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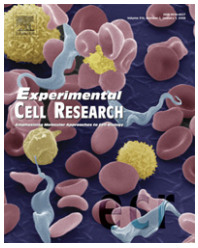

Research Article

Nicotinamide induces differentiation of embryonic stem cells into insulin-secreting cells

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ABSTRACT

The poly(ADP-ribose) polymerase (PARP) inhibitor, nicotinamide, induces differentiation and maturation of fetal pancreatic cells. In addition, we have previously reported evidence that nicotinamide increases the insulin content of cells differentiated from embryonic stem (ES) cells, but the possibility of nicotinamide acting as a differentiating agent on its own has never been completely explored. Islet cell differentiation was studied by: (i) X-gal staining after neomycin selection; (ii) BrdU studies; (iii) single and double immunohistochemistry for insulin, C-peptide and Glut-2; (iv) insulin and C-peptide content and secretion assays; and (v) transplantation of differentiated cells, under the kidney capsule, into streptozotocin (STZ)-diabetic mice. Here we show that undifferentiated mouse ES cells treated with nicotinamide: (i) showed an 80% decrease in cell proliferation; (ii) co-expressed insulin, C-peptide and Glut-2; (iii) had values of insulin and C-peptide corresponding to 10% of normal mouse islets; (iv) released insulin and C-peptide in response to stimulatory glucose concentrations; and (v) after transplantation into diabetic mice, normalized blood glucose levels over 7 weeks. Our data indicate that nicotinamide decreases ES cell proliferation and induces differentiation into insulin-secreting cells. Both aspects are very important when thinking about cell therapy for the treatment of diabetes based on ES cells.

Introduction

Nicotinamide, the amide derivative of nicotinic acid, has been used over the last few decades for a variety of therapeutic applications. High doses of nicotinamide have protective effects on β-cell survival and function in animal and *in vitro* studies [1]. In this regard, nicotinamide prevents the development of diabetes in experimental animals following administration of a range of β-cell toxins [2] and in mouse models of diabetes [3]. Nicotinamide has also been reported to affect insulin production and cell proliferation in adult mouse islets in culture and following transplantation [4,5]. Further studies have demonstrated that nicotinamide enhances the formation of β-cells in fetal porcine islet-like cell clusters grafted into diabetic nude mice [6]. Moreover, increases in insulin production and content of cultured fetal pig islet-like cell clusters were seen as a consequence of β-cell neoformation through differentiation [7]. Finally, nicotinamide stimulates

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formation [8] and endocrine differentiation [9] of cultured fetal human islet-like cell clusters and in human adult pancreatic cell culture [10].

These observations suggested the possibility of forcing *in vitro* differentiation of ES cells using nicotinamide as a differentiation agent. Thus, many strategies were developed to obtain insulin-producing cells from ES cells *in vitro* through the use of nicotinamide [11–17]. Unfortunately, none of these reports have investigated the differentiating effects of nicotinamide alone on ES cells. There is only one study addressing the effects of nicotinamide on ES cell differentiation [18], but this article only analysed cell proliferation and insulin content. Thus, in the present study, we completely explore the possibility of using nicotinamide alone as a differentiating agent to generate insulin-secreting cells from mouse ES cells.

Materials and methods

Generation of vector and transfection protocol

A DNA molecule containing the human promoter insulin/βgeo gene and a phosphoglycerate kinase–hygromycin-resistant gene (pGK-hygro) in a pBSII-SK (Stratagene, La Jolla, CA) common vector was constructed. The human promoter insulin gene was obtained by digestion with *Nco*I (Roche Diagnostics, Barcelona, Spain) from pHins300 cloned into pBSII-SK. Correct orientation was verified after ligation. The βgeo fragment was obtained after double digestion of the pSA-βgeo plasmid with *Hind*III–*Xho*I and subcloned into pBS-HIP digested with *Hind*III–*Xho*I. This new construct (GB2) was then digested with *Xho*I and blunted. Finally, the construct was digested with *Kpn*I in order to insert pGK-hygro (*Sma*I–*Kpn*I). The HIP/β-geo/pGK-Hygro^r construct was inserted into the *Sma*I–*Kpn*I binding site that exists inside the *LacZ* gene of the pBluescriptks vector. The plasmid was linearized with *Kpn*I digestion and transfected into D3 ES cells by electroporation. Transfected cells were selected for growth in the presence of 200 μg/ml hygromycin (Calbiochem, La Jolla, CA).

