Extracellular matrix molecules applied to promote functional survival of microencapsulated pancreatic islets

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Chap

Extracellular matrix components supporting human islet function in alginate-based immunoprotective microcapsules for treatment of diabetes

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Abstract

In the pancreas, extracellular matrix (ECM) components play an import role in providing mechanical and physiological support, and also contribute to the function of islets. These ECM-connections are damaged during islet-isolation from the pancreas and are not fully recovered after encapsulation and transplantation. To promote the functional survival of human pancreatic islets, we tested different ECMs molecules in alginate-encapsulated human islets. These were laminin derived recognition sequences, IKVAV, RGD, LRE, PDSGR, collagen I sequence DGEA (0.01-1.0 mM), and collagen IV (50-200 μg/ml). Interaction with RGD and PDSGR promoted islet viability and glucose induced insulin secretion (GIIS) when it was applied at concentrations ranging from 0.01- 1.0 mM (p<0.05). Also, the laminin sequence LRE contributed to enhanced GIIS but only at higher concentrations of 1 mM (p<0.05). Collagen IV also had beneficial effects but only at 50 μg/ml and no further improvement was observed at higher concentrations. IKVAV and DGEA had no effects on human islets. Synergistic effects were observed by adding Collagen(IV)-RGD, Collagen(IV)-LRE, and Collagen(IV)-PDSGR to encapsulated human islets. Our results demonstrate the potential of specific ECM components in support of functional survival of human encapsulated and free islet grafts.

Key words: pancreatic islets, extracellular matrix, laminin, encapsulation, glucose induced insulin secretion

Introduction

The use of therapeutic cells within semi-permeable immunoprotective capsules is proposed as a potential therapeutic option for many disorders in which a minute-to-minute regulation of metabolites is required [1]. The proof of principle of cell encapsulation has been demonstrated in a wide variety of diseases, including parathyroid cells [2], hemophilia B [3], anemia [4], dwarfism [5], kidney and liver failure [6, 7], pituitary and central nervous system insufficiencies [2, 8], and for treatment of type 1 diabetes [9-11]. During encapsulation, living cells are suspended in biomaterials, such as alginate [12, 13], agarose [12, 14], poly (ethylene glycol) (PEG) [15], or poly (vinyl alcohol)[16]. The cells-biomaterial suspension are transferred into droplets, and processed by specific procedures to create immunoprotective membranes [17]. The advantages of the system are, besides the ability of minute-to-minute regulation of metabolites, that the technique allows for transplantation in the absence of harsh immunosuppressive protocols and that it might provide a solution for the world-wide donor shortage since it allows for xenografting [18, 19].

Encapsulation in immunoprotective membranes has advanced rapidly in the field of islet transplantation for the cure of diabetes [20]. Successful efficacy studies have been performed in rodents [11, 21], large and small mammals [21], and even in humans [22]. However, a consistent issue with encapsulated grafts is the limited grafts survival time. Reportedly, graft survival varies from several days to months but is never permanent [23]. Many factors have been discussed as causative for this limited duration of graft survival such as low oxygen supply in the transplantation site [24], differences in biocompatibility of the applied capsules, and loss of islet cells due to inflammatory responses against islet-cell components [23, 25-27]. However even with adequate external oxygen supply [28], and in the absence

of any tissue response, graft survival is limited to periods of 42 till 200 days [29]. This implies that other factors also contribute to the limited duration of functional survival of islets in capsules.

A factor that might influence functional survival is the quality of the intracapsular environment and absence of specific extracellular matrix molecules (ECM). Islets in the pancreas have an extensive network of ECM molecules of a specific composition, which are involved in maintenance of survival and normal function of the tissue [30]. This network of ECM molecules are damaged during isolation of pancreatic islets by application of enzymes that selective breakdown the ECM molecules that connect the endocrine and exocrine tissue in the pancreas [31]. The most essential ECM structures in islet function are collagen type I and IV, and laminins [30]. The amount of collagen type VI expressed in islet–exocrine interface and basement membrane of islets regulates fibronectin assembly by restraining cell–fibronectin interactions [30, 32]. This influences cell functions such as migration [33]. Enzyme induced damage to collagen type VI in islets leads to recruitment of tensin at the fibrillar adhesion points via the activation of myosin light chain kinases (MLCKs), which mediate actomyosin contractility, extensive fibrillogenesis, and uncontrolled cell migration [33, 34].

Laminins are also essential [30, 32]; most of the functional studies with laminin have been performed with other epithelial cell sources than islet-cells. The tripeptide RGD is one of the most extensively studied laminin molecule [35]. RGD can be found in many ECM molecules involved in anchorage of cells, such as in fibronectin. This anchorage prevents a variant of apoptosis called anoikis in for example undifferentiated human intestinal epithelial crypt (HIEC) cells through the recruitment of vinculin and the activation of the PI3K–AKT signaling pathway [34]. Other known laminin adhesion sequences with possible beneficial effects for cell-survival are the

proteins that contains IKLLI [36], IKVAV [37], LRE [38], and PDSGR [39]. However, minor knowledge is available about the beneficial effect of these ligands for islet-cells.

