Journal of
Stem Cell Research & Therapy
ISSN: 2157-7633

Ngamjariyawat et al., J Stem Cell Res Ther 2016, 6:6
DOI: 10.4172/2157-7633.1000343

Research Article Open Access

Co-culture of Insulin Producing Human EndoC-βH1 Cells with Boundary Cap Neural Crest Stem Cells Protects Partially against Cytokine-induced Cell Death

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Abstract

We have recently observed that co-culture of mouse and rat beta-cells with mouse boundary cap neural crest stem cells (bNCSCs) protected against inflammatory cytokine-induced beta-cell death, possibly via direct cadherin-mediated cell-to-cell junctions. However, it has not been addressed whether also human beta-cells can be protected via this strategy. If possible it would be an important approach for development of new protocols for improved outcome of islet transplantation to Type-1 diabetes patients. The aim of this investigation was therefore to study the effect of bNCSC co-culture with insulin producing human EndoC-βH1 cells on cytokine-induced cell death. For this purpose GFP-positive bNCSCs were cultured together with GFP-negative human EndoC-βH1 cells in the presence of the cytokines IL-1β (50 U/ml) and IFN-γ (1000 U/ml). Cells were then stained with propidium iodide and trypsinized for flow cytometry analysis. Analysis of propidium iodide fluorescence in GFP-positive and GFP-negative cells revealed that EndoC-βH1 cells died to a lower extent when co-cultured with bNCSCs than when cultured without bNCSCs. We also observed that EndoC-βH1 cells formed N-cadherin, but not E-cadherin junctions with the bNCSCs. The bNCSC cell population contained a large proportion of beta-tubulin expressing cells indicating neuronal differentiation. A protective function of the N-cadherin junctions was verified by the finding that a neutralizing N-cadherin antibody counteracted the effect of co-culture. We conclude that the interaction between human insulin producing cells and bNCSCs results in a lowered susceptibility of insulin producing cells to pro-inflammatory cytokines *in vitro*.

Keywords: Autoimmune disease; Cytokines; Diabetes; Immunostaining; Cell death

Introduction

Type 1 diabetes is an autoimmune disease that results in destruction of the insulin-producing beta-cells. Pro-inflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α), and interferon-γ (IFN-γ), induce beta-cell death *in vitro*; and the local release of the same cytokines has been proposed to participate in pancreatic beta-cell destruction in vivo [1]. Indeed, levels of pro-inflammatory cytokines have been correlated to insulitis and beta-cell destruction in both NOD mice [2] and human pancreatic biopsies from patients with recent-onset type 1 diabetes [3]. After receptor activation, signal transduction by these cytokines involves the activation of mitogen-activated protein kinases (MAPK), c-Jun NH2-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 MAPK [4,5]. In addition to the mitogen-activated protein kinases, IL-1β and TNF-α–induced signaling results in activation of the pro-inflammatory transcription factor NF-κB [6]. It has been suggested that in rodent islets, cytokine-induced cell death is caused by increased nitric oxide production, which results from activation of NF-κB–mediated inducible nitric oxide synthase gene transcription [6,7].

Presently, transplantation of isolated islets has become an attractive approach to treat selected patients with Type 1 diabetes. However, adequate graft function is seen in less than 10% of patients after 5 years [8]. Immediate post-transplantation cell death and graft failure are likely due to hypoxia in combination with inflammatory events [9]. To increase beta-cell mass and dampen postgrafting inflammation are thus important goals for successful islet transplantation. It is possible that co-transplantation of islet cells with boundary cap neural crest stem cells (bNCSCs) will promote an improved islet transplantation outcome. Indeed, recent studies have demonstrated that bNCSCs have an important role in beta-cell differentiation by regulating beta-cell mass during development [10]. It has also been observed that embryonic dorsal root ganglia affect insulin secretion in co-cultured islets [11], that co-cultured islets and bNCSCs have mutual beneficial effects *in vitro* [12], and that co-transplantation of bNCSC-derived neurospheres with both mouse and human islets induces proliferation and promotes function of transplanted beta-cells [13,14], an effect that was recently reproduced also *in vitro* [15]. More recently, co-culture of insulin producing cells and bNCSC resulted in protection against cytokine-induced beta-cell death [16], possibly via direct cadherin-mediated cell-to-cell contacts [17]. Furthermore, the positive effects of bNCSCs on beta cells seems to be specific for boundary cap and not for other types of NCSCs.

