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RESEARCH PAPER

Collagen type VI interaction improves human islet survival in immunoisolating microcapsules for treatment of diabetes

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ABSTRACT

Collagens are the most abundant fibrous protein in the human body and constitute the main structural element of the extracellular matrix. It provides mechanical and physiological support for cells. In the pancreas, collagen VI content is more than double that of collagen I or IV. It is a major component of the islet-exocrine interface and could be involved in islet-cell survival. To test the impact of collagen VI on human encapsulated pancreatic islets-cells, we tested the effects of exogenous collagen type VI on in vitro functional survival of alginate encapsulated human islet-cells. Concentrations tested ranged from 0.1 to 50 μg/ml. Islets in capsules without collagen type VI served as control. Islet-cell interaction with collagen type VI at concentrations of 0.1 and 10 μg/ml, promoted islet-cell viability (p<0.05). Although no improvement in glucose induced insulin secretion (GSIS) was observed, islets in capsules without incorporation of collagen type VI showed more dysfunction and oxygen consumption rates was improved by inclusion of collagen type VI. Our results demonstrate that incorporation of collagen type VI in immunoisolated human islets supports in vitro viability and survival of human pancreatic islets.

Introduction

Islet transplantation is a promising method to cure patients with type I diabetes. Transplantation of allogeneic pancreatic islets can theoretically regulate glucose levels from a minute-to-minute level,[1,2] preventing the development of hypoglycemia and diabetic complications.[3] An advantage of implanting pancreatic islets over transplantation of the whole pancreas is that isolated islets can be modulated before transplantation to reduce the risk of graft rejection.[4,5] Moreover, islet transplantation is a minimal invasive surgical procedure with short hospitalization periods and can be repeated in cases of graft failure with minor adverse effects.[1,6] Isolated islets can also be enveloped in immunoisolating capsules that are impermeable to immunoglobulins and cells of the immune system but allow diffusion of nutrients, glucose, and insulin.[6-8] This could potentially lead to avoidance of the need to administer immunosuppressive drugs, which cause unwanted side-effects.[9,10] This technology of immunoisolation is subject of intense research, as it would make islet-transplantation available for a larger group of patients with type I diabetes.[6,11]

The proof of principle application of encapsulated islets for the treatment of type I diabetes has been shown in several studies in both experimental animals and in humans,[12,13] however, islet graft survival is still never permanent and in most cases limited to several months.[14] A factor considered to influence the duration of graft survival is the loss of interaction with the extracellular matrix (ECM) in isolated islets.[2,15-17] As a consequence of islet-isolation, the ECM molecules that surround the islets and interconnect the endocrine cells in the pancreatic islets are damaged by application of collagenases.[2,16,18,19] This enzymatical isolation process impacts laminins as well as collagen type I, III, IV, V and VI in islets.[20] Moreover, recently it has been shown that after isolation with ECM-degrading collagenases, the whole microvasculature of the islet is destroyed,[20] and that pancreatic islet-cells undergo different cell death processes such as anoikis, necroptosis, and necrosis.[18,19,21,22]

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A strategy of supplementation of islets with ECM molecules has been shown to enhance functional survival of encapsulated pancreatic islets.[23-25] Incorporation of ECM guides cellular development by mimicking the biochemical composition of ECM in the target organ, its fibrillar structure, and its viscoelastic properties.[26] Examples of molecules that are considered to enhance survival of islets are laminins and collagens.[27] Laminin may benefit islet-survival by modulating the expression of specific transcription factors and hormones such as Pdx-1, insulin 1 and insulin 2, glucagon, somatostatin, and GLUT-2.[28] Moreover, different adhesive laminin sequences such as IKVAV, VAYI, and IKLLI, YIGSR, PDSGR, located in α1 and β1chain have been shown to influence pancreatic islet-cell function.[29,30]

The most essential collagens used as supplement or adjuvant for cellular functions in biomedical applications are collagens type I and IV.[31,32] Collagen is present and located in the islet–exocrine interface and basement membrane of islets where it regulates fibronectin assembly by restraining cell–fibronectin interactions.[33-35] In contrast to collagen type IV, effects of collagen type VI on islet-survival and function has not been studied. Collagen type VI is composed of three polypeptide chains that form a short triple helix. These polypeptide chains are named α1 (VI), α2 (VI), and α3 (VI), and are encoded by three genes.[36] The importance of collagen type VI for tissue homeostasis is illustrated by the observation that a deficiency of collagen type VI results in death of muscle cells causing muscular dystrophy.[37] Since collagen type VI is abundantly present in the pancreas,[38,39] we investigated the effect of exogenous collagen VI on islet-cell survival and function.