In the present study, we investigated the effect of addition of type I collagen α1(I)-CB3 fragment (DGEA) and full type IV, also the synthetic laminin peptides such as IKLLI, IKVAV, LRE, and PDSGR on the functional survival of encapsulated human pancreatic islets *in vitro*. The ultimate goal of this study is to select ECM molecules that might promote functional survival of islets cells in capsules for immunoisolation of human pancreatic islets. We concentrated as much as possible on synthetic sequences of ECM components to facilitate reproducibility and regulatory matters. Functional survival was studied by determining the glucose induced insulin response (GIIS) and the oxygen consumption rate.

Material and methods

Study design

Extracellular matrix (ECM) components were added to human pancreatic islets enveloped in alginate based microcapsules. As reportedly, collagen type IV and the laminin types such as Laminin 111, 332, 411-421, and 511-521 might supports islet function [9, 30, 40], graded loads of the full or active sequences of these ECM components were added and tested in microencapsulated human islets. The active laminin sequences; IKVAV, RGD, LRE, PDSGR, and type I collagen α1 (I)-CB3 fragment (DGEA) synthesized by GenScript Corporation (Piscataway, USA) were tested in concentrations of 0.01, 0.1, and 1 mM respectively (n=6). Also, collagen type IV was tested at concentrations of 0.311, 0.621, and 1.242 x 10^{-3} mM (50, 100, 200 μg/ml) (n=5). After determining the most optimal concentration, ECM components were combined. Effects were determined at 3, 5, and 7 days after

addition of ECM combinations to the intracapsular environment of alginate microcapsules.

Functional effects were determined by subjecting the islets to a glucose induced insulin secretion (GIIS) test. In selected experiments live/dead staining was applied and measured at day 3, 5, and 7. Additionally, oxygen consumption rate (OCR) determinations were performed after 72h of incubation.

Human pancreatic islet isolation

Human islets were ordered from Prodo Laboratories Inc. (Irvine, USA). A dithizone (Merck, USA) staining was performed before shipment to determine the purity. Islets were shipped to the Groningen University Medical Center (Groningen, The Netherlands). After shipment islets were handpicked and washed five times with CMRL 1066 (Gibco, USA) before culture.

Alginate purification

Intermediate-G sodium alginate (44% G-chains, 56% M-chains, 23% GG-chains, 21% GM-chains, 37% MM-chains) obtained from ISP Alginates (Ltd, UK) was dissolved at 4°C in a 1 mM sodium EGTA solution, and filtered over 5.0, 1.2, 0.8, and 0.45 μm filters (Corning®, USA). The pH of the solution was carefully adjusted to 2.0 by addition of 2N HCl. Proteins were removed by extraction with chloroform/butanol (4:1). Next, the alginic acid was slowly adjusted to pH 7.0 by slowly adding 0.5 N NaOH. The obtained alginate solution was subjected to a chloroform/butanol (4:1) extraction inducing the formation of a separate chloroform/butanol phase by centrifugation. The supernatant was removed by aspiration. The alginate was suspended in cold ethanol for 10 min and washed twice, until all alginate had precipitated. Finally, the alginate was washed three times with twice the volume of diethyl

ether and freeze-dried overnight [41, 42].

Microencapsulation and extracellular matrix building

Purified alginate was mixed with the appropriate extracellular matrix components by physical entrapment within 3.4% purified alginate (Table 1). The alginate-matrix was mixed with human pancreatic islets in a ratio of 800 islets per ml alginate-ECM mixture. Alginate-solutions without ECM components served as control. Subsequently the solution was converted into droplets using an air-driven droplet generator as previously described [1, 24, 43]. Droplets were gelled in 100 mM $CaCl_2$ solution for at least 10 min [44]. The diameters of the droplets were between 500-600 μm and were controlled by a regulated air-flow around the tip of needle. All droplets were washed with KRH buffer containing 2.5 mM $CaCl_2$ for 2 min; subsequently, were cultured in CMRL 1066 (Gibco, USA) supplemented with 8.3 mM D-glucose, penicillin/streptomycin (1%) (Gibco, USA), and 10% fetal calf serum (FCS) (Gibco, USA) at 37 °C, 5% CO_2 till further use [45-47].

Table I. ECM components tested with human islets.

ECM protein	Vendor	Molecular Weights (Da)	Tested concentrations (mM)
IKVAV	GenScript[a]	529.0	0.01, 0.1, and 1
RGD	Sigma[b]	346.34	0.01, 0.1, and 1
LRE	GenScript[a]	457.53	0.01, 0.1, and 1
PDSGR	GenScript[a]	571.59	0.01, 0.1, and 1
DGEA	Sigma[b]	390.35	0.01, 0.1, and 1
Collagen, type IV	Sigma	160,615	0.311, 0.621 and 1.242 x 10^{-3}

[a] Synthetic peptides of >95% and [b] ≥97% purity were used to test the biological activity.

