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Selective effect of INGAP-PP upon mouse embryonic stem cell differentiation toward islet cells

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ABSTRACT

We evaluated the effect of islet neogenesis-associated protein pentadecapeptide (INGAP-PP) upon islet β- and non-β cell differentiation from mouse embryonic stem (mES) cells. ES-D3 cell lines were cultured following Lumelsky's protocol with or without INGAP-PP (5 μg/ml) at different stages. Gene expression was quantified using qPCR. mES cells were fixed and immunostained using anti insulin-, somatostatin-, glucagon-, Pdx-1-, Ngn-3-, Nkx-6.1 and PGP9.5 specific antibodies. PCNA was used to measure replication rate. Bcl_2 (immunostaining) and caspase-3 (enzyme activity and gene expression) were determined as apoptosis markers. INGAP-PP increased IAPP, Glut-2, Kir-6.2, SUR-1 and insulin gene expression, and the percentage of insulin-immunostained cells. Conversely, INGAP-PP reduced significantly glucagon and somatostatin gene expression and immunopositivity. While nestin gene expression was not affected, there was a significant reduction in the percentage of PGP9.5-immunostained cells. Pdx-1 gene expression increased by 115% in INGAP-PP treated cells, as well as the percentage of Pdx-1, Ngn-3 and Nkx-6.1 immunopositive cells. Neither caspase-3 (expression and activity) nor Bcl_2 positively immunostained cells were affected by INGAP-PP. Accordingly, INGAP-PP would promote stem cell differentiation into a β-like cell phenotype, simultaneously decreasing its differentiation toward non-β-cell precursors. Therefore, INGAP-PP would be potentially useful to obtain β-cells from stem cells for replacement therapy.

1. Introduction

Type 1 diabetes mellitus (T1DM) is the result of a progressive autoimmune destruction of β-cells [1]. This destruction is of such magnitude that the remaining β-cell mass is unable to maintain normal glucose homeostasis, so that patients are forced to receive continuous insulin treatment to survive [2]. Unfortunately, most patients treated with insulin do not attain an appropriate metabolic control to effectively prevent the development and progression of chronic complications [3]. Consequently, many researchers have actively attempted to replace the lost β-cells by pancreas or islet transplantation. Although significant progress has been made by the development of an islet transplant-protocol that successfully achieves insulin independence in transplanted patients [4], the scarce availability of tissue donors limits its wide application. The report that embryonic stem (ES) cells could differentiate into pancreatic islet-like structures [5] encouraged to use these cells as a potential source of surrogate β-cells for T1DM therapy [6–7]. Differentiation of stem cells toward a specific cell lineage requires the highly regulated as well as tightly and timely controlled transcription of several genes encoding structural and regulatory proteins. Thus, several attempts have been made to optimise the results by modifying the original protocol conditions and using several small bio-organic molecules [8–9].

During the last years our group has been studying one of these molecules, islet neogenesis-associated protein (INGAP), which was first identified in adult hamsters whose pancreas head was wrapped in cellophane [10]. Later, INGAP-immunopositive cells were identified in the pancreas of normal hamsters [11]. These immunoreactive INGAP-cells have also been reported during normal embryonic mouse pancreas commitment, thus providing evidence of its early presence and possible involvement in pancreas development and patterning [12]. Further, transgenic mice with targeted pancreatic expression of INGAP become resistant to diabetes induction by streptozotocin (SZT) injection [13]. Using a model of insulin resistance induced by dietary manipulation, we have consistently shown a simultaneous and significant increase in β-cell mass [11], INGAP-positive cell mass [14], glucose-induced insulin secretion, and the appearance of a possibly early islet precursor cell co-expressing INGAP/Pdx-1 [15]. On the other hand, it has been proved that a pentadecapeptide having the 104–118 amino acid sequence of INGAP (INGAP-PP) reproduces the effect of the intact molecule upon thymidine incorporation into ductal cells and a ductal cell line [16]. Injection of INGAP-PP to either normal or SZT diabetic mice is accompanied by an increase in β-cell mass and signs of islet neogenesis [17]. We have also shown that neonatal and adult

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rat islets cultured with INGAP-PP release more insulin in response to glucose and other secretagogues [18], and increase the expression of genes involved in the process of insulin secretion [19–20].

Based on these results, we have currently tested the effect of INGAP-PP upon differentiation of mouse ES (mES) cells toward insulin-producing and other islet cells.