The goal of this study was to investigate the effect of collagen type VI on the functional survival of human pancreatic islets in immunisolating alginate-based capsules. Functional survival of islets was studied by determining the glucose stimulated insulin secretion (GSIS) and the oxygen consumption rate (OCR) in islets encapsulated in alginate with or without collagen VI.

Materials and methods

Human islets culture

Human islets were obtained from Prodo Laboratories Inc. (Irvine, USA). A dithizone (Merck, USA) staining was performed before shipment to determine the purity. Islets with a purity over 80% were shipped to the Groningen University Medical Center (Groningen, The Netherlands). Immediately after arrival, islets were washed before handpicking and cultured in CMRL medium (Gibco, USA), supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin (Gibco, USA), 1% glutamax (Gibco, USA), 2% HEPES (Gibco, USA), and 8.3 mM D-glucose (Sigma-Aldrich, USA). Before encapsulation, islets were both micro- and macroscopically inspected. After encapsulation islets were tested for glucose stimulated insulin secretion (GSIS) and subjected to live-dead staining kit for mammalian cells to confirm viability and function. Human islets were cultured in a humidified 5% CO_2 incubator at 37°C.

Microencapsulation

Capsules were composed of purified 3.4% intermediate-G alginate (44% G-chains, 56% M-chains, 23% GG-chains, 21% GM-chains, 37% MM-chains), mixed with collagen type VI (Abcam, UK) by physical entrapment in concentrations of 0.1, 10, and 50 μg/ml. Capsules without type VI collagen served as controls. An air-driven droplet generator was used for the encapsulation of human islets.[40,41] Before encapsulation, per donor, islets were split up in different appropriate portions and mixed with 3.4% ultrapure alginate containing either 0.1, 10, or 50 μg/ml collagen VI. The alginate was purified as previously described[42] and tested for presence of endotoxins.[43,44] Islets were mixed in a ratio of 1000 islets/ml alginate and transferred into droplets[45,46] and collected in 100 mM $CaCl_2$. Droplets were gelled in 100 mM $CaCl_2$ solution for at least 10 minutes.[47] The diameters of the droplets were between 500–650 μm. All droplets were washed with KRH buffer containing 2.5 mM $CaCl_2$ for 2 minutes. Subsequently, encapsulated islets 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.[48-50]

Glucose-stimulated insulin secretion of encapsulated islets

Human pancreatic islets were tested for GSIS at days 3, 5, and 7. The production of insulin was measured in response to low and high glucose solutions. Twenty-

five encapsulated islets were transferred to glass incubation tubes. The first incubation consisted of a low glucose concentration solution in KRH (2.75 mM) for 1 hour, followed by a high glucose concentration buffer in KRH (16.7 mM) for 1 hour, and another 1-hour 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 previously described.[41] 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 a nanogram of insulin.ml^{-1}. μDNA^{-1}. hour^{-1}.

Live/dead cell viability assay

Viability of the encapsulated islets was tested using a LIVE/DEAD Cell Viability/Cytotoxicity Assay Kit (Thermo Scientific). Encapsulated islets were incubated in culture media with Calcein AM (1 mM) and Ethidium homodimer (EthD) (2 mM) (concentration according to manual) for 30 minutes in the dark. After the incubation period, islets were washed with 25 mM KRH buffer, pH 7.4 prior to imaging.

A helium-neon and an argon-krypton laser were used in combination with a confocal microscope (Leica) to quantify islet-cell viability at days 3, 5, and 7. The data was analyzed in ImageJ (v1.48b; National Institutes of Health), and viable or dead cells was expressed as the percentage the total number of stained cells. Imaris 664 version 7.6.4 software was applied.[45]