Glucose-stimulated insulin secretion of encapsulated islet

Islets were tested for glucose stimulated insulin secretion at day 3, 5, and 7 after encapsulation. The encapsulated islets (25 islets) were preincubated for 1.5 hours in 2 ml Krebs-Ringer HEPES (KRH), gassed with 95% O_2 and 5% CO_2, containing 0.25% BSA, and 2.75 mM glucose. The incubations were performed in an incubator at a stirring rate of 120 cycles/min at 37°C. The quantitative insulin secretion was then assessed by three consecutive incubations of (i) low glucose concentration solution in KRH (2.75 mM) for 1 h, (ii) high glucose concentration buffer in KRH (16.5 mM) for 1 h, and (iii) another 1 h incubation in 2.75 mM glucose in KRH. At the end of each incubation, the incubation media were removed and frozen for insulin measurement via Enzyme-Linked Immunosorbent Assay (ELISA) (Mercodia AB, Sweden) using a spectrophotometric plate reader as described previously [1]. Finally, insulin concentrations were calculated through the interpolation of sample absorbance values from the standard curves.

DNA content of islets was quantified with a fluorescent Quant-iT PicoGreen double-strand DNA (dsDNA) assay kit (Invitrogen, United States). The insulin secretory responses were expressed as nanogram of insulin.ml^{-1}. $\mu gDNA^{-1}$. $hour^{-1}$.

Live-dead staining

Viability of encapsulated islet was test using a LIVE/DEAD Cell Viability/ Cytotoxicity assay Kit (InvitroGen, USA). To this end, a stock solution of Calcein AM (4 mM) and Ethidium homodimer (EthD) (2 mM) was added. Encapsulated islets were incubated at room temperature for 30 min in darkness, and then washed with Krebs–Ringer–Hepes (KRH), pH 7.4 prior to imaging. Live cells were identified by green fluorescence (Ext/Abs 490/515 nm) while dead cells were recognized by red fluorescence upon binding of

ethidium homodimer to exposed DNA (Ext/Abs 528/617 nm).[48]

Fluorescent confocal microscopy was applied to study islets stained with either assay within alginate capsules. Stained islet-cells were manually counted in images (n≥3) of each sample. Data viability was expressed as the percentages of live cells relative to the total number of stained cells and analyzed by Imaris 664 version 7.6.4 software [41].

Islet oxygen consumption rate

The oxygen consumption rate (OCR) was measured in human pancreatic islet using the extracellular flux analyzer XF24 (Seahorse Bioscience, USA), as previously described in detail [49]. This was done after removal of the alginate capsule with 25 mM citrate solution at 37°C as this interfered with the measurements. After that, between 120-160 islets per condition were incubated overnight in CMRL 1066 (Gibco, USA) with 8.3 mM D-glucose, penicillin/streptomycin (1%) (Gibco, USA), and 10% fetal calf serum (FCS) (Gibco, USA) at 37°C. After one washing step with RPMI (Gibco, USA) islets were resuspended in 2 ml of modified XF assay media (MA media) at 37°C, supplemented with containing 3 mM glucose, 1% FCS used in the XF analysis. Islets were subsequently plated by pipetting into the wells, together with 500 µl of MA media. Four wells were kept as blank, empty controls. To avoid bubble formation in the screen-net, provided in the XF sensor cartridge, screens were pre-wetted with MA media. The plates were then incubated for 60 minutes at 37°C before it was loaded into the XF24 machine. To adjust for the variation in islet number, OCR of each individual well was normalized for DNA content. Data was expressed as normalized oxygen consumption rate at intervals of approximately 5-8 minutes OCR/DNA (*p*mol $O2 \cdot min^{-1} \cdot \mu gDNA^{-1}$), to compare different experimental conditions, and analyzed by Seahorse XF24 software. An initial drift in OCR was typically observed in

the first 1–2 measurements until steady state was reached. Therefore, these initial data points were always disregarded.

Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). Normal distribution of the data sets was determined using the Kolmogorov-Smirnov test. If the data sets were not normally distributed, they were log transformed and reanalyzed using the Kolmogorov-Smirnov test. Statistical comparisons between experimental conditions in each study were performed by two-tailed ANOVA using GraphPad Prism 5.0. *P*-values <0.05 were considered to be statistically significant.

Results

Single synthetic laminin peptides and GIIS of encapsulated human islets

A selection of laminin sequences known to be present in islets in the native pancreas, i.e. IKVAV, RGD, LRE, and PDSGR [30, 50] were tested for potential beneficial effects on encapsulated human pancreatic islets (Table 2). Also, type I collagen α1 (I)-CB3 fragment (DGEA) was studied in this comparison. Graded loads of 0.01, 0.1, and 1 mM were tested. The effects of individual ECM components on human islet GIIS were tested at 3, 5, and 7 days after encapsulation.

