig. 2A). Amounts of C-peptide released into the medium were 9.4 ± 1.5 , 12.4 ± 5.1 and 9.4 ± 11.1 pg/ml for control group, and 6.7 ± 4.0 , 96.6 ± 36.3 and 242.9 ± 40.0 pg/ml N group,

and 17.1 ± 7.0 , 407.1 ± 17.4 and 1147.1 ± 220.0 pg/ml IGF2 group, respectively (Fig. 2B).

RT-PCR analyses showed that control group HEAC expressed genes coding for glucokinase, NeuroD1, and Isl1, but did not express other β -cell-related genes such as *Ngn3*, *Nkx2.2*, *Pdx1*, *glut2*, *Pax6*, *PC1/3*, *Nkx6.1* and *Ins* (Fig. 3A). In contrast, HEAC of IGF2 group began to express *Ngn3*, *Nkx2.2*, *Pdx1* and *glut2* genes during the first week of insulinogenic differentiation, *Pax6* gene during the second week, and *PC1/3*, *Nkx6.1* and *insulin* genes during the (final) third week. Although HEAC of N group also showed a similar expression pattern, IGF2 group HEAC had a greater gene expression of *Pdx1*, *Isl1*, *Pax6* and *PC1/3*. Compared to human pancreatic tissue (hPAN), IGF2 group HEAC of had weaker expression of *glut2* gene, but enhanced expression of *Isl1* and *Pdx1*. Immunocytochemical staining showed that IGF2 group

(A)

1 week	2 weeks
DMEM-HG + 10% FBS	DMEM-LG + 10% FBS : C group
+ activin A	+ nicotinamide + activin A + GLP-1 : N group
+ bFGF	+ nicotinamide + activin A + GLP-1 + insulin : Ins group
+ GLP-1	+ nicotinamide + activin A + GLP-1 + IGF1 : IGF1 group
	+ nicotinamide + activin A + GLP-1 + IGF2 : IGF2 group

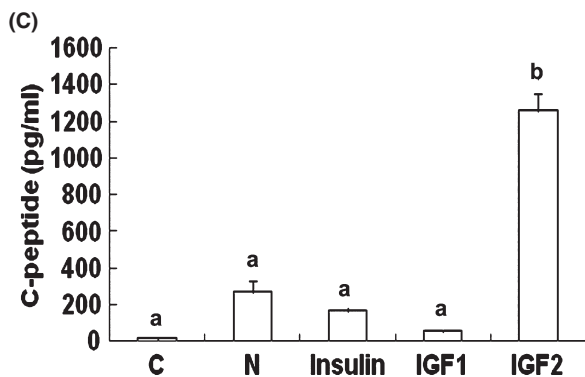
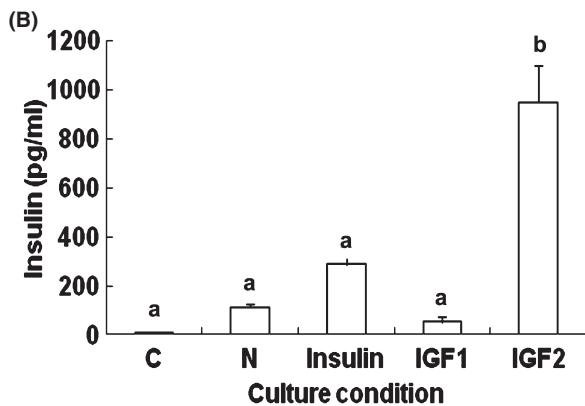


Figure 1. Effects of insulin families on differentiation of HEAC into insulin-secreting cells. (A) Schematic diagram of experimental procedure. (B, C) ELISA analyses of insulin and C-peptide release from various adult stem cells after culture in control or insulinogenic differentiation media. Insulin (B) and C-peptide (C) released from HEAC after culture in various differentiation media for 3 weeks as described in the Materials and methods section. C, control group; N, NAG group; Insulin, Insulin group; IGF1, IGF1 group; IGF2, IGF2 group. Values were obtained from three independent experiments. a and b, $P < 0.01$ compared to C group, by ANOVA testing.