Islet oxygen consumption

The oxygen consumption rate (OCR) was measured using an XF24 Extracellular Flux Analyzer (Seahorse Bioscience, USA).[51] This experiment was performed at 72 hours after encapsulation and for one-time point only as the assay requires the use of high numbers of islets. To perform OCR, human islets were retrieved from the capsules by dissolving the capsules in 25 mM citrate solution at 37°C. The islets were transferred to 6-well plates, and fresh medium was added before putting the plate at culture conditions. After that, human

islets were transferred to the microplate reader provided with the XF24 Islet FluxPak (Seahorse Bioscience, USA). For the correct measurement, at least 80 islets were inserted in every well of the XF24 islet capture microplate. Experiments were performed in triplo. The islets were centered in the wells and a screen was fixed upon each islet-containing well. The medium was removed and the islets were washed 3 times with MA medium (XF assay media) and incubated for 60 minutes at 37°C. After addition of 500 μl of MA medium, the plate was placed into the XF24 Extracellular Flux Analyzer. At the end of the experiment, islets were transferred to Eppendorf cups and stored at −20°C for DNA content analysis. OCR data was normalized for DNA content to allow for comparison of different experimental conditions and adjust for the variation in islets numbers. Oxygen consumption rate was expressed as minutes OCR/DNA ($p$mol O2·min^{-1}·mg^{-1} DNA) and analyzed by Seahorse XF24 software. An initial drift in OCR was typically observed in the first 1–2 measurements. Analysis started at an interval of 5–8 minutes after steady state was reached.

Statistical analysis

Results were expressed as a mean ± standard error of the mean (SEM). A Two-way ANOVA followed by Tukey's secondary tests for significance was used for GSIS, stimulation index and live-dead staining data. Paired t-test was applied in order to evaluate the OCR. The analysis was performed using GraphPad Prism 5.0 (GraphPad Software, USA). A value of $p < 0.05$ was considered statistically significant.

Results

Effect of collagen type VI on GSIS on encapsulated human islets

We investigated the effect of collagen type VI on islet-cell functionality and survival, as collagen type VI is an ECM component and basement membrane (BM) anchoring molecule in human islets.[35,52] To this end, collagen VI was incorporated into islet-containing alginate microcapsules. We tested the effects of 0.1, 10, and 50 μg/ml on GSIS of human islets encapsulated in alginate at days 3, 5, and 7. The GSIS data for encapsulated islets with and without collagen type VI is shown in Fig. 1. GSIS was not statistically influenced

by collagen type VI addition, but there was a trend toward higher GSIS in encapsulated islets with 10 μg/ml and 50 μg/ml at day 7.

Interestingly, stimulation index of pancreatic islets entrapped with 10 μg/ml collagen type VI was significantly higher ($p < 0.05$) than that of control groups (Fig. 2). The glucose stimulation index significantly increased 1.8 and 2.3 time-fold at day 5 and 7 for islets encapsulated in alginate with 10 μg/ml collagen type VI ($p < 0.05$).

Collagen type VI improves cell viability of human islets

Despite the absence of statistically significant effects on GSIS, we did find significant effects of collagen type VI incorporation on the viability of encapsulated
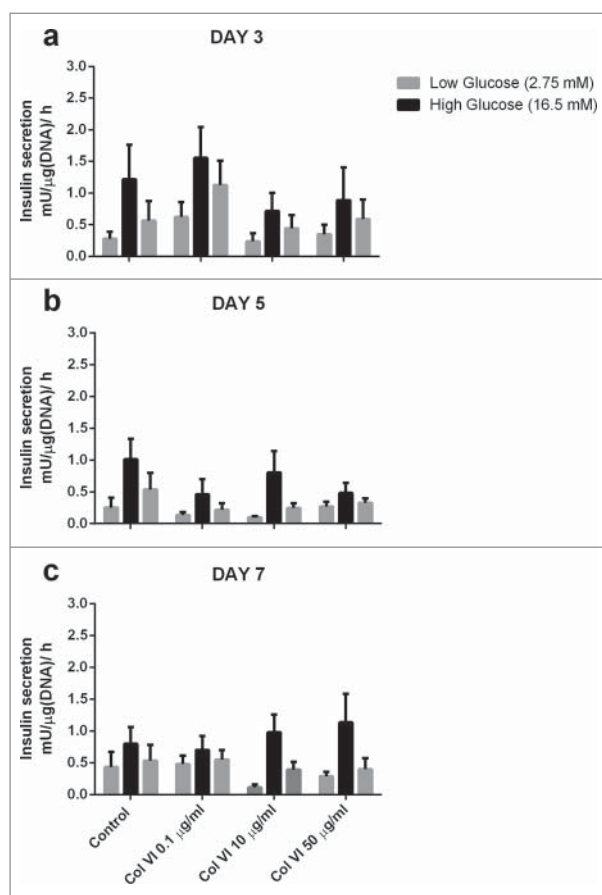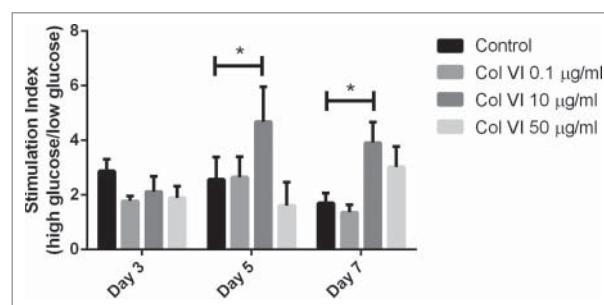