However, the mechanisms by which bNCSCs mediate positive effect on beta cells are not well characterized, especially in human insulin producing cells. Thus, the aim of the present investigation was to closer study *in vitro* interactions between bNCSCs and the human insulin producing cell line EndoC-βH1. These cells are a recently generated human β-cell line that displays a glucose-sensitive insulin

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Received June 04, 2016; **Accepted** June 20, 2016; **Published** June 27, 2016

Citation: Ngamjariyawat A, Vasylovska S, Turpaev K, Ravassard P, Welsh N, et al. (2016) Co-culture of Insulin Producing Human EndoC-βH1 Cells with Boundary Cap Neural Crest Stem Cells Protects Partially against Cytokine-induced Cell Death. J Stem Cell Res Ther 6: 343. doi:10.4172/2157-7633.1000343

release [18] and exhibit glucose-induced respiration and stimulus-secretion coupling responses similar to human islets [19]. A better understanding of the underlying mechanisms for neural crest stem cells and beta-cell interaction is crucial for development of new protocols for improved outcome of islet transplantation to diabetes patients.

Material and Methods

Mice

Transgenic heterozygous C57BL/6-β-actin enhanced green fluorescent protein (EGFP) mice (Jackson Laboratories, Bar Harbor, ME, http://www.jax.org) were used to generate bNCSCs.

Ethic statement

All procedures were approved by the Regional Ethics Committee for Research on Animals (The Uppsala County Regional Ethics Committee for Research on Animals).

EndoC-βH1 cells

Human EndoC-βH1, a kind gift from Profs. Scharfmann and Ravassard, INSERM, Paris, France, cells were cultured in ECM/fibronectin-coated plates in low-glucose DMEM with supplements as previously described [18]. All cells were grown at 37°C in a humidified air incubator with 5% CO_2.

Preparation of bNCSC culture

Dorsal root ganglia from E11.5 day old EGFP mouse embryos were isolated and used to generate bNCSCneurospheres (NL38 cell line) from the so-called boundary cap (20, 21). Briefly, mice were anesthetized by intraperitoneal injection of xylazine (Rompun®vet.; http://www.bayer.com) and ketamine (Ketaminol®vet.; http://www.intervet.com) (10 and 100 ng per gram body weight, respectively) and the uterus was removed from the anaesthetized pregnant mouse and placed in cold PBS. Embryos were separated and rinsed in PBS, placed in DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA), supplemented with N-2 (Invitrogen, Carlsbad, CA, USA) (N-2 medium), after which the dorsal root ganglia were removed and collected. Collected dorsal root ganglia were allowed to settle before removing the supernatant fractions and adding a collagenase/dispase (1 mg/mL) (Roche Diagnostics Scandinavia, Bromma, Sweden) and DNase solution (0.5 mg/mL) (Sigma-Aldrich) in N-2 medium and incubating for 20 to 30 min in a 37°C water bath. This was followed by rinsing in N-2 medium supplemented with B-27 (1:50) (Invitrogen, Carlsbad, CA, USA) titrating and plating of 1 to 2 × 105 cells/well in a 24-well dish after dissociation. Cells were placed directly into 500 µL N-2 medium containing B-27, epidermal growth factor (20 ng/mL) (R&D Systems, Minneapolis MN, USA) and basic fibroblast growth factor (20 ng/mL) (R&D Systems). After 12 h, non-adherent cells were removed together with half of the medium before adding 250 µL of fresh medium. The medium was then changed every other day (50% of the medium replaced with fresh medium) before neurospheres began to form. Neurospheres from passages 4 to 5 were treated with TrypLE (Invitrogen) for 7 min to generate free single cell suspensions for subsequent co-culture with EndoC-βH1 cells.

In vitro treatment of cells

105 dispersed bNCSCs were plated in 24-well plates pre-coated ECM/fibronectin. At the same time 105EndoC-βH1 cells were plated either alone or together with the bNCSC cells. At this stage the culture medium was changed to RPMI-1640 medium containing the same supplements as given above. After two days of co-culture, cells were either left untreated or treated with a mixture of cytokines (50 U/mL IL-1β + 1000 U/mL IFN-γ; Peprotech) for an additional 24 hours. When pre-treating cells with antibodies against cell contacts, EndoC-βH1 cells where seeded in a ratio of 1:5 with bNCSCs to ensure contacts between the two cell types. At the time of seeding the medium was supplemented with a function-blocking anti-N-cadherin antibody (25 µg/ml) (Zymed) or control IgG at the same concentration. To avoid degradation of the antibody were cultivated for only four hours before cytokines where added as afore mentioned.

