Hepatocyte growth factor regulates proliferation and differentiation of epithelial monolayers derived from islets of postnatal rat pancreas

R Wang1,2, N Yashpal1, F Bacchus1 and J Li1

1Department of Physiology and Pharmacology, Lawson Health Research Institute, University of Western Ontario, London, Ontario, Canada
2Department of Medicine, Lawson Health Research Institute, University of Western Ontario, London, Ontario, Canada

(Requests for offprints should be addressed to R Wang, Child Health Research Institute, 5th Floor, 8000 Commissioners Road East, Room A5-146, London, Ontario N6C 2V5, Canada; Email: rwang@uwo.ca)

Abstract

Hepatocyte growth factor (HGF) has been suggested to be a potent regulator of β-cell function and proliferation. The purpose of this study was to investigate whether HGF could regulate the proliferation and differentiation of islet-derived epithelial monolayers into insulin-producing cells. We have generated islet-derived epithelial monolayers that are enriched with cells expressing c-Kit, a tyrosine kinase receptor and putative marker, from isolated postnatal rat islets. Monolayers were cultured on type I collagen gel and treated in defined differentiation medium with or without HGF (50 ng/ml) for 7 days. Subsequently, the expression of transcription factors and pancreatic endocrine cell markers as well as c-Kit expression were compared between the HGF (HGF+), no HGF treatment (HGF−) and monolayers without differentiation medium (control) groups, using immunocytochemical and RT-PCR approaches. We observed that the number of c-Kit-, glucose transport type 2 (Glut2)- and the transcription factor pancreatic duodenal homeobox-1 (PDX-1)-expressing cells were significantly increased in the HGF+ group. The expression of insulin at the mRNA and protein level was also increased in this treatment group with a 1.7-fold increase in basal insulin release and a 2.3-fold increase in insulin content in comparison with the HGF− group. A high proliferative capacity was also found in the HGF+ group. Co-localization of insulin and PDX-1 or Glut2 was revealed frequently in cells treated with HGF+ with occasional co-staining of c-Kit and insulin observed. This study showed that HGF can activate the proliferation and differentiation of islet-derived epithelial monolayer into insulin-producing cells. However, no formation of islet-like clusters was observed. Taken together, this study implies that HGF mediates differentiation of immature cell types into insulin-expressing cells; however, HGF supplementation alone is insufficient in restoring full β-cell function.


Introduction

Hepatocyte growth factor (HGF) is a mesenchyme-derived large multifunctional polypeptide paracrine growth factor. It exerts its effects through the heterodimeric transmembrane tyrosine kinase receptor, c-met, and has pleiotropic effects, inducing different cellular responses such as cell survival, cell proliferation, migration and differentiation (Gherardi & Stoker 1991, Zarnegar & Michalopoulos 1995, Wang et al. 2003). HGF in the pancreas is produced in pancreatic mesenchyme-derived cells and in islet cells. Previous studies have shown that HGF and its receptor are expressed preferentially in a specific spatial and temporal pattern in which maximal HGF and c-met expression correspond to a period of rapid proliferation and development of the endocrine and exocrine compartments, highlighting their role in pancreatic development (Beattie et al. 1996, Calvo et al. 1996).

Both in vivo and in vitro studies have demonstrated that HGF functions as an insulinotropic factor and promotes β-cell proliferation and regeneration of fetal and adult islets (Otonokoski et al. 1994, 1996, Hayek et al. 1995, Beattie et al. 1996, Gahr et al. 2002). In vitro, HGF has been shown to increase insulin content and proliferation of islets. In addition, intraperitoneal injection of HGF or exogenous HGF gene introduction in mouse models of diabetes has been shown to mitigate hyperglycemia (Nakano et al. 2000, Dai et al. 2003). Furthermore, transgenic mice that over-express HGF in islets are resistant to the diabetogenic effects of the β-cell toxin streptozotocin (Garcia-Ócana et al. 2001), and β-cell proliferation, islet cell mass and
hypoglycemia are increased. Thus it is speculated that HGF not only preserves β-cell mass but also protects pancreatic β-cells from destructive death, promotes β-cell proliferation and preserves insulin production. Finally, HGF alone or in combination with activin A is also able to induce the transdifferentiation of the amylase-secreting pancreatic acinar AR42J cells into insulin-producing cells (Mashima et al. 1996, 1999), suggesting that HGF may also be a β-cell differentiation factor.