The GIIS for islets encapsulated with and without ECM proteins are shown in Figure 1. The stimulated insulin secretion at day 3 was statistically significantly enhanced by 1 mM RGD ($p<0.001$), 0.1 mM LRE ($p<0.05$), 1 and 0.01 mM PDSGR ($p<0.05$, $p<0.001$). The most pronounced effect was obtained with RGD where a two and half fold increase of GIIS was observed. At day 5 only islets encapsulated with 1 mM RGD ($p<0.05$) and 0.01 mM PDSGR ($p<0.005$) demonstrated a statistically enhanced response when

compared to controls (Fig. 1B). At day 7, the GIIS of the controls were two-fold reduced compared to day 3. However, also here we found statistically significant enhanced responses of islets in capsules containing either 0.1 mM or 0.01 mM RGD ($p<0.001$, $p<0.05$), 1 and 0.1 mM LRE ($p<0.005$, $p<0.03$), and 0.01 mM PDSGR ($p<0.05$) (Fig. 1C). In addition, islets in capsules containing 1 mM LRE showed an increase of GIIS over two and half fold compared to controls.

Table II. Matrix proteins and laminin peptide sequences incorporate into encapsulated human islets.

Matrix proteins	ECM protein/ localization	Receptor details
IKVAV	Laminin 111	110kda laminin receptor protein
RGD	Laminin 511	α5β3, α5β1
LRE	Laminin γ1	Unknown
PDSGR	Laminin 511-β1	Unknown
DGEA	Type I collagen α1	α2β1
Collagen, type IV	Human placenta tissue	400 nm long chain, nonfibrillar: α1β1 α1β2

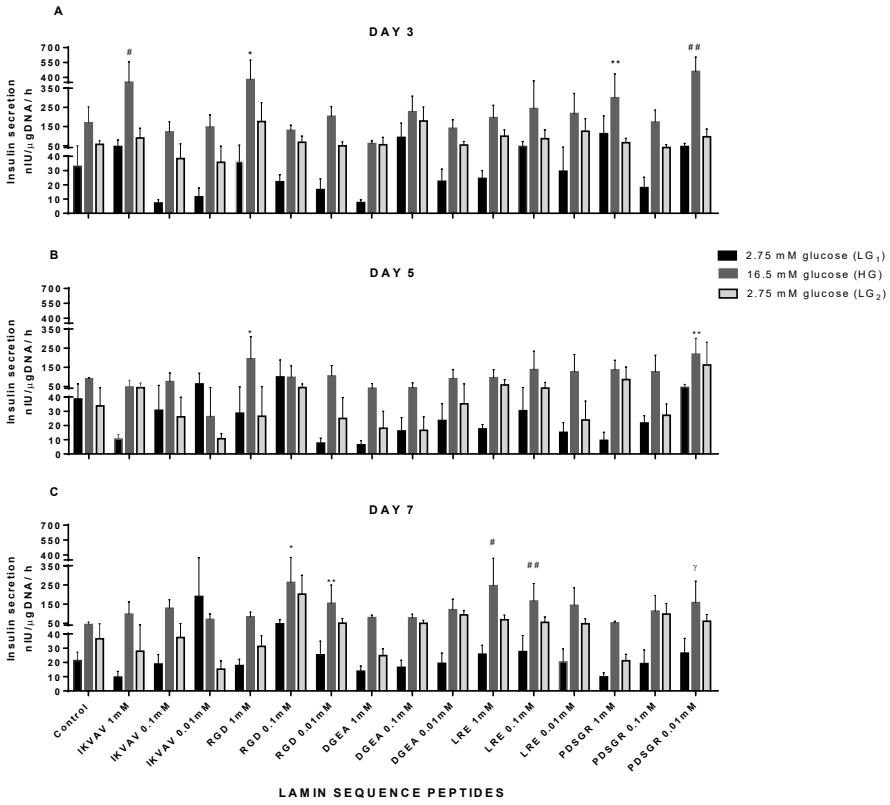

Figure 1. Glucose stimulated insulin secretion of human islets encapsulated in alginate based capsules with incorporated IKVAV, RGD, LRE, DGE, and PDSGR at concentrations of 0.01 mM, 0.1 mM and 1 mM. Independent sets of 25 islets for each condition were treated with low (2.75 mM, LG_1 and LG_2) and high (16.5 mM, HG) glucose for 1 hour at day 3(A), day 5 (B), and day 7 (C). Values represent mean ± SEM. *, **, #, ## and γ indicate statistical significant differences ($p < 0.05$) when compared to control islets (n=5, different donors).