Flow cytometry analysis of cell viability

In vitro cultures of EndoC-βH1 cells, bNCSCs or EndoC-βH1 + bNCSCs were labelled for 10 min at 37°C with 10 µg/ml of propidium iodide (Sigma-Aldrich). The cells were trypsinized for 5 min at 37°C. Cell suspensions were analyzed in a Becton Dickinson FACSCalibur flow cytometer for FL1 (GFP) and FL3 (propidium iodide) fluorescence. Cell death frequencies were quantified for GFP positive and GFP negative cells separately, and expressed as percentage of total GFP positive and negative cell numbers, respectively.

Propidium iodide positive-beta cell counting

To evaluate whether direct contact between bNCSC-beta cells plays a role behind beta-cell viability, EndoC-βH1 cells were cultured either with or without bNCSCs and treated with cytokines as afore mentioned. The cells were stained with 10 µg/ml propidiumiodide and Hoechst (Sigma-Aldrich) for 10 min at 37°C after which the cells were washed. Fluorescent images of labeled EndoC-βH1 cells were taken using a 40X lens. The numbers of labeled EndoC-βH1 cells which were in direct contact with bNCSCs were counted and compared with the numbers of EndoC-βH1 cells cultured alone by using ImageJ software.

Immunostaining

Cells were fixed in 4% buffered paraformaldehyde at room temperature for 5 minutes then washed with PBS prior to permeabilization and blocking using PBS with 0.1% triton® X-100 (Sigma), 1% BSA (Sigma), and 3% fetal calf serum. The cells were incubated with primary antibodies in PBS with 1% BSA and 1% fetal calf serum for 30 minutes at 37°C before washing two times with PBS. The cultures were then incubated with secondary antibodies for 30 minutes at 37°C and rinsed three times in PBS for 15 minutes, the second wash included Hoechst 33242 (11 ng/mL, Invitrogen). Coverslips were mounted on glass slides with DakoCytomation fluorescent mounting solution. Primary antibodies were as follows: anti-beta-tubulin, (polyclonal rabbit, 1:800, Covance Research Products), anti-GFAP (polyclonal rabbit, 1:500, Dako), anti-N-cadherin (polyclonal rabbit, 1:100, Abcam) and anti-E-cadherin (monoclonal mouse, 1:100, Abcam). Secondary antibodies were Cy3 (donkey anti-mouse, 1:500, Jackson laboratories), Alexa flour 555 (goat anti-rabbit, 1:600, Invitrogen), and Alexa flour 594 (goat anti-mouse, 1:800, Invitrogen).

Microscopic analysis

Immunolabelled slides were analyzed in a Nikon Eclipse E800 fluorescence microscope and Zeiss LSM 780 confocal microscope. The images were analyzed with ZEN software.

Plasma membrane E/N-cadherin intensity measurement

Fluorescent images of E- and N-cadherin labeled beta-cells were analyzed using ImageJ software. Based on bNCSC-beta cell

connections, we divided the labeled beta-cells into four groups (N=100 cells/group): direct bNCSC-beta cell contact, indirect bNCSC-beta cell contact, beta-cell alone in group, and single beta-cell. For calculation of plasma membrane E- and N-cadherin intensity, we used the following formula:

Plasma membrane intensity = Mean gray value of selected area-Background, where background is the mean gray value for a region selected just beside the cell.

Statistical analysis

Data were analyzed using Student's paired t-test or ANOVA followed by Tukey's post hoc test. For both tests significance was set at $p < 0.05$.

Results and Discussion

bNCSC co-culture protects EndoC-βH1 cells from cytokine induced cell death

In the present investigation we plated human EndoC-βH1 cells and murine bNCSCs either alone or together in co-culture and analyzed cell death in response to pro-inflammatory cytokines IL-1β and IFN-γ. Our previous studies have demonstrated beneficial effects of bNCSC–beta-cell co-culture when using mouse or rat insulin producing beta-cells [15,16], which warranted us to investigate whether also human beta-cells are protected. The cells were co-cultured in RPMI1640 since bNCSCs do not survive well in low-glucose DMEM (results not shown), which is the preferred culture medium for EndoC- β H1 cells [18]. During culture in RPMI1640 EndoC- β H1 survival was decreased, resulting in basal cell death rates of >30% (Figure 1), which is more than twice that of what is usually observed during culture in DMEM.