HEAC were strongly immunoreactive using antibodies to human proinsulin, insulin and C-peptide, compared to controls (Fig. 3B). DTZ, known to discriminate β cells from others, intensely stained cells of colony-forming area of IGF2 group (Fig. 3C). These results suggest that IGF2

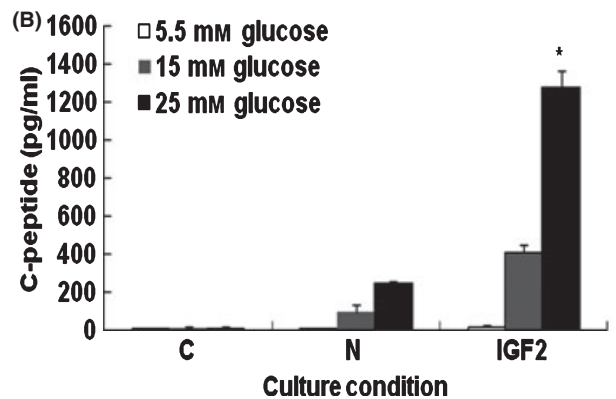
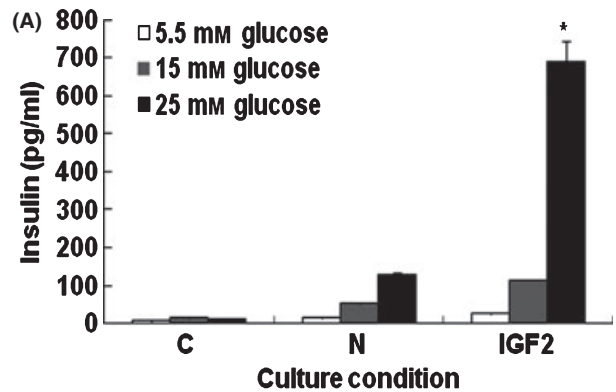


Figure 2. ELISA analyses of insulin and C-peptide release from HEAC in response to different concentrations of glucose. HEAC cultured in either DMEM containing FBS (control), DMEM containing FBS and NAG (N), or DMEM containing FBS, NAG and IGF2 (IGF2). Then cells were incubated in DMEM containing 5.5, 15.0 or 25.0 mM glucose concentration. Amounts of insulin (A) and C-peptide (B) released into each medium was measured. Values were obtained from three independent experiments. a and b, $P < 0.01$ compared to C group, by ANOVA testing.

group differentiated cells could functionally resemble β cells.

We also examined whether increased insulin and C-peptide secretion from IGF2 group HEAC was due to either enhanced differentiation or proliferation by IGF2. Cells were divided into four groups and then cultured. At the end of culture time, cell number of each group increased from the initial 5×10^3 to $5.0 \pm 1.1 \times 10^4$ control C group, $6.6 \pm 1.2 \times 10^4$ N group, $6.7 \pm 1.2 \times 10^4$ Insulin group and to $7.5 \pm 1.0 \times 10^4$ IGF2 group. To make satisfactory comparison, cell number of all groups was normalized to 5×10^4 cells and amounts of insulin and C-peptide secretion were analysed. When undifferentiated cells of C group secreted insulin of 9.6 ± 5.1 pg/ml/ 5×10^4 cells and C-peptide of 21.1 ± 2.0 pg/ml/ 5×10^4 cells at 25 mM glucose concentration, differentiated cells of N group secreted 94.5 ± 26.6 pg of insulin and 293.2 ± 50.9 pg of C-peptide under the same