Effect of collagen type IV on GIIS of encapsulated human islets

Collagen type IV is reportedly an essential component of the peri-islet area of human islets [51] and contributes to β-cell motility [52], and is largely destroyed during the enzymatic isolation of the islets from the pancreas [30]. Therefore, it was investigated whether addition of graded loads of human collagen IV enhances the GIIS of microencapsulated human islets. To this end, 50, 100, and 200 µg/ml of collagen type IV was built into the intracapsular environment.

As shown in Figure 2, an elevation of over two times fold on GIIS within 50 µg/ml (p<0.01) and, around one an half times fold with 100µg/ml in encapsulated islets entrapped collagen type IV was observed at day 3. Higher concentrations of collagen type IV did not further enhance the GIIS. It even decreased compared to islets exposed to 50 µg/ml collagen type IV but never came below the GIIS of the control islets. Also at day 5 and 7, we found a two-fold increase of GIIS for 50 µg/ml, and a one and half fold increase for 100 µg/ml collagen type IV (p<0.05). Interestingly, encapsulated islets entrapped within 50 µg/ml collagen type IV enhance their GIIS at least two-fold over the culture days in comparison to encapsulated islets in just alginate without any ECM.

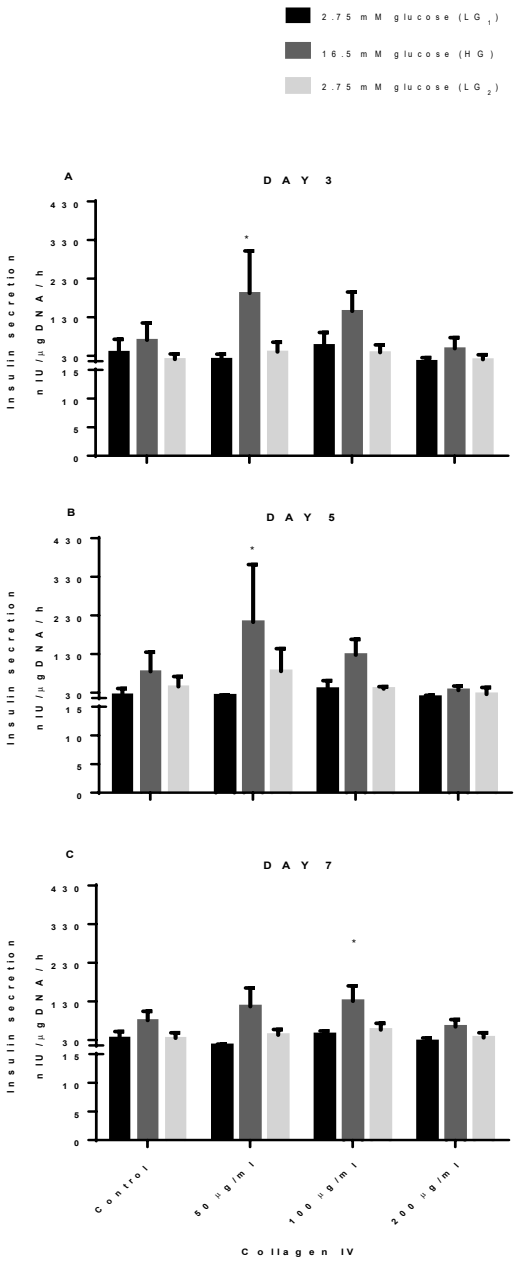

Figure 2. Glucose induced insulin secretion from encapsulated human islets with incorporated collagen type IV concentration. Independent sets of 25 islets for each condition were treated with low (2.75 mM, LG1 and LG2) and high (16.5 mM, HG) glucose for 1 hour at day 3 (A), 5 (B), and day 7 (C). Values represent mean ± SEM. * indicates statistical significant differences ($p<0.05$) when compared to control islets (n=4, different donors).

Synergistic effects of ECM combinations on human islets

From the foregoing follows that GIIS stimulating effects were consistently observed for RGD, LRE, and PDSGR at all days tested. To determine whether the effects are synergistic we combined 50 µg/ml collagen type IV with either the stimulating laminin sequences 0.01 mM RGD, 1 mM LRE, and 0.01 mM PDSGR. As shown in figure 3, at day 3 some synergistic effects were observed for the combination of 50 µg/ml collagen type IV and 1 mM LRE ($p<0.01$). In all cases the GIIS was better maintained during the 7 days culture period than in islets encapsulated without ECM components. The effects were however dependent on the duration of culture. Addition of 1 mM LRE in combination with 50 µg/ml collagen type IV had the most pronounced effects on GIIS at day 3. Both the basal and stimulated insulin secretion was consistently higher in capsules containing this combination of ECM components. Although, the stimulating effect remained it was less pronounced at day 5 and 7. As of day 5, 50 µg/ml collagen type IV and 0.01 mM RGD ($p<0.01$) as well as 50 µg/ml collagen type IV and 0.01 mM PDSGR ($p<0.001$) had a stimulating effect similar to that of collagen type IV and 1 mM LRE.