Nevertheless, cytokine addition resulted in an increased cell death of EndoC-βH1 cells when cultured alone (Figure 1), which concurs well with the well established understanding that beta-cells are highly susceptible to pro-inflammatory cytokines. Interestingly, co-culture of EndoC- β H1 together with bNCSCs reduced EndoC- βH1 cell death, both at basal and cytokine-stimulated conditions (Figure 1). This indicates that also human beta-cells benefit from co-culture with bNCSCs, even though the bNCSCs were from mouse.

In our previous studies we found evidence for an important role of direct cell-to-cell contacts for positive effects of bNCSCs on beta-cells [14,17]. To evaluate whether the protection of the EndoC- βH1 is mediated through direct cell-cell contact rather then secretion of protective substances we stained cells from either co-culture or pure EndoC-βH1 cell cultures with Hoechst and propidium iodide. By counting the number of beta-cells in contact with either other beta-cells or bNCSCs we found that cytokine induced beta-cell death was completely abolished when the cells were in contact with bNCSC (Figure 2). This result showed that the protective effect was mediated through cell-to-cell contact while indicating that the EndoC-βH1 cells that undergo cytokine induced cell death were either alone or in contact with other EndoC-βH1 cells. Based on previous observations [17], we continued to investigate if this cell contact mediated protection was mediated by cadherins.

EndoC-βH1 and bNCSC cells form numerous N-cadherin junctions but less E-cadherin junctions

To investigate whether human beta-cells can form cadherin junctions with murine bNCSCs, we immunostained the two cell types in co-culture for N- and E-cadherin subcellular localization, as beta-cells have been reported to express these two forms of cadherins [20-24].

We observed that the EndoC-βH1 and the bNCSCs were often in direct contact, thereby enabling cell-to-cell junctions (Figure 3). In addition, both bNCSCs and EndoC-βH1 cells displayed clear N-cadherin signals, and we often observed accumulation of N-cadherin at cell-to-cell junctions between non-GFP positive EndoC-βH1 cells and bNCSCs (Figure 3, white arrows), suggesting that mouse bNCSCs and human EndoC-βH1 cells can form homophilic N-cadherin junctions, despite the species difference.

On the other hand, immunostaining of E-cadherin revealed strong E-cadherin expression in EndoC-βH1 cells, but not in bNCSCs (Figure 4). In addition, we could not observe any accumulation of E-cadherin at beta-cell to bNCSC junctions, most probably due to the lack of E-cadherin expression in bNCSCs.

We next quantified N- and E-cadherin expression intensities at the plasma membrane region by ImageJ analysis. The plasma membrane regions of EndoC-βH1 cell either in direct contact with bNCSCs, in indirect contact with bNCSCs (via another beta-cell in direct contact with bNCSCs), growing in groups without any contact with bNCSCs, or in contact with no other cells were selected and quantified.

We observed that EndoC-βH1 plasma membrane N-cadherin intensities were lower in cells with no direct contact with bNCSCs (Figure 5). Especially single EndoC-βH1 cells showed no N-cadherin expression at the plasma membrane, which is compatible with the