Cell-based therapeutics such as islet cell transplantation offer a promising approach to the treatment of diabetes. However, for such therapies to be feasible a better understanding of the role of peptide growth factors such as HGF in the regulation of mature β-cell function and differentiation are necessary. Several studies have shown that it is possible to convert an acinar to duct cell phenotype from the pancreas of different species (Hall & Lemoine 1992, Arias & Bendayan 1993) or an acinar to islet cell phenotype under the influence of HGF (Mashima et al. 1996); however, the possibility of HGF influencing the change in phenotype from a ductal to islet cell type is limited.

It is well documented that islets in vitro become senescent, lose their function or, under certain conditions, transdifferentiate (Beattie et al. 1996, 2002). Our previous studies showed that islet isolation results in a loss of islet–matrix interactions, stability and the in vitro transdifferentiation of islets, with a loss of islet hormone expression and the concomitant up-regulation of cytoskeletal proteins characteristic of duct epithelial cells (Wang et al. 2001). Although these studies reveal the plasticity of islet cell phenotype, little is known of the factors or the potential to transform the epithelial phenotype back into an islet one. Our more recent study showed that these cytokeratin-expressing monolayers are enriched with cells that express markers of potential islet precursors such as the well-known hematopoietic stem cell marker c-Kit. Under the appropriate trophic support, we previously described that these c-Kit-expressing cells can differentiate and re-express insulin (Wang et al. 2004, Yashpal et al. 2004). Given HGF’s role in β-cell differentiation and function in islets, the present study has examined the potential of HGF to mediate the differentiation of islet-derived epithelial monolayers into insulin-producing cells. We hypothesized that supplementation of HGF might provide a novel strategy for increasing insulin-positive cells in vitro and regulate the differentiation of new islet cell formation.

**Material and Methods**

**Tissue culture**

Timed pregnant Wistar rats were obtained for the isolation of islets on postnatal day 7 (Charles River, Quebec, Canada). All protocols were approved by the Animal Care Committee at the University of Western Ontario, and were in accordance with the guidelines of the Canadian Council on Animal Care. Rat pancreata were dissected and islet isolation was carried out as described previously (Wang et al. 2004). To promote epithelial monolayer formation, freshly isolated islets were placed on type I collagen gel and cultured in Dulbecco’s modified Eagles’s medium (DMEM)/F12 (17·6 mM glucose; Gibco, Burlington, ON, Canada) serum-free medium supplemented with transferrin (10 µg/ml), insulin (1 µg/ml), and epidermal growth factor (10 ng/ml) (Sigma, St Louis, MO, USA) (Bonner-Weir et al. 2000, Wang et al. 2001). The cells of the epithelial monolayers, which had no detectable expression of endocrine hormones were sub-cultured into two experimental groups: an HGF-treated (HGF+; 50 ng/ml) (ID Laboratory Inc. London, ON, Canada) and a non-HGF-treated (HGF−) group cultured on type I collagen with a modified DMEM/F12 as a defined differentiation medium which contained 6·3 mM glucose and 5% fetal bovine serum (FBS) for a week (Wang et al. 2004). The epithelial monolayers cultured in DMEM/F12 serum-free medium served as a control group. Cultures were maintained in 95% air/5% CO2 at 37 °C, and the medium was renewed on alternate days. After 7 days of differentiation in culture, cells and medium were collected for the following investigations.