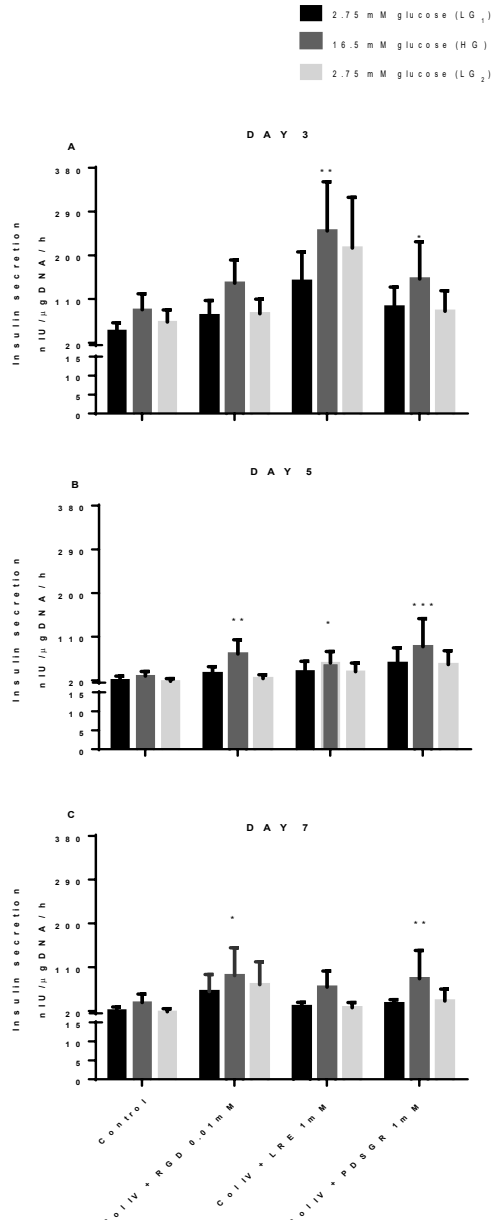

Figure 3. Glucose induced insulin secretion of human islets encapsulated in alginate-based capsules supplemented with combinations of ECM proteins. Capsules contained combinations of 50 µg/ml collagen type IV with 0.01 mM RGD, 0.1 mM LRE, or 0.01mM PDSGR were treated with low (2.75 mM, LG_1 and LG_2) and high (16.5 mM, HG) glucose for 1 hour at day 3(A), day 5 (B), and day 7(C). Values represent mean ± SEM. *, **, and *** indicates statistical significant differences (p <0.05) when compared to control islets (n=5, different donors).

Cell viability and oxygen consumption rate of human islets in alginate capsules with ECM

We determined whether the differences in function could be explained by differences in cell-survival within islets and/or whether the addition of ECM impacts the oxygen consumption rate of islets. A higher oxygen consumption rate reflects a better function and is correlated with a higher success rate of islets after implantation [53, 54].

Figure 4 demonstrates that the percentage of living cells in the islets encapsulated in capsules containing 50 µg/ml collagen type IV with either laminin sequences 0.01 mM RGD, 1 mM LRE, and 0.01 mM PDSGR was always higher than in pancreatic islets in the control capsules without ECM molecules. At day 3, the percentage of living cells was statistical significantly higher with 50 µg/ml collagen type IV and 0.01 mM RGD (81.1% (p<0.05)) and 50 µg/ml collagen type IV and 1 mM LRE (83.2% (p<0.03)), than with the combination of 50 µg/ml collagen type IV with 0.01 mM PDSGR (78.6%). At day 5 a general drop in viability was observed. However, also here and at day 7 the survival rates of islet-cells in capsules was consistently higher in capsules with ECM molecules compared to controls without ECM molecules and reached statistical significant differences in case of 50 µg/ml collagen type IV with 0.01 mM PDSG enhancing the percentage of living cells from 62.5% to 77.4% (p<0.05). The combination of 50 µg/ml collagen type IV with 1 mM LRE, and 50 µg/ml collagen type IV with 0.01 mM RGD enhanced survival rates of islet-cells from 62.5% to 75.9% and 75.2% respectively, however this did not reach statistical significance. Islets in capsules without ECM functionality exhibited a cell survival of only 62.5% after 7 days.

The oxygen consumption rate (OCR) was only determined at 72 hours as large numbers of islets were required. As shown in figure 5, addition of ECM does enhance OCR but this only reached statistical significant

differences with 50 µg/ml collagen type IV with 1 mM LRE. As shown in Figure 5, the OCR was 76.7 *p*mol $O_2 \cdot min^{-1}.mgDNA^{-1}$ for islets encapsulated in 50 µg/ml collagen type IV with 1 mM LRE which was three-fold higher than that of the control islets ($p<0.05$). Although, 50 µg/ml collagen type IV with 0.01 mM PDSGR, and 50 µg/ml collagen with 0.01 mM RGD enhanced the OCR, this never reached statistical significant differences and was not of the impact of 50 µg/ml collagen type IV with 1 mM LRE.