Figure 2. The figure shows the stimulation index (SI) of encapsulated islet in response to low (2.75 mM), and high (16.7 mM) glucose. Results from human islets encapsulated in alginate-based capsules supplemented with collagen VI at concentrations of 0.1, 10 and 50 μg/ml collagen VI tested at day 3, 5 and 7. Error bars represent standard error. Statistical significance is indicated by stars ($p < 0.05$).

pancreatic islets, as shown in Fig. 3b. From day 3 of culture and onwards, cell-survival enhancing effects of collagen type VI were observed. At day 5, all the tested groups containing collagen type VI had strong and statistically significant effects on islet-cell viability ($p < 0.01$). The percentage of surviving cells was 87.7 $\pm$ 6.1%, 86.5 $\pm$ 2.4% and 85.6 $\pm$ 1.9% ($p < 0.01$), when collagen type VI at 0.1, 10 and 50 μg/ml were added to the alginate matrix respectively. Moreover, a significant increase of 11% on islet viability was observed in encapsulated islets entrapped with 10 μg/ml collagen type VI at day 7. Although, no differences between higher collagen VI concentrations at any time-point was observed, in general, the strongest and most persistent effects were observed with encapsulated islets containing 10 μg/ml collagen type VI ($p < 0.05$).

Interestingly, islet-containing capsules with collagen type VI showed a diminished cell death trend compared to controls during the culture days (Fig. 3c). Although, not statistically significant at day 3, all tested groups containing collagen type VI demonstrated a trend to inhibition of cell death. At day 5, pancreatic islets entrapped with 0.1 μg/ml collagen type VI have shown a 16 $\pm$ 7.6% on death cells rate, presenting a decrease of 12.2% when compared to capsules without collagen type VI ($p < 0.05$).

Oxygen consumption rate analysis

The effect of collagen VI on oxygen consumption rate (OCR) of islet-cells was tested only at 72h of culture because we needed to use relatively high amounts of islets. The addition of collagen type VI does enhance