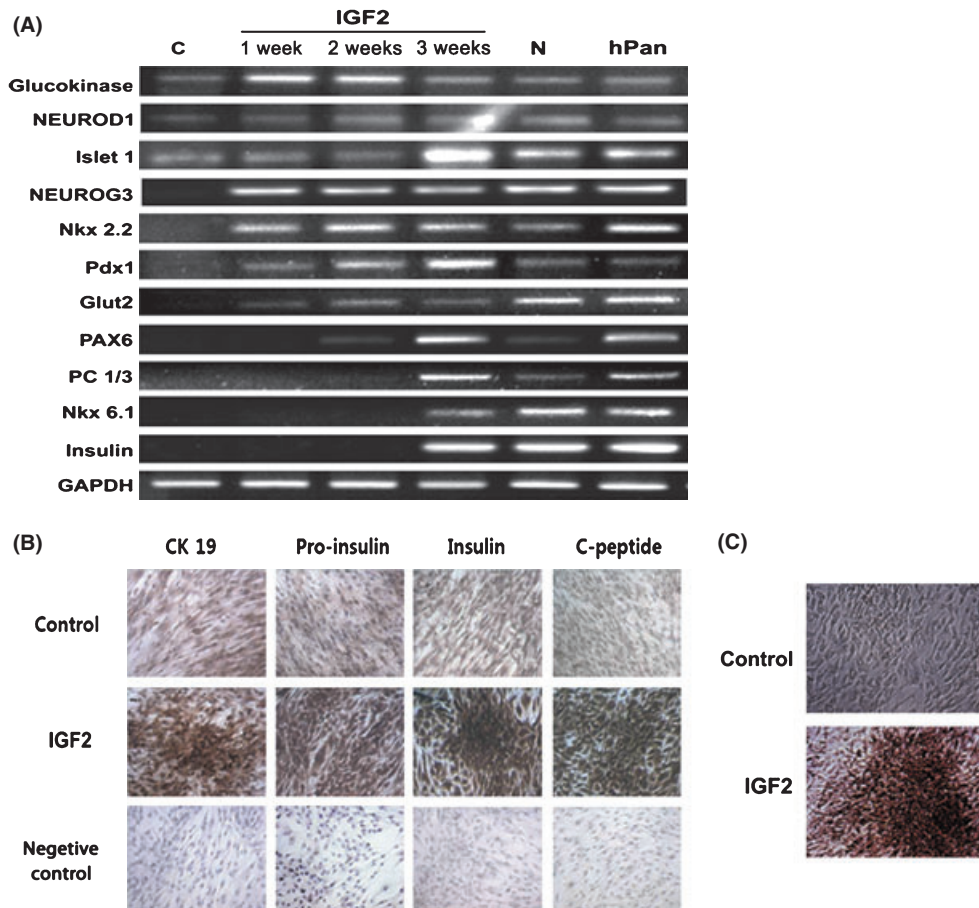


Figure 3. Gene expression profile of HEAC after culture in various differentiating media. C, HEAC cultured in DMEM containing FBS alone for 3 weeks; IGF2, HEAC cultured in DMEM containing FBS, NAG and IGF2 for 1 week (1 w), 2 weeks (2 w) or 3 weeks (3 w); N, HEAC cultured in DMEM containing FBS and NAG only; hPAN, human pancreatic cell mRNA.

conditions. In contrast, differentiated cells of the Insulin group secreted 196.5 ± 43.3 pg insulin and 261.2 ± 26.2 pg C-peptide, and cells of IGF2 group secreted 504.3 ± 76.2 pg insulin and 812.2 ± 174.2 pg C-peptide (Fig. 4A,B). Based on secretions of both insulin and C-peptide, the results show that insulin treatment did not enhance insulinogenic differentiation of HEAC, whereas IGF2 greatly enhanced it.

To find target receptor molecules that mediate the IGF2 effect, antibody to IR and/or IGF-1R were added to culture medium of IGF2 group during differentiation culture. After 3 weeks, amounts of insulin and C-peptide released by HEAC into the medium were analysed (Fig. 5). IGF2 group HEAC without antibody treatment demonstrated greatly enhanced insulin and C-peptide secretion in a glucose-dependent manner. In contrast, those treated with anti-IR antibody mostly had diminished secretion of both insulin and C-peptide such that amounts of both proteins were near quantities of the control group.

In contrast, both secretions from HEAC treated with anti-IGF1R antibody were not affected. Combined treatment of both anti-IR and anti-IGF1R antibodies resulted in diminished secretion similar to anti-IR antibody treatment alone. These results demonstrate that IGF2 signalling might be mediated *via* IR in HEAC during insulinogenic differentiation *in vitro*.

Finally, we investigated expression profiles of insulin- and its receptor-related genes including *IGF1*, *IR-A*, *IR-B* and *IGF1R* in IGF2 group HEAC, before and after differentiation (Fig. 6). Undifferentiated HEAC strongly expressed *IR-A* gene and showed weak expression of *IR-B*, *IGF1R* and *IGF2R*. They expressed neither *IGF1* nor *IGF2* genes. During the first week of differentiation, HEAC began to express *IGF1* and increased expression of *IR-B*, *IGF1R* and *IGF2R*. During second and third weeks of culture, interestingly, HEAC exhibited little expression of *IGF2R* and diminished expression of *IR-B*, *IGF1* and *IGF1R*, whereas they consistently exhibited strong