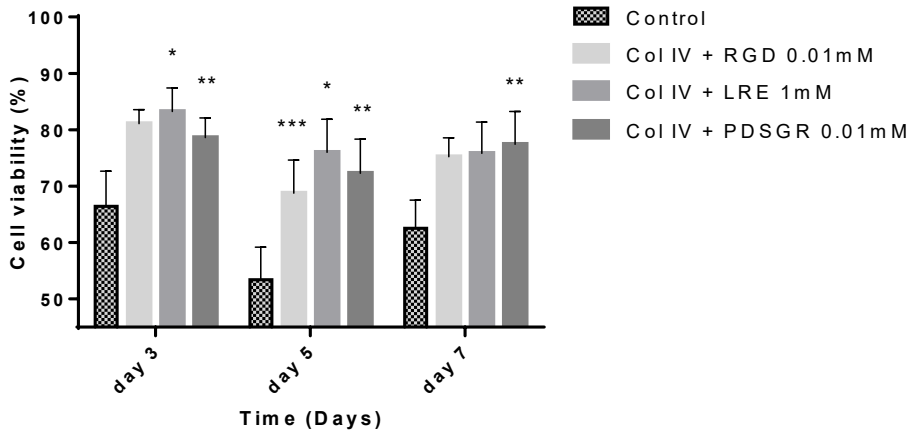

Figure 4. Viability of human islets encapsulated in alginate capsules containing 50 µg/ml collagen type IV and either 0.01 mM RGD, 1 mM LRE, and 0.01 mM PDSGR. Alginate capsules without ECM components served as control. Values represent mean ± SEM (n=5, different donors). *, **, and *** indicates statistical significant differences ($p<0.05$) when compared to control islets. (Col IV, collagen type IV).

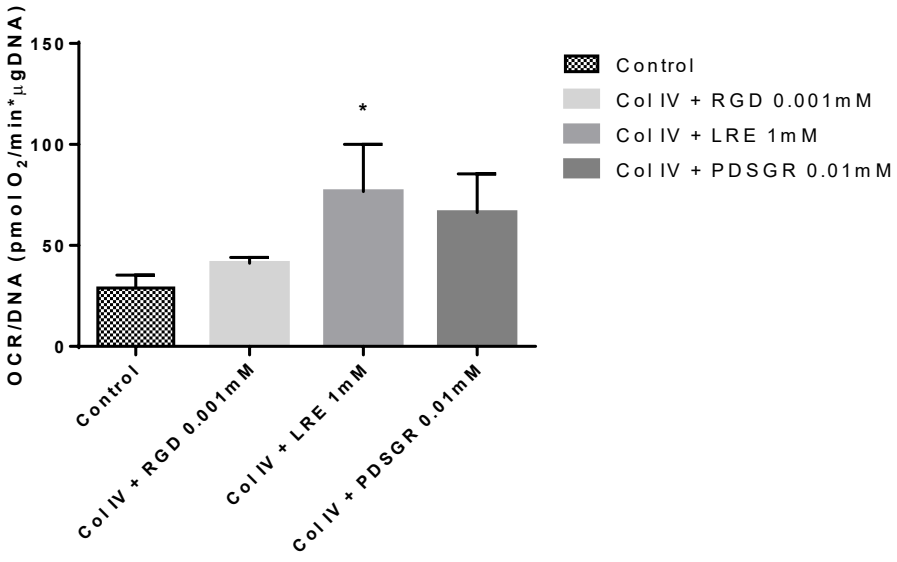

Figure 5. Oxygen consumption rate (OCR) of human islets encapsulated in alginate capsules containing combination of 50 µg/ml collagen type IV and either 0.01 mM RGD, 1 mM LRE, and 0.01 mM PDSGR after 72 hours in culture. OCR is expressed after correction for DNA content (OCR/DNA). Values represent mean ± SEM. * indicates statistical significant differences (p <0.05) when compared to control islets (n=5, different donors).

Discussion

To the best of our knowledge this is the first study demonstrating the beneficial effects of specific ECM components on functional survival of human islets in capsules for immunoprotection. An increase of as much as 300% in GIIS could be obtained by adding laminin α1 chain (Fig. 1) and 140% with collagen type IV (Fig. 2) compared to islets encapsulated in just an alginate matrix without ECM. This beneficial effect corroborates the findings of Nikolova

et al [55]. This author demonstrated that increasing collagen type IV and laminin localized in the vascular basement membrane surrounding intra-islet capillaries *in vivo*, resulted in enhanced insulin gene expression both on matrix-coated culture substrate and when soluble matrix protein was added to the culture medium [52].