2. Materials and methods

2.1. Reagents

DMEM and DMEM/F-12 tissue culture media, glutamine, non-essential amino acids, bFGF and Pluronic F-68 were obtained from Invitrogen (Karlsruhe, Germany). Fetal calf serum (FCS) embryonic stem cell grade and gentamicin were purchased from PAA (Vienna, Austria); leukemia inhibitory factor (LIF) was obtained from Chemicon (Temecula, CA, USA). Insulin, transferrin, sodium selenite, putrescine and progesterone were from Sigma (St. Louis, MO, USA). All primer pairs, including random hexamer primers, were synthesized by MWG (Munich, Germany). RevertAid™ H⁻M-MuLV reverse transcriptase was from Fermentas (St. Leon-Rot, Germany). Biotherm™ Taq-polymerase as well as the dNTP was from Genecraft (Münster, Germany). SYBR Green I was from Biozym (Hess. Oldendorf, Germany) and the plastic ware for the qPCR reaction was from Abgene (Hamburg, Germany). Ac-DEVD-AMC was obtained from Biosource International (Camarillo, Ca, USA) and AMC from Merck (Darmstadt, Germany). INGAP-PP – a pentadecapeptide with the 104–118 amino acid sequence of INGAP (NH_2-Ile-Gly-Leu-His-Asp-Pro-Ser-His-Gly-Thr-Leu-Pro-Asn-Gly-Ser-COOH) – and a scrambled peptide containing the same INGAP-PP amino acids but in a different sequence (NH_2-Thr-Ser-Asn-Leu-Ile-Gly-Gly-Asp-Pro-His-Gly-Pro-Leu-Ser-His-COOH) were provided by GenScript (Scotch Plains NJ, USA). Quality control of the peptides (amino acid analysis and mass spectrometry) indicated >95% purity and a molecular weight of 1501.63. Unless otherwise mentioned, chemicals of analytical grade were obtained from Sigma or Merck (Darmstadt, Germany).

2.2. Cell lines and culture conditions

The mES cell line ES-D3 [21] was kindly provided by Dr. S. Lenzen (Institute of Clinical Biochemistry, Hannover Medical School, Hannover, Germany). In order to maintain these cells in an embryonic state, they were cultured on a feeder layer of mouse embryonic fibroblasts in stem cell medium (DMEM) containing 25 mM glucose and supplemented with 15% (v/v) FCS, 2 mM L-glutamine, 100 mM non-essential amino acids, 0.1 mM β-mercaptoethanol, 50 μg/ml gentamicin and 1000 U/ml LIF in a humidified atmosphere at 37 °C and 5% CO_2. The cells were transferred for two passages on gelatin-coated tissue culture dishes to remove the feeder layer.

To promote differentiation, cells were cultured following the Lumelsky's protocol [5]. Briefly, one million cells were transferred onto a bacterial culture dish in medium as described above but devoid of LIF. Cells were then grown in suspension for up to 5 days (stage 2). During this time, cells formed embryoid bodies (EBs) which were allowed to settle down on gelatin-coated dishes in serum-free DMEM/F-12 medium supplemented with 25 μg/ml insulin, 50 μg/ml transferrin, 30 nM sodium selenite, 20 nM progesterone, 100 μM putrescine, 2 mM L-glutamine, and 100 mM non-essential amino acids for 7 days (stage 3). Thereafter, EBs were dissociated by adding 2 ml trypsin and further incubated for 5–10 min at 37 °C. Fifteen million cells were then cultured for 4 days in the same medium but supplemented with 10 ng/ml bFGF (stage 4). The cells were finally cultivated 7 days in DMEM/F-12 medium supplemented with 25 μg/ml insulin, 50 μg/ml transferrin, 30 nM sodium selenite, 20 nM progesterone, 100 μM putrescine, 5% FCS, 2 mM L-glutamine, 100 mM non-essential amino acids and 10 mM nicotinamide (stage 5). At every stage the medium was changed daily.

Regarding treated cells, 50 μl of the INGAP-PP stock solution freshly prepared (1 μg/μl in DMEM-F12 medium) was added daily to a 10 ml plate (5 μg/ml final concentration in the plate) from stage 3 to the end of the experiment. In order to discard any possible general unspecific peptide effect upon the cultures, the scrambled pentadecapeptide was used as negative control. This peptide was prepared and added to the control plates in the same way as INGAP-PP.

