**INTRODUCTION**

Diabetes mellitus is a metabolic disorder that affects millions of people. The number of patients suffering from diabetes continues to increase all over the world. Both type 1 and type 2 diabetes results from an inadequate mass of functioning beta cells. The prevalence of both type 1 and type 2 diabetes mellitus is increasing throughout the world along with the ensuing morbidity and early mortality because of premature microvascular and macrovascular disease.

Type-I diabetes is a consequence of self destruction of insulin producing pancreatic β cells which creates a chaos state for which there is presently no cure. Clinical trials indicate that, in some instances, control of blood glucose can be restored by transplantation of cadaveric derived islets (Korsgen et al., 2005). Stem cells both embryonic and adult open new vistas in islet therapy. Recently researcher look for fibroblast-like cells emerging from islets in culture due to gradual loss of endocrine cells as an alternative and potential source of beta cells (Scharfmann et al., 2008; Schmied et al., 2001; Zulewski et al., 2001). Proper lineage, nature and origin of these fibroblast-like cells are still a matter of conflict. Various groups now started characterizing these cells as endocrine precursors (Dorisetty et al., 2008; Zulewski et al., 2001; Lars and Helena, 2002) but not many evidences have been generated so far to value them as islet progenitor cells. In fact queries still persist that whether these cells do have proliferative potencies or not and are they originate from beta cells or are they really nestin positive cells (NPC’s). These cell based therapies may eventually provide new rays of hopes for a curative treatment. However, even if the hurdles in islet transplantation can be significantly improved, the availability of this treatment option will always be limited by the (1) dearth of cadaveric islet donors (Holland et al., 2009), (2) generation of new islets from stem cell pool, (3) availability of islet neogenic or differentiating agents (Zhang et al., 2005).

Medical practitioners have started using islet transplantation therapy to treat diabetes in year 2000 (Bretzel et al., 2004). Stem cell pool is prodigious target that is being exploited to generate islets for clinical intention. Various differentiating agents for this rationale are now being rummage around. Biological growth factor like...
Enicostemma Littorale: A new therapeutic target for islet neogenesis

KGF, FGF, GLP-1, and betacellulin (Abraham et al., 2002; Katdare et al., 2004; Meenal Banerjee et al., 2005) and chemical agents like Nicotinamide, Activin-A, Exendin-4 (Chandra et al., 2009; Banerjee and Bhonde, 2003; Xu et al., 1999) are conveniently used up by researchers. But the yield of islets after differentiation is not sufficient to overcome the verge of demand and also the cost of therapy is very high. So people are now shifting to uncover the dramatic medicinal properties of herbal plants that may possess islet neogenic activity. Much work has not been done on this line, except a very recent work from a Japanese group (Itaru and Kazuo, 2006). One compound conophyllin isolated from plant Evertiamia microphylla do show islet regeneration. Kojima’s idea was that the generation of new islets is via Activin-A signaling. Conophyllin acts as a ligand for Activin-A was proven by performing differentiation in presence of Activin-A antagonist. The antagonist blocked islet generation when incubated with Conophyllin (Itaru and Kazuo, 2006). This clearly provides evidence that herbal compounds do have potent role in islet differentiation.

Enicostemma littorale (EL) is another such plant which is well characterized by the author. Antidiabetic, hypolipideamic, and antioxidant properties of Enicostemma littorale (EL) are very well established (Maroo et al., 2003a; Maroo et al., 2003b; Vijayvargia et al., 2000; Vasu et al., 2005). Author also demonstrated earlier that Enicostemma littorale have insulin secretory activity in isolated islets (Maroo et al., 2002). Preliminary experiment with extract treated alloxan induced diabetic rats not only showed improved glucose status but also enlightened some blueprints for new islet generation histologically. This led the authors to hypothesize that there are some active components in either aqueous or methanolic extracts of the plant which are responsible for its islet neogenic activity.

MATERIALS AND METHODS

Chemicals and medium

All chemicals, medium and antibodies used in this study were purchased from Sigma Aldrich, St. Louis, MO, USA. Molecular biology reagents and cDNA and PCR kits were procured from Fermentas Inc USA. All plastic wares were purchased from NUNC, USA (Naperville, IL, USA).