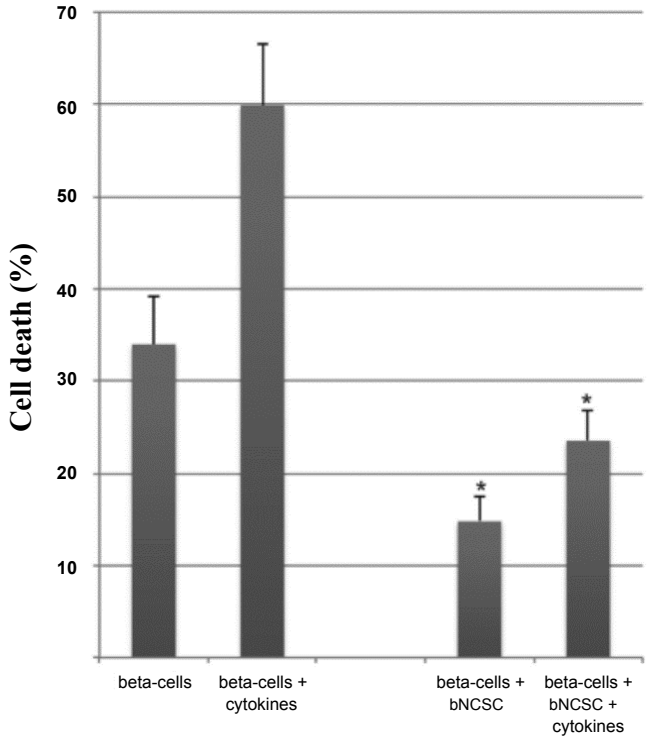

Figure 1: Flow cytometric analysis of cytokine-induced cell death rates in EndoC-βH1 and EndoC-βH1 + bNCSC cell cultures. EndoC-βH1 cells and EndoC-βH1 + bNCSC cells were cultured for two days and then exposed to IL-1β (50 U/ml) + IFN-γ (1000 U/ml) for 24 hours. Cells were labeled with propidium iodide, trypsinized, and then analyzed by flow cytometry. Results are means ± SEM for 5 observations. * denotes $p<0.05$ using Students t-test compared the corresponding beta-cell group.

requirement for cell-to-cell contacts for the formation of cadherin accumulation. The expression of E-cadherin, however, was higher between beta-cells than between EndoC-βH1 cells and bNCSCs. This corresponds to our observation that E-cadherin accumulation was not observed at EndoC-βH1-to-bNCSC junctions (Figure 4). We next verified the importance of N-cadherin junctions in cytokine mediated cell death.

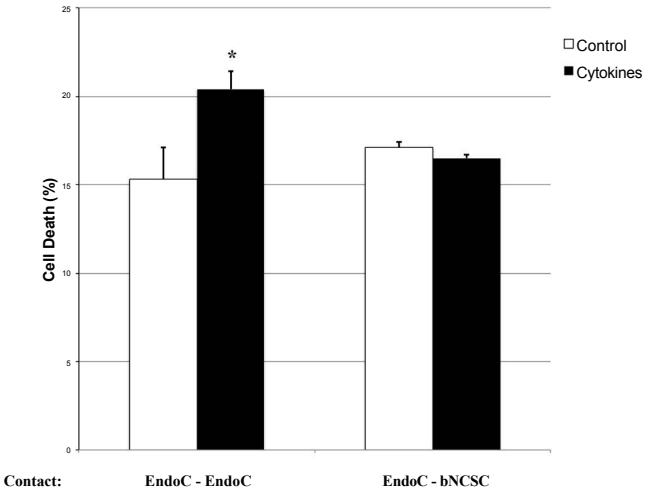

Figure 2: Fluorescence microscopy analysis of cytokine-induced cell death rates in EndoC-H1 cells in contact with EndoC-βH1 cells or bNCSCs. EndoC-βH1 cells and EndoC-βH1 + bNCSC cells were cultured for two days and then exposed to IL-1β (50 U/ml) + IFN-γ (1000 U/ml) for 24 hours. Cells were labeled with propidium iodide and Hoechst.Images were then acquired by fluorescence microscopy and analyzed using the ImageJ software. Results are means ± SEM for 4 observations. * denotes p<0.05 using Students t-test compared to control.

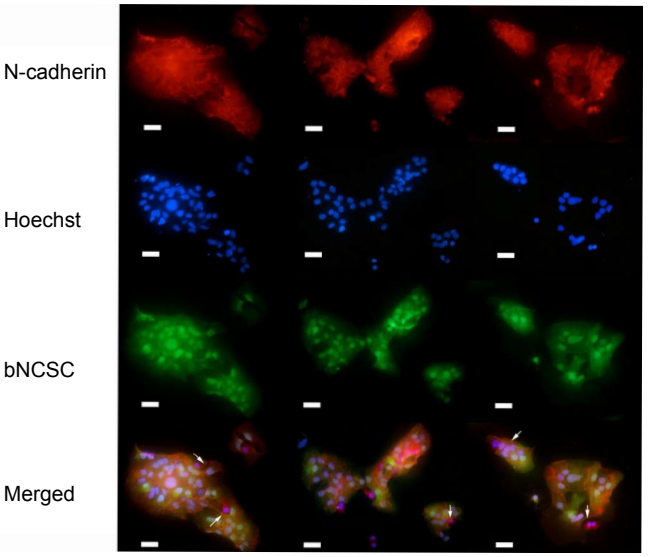

Figure 3: EndoC-βH1 cells and bNCSCs form N-cadherin junctions. Non-GFP positive EndoC-βH1 cells were cultured together with GFP-positive bNCSC cells (green cells) on ECM/fibronectin-coated cover slips. Cells were then fixed, permeabilized, and stained for N-cadherin (red). Nuclei were visualized by Hoechst. Fluorescence was then photographed in a fluorescence microscope using a 40X lens. Arrowheads indicate positions of N-cadherin junctions between EndoC-βH1cells and bNCSCs. Results are representative for three independent experiments. Scale bar is 20 μm.

