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Nicotinamide Induces Both Proliferation and Differentiation of Embryonic Stem Cells Into Insulin-Producing Cells

P. Vaca, G. Berná, F. Martín, and B. Soria

ABSTRACT

In contrast to the consistent observation that methods that promote proliferation also dedifferentiate insulin-producing cells, useful in vitro differentiation protocols must drive both proliferation and differentiation. We herein describe an strategy in which the combination of nutrient restriction and nicotinamide supplementation results in a consistent increase in the mass of insulin-producing cells.

ISLET TRANSPLANTATION as a potential treatment for diabetes has been investigated extensively during the past 15 years, with limited success; less than 10% of patients who underwent transplantation were insulin-independent after 12 months. Recent results from the Edmonton group suggest, for the first time, that insulin-independence can be achieved in more than 80% of type 1 diabetic patients who received 2 subsequent transplants of freshly prepared islets together with nonsteroid immunosuppression.[1] However, this approach is always limited mainly because of the difficulty to obtain sufficiently large numbers of purified islets from cadaveric donors. Even in countries such as Spain, which ranks number 1 in organ procurement and does not have a high prevalence of diabetes, no more than 2% to 4% of type 1 diabetic patients could undergo transplantation. To make this therapy readily available, new sources of insulin-producing cells must be identified.

Recently, attention has focused on the use of stem cells to obtain specialized cells for treating a variety of diseases, including diabetes. Embryonic stem cells (ESC) have the potential to proliferate, differentiate into many types of cells, and be genetically modified in vitro, thus providing cells that can be isolated and used for transplantation. In this regard, we have previously reported a well-defined differentiation protocol, which can be used to guide stem cells into insulin-secreting cells. Moreover, these cells have proven effective in a diabetic animal model.[2,3] This study used a 3-step in vitro differentiation method consisting of directed differentiation, cell-lineage selection, and maturation.

An overriding principle of development is that most cells become permanently postmitotic once they differentiate. Thus, on the one hand, when cells are forced to proliferate, they phenotypically dedifferentiate. Keep, the differentiation process results in cessation of cell proliferation. This rule has been proven during in vitro differentiation but not during in vivo development. For example, during pancreas development, the organ increases 1000-fold in size, thus proliferation coexists with differentiation.[4] ESC seem to recapitulate many processes of early embryonic development, combining the ability to proliferate and differentiate. However, this fact can be a disadvantage when ESC are used for transplantation, because proliferating cells may potentially become malignant, rather than mature postmitotic cells.

Nicotinamide has been used to generate insulin-producing cells from stem cells.[2,3,5] The aim of this article is to analyse both proliferation and differentiation under the presence of nicotinamide.

MATERIALS AND METHODS

Culture of mES Cells and Differentiation Protocol

D3 mESC line was cultured on gelatin-coated flasks with high glucose Dulbecco's modified Eagle's medium (D-MEM) (GIBCO/BRL, Grand Island, NY, United States), containing 15% fetal bovine serum (FBS) (Hyclone, Logan, Utah, United States), 1%

From the Institute of Bioengineering, University Miguel Hernandez, San Juan de Alicante, Alicante, Spain, and Department of Surgery, National University of Singapore, Singapore, Singapore.

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Address reprint requests to Dr Bernat Soria, Institute of Bioengineering, Campus de San Juan, University Miguel Hernández, 03550 San Juan, Alicante, Spain. E-mail: bernat.soria@umh.es

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nonessential amino acids (GIBCO/BRL), 0.1 mmol/L 2-mercaptoethanol (GIBCO/BRL), 1 mmol/L sodium pyruvate, 1000 U/mL mouse recombinant leukemia inhibitory factor (LIF) (GIBCO/BRL), 100 IU/mL penicillin, and 0.1 mg/mL streptomycin. To direct the differentiation, besides eliminating LIF from the medium, we combined a reduced serum concentration (from 15% to 10% and 3%) with the addition of selected factors. Briefly, D3 cells cultured for 7 days to form embryoid bodies (EB period) were plated for 7 days in gelatin-coated dishes (P period) for 14 days, during which they were treated with different factors. The differentiation protocols included: (1) D3: control D3 mESC cultured as described above; (2) M1: D3 mESC cultured in 10% FBS during EB and P periods; (3) M2: D3 mESC cultured in 3% FBS during EB period and 10% FBS during P period, and (4) M3: D3 mESC cultured in 3% FBS during EB period and 10% FBS during P period, plus 10 mmol/L nicotinamide during both periods.

Cell Proliferation Analysis

After trypsinization of undifferentiated stem cells, a cell count was performed prior to the cells being cultured using different protocols. At the end of the differentiation protocols, mESC were harvested using 0.25% trypsin/1 mmol/L EDTA for cell counts. The number of population doublings were calculated as $\log_2$ (nº of harvested cells/number of seeded cells).

Insulin Content Assay

To determine total insulin content, 1×10^6 cells were sonicated in 1 mmol/L acetic acid containing 0.1% bovine serum albumin (BSA) (Sigma-Aldrich) at 4°C. Cellular extracts were assayed for insulin determined by RIA using the Coat a-Count kit (DPC, Los Angeles, Calif, United States) as previously described.[6]

RESULTS

Analysis of Cell Proliferation

D3 mESC from different differentiation protocols were assayed for growth. Figure 1A shows the population doubling after different protocols. Undifferentiated ESC (D3 condition) duplicated their population approximately once in 24 hours (1.07 ± 0.08; $n = 6$). Compared with this control, all differentiation protocols showed a significant decreased ($^*P < -.001$) value of 70%. On the other hand, the other protocols, including nicotinamide addition (M3), were not significantly different in their proliferation analysis.