**Immunofluorescence and orphometric analysis**

Cells from HGF+ and HGF− control groups were fixed in situ with 4% paraformaldehyde (Wang et al. 2004) and immunostained using immunofluorescence, as described previously (Yashpal et al. 2004). The antibodies used in this study were as follows: mouse anti-human insulin and glucagon, mouse anti-5-bromo-2′-deoxyuridine (BrdU; Sigma), rabbit anti-c-Kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-Glut2 (Chemicon, Temecula, CA, USA) and rabbit anti-pancreatic duodenal homeobox-1 factor (PDX-1; a gift from Dr C Wright, University of Vanderbilt, Nashville, TN, USA). The sections were incubated overnight at 4 °C with the appropriate dilution of primary antibody. To evaluate cell proliferation, cells from all three groups were incubated with 10 µM BrdU for 4 h at 37 °C, followed by immunostaining for BrdU (Wang et al. 1994, 2004). To identify co-expression of phenotypes in both HGF+ and HGF− groups, double immunofluorescence staining was performed. Double-labeled images were recorded by a Leica DMIRE2 fluorescence microscope with the Openlab image software (Improvision, Lexington, MA, USA). Negative controls included the omission of the primary antibodies or secondary antibody. No staining was observed under the negative control conditions.

The number of cells expressing endocrine hormones, as well as c-Kit, PDX-1 and Glut2, and the BrdU-labeling index was determined using a Leica DMIRE2 microscope.
connected to computer-assisted image analysis software (Openlab version 1·3). The percentage of expression cells was determined by counting, at least 1000 cells for each experimental group was repeated six times. The data are expressed as a percentage of the total number of cells counted. Data are expressed as means ± s.e.m. and compared using a Student’s t-test, with differences considered to be statistically significant when \( P<0·05 \).

**Measurement of insulin secretion**

Insulin content and basal insulin release from HGF+ and HGF− control groups were measured using a rat insulin ELISA kit (DRG Diagnostic Inc. Mountainside, NJ, USA). The insulin content within the cells from each experimental group was determined and normalized to the DNA content and expressed as ng/µg DNA (Wang & Rosenberg 1999). Glucose responsiveness of insulin-expressing cells from both the HGF+ and HGF− groups was examined using an acute glucose challenge, as described previously (Wang & Rosenberg 1999).

**RT-PCR**

Total RNA was extracted at the end of the experiment using the RNAqueous-4 PCR kit (Ambion, Austin, TX, USA). cDNAs were synthesized from 2µg total RNA using oligo(dT) with the SuperScript First Strand Synthesis System (Invitrogen, Burlington, ON, Canada). Subsequent PCR was carried out in a Bimetra Tgradient (Montreal Biotech Inc., Montreal, QC, Canada) for 33 cycles. The following PCR oligonucleotide primers were used: (1) for insulin: forward-5′-TCACACCGT GTGGAAGCTC-3′, reverse-5′-AACTGGCCACGGCT TCTGC-3′ (28 cycles); (2) for glucagon: forward-5′-ATGAAGGAGCAAGCGC-3′, reverse-5′-TTCAC CAGCCAAGCAATG-3′; (3) for c-Kit (rat): forward-5′-AGCAAGAGTTAAGTGGCAGGAG-3′, reverse-5′-CCAGAAAAGTGTAAAGGCTCTCCT-3′; (4) for PDX-1: forward-5′-GGTGCCAGAGTTCCGAGTCTA-3′, reverse-5′-TTATTCCTTCCGTTGTTGC-3′; (5) for Neurogenin 3 (Ngn3): forward-5′-TGCGGCCT CATCCTTTGGGT-3′, reverse-5′-CAGTCACC ACCTTCTGCTCG-3′; and (6) β-actin: forward-5′- GACGCGGTCAACCAACTGTGCCCCATC-3′, reverse-5′-CTAGAAGCATTTGCGTGGAGCATGGA GG-3′ (28 cycles). The amplified products were analyzed on 1·5% agarose gels and visualized by ethidium bromide staining. The amount of PCR products generated was quantified by densitometric scanning of band intensities and normalized to the levels of the housekeeping gene β-actin using the Syngenetool gel analysis software (Syngene version 3·0, Cambridge, Cambs, UK) and expressed as relative expression units (Yashpal et al. 2000).