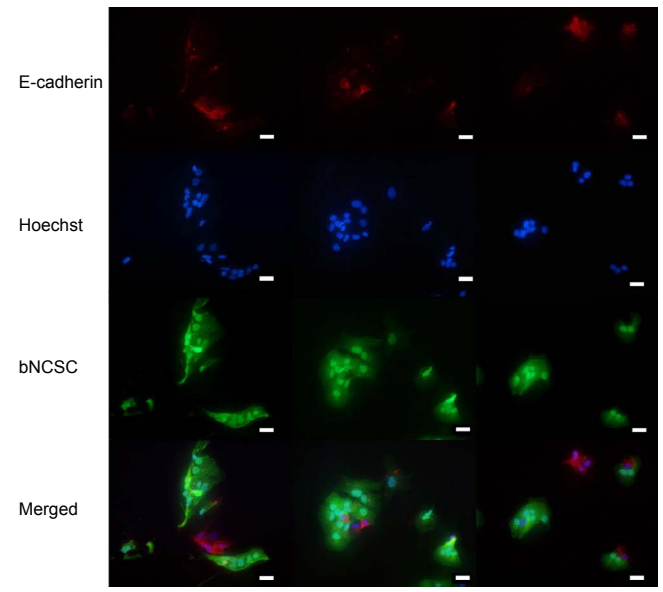

Figure 4: EndoC-βH1cells and bNCSCs do not form E-cadherin junctions. Non-GFP positive EndoC-βH1cells were cultured together with GFP-positive bNCSC cells (green cells) on ECM/fibronectin-coated cover slips. Cells were then-fixed, permeabilized, and stained for E-cadherin (red). Nuclei were visualized by Hoechst. Fluorescence was then photographed in a fluorescence microscope using a 40X lens. Results are representative for three independent experiments. Scale bar is 20 μm.

Blocking the formation of N-cadherin junctions partially inhibits the protective effect of EndoC-βH1- bNCSC co-culture on cytokine induced cell death

To analyze the role of N-cadherin in cytokine induced cell death the cells were seeded in the presence of a function-blocking antibody. The antibody binds to the extracellular domain of N-cadherin thereby blocking the formation on N-cadherin junctions. To avoid degradation of the antibody the cells were cultured for only four hours before adding the cytokines and cell death was measured after approximately 18 hours. In accordance with the results in Figure 2, which indicated that the beta-cells affected by the cytokines were those without contact with bNCSC, we now seeded the cells in a 1:5 ratio to increase the probability of EndoC-βH1 – bNCSC contacts. The results showed that the shorter culture and incubation time generated both a lower basal cell death and lower cytokine-induced cell death rate (Figure 6). Nevertheless, addition of cytokines led to an increase in cell death of EndoC-βH1 cells when cultured alone. The protective effect of the co-culture with bNCSC cells was partially counteracted in the group that has been cultured in presence of the N-cadherin blocking antibody. The reason for the small, but still significant, inhibition may probably be that theformation of N-cadherin junctions is only to a certain extent prevented by the anti-N-cadherin antibody. Finally, we wanted to study if the protective effect of the co-culture was due to differentiation of the bNCSCs during culture.

The protective effect of bNCSCs is mediated by differentiating cells

Co-culture of bNCSCs with EndoC-βH1 cells will result in differentiation of the bNCSC into neuronal and glial cell types. To characterize this process we stained the co-cultures with an anti-beta-tubulin antibody, which recognizes immature neurons, and

