Mass Production of Embryoid Bodies in Microbeads

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ABSTRACT: Embryonic stem cells (ESC) are totipotent cells that can differentiate into a large number of different cell types. Stem cell-derived, differentiated cells are of increasing importance as a potential source for non-proliferating cells (e.g., cardiomyocytes or neurons) for future tissue engineering applications. Differentiation of ESC is initiated by the formation of embryoid bodies (EB). Current protocols for the generation of EB are either of limited productivity or deliver EB with a large variation in size and differentiation state. To establish an efficient and robust EB production process, we encapsulated mouse ESC into alginate microbeads using various microencapsulation technologies. Microencapsulation and culturing of ESC in 1.1% alginate microbeads gives rise to discoid colonies, which further differentiate within the beads to cystic EB and later to EB containing spontaneously beating areas. However, if ESC are encapsulated into 1.6% alginate microbeads, differentiation is inhibited at the morula-like stage, so that no cystic EB can be formed within the beads. ESC colonies, which are released from 1.6% alginate microbeads, can further differentiate to cystic EB with beating cardiomyocytes. Extended supplementation of the growth medium with retinoic acid promotes differentiation to smooth muscle cells.

KEYWORDS: embryonic stem cells; embryoid bodies; microbeads

INTRODUCTION

Organ transplantation, as today's standard surgical treatment of organ failure, is mainly limited by the availability of donor organs. In recent years tissue-engineered artificial organs, generated from autogeneic or allogeneic cells, have been shown to provide a true alternative to organ transplantation. Living tissue equivalents are available for skin,[1] cartilage,[2] and bone,[3,4] and many more equivalents are in development.[5] This approach is successful for cell types that are capable of proliferation, for example, *in vitro* cultures provide a controlled and renewable reservoir for these cells. In case of non-dividing cells, however, other ways and sources need to be found. There are two principal ways: either a temporal release of postmitotic cells

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from quiescence or the isolation of the stem cells of the cell type of interest. In the case of cardiomyocytes, neither of these possibilities were successful until now.[6]

Embryonic stem cells (ESC) are totipotent cells, originating from the inner cell mass of blastulas. They are able to differentiate to virtually all cell and tissue types *in vivo* as well as *in vitro*.[7,8] Therefore, ESC could serve as a source for the *in vitro* generation of postmitotic cells like cardiomyocytes. The full differentiation potential, however, is only achieved if the ESC are forced to differentiate both by morphological cues (embryoid body [EB] formation) as well as by growth- and differentiation-inducing factors. Steps for the induction of ESC differentiation include the production of morula-like aggregates, followed by the formation of blastula-like structures (cystic embryoid bodies) in suspension cultures and final differentiation in adhesion cultures. However, even under optimized conditions, only part of the ESC differentiate to cardiomyocytes[9,10] and even these cardiomyocytes are limited in their capability to proliferate.[11–13] Therefore, for the development of myocardial tissue engineering approaches it is important to establish controllable methods that are suitable for the large-scale production and differentiation of embryoid bodies.

Microencapsulation of animal cells into alginate hydrogels has been used with a large number of different cell types.[14–16] Immobilization of ESC into such gels, and their proliferation, should give rise to a large number of isolated ESC aggregates and later to embryoid bodies that might be able to differentiate to cells of all three germ layers. The number of equally developed embryoid bodies that could be generated by using this technology cannot be reached with any other current method. Here we describe such a large-scale production of EB by immobilization of ESC into alginate microbeads. Microencapsulated ESC can grow as compact colonies within the beads and give rise to morula- and subsequently to blastula-like structures. The ability to generate large number of EB by microencapsulation makes microbead-grown EB a potential *in vitro* source of cardiomyocytes for tissue engineering approaches. The differentiated EB can then easily be released from the beads by Ca^{2+}-depletion.

MATERIAL AND METHODS

Culture and Microencapsulation of Embryonic Stem Cells

Undifferentiated ESC of the mouse R1 cell line[17] were cultured on mouse feeder fibroblasts according to established procedures.[18] For microencapsulation, single cell suspensions of ESC were mixed with sterile filtered alginate (UP-LVG alginate; Pronova Biomedical A.S., Oslo, Norway) dissolved in Joklik medium (Cell Culture Technologies Inc., Zurich, Switzerland). Drops were formed from the alginate-ESC suspension using either the Nisco Var.A, laminar-jet-breakup microencapsulator (Nisco Engineering Inc., Zurich, Switzerland) or the Nisco Var.V high-voltage-driven microencapsulator (Nisco) into a hardening bath (100 mM $CaCl_2$, 10 mM morpholinoethanesulphonic acid (MOPS), pH = 7.4). After 10 minutes of hardening, beads were washed three times with Joklik medium and once with M199 medium (Amimed AG, Basel, Switzerland) and were cultured in M199 medium, supplemented with

10% FCS (PAA, Vienna, Austria), 1% penicillin/streptomycin (Gibco Laboratories, Grand Island, NY).