Capsules for islet encapsulation are usually only containing biomaterials and no support of ECM components. Only one group has applied RGD in encapsulated islet grafts [54], but to the best of our knowledge a systemic comparison of its effects between capsules without RGD or with other ECM molecules have not been performed. As shown here only a limited number of four out of six ECM molecules tested had a beneficial effect and RGD was not the most efficacious ECM. This illustrates that a systematical approach is necessary to select ECM components with an enhancing effect on functional survival of islets in capsule by this approach. Notably, this is a first screening for efficacious ECM molecules *in vitro*. Long-term efficacy studies of smaller amounts of ECM molecules *in vivo* will be part of future efforts.

The beneficial effects of ECM components should be attributed to improved cell-matrix interactions that benefits processes such as cell-cell adhesion and gene expression or the coordinated behavior of cell groups. [56] The presence of ECM signaling can influence cellular differentiation, apoptosis (anoikis), and function. However not all ECM components enhanced GIIS. The type 1 collagen α1 (I)-CB3 fragment (DGEA) had no effect on GIIS while ECM-derived peptide fragments located on laminin α-1 chain (RGD, and IKVAV); laminin γ-1 chain (LRE); laminin β-1 chain located (PDSGR) in specific concentrations did enhance the responses. This enhancing effect of specifically insulin secretion should be explained by the fact that presence of several integrin variants found in laminin α-1, e.g. $\alpha_v\beta_3$, $\alpha_v\beta_{5,}$ regulate adhesion of islet-cells, and also by presence of $\alpha_3\beta_1$ and $\alpha_6\beta_1$ that

are involved in regulation of insulin secretion [57, 58]. In addition, integrins present in specific regions of laminin β-1 and γ-1 may prevent programmed cell death (anoikis) resulting from inadequate or inappropriate matrix signaling enzymatic isolation of islets from the pancreas. This is corroborated by our observation that presence of laminin γ-1 was associated with less cell-death. Also, the matrix-cell interactions might benefit cytoskeletal organization throughout the islets. Relative large portions of beta-cells are in the interior of the islet and do not have direct contact with the peripheral ECM. Therefore, any benefit from ECM addition must derive from indirect matrix restoration [59], which have been shown to influence expression of genes implicated in GSIS [60].

Collagen type IV and the GIIS promoting laminin sequence were combined to study synergistic effects. To our surprise higher concentrations of collagen type IV induced a lowering instead of an enhanced GIIS of encapsulated islets. This observation is in accordance with the observations of Lucas-Clerc C *et al* [61], who also demonstrated collagen type IV dependent suppression of GIIS at higher concentrations. Moreover, this should be explained by the suggestion that supraphysiological collagen type IV concentration by overexpression enhances cell survival rather than supporting metabolic processes such as insulin release [34]. Preferably the concentration of collagen type IV should approach the concentration in the pancreas in order to have beneficial effects. The 50 μg/ml collagen type IV combination with other ECM molecules is indeed similar to the relative amount of these proteins in the normal basement membrane [62].

It might be suggested that the profound positive effects on islet function might be caused just by ECM-signals at the exterior of the islets and communication of the stimulated cells with the interior of the islets. We feel, however, the process is more complicated and that islet are dynamic-organs

in which cells migrate instead of behave as static cell-clumps [52, 63]. As the consequence of this more islet-cells will benefit from ECM contact. Also, it is plausible that ECM-sequences will migrate into the interior and support beneficial processes. The fact that ECM molecules can stimulate the GIIS with as much as 300% supports this suggestion as this cannot be explained by just stimulation of cells in the periphery of islets.

Oxygen consumption rate (OCR) of islets is an emerging technology for assessing human islet-functionality as it correlates with functionality of grafts after transplantation [64]. The higher the OCR the better the metabolic responsiveness of the islets [65]. Unfortunately measuring OCR is laborious and still requires significant amounts of islets. We therefore applied it only for one-time point and for the ECM components with a proven efficacy in GIIS. The OCR data underscore the beneficial findings of ECM components illustrating that ECM addition may not only be beneficial for encapsulated islets but possibly also for free human islet grafting in which OCR correlated well with graft function *in vivo* [64].

Conclusions

Not all but only a selection of laminin sequences do support function of human pancreatic islets in alginate-based microcapsules applied for immunoprotection of islets. The laminin recognition sequence, RGD, and PDSGR promote islet viability and insulin secretion when immobilized at concentrations of 0.01 to 1 mM while the laminin sequence LRE contributes to enhanced GIIS only at higher concentrations of 1 mM. When this was combine with collagen type IV in concentrations similar to that found in the pancreas we found synergistic beneficial effects. However, at higher collagen type IV concentrations adverse effects were observed. Our data underscores that the intracapsular environment should receive more attention in efforts

to support longevity of encapsulated pancreatic islets. This is a pertinent consideration as islets are obtained from rare cadaveric donors. Therefore, loss of functionality or viability should be reduced to a minimum.

Acknowledgments The author gratefully acknowledges the financial support of Erasmus Mundus Lindo (scholarship ML12FD0331).

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