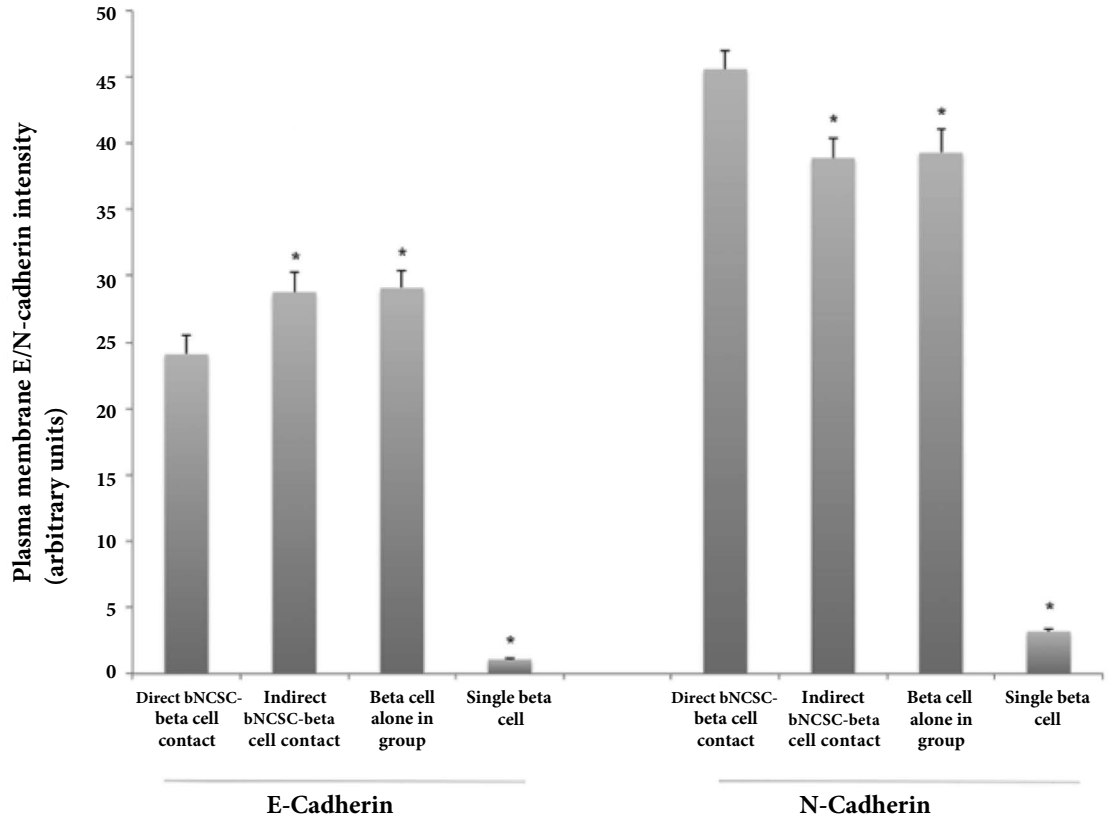

Figure 5: EndoC-βH1cells in direct contact with bNCSCs form N-cadherin junction, but not E-cadherin junctions. Non-GFP positive EndoC-βH1cells were cultured together with GFP-positive bNCSC cells on ECM/fibronectin-coated cover slips. Cells were then fixed, permeabilized, and stained for E-cadherin and N-cadherin. Nuclei were visualized by Hoechst. Fluorescence was then photographed in a fluorescence microscope using a 40X lens. Beta-cells were then grouped according to their vicinity to bNCSCs and the intensity of the N-cadherin and the E-cadherin signals at the plasma membrane region was calculated. Results are means ± SEM for 3 observations. * denotes $p<0.05$ using ANOVA followed by Tukey's post hoc test compared to direct contact.