Induction of Differentiation

Colonies of ESC, grown in 1.6% alginate microbeads were released by incubation of the beads in depolymerization buffer (50 mM Na-citrate, 77 mM NaCl, 10 mM MOPS, pH = 7.4) until the bead matrix was completely depolymerized. Bead-released ESC colonies were washed once with Joklik medium and once with M199 medium and plated onto gelatinized tissue-culture plates. Formation of spontaneously beating cardiomyocytes was promoted by supplementation of the medium with retinoic acid to a final concentration of 10^{-8} M[19] (RA; Sigma Chemical Co., St. Louis, MO). In case of ESC colonies grown in 1.1% alginate beads, differentiation within the beads was only promoted by supplementation of the medium with RA.

Immunofluorescence Staining and Microscopic Analyzes

Fixation and immunostainings were performed as described previously.[20] Cells were monitored by staining of the DNA with Pico-green or by staining of the actin cytoskeleton with rhodamine-phalloidin (both from Molecular Probes Inc., Eugene, OR). For cardiomyocyte specific staining monoclonal mouse antibodies directed against sarcomeric α-actinin (clone EA-53; Sigma) and for smooth muscle cell specific staining monoclonal mouse antibodies directed against α-smooth muscle actin (clone 1A4; Sigma) or polyclonal rabbit antibodies against calponin (generously donated by Dr. Mario Gimona, Salzburg, Austria) were used. Optical analysis was performed by using a Leica TCS-NT confocal microscope (Leica, Mannheim, Germany) equipped with an argon-krypton mixed gas laser. Image analysis was performed using the Imaris software package (Bitplane AG, Zurich, Switzerland). Cellular and tissue contractions were documented using real-time videoimaging of beating or peristaltically contracting areas using a Zeiss Axiovert microscope (Zeiss, Oberkochen, Germany) combined with a CCD camera (C-5810; Hamamatsu Photonics K.K., Hamamatsu, Japan) and a video recorder (Panasonic AG-7350-E; Matsushita Electronics Co. Ltd., Kyoto, Japan).

RESULTS

We have analyzed growth and differentiation of ESC inside alginate microbeads as a potential tool for large scale production of ESC derived cardiomyocytes. When encapsulated into 1.6% alginate beads, ESC grew to compact, lens-shaped colonies (see FIGURE 1A). The number of ESC per bead and the survival rate of the cells determined the number of embryoid bodies growing in the beads. Even extended culturing did not result in the development of blastula-like structures or in the differentiation to spontaneously beating cardiomyocytes and the lens-shaped morphology was retained in all preparations throughout the entire culturing period. Therefore, we released the ESC colonies from the alginate beads by calcium chelation. Freshly

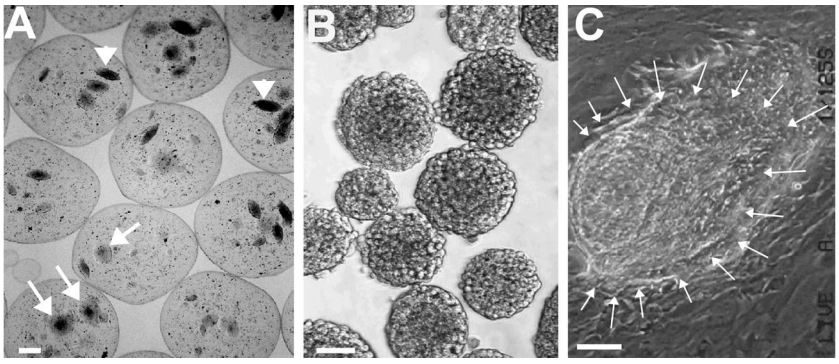

FIGURE 1. Morphology of bead-grown ESC colonies and EB. (**A**) Phase contrast image of ESC colonies grown in 1.6% alginate microbeads. Note that the oval appearance of the colonies is a chance result of the adjusted lateral view (*arrowheads*). A spherical appearance of colonies is due to the perpendicular view angle (*arrows*). (**B**) Freshly released ESC colonies have an apparent spherical form, whereas this is only due to the fact that the lens-shaped colonies lie on their broad side. (**C**) Beating area in a differentiated EB. Boundary of the area is depicted by *arrows* pointing toward its center.

released colonies remained compact and retained their lens-shaped morphology. Culture of these colonies in suspension in bacterial dishes resulted in the formation of spheroid, morula-like structures within one to two days. Additional culture for one to two days led to the differentiation to blastula-like structures (cystic embryoid bodies) in the vast majority of the colonies. On further culturing in gelatin-coated tissue culture dishes, these structures adhered to the substratum and differentiated into various cell types, as judged by the multiform appearance of the colonies. A varying number of these colonies showed regions that differentiated into cardiomyocytes, as judged by the spontaneous periodic beating of the cells.