**Results**

**Effect of HGF on differentiation of epithelial monolayers into pancreatic endocrine cell phenotypes**

Previously we found that freshly isolated islets from postnatal day 7 cultured islets on type I collagen gel form epithelial monolayers that lack expression of islet hormones and express immature cell phenotypes such as c-Kit (Wang et al. 2001, 2004). Therefore, we examined the effect of HGF supplementation on the differentiation of islet-derived epithelial monolayers and evaluated differentiation through immunocytochemical staining for insulin and glucagon. In the control group, the epithelial monolayers contained only a few insulin- and glucagon-positive cells with the percentage being 1·2 ± 0·5% and 4·1 ± 1% respectively (Fig. 1). On the other hand, monolayers treated with supplemented 5% FBS plus HGF (HGF+ group) had a significant increase in the proportion of insulin- and glucagon-expressing cells with a 35-fold increase in the percentage of insulin (42 ± 3%)- and a 7-fold increase in the percentage of glucagon (28 ± 2%)-immunoreactive cells compared with control monolayers (P<0·001; Figs 1 and 2). In the HGF− group, a significantly high proportion of insulin- (P<0·001) and glucagon-immunopositive cells (P<0·01) were present in comparison with the percentage of control monolayers with 31 ± 2% insulin-expressing cells and 27 ± 4% glucagon-expressing cells (Figs 1 and 2). Both the HGF+ and HGF− groups had no islet-like clusters present (Fig. 2). In the HGF− group, there was no change in the expression of c-Kit (44 ± 2%), and a significant 2-fold decrease in the percentage of c-Kit-expressing cells was
observed in the HGF− group (23 ± 3%) compared with the expression of c-Kit in the control monolayers (Fig. 1; P<0·001).

Both PDX-1 and Glut2 are normally expressed in mature β-cells and are associated with early endocrine cell development (Pang et al. 1994, McKinnon & Docherty 2001). To delineate the morphometric changes in the expression of PDX-1 and Glut2, and their linkage to the HGF stimulation, we examined the number of cells expressing PDX-1 and Glut-2 in both the HGF+ and HGF− groups (Fig. 3). In a defined differentiation medium, the addition of HGF resulted in a 1·5- and 2-fold...
increase in the number of PDX-1- and Glut2-expressing cells compared with the group with no HGF supplementation (Fig. 3; P<0.05). Co-expression of PDX-1 or Glut2 with insulin was observed frequently in the HGF+ groups (Fig. 4). Interestingly, co-expression of c-Kit with insulin which is frequently observed in fetal rat islet cell development (Yashpal et al. 2004) was also detected after 1 week in the differentiation culture (Fig. 4).

HGF alters the expression of genes in epithelial monolayers

Given that HGF is a potent differentiation factor, we examined at the mRNA level whether or not HGF altered the expression of insulin or glucagon, the expression of the progenitor marker c-Kit and the transcription factors PDX-1 and Ngn3 at the mRNA level. With the addition of the growth factor HGF, the monolayers had increased expression of insulin and glucagon mRNA, a 6.5- and 2-fold increase respectively in comparison with control monolayers (Fig. 5). However, the increase was only significant for insulin mRNA levels (P<0.001). A similar expression pattern was observed in the HGF− group with slightly lower expression than that of the HGF+ group (Fig. 5). We also examined the expression of PDX-1 and Ngn3, transcription factors importantly expressed during pancreatic development (Pang et al. 1994, Sander & German 1997, McKinnon & Docherty 2001, Gu et al. 2003). Upon closer inspection, PDX-1 levels were only slightly lower expression than that of the HGF+ group. Ngn3 was highly expressed in the control monolayers and weakly in both the HGF+ and HGF− groups (Fig. 5). The expression of c-Kit mRNA reached a maximum in the control and HGF+ group, but was also expressed in the HGF− group (Fig. 5).