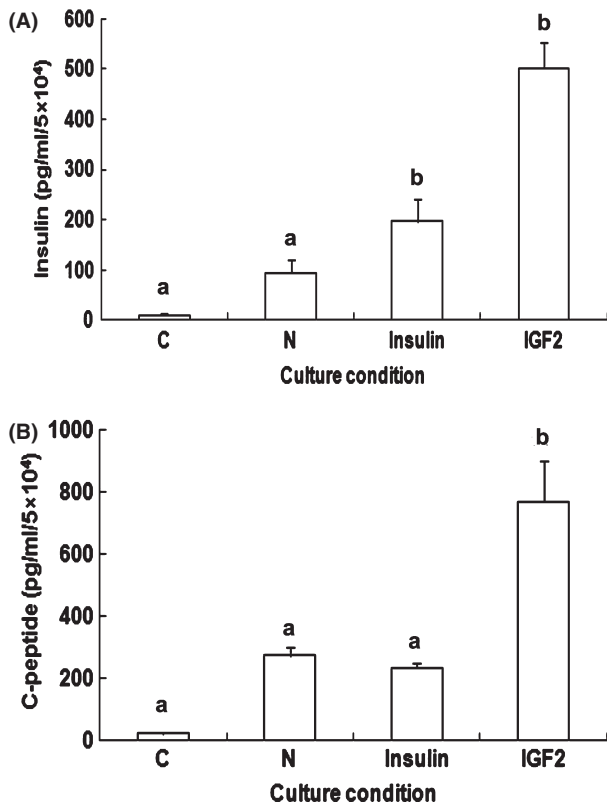


Figure 4. ELISA analyses of insulin and C-peptide release from various adult stem cells after differentiation. Cell number of all groups was normalized to 5×10^4 cells and amounts of insulin (A) and C-peptide (B) secretion in response to 25 mM glucose, compared to each other. C, control group; N, NAG group; Insulin, Insulin group; IGF2, IGF2 group. Values were obtained from three independent experiments. a and b, $P < 0.01$ compared to C group, by ANOVA testing.

expression of *IR-A*. HEAC never expressed *IGF2* gene whether differentiated or not. The human HepG2 hepatocyte cell line showed a distinct expression of all these genes. These results demonstrate that IGF2's effect cannot be mediated *via* IGF2R in HEAC during insulinogenic differentiation *in vitro*.

Discussion

In this study, we observed that IGF2 *in vitro* greatly enhanced differentiation of human somatic stem cells derived from eyelid adipose tissues, into insulin-secreting cells, while insulin had little effect and IGF1 inhibited their differentiation. Furthermore, IGF2-enhanced differentiation appeared to be mediated *via* IR of HEAC.

Many studies have shown that IGF2 has anti-apoptotic activity and/or increases survival of pancreatic β cells. IGF2 protects against cytokine-mediated apoptosis in primary pancreatic islets of neonatal rat (11), and increases the pool of β cells in pancreatic rudiments isolated from

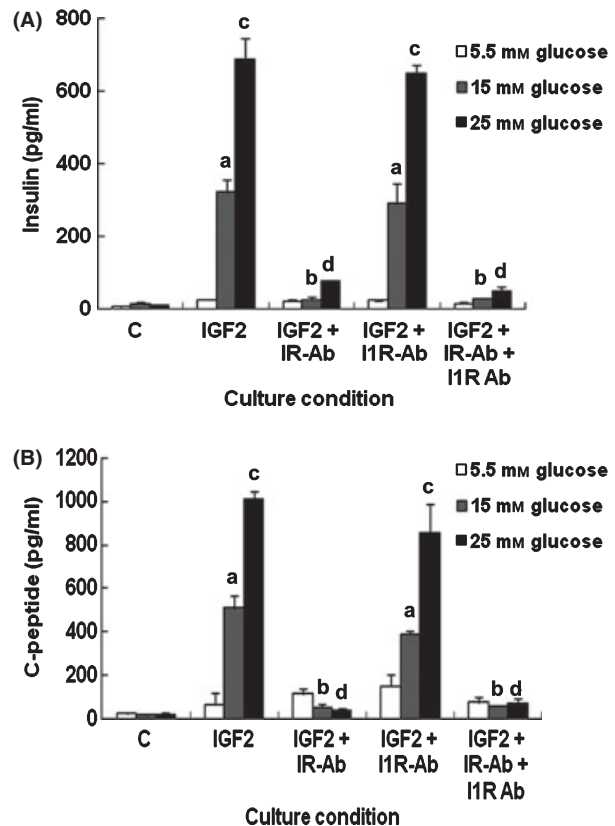


Figure 5. Effects of antibodies to IR and IGF1R on secretion of insulin and C-peptide by HEAC in the presence of IGF2. During the last 2 weeks of culture, for insulinogenic differentiation, 1 nM IR antibody, 1 μ g/ml IGF1R antibody or both antibodies were added to culture media. After culture, amounts of insulin and C-peptide release from cell into media were measured using ELISA. Values were obtained from three independent experiments. a and b, $P < 0.05$, c and d, $P < 0.01$ compared to IGF2 group, by ANOVA testing.