Insulin Content Study

Intracellular insulin levels analyzed using RIA were significantly increased in the M3 condition (Fig 1B) (1.78 ± 0.46 ng/μg of protein; n = 3; $^*P < -.001$), compared with the rest of the protocols, including undifferentiated cells (D3 condition). M1 and M2 differentiation protocols showed only a moderate but significant increase (0.29 ± 0.09; n = 3; and 0.37 ± 0.05; n = 4, respectively; $^{**}P < -.001$), compared with undifferentiated D3 cells, although the differences were not significant. Finally, undifferentiated D3 cells did not produce insulin.

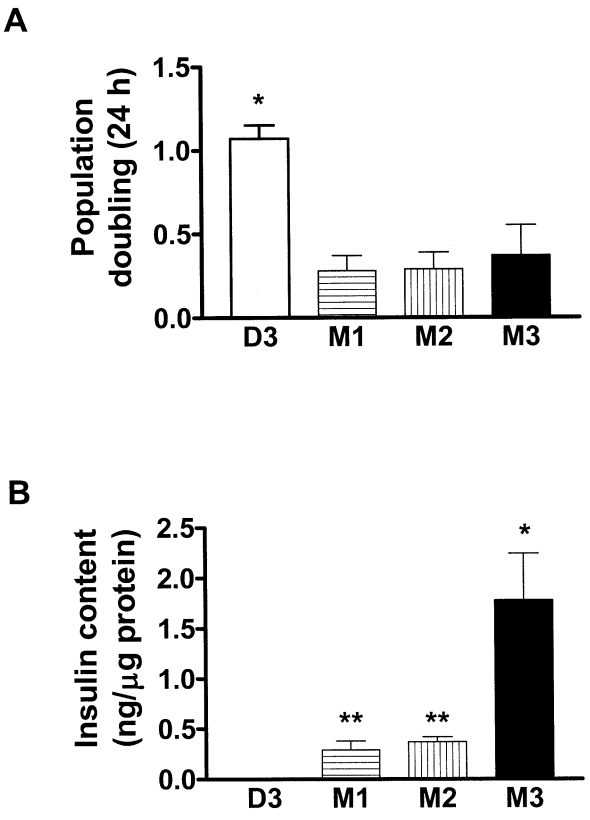

Fig 1. (A) Cell proliferation in mESC differentiated with different protocols. Population doubling time. The Y axis indicates the number of population doublings in each of the protocols analysed. D3: undifferentiated D3 mESC (n = 6); M1: D3 mESC cultured in 10% FBS during EB and P periods (n = 3); M2: D3 mESC cultured in 3% FBS during EB period and 10% FBS during P period (n = 4); and M3: D3 mESC cultured in 3% FBS during EB period and 10% FBS during P period, plus 10 mmol/L nicotinamide during both periods (n = 3). Each value represents mean ± SEM. Statistical significant was tested using Student test.$^*P < .001$. (B) Insulin content of mESC submitted to differentiating protocols. Intracellular insulin levels was determined using RIA and determinations were run in triplicate. The Y axis indicates the insulin content in each protocol studied. D3: undifferentiated D3 mESC (n = 3); M1: D3 mESC cultured in 10% FBS during EB and P periods (n = 4); M2: D3 mESC cultured in 3% FBS during EB period and 10% FBS during P period (n = 5); and M3: D3 mESC cultured in 3% FBS during EB period and 10% FBS during P period, plus 10 mmol/L nicotinamide during both periods (n = 4). Each value represents mean ± SEM. Statistical significant was tested using Student test. $^*P < -.001$ vs the rest of conditions. $^{**}P < -.001$ vs D3.

DISCUSSION

It has been demonstrated that glucose normalization requires an islet mass of 10,000 to 12,000 islet equivalents per kilogram.[1] The use of insulin-producing cells for diabetes cell therapy requires in vitro procedures to generate sufficient mass of insulin-producing cells that display regulated

release.[2,3] Exposure to 10 mmol/L nicotinamide, as previously described, decreases the rate of proliferation and substantially increases the insulin content (1.780 ng insulin/mg protein), Although this figure only represents 7% of the insulin content of control islets, it is 3-fold greater than the values obtained by other authors.[5] Population doublings combined with increased insulin content may allow investigators to engineer the process to obtain the desired mass of differentiated cells.

We have previously shown that both nicotinamide plus nutrient restriction increase insulin content and improve the last steps in the maturation process under experimental conditions in which undesired proliferation is controlled.[2] Growth retardation using inhibitors of PI3 kinase also increase insulin content and force maturation into insulin-producing cells with regulated release.[5,7] A recent report by Rajagopal et al[8] describes the uptake of insulin from the culture media under conditions of nutrient restriction, thereby introducing artefacts. Figure 1B shows that the insulin content was always 1.400 ng insulin/mg protein above other controls. Thus, nicotinamide may either induce insulin uptake or increase insulin synthesis. Our results support the late hypothesis. Using a cell selection system based on the expression of the insulin gene, we[2,3] and others[9] have shown that this system yields insulin-producing cells, which are also positive for other markers such as Glut-2. It is suggested that the combination of these maturation strategies with the cell selection method[2,3,9] may improve the capacity of stem cells to generate and release insulin.

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