HGF promotes proliferation of epithelial monolayers

Given that HGF is a regulator of cell proliferation (Beattie et al. 1996, Burr et al. 1998, Kokuzawa et al. 2003), we also tested the ability of HGF to regulate proliferation of epithelial monolayers derived from postnatal rat islets. In the control monolayers, a highly proliferative population of cells was detected with a 34±3% BrdU-labeling index (Wang et al. 2004). There was a lower proliferative capacity present in both the HGF+ and HGF− groups in comparison with the control monolayers (Fig. 6A; P<0.01). HGF was able to stimulate a higher proliferation as a 1.8-fold increase in comparison with cells with no HGF treatment was observed (18±2 vs 10±3%); however, this increase was not statistically significantly different. Double immunostaining for BrdU and c-Kit in the HGF+ group showed that c-Kit-positive cells frequently labeled for BrdU after 1 week of culture (Fig. 6B).

HGF increases insulin content and secretion of epithelial monolayers

We also examined the ability of epithelial monolayers treated with HGF to re-store and secrete insulin. In control monolayers, basal insulin release was compromised such that insulin in the media was not detected. However, upon treatment with HGF, there was an up-regulation in insulin content present in cells within the control monolayers (0.49±0.18 vs 0.15±0.01 ng/µg DNA; P<0.01). No difference in basal insulin release was observed between the HGF+ and HGF− groups; however, there was 2-fold increase in the insulin content in the HGF+ group, significantly higher than that of the HGF− insulin levels (Fig. 7; P<0.05). Glucose stimulation studies showed that insulin-expressing cells present in the HGF+ and HGF− groups failed to respond to a 22 mM glucose challenge.

Discussion

HGF has previously been shown to be a potent regulator of β-cell function and to maintain mature β-cell phenotypes (Otonkoski et al. 1994, 1996, Hayek et al. 1995). Previous studies have demonstrated that islets in vitro on a type I collagen gel transdifferentiate into pancreatic ductal cells (Yuan et al. 1996, Wang et al. 2001), but the factors which confer morphogenetic stability on isolated islets and the possibility of reversing an islet from ductal phenotype has remained limited (Wang et al. 2004). Given the rather
poor stability of islets in vitro, the present study has demonstrated that peptide growth factors such as HGF may guide the differentiation of putative islet progenitors such as c-Kit-expressing cells into insulin-expressing cells. Taken together, this study suggests that HGF is involved in proliferation, differentiation and the partial restoration of β-cell function and islet cell phenotype.

The reversibility of transdifferentiation has been reported in other systems (Erenpreisa & Roach 1996). Stability of cellular phenotype is most likely regulated by cell–cell interactions or cell–substrate interactions, thus a disruption in cellular milieu may induce cells to change their commitment. Growth factors may essentially play a bi-potential role in either mediating the transdifferentiation of one cell phenotype into another or the reversibility of transitory unstable changes in cell phenotype. Our present studies have been concerned with the latter effects of growth factor function. Given the repertoire of cellular events that HGF regulates in the pancreas, it is plausible that HGF acts as an insulinotropic and β-cell-differentiating factor within our own monolayer system (Otonkoski et al. 1994, 1996, Hayek et al. 1995, Beattie et al. 1996, Mashima et al. 1996, 1999, Gahr et al. 2002). This role in our study is exemplified by insulin- and glucagon-expressing cell phenotypes subcultured in a modified defined differentiation medium (Wang et al. 2004) showing an increased proportion of insulin- and glucagon-expressing cells in comparison with the control monolayers and an increase in their mRNA expression with the addition of HGF.