Cell culture and "in vitro" differentiation procedure

Undifferentiated GB2-transfected ES cells (D3 cell line, passage 23) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY) supplemented with 15% fetal bovine serum (FBS) (Gibco BRL), nonessential amino acids (1%), 2-mercaptoethanol (0.1 mM), L-glutamine (4 mM), sodium pyruvate (1 mM), penicillin (100 IU/ml) and streptomycin (0.1 mg/ml). The undifferentiated state was maintained by 1000 U/ml recombinant leukemia inhibitory factor (LIF) (Gibco BRL). To direct differentiation, hygromycin resistant cells were grown for 7 days in suspension (1.5×10^6 cells/ml) in non-adherent Petri dishes to allow formation of embryoid bodies (EB). During this period, the FBS concentration was reduced to 3%, and LIF was withdrawn. The EB were then plated for an additional 7 days, and FBS was increased to 10%. During this 14-day period, the cells were treated with 10 mM nicotinamide (Sigma, St. Louis, MO). Finally, for ES-Ins/βgeo selection, the differentiated cultures were grown in the same differentiation medium in the presence of 2.3 mg/ml G418 (Gibco BRL).

X-gal staining

Cells were fixed in 4% paraformaldehyde for 5 min and washed with PBS. Following fixation, cells were incubated overnight at RT in the X-gal reaction solution and then washed with PBS.

BrdU incorporation

For BrdU staining, the cells were incubated at 37 °C with 10 μM BrdU (Sigma) for 20 h. Cells were then fixed with 4% paraformaldehyde for 5 min and washed with phosphate buffer saline (PBS), and the DNA was denatured with 2 M HCl for 30 min at RT. The rest of the protocol used standard immunocytochemistry. A mouse monoclonal BrdU antibody (Sigma) was used at 1:500 as the primary antibody, and anti-mouse TRITC (Sigma) was used at 1:300 as the secondary antibody. Nuclear staining was performed by adding 300 nM DAPI (Sigma) for 5 min at RT before visualisation.

Immunocytochemistry

A standard immunocytochemistry protocol was used. Cells were fixed with 4% paraformaldehyde for 4 min, washed with PBS and permeabilized with 0.02% Triton X-100 overnight. Primary antibodies and dilutions were as follows: insulin mouse monoclonal (1:250; Sigma), C-Peptide guinea pig polyclonal (1:100; Linco Research, St. Louis, MO) and Glut-2 rabbit polyclonal (1:250; Chemicon International, Temecula, CA). Primary antibody localisation was done using anti-mouse TRITC or FITC (1:200; Sigma), anti-guinea pig FITC (1:50; Dako, Kyoto, Japan) and anti-rabbit TRITC (1:125; Sigma). Proper controls for secondary antibodies revealed no non-specific staining. Cells were counter-stained with 300 nM DAPI (Sigma) for 5 min before visualisation. Samples were analysed using fluorescence (Olympus, Hamburg, Germany) and laser scanning (LSM-510; Zeiss, Jena, Germany) confocal microscopy. Metamorph software was used to quantify insulin and C-peptide staining.

Insulin and C-peptide secretion assays

Secretion studies were performed as previously described (1). In summary, 2.5×10^5 cells were cultured overnight in D3 culture medium supplemented with 10% FBS and 10 mM nicotinamide in 24-well dishes. The cells were then washed three times with Krebs buffer for 5 min each and incubated for 4 h in 500 μl of fresh modified Krebs buffer with 0.1% bovine serum albumin (BSA) and 3 mM glucose. The temperature of the Krebs buffer was held constant at 37 °C and was continuously gassed with a mixture of O_2 (95%) and CO_2 (5%) for a final pH of 7.4. Afterwards, the supernatant was discarded, and the cells were incubated for 1 h in 250 μl of the same Krebs buffer, at which point the culture supernatants were collected. Cells then received a final 1-h incubation in 250 μl of the same fresh modified Krebs buffer, containing 22 mM glucose or 3 mM glucose plus 25 μM tolbutamide. At the end of the incubation period, the buffer was collected. In the last two incubations, buffer supernatants were centrifuged at 3000 rpm for 5 min. Insulin and C-peptide were assayed by radioimmunoassay (RIA) using two different