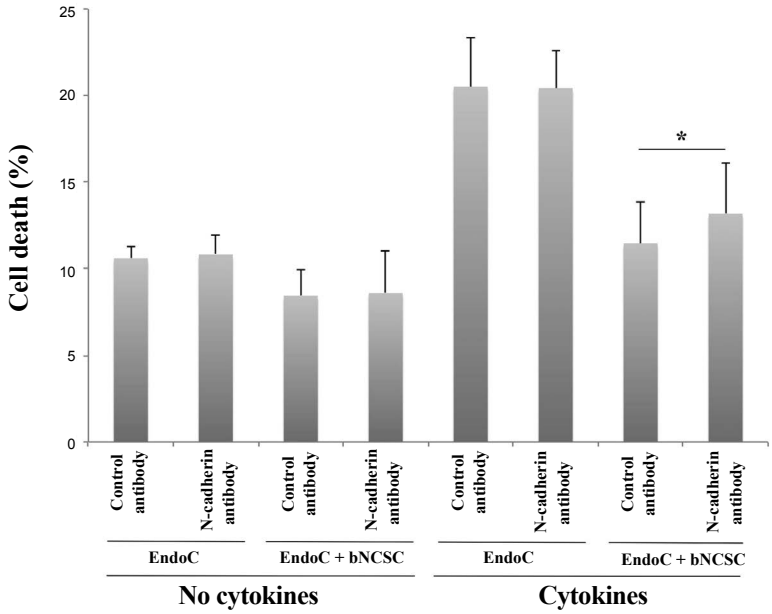

Figure 6: EndoC-βH1/bNCSC cell co-cultures are more sensitive to cytokines in the presence of an N-cadherin antibody.EndoC-H1 cells and EndoC-βH1 + bNCSC cells were seeded in the presence of a control antibody or an anti-N-cadherin antibody. After 4 hours of co-cultivation cytokines, IL-1β (50 U/ml) + IFN-γ (1000 U/ml), were added as indicated and the cells were cultured o/n (18h). Cells were labeled with propidium iodide, trypsinized, and then analyzed by flow cytometry. Results are means ± SEM for 4 observations. * denotes $p<0.05$ using Students t-test.

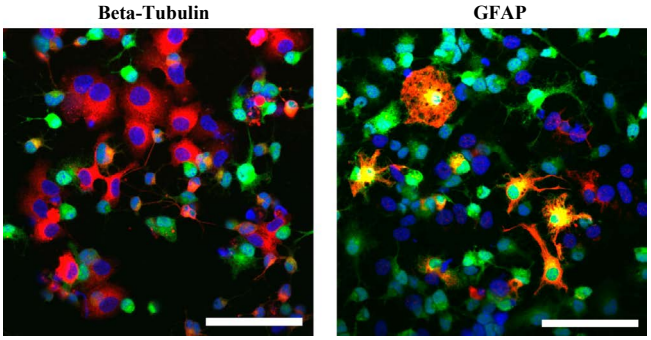

Figure 7: EndoC-βH1/bNCSC cell co-cultures contain beta-tubulin and GFAP positive cells.EndoC-βH1 cells and bNCSCs were co-cultured for 48 hours on cover slips coated with ECM/fibronectin and then stained for beta-tubulin (red) or GFAP (red). bNCSCs are green and nuclei areblue. Scale bar is 50 μm.

with an anti-GFAP antibody, which recognizes cells differentiating into glial cells. We observed that EndoC-βH1 cells strongly expressed beta-tubulin (Figure 7), which is in line with the strong resemblance between beta-cells and neurons, as previously reported [25]. Among the GFP-positive bNCSCs approximately 40% expressed beta-tubulin, and these cells often extended limited axon-like extensions typical for immature neurons. This observation is in line with our previous finding that beta-cell protection is mediated by differentiating rather than mature bNCSCs [15]. GFAP was not expressed in the EndoC-βH1 cells (Figure 7). Instead approximately 10% of the GFP-positive cells exhibited strong GFAP positivity and a ramified morphology, indicating differentiation to Schwann cells and/or astrocytes. All of these cells are known to express N-cadherin [25-27] and can therefore potentially form N-cadherin junctions with beta-cells. However, as the beta-tubulin cells are in majority, it is likely that these cells form the bulk of the cadherin junctions with EndoC-βH1 cells.

Conclusion

We report here that bNCSC – EndoC-βH1 cell interactions result in reduced sensitivity to cytokines *in vitro*. Furthermore, bNCSCs are able to promote overall survival of co-cultured beta-cells in the absence of cytokine challenge [24]. The protective effect of bNCSCs might involve the formation of homophilic N-cadherin junctions, as indicated by the results of experiment with a neutralizing N-cadherin antibody (Figure 6). This notion is supported by our finding that hair follicle-derived NCSCs do not express N-cadherin when cultured with beta-cells [28]. It has been proposed that co-transplantation of islets with bNCSCs will improve transplantation outcome by enhancing beta-cell proliferation and function [13]. Techniques for coating islets with NCSCs in order to successfully transplant them to the clinically used liver sitewere recently developed [29] proving that this is indeed a feasible approach to improve islet engraftment and function. The findings of this study indicate that bNCSCs may also dampen the negative effects of inflammatory reactions in the vicinity of human beta-cells, thereby further enhancing the ability for beta-cells to survive after transplantation.

Acknowledgement

EndoC-βH1 cells were kindly provided by Raphael Scharfmann and Philippe Ravassard, Paris, France. This work was supported in part by the Swedish Diabetes Association, the Family Ernfors Fund, Barndiabetesfonden, SvenskaLäkaresällskapet, the Novo-Nordisk Foundation, Swedish Research Council, proj 20716, and the Swedish Institute's Visby program 00613/2011, Stiftelsen Olle Engkvist Byggmastare and Signhild Engkvist's Stiftelse.

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