Plant material

Plant material was procured from sourastra region of Gujarat after proper identification with voucher specimen (Oza 51, 51 (a)) kept in the herbarium of Department of Botany MS University of Baroda, Vadodara.
Enicostemma Littorale: A new therapeutic target for islet neogenesis

Extract Preparation and Isolation of SGL-1

Plant material was dried and cut into small pieces. Methanolic extract from EL plant was prepared according to Maroo et al. (2003a). Compound SGL-1 was isolated by fractionating methanolic extract successively with chloroform, ethyl acetate and butanol. The precipitated powdered compound obtained was named SGL-1 (characterization is under process).

Cell culture and Induction of Differentiation

NIH3T3 and PANC-1 cell lines (generous gift from Dr. R.R. Bhonde, NCCS, Pune) was maintained and cultured in DMEM high glucose medium with 10% Fetal Bovine Serum. Cells were passaged and splitted into 1:1 ratio in new tissue culture flask upon reaching 90% confluence. Once confluent, the cells were then washed twice with DMEM medium and replaced by neogenic differentiating medium prepared with DMEM Ham’s F-12 (1:1, 8mM Glucose) medium with no serum, containing cocktail supplements of Insulin 5mg/L, Transferrin 5ug/L and Selenite 5 ug/L (Sigma Aldrich, USA) and BSA 1.5g/L with antibiotics Penicillin 25ug/ml, Streptomycin 25ug/ml, Gentamycin 25ug/ml, Oxy-tetracyclin 25ug/ml and Amphotericin B 25ug/ml. The differentiation was carried out with control (SFM), positive control KGF (10ng/ml), EL extracts and SGL-1 compound (15ug/ml). The medium was changed every alternate day till 6 days. Finally on 8th day cells were observed for islet like cellular aggregates (ICA’s) in phase contrast inverted microscope (Nikon TE200, Japan) and confirmed with DTZ staining.

Morphometric analysis

To estimate the total yield of ICA’s generated, medium along with suspended ICA’s were centrifuged and resuspended in 1ml PBS and 100ul drops of this suspended clusters was stained with 10ul of DTZ. Brick red or crimson red stained clusters were counted in bright field inverted microscope in three independent experiments.

Molecular and Immunological characterization

ICA’s were spun at 1500rpm (Eppendorf 5415R) and total RNA was isolated using TRizol (Sigma Aldrich). 1µg RNA was reverse transcribed using Muv Reverse transcriptase, oligo dT, dNTPs, and ribolock inhibitor using manufacturer’s manual instructions (Fermentas). Gene specific forward and reverse primer for insulin was used to amplify the desired cDNA product at 56°C annealing temperature. Amplified product was confirmed on 15% polyacrylamide gel.

For immunocharacterization ICA’s were fixed on glass coverslip with DMEM and 10% FBS for 30 min. Upon attachment on cover slip clusters were immediately fixed with 3.7% paraformaldehyde for

---

*Figure 3: a) Islet like cluster generated from PANC-1 cells stained for guinea pig anti-insulin (red-Alexa-546), mouse anti-glucagon (green-FITC) and mouse anti-somatostatin (pink- alexa-633). ICA obtained was positive for Insulin and Glucagon but did not show any fluorescence for somatostatin. Fig-3b shows ICA generated from NIH3T3 cells stained for mouse anti-insulin (green-FITC), mouse anti-Glucagon (green-FITC).*
Enicostemma littorale: A new therapeutic target for islet neogenesis

Immunocytochemistry. Fixed cells were incubated for 1 hour at room temperature in blocking solution (1% BSA and 1% horse serum in PBS). After incubation ICA were stained with anti-Insulin, Glucagon and Somatostatin primary antibodies (dilutions are 1:100, 1:4000 and 1:100 respectively) overnight at 4°C. Coverslips were then washed for three times with washing buffer and further incubated with anti-mouse FITC (1:400), anti-guinea pig alexa 546 (1:100), anti-mouse FITC (1:400) and anti-mouse alexa-633 (1:100) conjugated secondary antibodies for 1 hour at room temperature and finally stained with DAPI and mounted with DABCO and glycerol mountant.

Functional characterization

To check the functional status of newly differentiated ICA’s from both control and SGL-1 group, 30 ICA’s from each group were incubated with 100µl of 10mM L-arginine and 20mM glucose respectively for 2 hours at 37°C. After incubation supernatant was used to estimate insulin content released from the ICA’s using Mercodia rat insulin ELISA kit according to manufacturer’s manual instructions.