Figure 1. Glucose induced insulin secretion (GSIS) of human islets encapsulated in alginate-based capsules supplemented with collagen VI. Concentrations of 0.1, 10 and 50 μg/ml collagen VI tested at day 3 (a), 5 (b) and 7 (c). The bars from left to right indicate insulin production upon incubation with low (2.75 mM), high (16.7 mM), and low glucose solution of sets of 25 pancreatic human islets for each condition. Values represent mean $\pm$ SEM (n = 4, different donors).
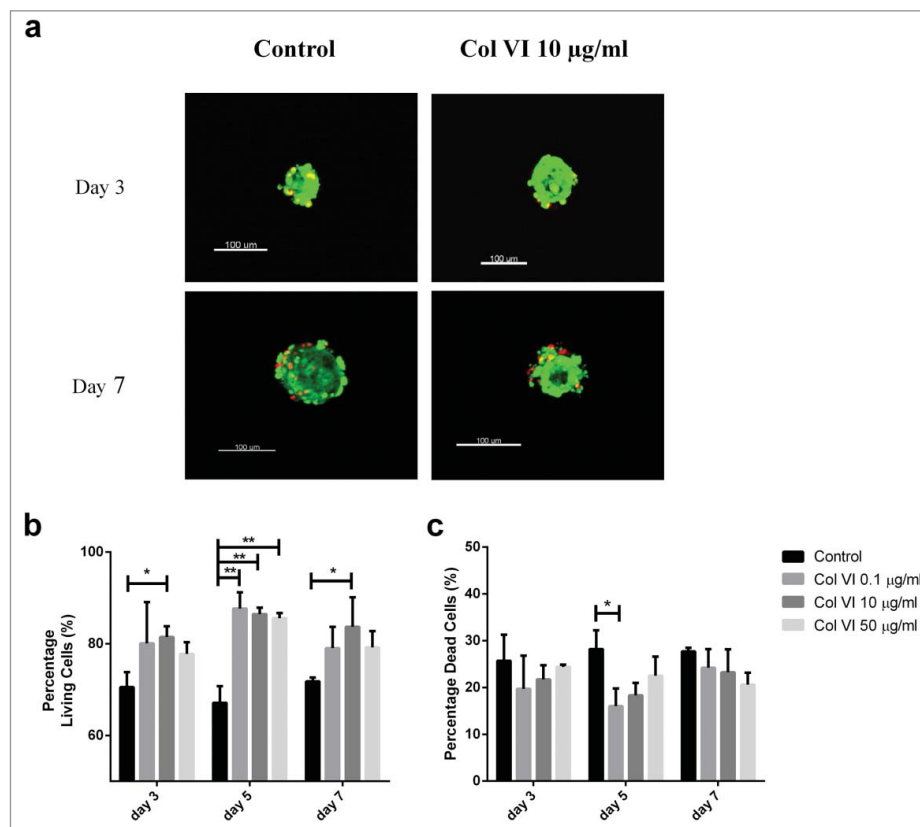
Figure 3. Live/dead staining of human islet-cells encapsulated in alginate-based capsules containing either 0.1, 10, and 50 μg/ml collagen type VI. Illustration of islets in control condition (in alginate capsule without collagen), and a capsule containing 10 μg/ml collagen VI (a). Islet-cell viability was enhanced by inclusion of collagen VI in the immunoprotective capsule at all-time points tested (b). Encapsulated human islets were stained with ethidium homodimer-1 to quantify the percentage of dead cells at days 3, 5, and 7 of culture (c). Alginate capsules without ECM components served as control. Values represent mean $\pm$ SEM (n = 4, different donors). *, and ** indicates statistical significant differences ($p < 0.05$), and ($p < 0.01$) when compared to control islets respectively (Col VI, collagen type VI).

OCR but this only reached statistically significant differences when applying 0.1 μg/ml ($p < 0.01$) and 10 μg/ml ($p < 0.05$). The oxygen consumption rate gives an indication of the viability and functionality of the islets and correlates with graft survival after implantation.[53,54] As shown in Fig. 4, islets encapsulated in 0.1 μg/ml collagen type VI had an OCR of 769.07 $\pm$ 82.9 $p$mol $O_2 \cdot min^{-1} \cdot mg^{-1}$ DNA which was nearly two-fold higher than the OCR of the control group ($p < 0.01$). OCR of islets entrapped with 10 μg/ml collagen type VI was 1.5 fold higher than the control group ($p < 0.05$). Additionally, there was a trend towards higher OCR using higher concentrations of collagen type VI.

Discussion

Various ECM molecules have shown to enhance the survival of islets. Examples of these molecules are laminin and several collagens.[27] Collagens type I and type IV have been studied extensively for their role in enhancing survival of pancreatic islet-cells[2,55] but collagen type VI has not been studied yet. Collagen type VI is expressed in human connective tissues, and in some specialized regions such as the pancreas islet–exocrine interface,[35] but its presence in the basement membrane is less recognized.[52] Just like other collagen types in islets, collagen VI is structurally damaged and digested during the enzymatic isolation process of islets from the pancreas.[20] To the best of our knowledge, this study is the first to demonstrate the beneficial effects of collagen type VI on functional survival of human islets. Collagen type VI can provide advantages over other types of ECM, as collagen type VI is the predominant constituent subtype immediately surrounding islets in the pancreas.[38] Previous studies have shown that collagen type VI is present in the peri-islet capsule in the porcine pancreas,[33] whereas
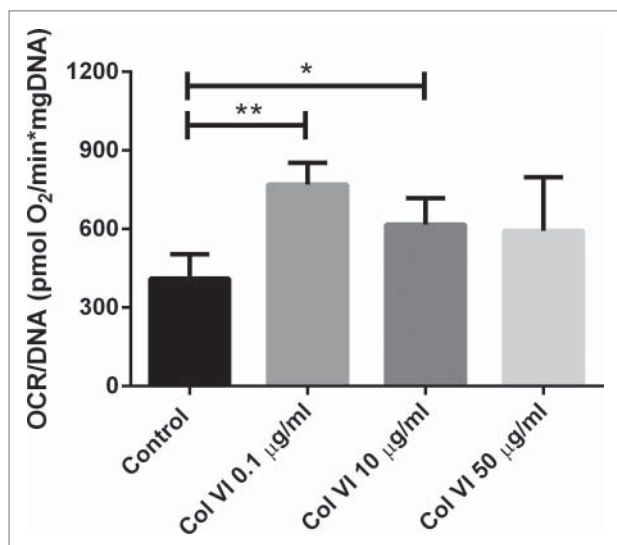
Figure 4. Oxygen consumption rate (OCR) of human islets encapsulated in alginate capsules containing a combination of 0.1, 10 or 50 μg/ml after 72 hours in culture. OCR was expressed after correction for DNA content (OCR/DNA). Values represent mean $\pm$ SEM. *, and ** indicates statistical significant differences ($p < 0.05$), and ($p < 0.01$) when compared to control islets respectively (n = 4, different donors).