Six different experiments were done, including in each one three plates for each condition tested.

2.3. qPCR analysis

Total RNA was isolated from mES cells using the Chomczynski protocol [22]. RNA was quantified photometrically and analysed on a denaturing agarose gel. For cDNA synthesis, random hexamers were used to prime the reaction of the RevertAid™ H⁻M-MuLV reverse transcriptase. The QuantiTect SYBR Green™ technology (Qiagen, Hilden, Germany), which uses a fluorescent dye that binds only double-stranded DNA, was used. The reactions were performed using the DNA Engine Opticon™ Sequence Detection System (Biozym Diagnostik, Hess. Oldendorf, Germany). Samples were first denatured at 94 °C for 3 min followed by 40 PCR cycles. Each cycle comprised a melting step at 94 °C for 30 s, an annealing step at 62 °C for 30 s, and an extension step at 72 °C for 30 s. Each PCR amplification was performed in triplicate. The optimal parameters for the PCR reactions were empirically defined. The purity of the amplified PCR product was verified by melting curves. Data are expressed as relative gene expression (rge) after normalisation to the beta-actin housekeeping gene, using the *Qgene96* and *LineRegPCR* softwares [23–24]. The sequence of every pair of primers used for q-PCR is listed in Table 1.

2.4. Cell processing and immunocytochemical studies

Pelleted mES cells were fixed in 4% paraformaldehyde in 0.15 M phosphate buffered saline (PBS), pH 7.3, and embedded in paraffin. At

Table 1
Sequence of pair of primers used for qPCR

Gene		Primer sequence
Insulin	Fw	5′-CCCACCCAGGCTTTTGTCAAACAGC-3′
	Rv	5′-TCCAGCTGGTAGAGGGAGCAGATG-3′
IAPP	Fw	5′-GATTCCCTATTTGGATCCCC-3′
	Rv	5′-CTCTCTGTGGCACTGAACCA-3′
Glucagon	Fw	5′-CAGGGCACATTCACCAGCGACTAC-3′
	Rv	5′-TCAGAGAAGGAGCCATCAGCGTG-3′
Somatostatin	Fw	5′-ATGCTGTCCTGCCGTCTCCA-3′
	Rv	5′-TGCAGCTCCAGCCTCATCTCG-3′
Glut-2	Fw	5′-GAAGACAAGATCACCGGAACCTTGG-3′
	Rv	5′-GGTCATCCAGTGGAACACCCAAAA-3′
Kir-6.2	Fw	5′-TGCTGTCCCGAAAGGGCATTATC-3′
	Rv	5′-TGCAGTTGCCTTTCTTGGACACG-3′
SUR-1	Fw	5′-ACCAAGGTGTCCTCAACAACGGCT-3′
	Rv	5′-TGGAGCCAGGTGCTATGGTGAATG-3′
Pdx-1	Fw	5′-ACCGCGTCCAGCTCCCTTTC-3′
	Rv	5′-CAACATCACTGCCAGCTCCACC-3′
Nkx-6.1	Fw	5′-AGAACCGCAGGACCAAGTGGAGAA-3′
	Rv	5′-TCGTCATCCTCCTCATTCTCCGAAG-3′
Ngn-3	Fw	5′-TAGCAGAACTTCAGAGGGAGC-3′
	Rv	5′-GGGAAAAGGTTGTTGTGTCTC-3′
Nestin	Fw	5′-GAGAGTCGCTTAGAGGTGCA-3′
	Rv	5′-CCACTTCCAGACTAAGGGAC-3′
Caspase-3	Fw	5′-CCTCAGAGAGACATTCATGGGCC-3′
	Rv	5′-GCTGCTCCTTTTGCTATGATCTTCC-3′
Cdc-1	Fw	5′-ACAGCAGGTCTTCGTGCAGA-3′
	Rv	5′-ACTGGATGTTGGCTGCTTCAC-3′
Beta actin	Fw	5′-AGAGGGAAATCGTGCGTGAC-3′
	Rv	5′-CAATAGTGATGACCTGGCCGT-3′

Fw, forward (sense) primer; Rv, reverse (antisense) primer. All amplicons were designed intron-spanning and were in a size ranging from 100–300 bp. Beta actin was used as housekeeping gene.