RESULTS

Cell lines NIH3T3 (mouse embryonic skin fibroblast) of extra-pancreatic nature and PANC-1 (Human pancreatic cancer stem cell) of pancreatic nature having stem cell like property were chosen to prove islet neogenic activity. The efficacy for islet differentiation from different compounds isolated from EL extract was then tested. The compounds were isolated in sequential fractionization. Three different compounds SGL-1, 2 and 3 were isolated. These compounds were tested for differentiation for new islet like clusters (ICA’s) formation in four stage protocol in DMEM-F12 medium along with BSA and Insulin, Transferrin and Selenite cocktail with and without growth factors as control.

The generated ICA were deeply stained crimson red when stained with DTZ showed presence of insulin (Fig. 2). Morphometric analysis of these ICA’s in all groups (Aqueous, Methanolic, SGL-1, 2 and 3 and KGF as positive control) were statistically compared with control (SFM). SGL-1 found to be the most potent compound and showed 375% higher yield of ICA’s as compared to control with NIH3T3 cells and 205% in case of PANC-1 cell line. The ICA’s with size 300-3000 µm were 138% more with NIH3T3 cells and 230% with that of PANC-1 cells. Furthermore the average area of these newly generated ICA’s was 110% and 140% more with NIH3T3 and PANC-1 cells respectively, when compared with control (Table1). Following data provide evidences for the higher yield, intactness, maturity and normal tissue architecture of differentiated ICA’s.

Molecular characterization of ICA’s by Reverse Transcriptase PCR for Insulin mRNA expression profile of newly differentiated ICA’s further confirmed the expression of endocrine markers. Insulin expression at 6 day was found to be 1.6 and 5 fold higher in PANC-1 and NIH3T3 differentiated ICA’s respectively (Table 1).

Immunopositivity of ICAs for islet hormones proved them differentiated. Prominent insulin staining was seen in whole clusters whereas little stain for few glucagon positive cells was observed at the periphery of the islets. Stain for somatostatin was negative in these aggregates (Fig. 3a and 3b). This characterization implies that newly generated islets are more or less identical to that of normal islets. Functionality assessment of the ICAs was achieved by challenging them for glucose and L-arginine thereby measuring insulin release by ELISA. It has been reported that mature islets responds more to glucose while that of fetal nature responds to arginine (Banerjee and Bhonde, 2005) The newly generated ICAs from both cell lines, when challenged to glucose and arginine, showed higher response to glucose and not to arginine. NIH3T3 showed 162 fold while PANC-1 showed 1.6 fold higher response to glucose when compared with positive control (Table1). This clearly implies that newly generated islets are mature and fully functional to sense glucose shift extracellularly.

Hence SGL-1 compound isolated from Enicostemma littorale found to be the most promising candidate for differentiation of stem cells and endogenous pancreatic progenitor cells into islets from cell lines of both pancreatic and extra-pancreatic lineages, which provides new hopes for alternate medicine and therapeutic agent to increase islet mass which can be used for treatment of diabetic patients.

### Table 1: Assessment of Islet like aggregates compared with control.

<table>
<thead>
<tr>
<th>Islet like cellular aggregates with SGL-1 compound</th>
<th>Total % yield of ICA</th>
<th>% yield of 300-3000 µm size ICA</th>
<th>% increase in average area of ICA</th>
<th>Fold increase in insulin mRNA expression</th>
<th>Fold increase in glucose responsive insulin release</th>
</tr>
</thead>
<tbody>
<tr>
<td>PANC-1</td>
<td>205</td>
<td>230</td>
<td>140</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>NIH3T3</td>
<td>375</td>
<td>138</td>
<td>110</td>
<td>5</td>
<td>162</td>
</tr>
</tbody>
</table>
Acknowledgement

The authors would like to acknowledge Department of Biotechnology, Govt. of India, New Delhi for funding the project (Grant Number: BT/TR-7721/MED/14/1071/2006). Authors also thank Dr. Anandwardhan Hardikar for their generous help and discussions. Confocal facility was availed at National Centre for Cell Science, Ganeshkhind, Pune, Maharashtra.

Abbreviations

EL, *Enicostemma littorale*, ICA’s, Islet like Clusters Aggregates, NPC’s, Nestin Positive Cells, KGF, Keratinocyte Growth Factor, FGF, Fibroblast Growth Factor, GLP-1, Glucagon Like Peptide-1, SFM, Serum Free Medium.

References