collagen type I, III, and IV are expressed predominantly in the peri-islet region.[56]

Although not statistical significant, effects on GSIS were found. We observed significant survival enhancing effect of up to 20% when collagen type VI was incorporated in the alginate-based capsules. In addition, beneficial, enhancing effects were observed on OCR. The latter is an important measure for viability after implantation as higher OCR values correlate with higher graft survival rates after implantation.[54] OCR represents the metabolic responsiveness of the islets, and correlates better with functionality of the graft than GSIS.[54] Notably, our results are obtained with islet-preparations with minimal donor-variations in viability. Results may be different with islet-sources of lower quality or preparations where larger donor-variability is present. In all cases however collagen VI may enhance viability.

Higher concentrations of collagen type VI were associated with a negative impact on survival of islet-cells. A similar concentration dependent effect on islet-OCR has been reported for collagen type IV.[2] This can be explained by the fact that supraphysiological collagen concentrations diminish metabolic capacity such as insulin release.[2,23,57] Negative effects of too high collagen concentrations have also been reported in cancer studies. Tumor-promoting effects of supraphysiological high concentrations of collagen type VI

were observed,[58] suggesting that increased concentrations enhance cell survival at the expense of cell functioning. Thus, increasing the concentration of type VI collagen beyond the tissue-concentration could induce toxicity and negatively impact islet-cell survival.[2,57-59] The effect of collagen VI on islet-cell survival is different from that of collagen IV. Collagen IV impacted both GSIS and viability while collagen VI only impacts viability. Collagen IV enhanced GSIS in a concentration dependent fashion and was most effective at a concentration of 50 μg/ml.[2,25] The positive effect on viability was similar for the two collagen types.

We recently have shown that inclusion of ECM is an efficacious strategy to promote islet-cell survival after isolation of human islets from the pancreas.[2,57] Here we have focused on the efficacy of collagen type VI. We show it has a positive impact on islet-cell viability. It did not influence GSIS but did enhance cell-survival and OCR. Moreover, as collagen VI specifically stimulates the cell death inhibitor β1 integrin it is probably this interaction that is partly responsible for the beneficial effect of collagen VI on islet-cell survival. However, as ECM act in concert with other collagens and laminins it might also be that addition of collagen VI to the intracapsular environment contributes to re-assembly of the ECM islet-network and by that contributes to prevention of cell death. A third explanation is that islet-isolation is associated with release of deleterious cytokines that damage islet-cells in an auto- or paracrine fashion.[18] Addition of ECM might reduce cytokine susceptibility of islets[57] and thereby contribute to better survival of islet-cells.[57] Which ECM components is most effective in supporting these beneficial effects is still subject of investigation.[2,57,59] In previous studies, we have shown that the interaction and beneficial effects of ECM of islets is highly ECM dependent and even concentration dependent.[2,57] Our current study extends these investigations and show that collagen VI contributes to survival of islet-cells after isolation from the pancreas.

Conclusions

Not all but only a few ECM molecules support function and survival of human pancreatic islet-cells in alginate-based microcapsules applied for immunoprotection of islets.[2] Here we show that collagen VI is one of these survival promoting ECM molecules. Incorporation of 0.1 to 10 μg/ml collagen type VI in the

alginate-capsule microenvironment promotes islet-cell viability. The present data underscore that the intra-capsular environment should receive more attention in effort to support longevity of encapsulated islet.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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