diabetic GK rats (13,14). Its overexpression during foetal development results in islet hyperplasia and reduced islet cell apoptosis (12). In the present study, IGF2 treatment caused marked increase in insulin secretion by HEAC following differentiation culture. Compared to our N group in which IGF2 was omitted, amounts of insulin and C-peptide secreted in response to high glucose increased more than 3-fold of each. Cell numbers in the same culture, however, increased only slightly. These observations demonstrate that the enhancing effect of IGF2 on insulinogenic differentiation of HEAC is caused mainly by its differentiation-inducing ability and little by cell proliferation-promoting activity.

HEAC differentiated in the presence of IGF2 showed no expression of either IGF2 or IGF2R, but distinctly expressed mRNAs of insulin, IGF1 and their receptor genes including *IR-A*, *IR-B* and *IGF1R*. Thus, it is not likely that IGF2 elicited its differentiation-enhancing

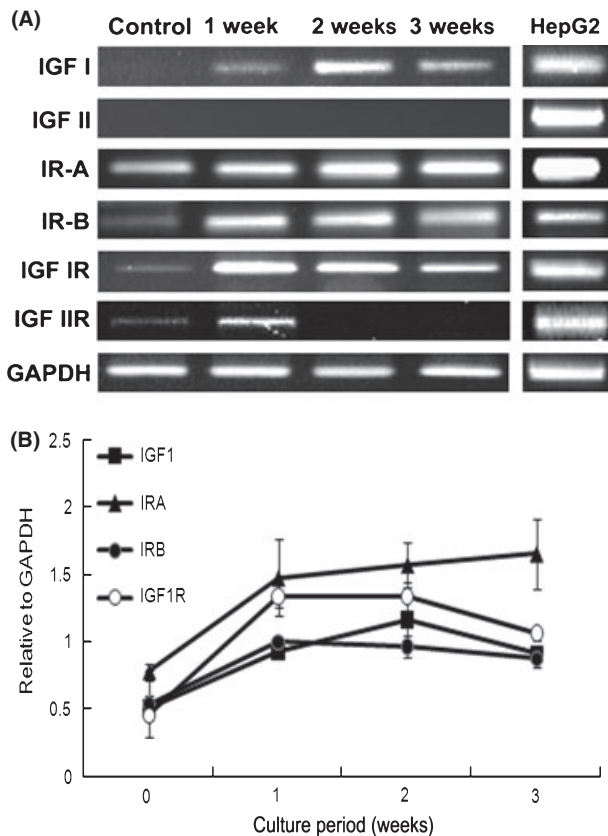


Figure 6. Insulin-related gene expression profile of HEAC during insulinogenic differentiation culture. (A) Gene expression profile of insulin and IR receptor families by HEAC during insulinogenic differentiation culture. (B) Semiquantitative densitometric analysis of selected products in (A). Values standardized to corresponding GAPDH. HEAC were prepared as shown in Fig. 3. For comparison, human HepG2 mRNA was used as human pancreatic cell mRNA library was devoid of both IGF2 and IGF2R mRNAs.

effect *via* IGF2's receptor, which serves as a clearance receptor that can modulate availability of extracellular molecule (33,34). IGF2 is more likely to act *via* other types of receptor. Insulin and IGFs act through their homo-receptors including IR-A, IR-B, IGF1R and IGF2R, or hybrid receptors including IR-A/IR-B, IR-A/IGF1R and IR-B/IGF1R. Of these receptors, IGF2 could bind IR-A, IGF1R and hybrid receptor of IR-A/IGF1R, but not bind IR-B and hybrid receptor of IR-B/IGF1R (35,36). However, antibody treatment experiments showed that anti-IGF1R antibody did not affect IGF2-induced differentiation, whereas anti-IR antibody mostly inhibited IGF2-induced differentiation. These results suggest that the IGF2 effect might be mediated by IR-A rather than by IR-B, IGF1R or hybrid receptors. IGF2 also plays a role in other differentiation processes; in mouse embryonic fibroblasts, interference of IGF2 production can inhibit production of both muscle-specific structural proteins and of

myocyte fusion to form multinucleate myotubes, suggesting that IGF2 plays a key role in satellite-cell differentiation (37). In this model of myogenic differentiation, however, IGF2 signals through IGF1R.