Mashima et al. (1996, 1999) had previously revealed that, in combination with activin A, HGF is capable of directing the differentiation of the rat acinar cell line, AR42J, into insulin-producing cells with an altered expression profile of genes, including the up-regulation of islet cell genes. In the present study, monolayers that were cultured with a defined differentiation medium had a decreased expression profile of early embryonic β-cell

Figure 4 Co-expression of PDX-1, Glut2 or c-Kit (FITC) with insulin tetramethyl rhodamine isothiocyanate (TRITC) in epithelial monolayers cultured with HGF (HGF⁺) for a week determined by double immunofluorescence staining. Nuclei were counterstained by DAPI (arrows indicate the co-localization). Original magnification ×400.
transcription factors such as PDX-1 and Ngn3 in both HGF+ and HGF− groups, but an increase in insulin mRNA expression. In addition, HGF supplementation also results in a significant increase in the proportion of Glut2 immunopositive cells and a slight increase in PDX-1 expression, two important mature β-cell markers. Furthermore, we showed that HGF promotes the restoration of β-cell function identified by increased insulin content and basal insulin release but there was an inability of the HGF+ group to respond to a high glucose challenge.

Transdifferentiation involves cell proliferation and the appearance of a multipotential transdifferentiated intermediate cell (Yuan et al. 1996, Wang et al. 2001) which can express markers characteristic of several alternative phenotypes. This intermediate cell type may be c-Kit-expressing cells which either co-localize with the ductal marker cytokeratin 20 (Wang et al. 2004) or upon stimulation with HGF co-localize with insulin. c-Kit-expressing cells during early embryonic rat pancreatic development in our recent findings were shown to frequently co-localize with insulin and later lose their insulin expression in postnatal life, suggesting that mature β-cells may derive from these c-Kit-expressing cells and can be expanded in epithelial monolayer culture (Wang et al. 2004, Yashpal et al. 2004). c-Kit-expressing cells can also be immunomagnetically sorted from these monolayers and under the appropriate environmental cues can re-express insulin mRNA (Wang et al. 2004). Interestingly, in this study, we have revealed that HGF enhances the proliferative capacity of our monolayer system in comparison with HGF and augments the frequent co-localization of c-Kit with BrdU. Thus HGF may be responsible for directing a greater number of cells along their differentiation pathway towards a mature insulin-producing β-cell. Thus these results have demonstrated that it may be possible to expand a population of multipotential cells and then induce guided differentiation to a desired phenotype – in this case a mature insulin-producing β-cell.

The ability to manipulate and reconstitute physiological processes has tremendous clinical applications and is likely to play a relevant role in cell therapy. Our study has demonstrated that HGF is a potent growth factor that is

Figure 5 Semi-quantitative RT-PCR analysis of endocrine signals, developmental transcription factors and c-Kit mRNA in epithelial monolayers cultured with HGF (HGF+) or without HGF (HGF−) and control monolayers (Ctrl) on a type I collagen matrix for a week. Data are given as means ± S.E.M. *P<0.05, **P<0.01, ***P<0.001.
capable of partial restoration of β-cell function in epithelial monolayers and a stimulator of β-cell differentiation. However, it must be noted that complete restoration of β-cell function does not occur as no islet clusters form, and furthermore these cells respond poorly to a glucose challenge. Taken together, these results suggest that in vitro differentiation protocols must be further examined and may be facilitated by the addition of exogenous HGF to create conditions that match embryonic and fetal development such as the polarization/presence of other growth factors and suitable extracellular matrix proteins. This study clearly revealed that along with HGF’s known pancreatic roles, this peptide growth factor may also prove to be a useful tool to augment islet progenitor markers into insulin-expressing cells. Although further improvement is needed for the complete functional restoration and maturation in vitro, these results suggest and demonstrate that HGF supplementation offers a promising culture condition for the in vitro maturation of cytokeratin-expressing cells that express islet progenitor markers into insulin-expressing cells.

Acknowledgements

This work was supported by grants from the Canadian Institute of Health Research (CIHR). R W is supported by a University Faculty Award from the Natural Sciences and Engineering Research Council of Canada (NSERC).
References


Pang K, Mukonoweshuro C & Wong G 1994 Beta cells arise from glucose transporter type 2 (Glut2)-expressing epithelial cells of the developing rat pancreas. PNAS 91 9559–9563.

Sander M & German MS 1997 The beta cell transcription factors and development of the pancreas. Journal of Molecular Medicine 75 327–340.


Received 9 April 2004

Accepted 22 June 2004

Made available online as an Accepted Preprint 7 July 2004