Unlike IGF2, insulin treatment did not elicit significant enhancing effects on insulinogenic differentiation of HEAC even though it has higher affinity to IR-A than IGF2 (36). One explanation is that insulin may act on HEAC in other ways from that of IGF2. An earlier study using IR⁻ mouse embryonic fibroblasts transfected with *IR-A* gene indicated that IGF2 was more effective than insulin in stimulating cell proliferation, while insulin was more potent than IGF2 in stimulating glucose uptake (35). Moreover, two types of IR have been proposed to play different roles in pancreatic β cells of β IRKO mice. Insulin secreted by pancreatic β cells on glucose stimulation activates its own insulin gene *via* IR-A, while it simultaneously stimulates transcription of β -cell *glucokinase* gene *via* IR-B (38). Microarray studies of R⁻/IR-A cells has revealed that while 214 transcripts were similarly regulated by either insulin or IGF2, 45 genes were differentially transcribed in response to only one of the two molecules (39). Differential roles of IR isoforms have also been demonstrated in other types of cells. Data obtained using murine 32D haematopoietic cells has indicated that IR-A responded to IGF2 and preferentially sent mitogenic, anti-apoptotic signals, whereas IR-B, poorly responsive to IGF2, tended to send differentiation signals (40). Immortalized neonatal hepatocytes from IR-knockout mice have shown that hepatocytes reconstituted with IR-A could fully restore glucose uptake up to levels observed in wild-type hepatocytes, whereas those with IR-B failed to do so (41). From these observations, it is suggested that binding of insulin to both IR-A and IR-B receptors during differentiation of HEAC might elicit different responses, unlike IGF2, which acts mainly through the IR-A receptor.

Insulin or IGFs have been frequently used in insulinogenic differentiation media for various human cells, *in vitro* (21,22,24,26,28). These studies demonstrated generation of insulin-secreting cells, as revealed by secretion of insulin or C-peptide from differentiated cells. However, these studies did not examine effects of removal of insulin or IGFs from medium upon differentiation. Thus, it is not clear whether such molecules actually promoted insulinogenic differentiation or not. In the present study, we have added a control group whose medium did not contain insulin or IGFs. Compared to this control group, we observed that insulin was hardly effective, and IGF1 was inhibitory in promoting differentiation in contrast, IGF2 markedly enhanced differentiation. Further studies will clarify whether these differential effects of insulin and IGFs on insulinogenic differentiation are limited to HEAC or exist in other types of cells too.

While a variety of human cell types have been examined for ability of differentiating into insulin-secreting cells, fewer studies have been conducted using human normal somatic cells. As use of adult stem cells is free from ethical issue associated with embryonic stem cells, and are not tumorigenic *in vivo*, insulinogenic cells produced from them seem to be plausible for therapeutic applications. Some of these studies involve normalized or reduced blood glucose level following transplantation to diabetic animal recipients, and we have observed presence of only human insulin and C-peptide in blood of normalized mice, suggesting that human insulin was solely responsible for the normoglycemia (20). However, even in this study, success rate was limited to 50%, which was possibly due to insufficient amounts of insulin secretion from the differentiated cells compared to normal human islet cells (42). In the present study, using the same cells and culture conditions, we have observed that IGF2 treatment resulted in more than 3-fold increase in insulin secretion after insulinogenic differentiation of HEAC. Further studies will verify whether these *in vitro* differentiated cells could provide better results with respect to treatment of diabetic animals.

In conclusion, we have shown that IGF2 treatment greatly enhanced functional differentiation of HEAC into insulin-secreting cells *in vitro*, as seen in expression of β -cell-specific genes, β -cell-specific staining of cells, and particularly, markedly increased, high glucose-dependent secretion of both insulin and C-peptide from the differentiated cells. The effect of IGF2 appeared to be mediated *via* IR-A alone, as anti-IR antibody treatment almost ablated the enhancement effect of IGF2 while anti-IGF1R antibody treatment did not, and *IGF2R* gene was not expressed. These findings may be used not only for preparation of therapeutic cells applicable to type 1 diabetes but also for research into β -cell differentiation.

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Author disclosure statement

Authors confirm no competing financial interests exist.

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