Since our aim was to establish a method suitable for the large-scale differentiation of ESC to EB and to EB with spontaneously beating cardiomyocytes, we tested whether a minimization of alginate concentration would permit a differentiation already inside the beads. Due to the weak mechanical resistance of these beads during the hardening process, microencapsulation of ESC into monodisperse and spherical 1.1% alginate microbeads was only possible using a high-voltage-driven microencapsulator. Similarly to ESC colonies grown in 1.6% alginate microbeads, in 1.1% alginate gels the initial form of the colonies was lens-shaped. After four days in culture, however, some of the ESC colonies became cystic, indicating that differentiation could proceed further than in 1.6% alginate beads. Cystic colonies also developed spontaneously beating areas, similarly to adherent cultures of 1.6% alginate bead-grown EB on gelatinized tissue culture dishes. Therefore, microencapsulation of ESC into 1.1% alginate microbeads allowed the generation of a large number of differentiated EB with spontaneously beating areas in a small culture volume.

In long-term (more than 10 days) cultures of adherent EB in the presence of RA showed large areas of slow, spontaneous, wave-like contractions. If analyzed by smooth muscle cell-specific immunostaining (see FIGURE 2B, C, and D), these areas contained large numbers of smooth muscle cells, aligned in an organized, parallel

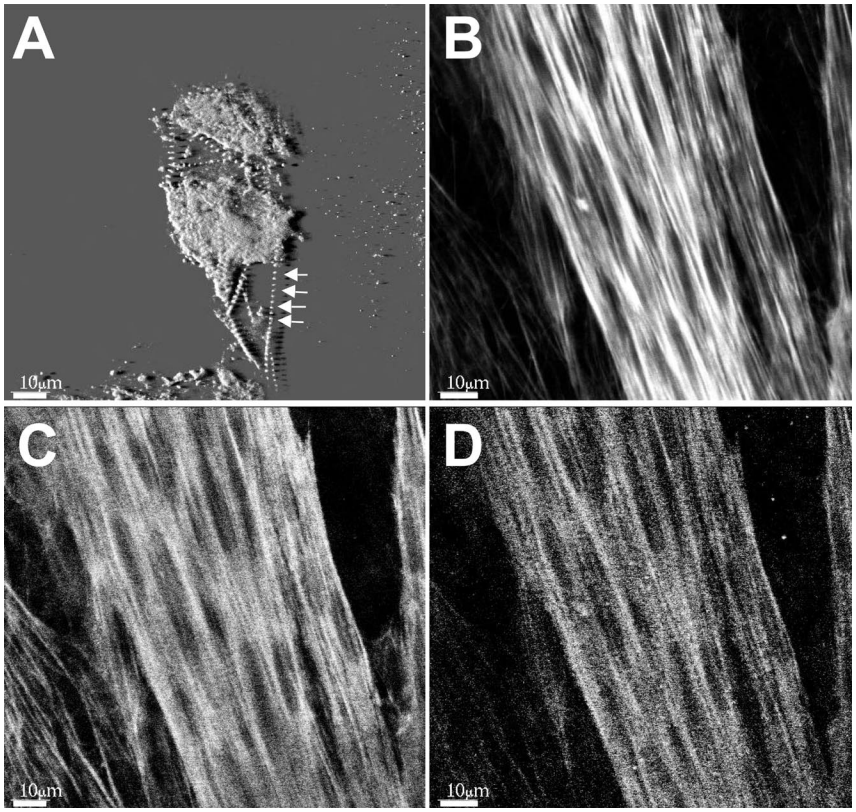

FIGURE 2. Differentiation of bead-grown EB to cardiomyocytes and smooth muscle cells. (**A**) Immunofluorescence staining of differentiated EB, grown in 1.6% alginate beads and differentiated in adherent cultures on gelatin-coated cell culture dishes. Striated appearance of the staining and expression of sarcomeric α-actinin clearly identifies cardiomyocytes in a region that was selected based on the basis of beating activity before fixation. (**B**, **C**, and **D**) Smooth muscle cells differentiated in the same culture were also identified by their slow contractions. Coimmunostaining for α-smooth muscle actin (**C**), and calpain (**D**) confirmed the presence of smooth muscle-specific proteins in these cells. Note the self-organization of the smooth muscle cells into parallel strands, best visualized by staining for f-actin (**B**: phalloidin stain).

fashion. Interestingly, these smooth muscle cells expressed, in addition to the smooth muscle actin α-isoform (FIG. 2C), calponin (FIG. 2D), a marker protein for smooth muscle cells that is normally rapidly downregulated in culture.[21] Therefore, the presence of calponin likely indicates the high degree of differentiation of these cells. The respective areas were much larger than the areas comprising of cardiomyocytes, indicating that the ESC derived smooth muscle cells could either be faster or longer proliferation competent than ESC derived cardiomyocytes. Indeed, the number of beating areas, attributed to cardiomyocytes, seemed to decrease in long-term cultures, as had been similarly reported for systems not involving microencapsulation.[9,22]

DISCUSSION

The capacity of ESC to form embryoid bodies and to differentiate *in vitro* to all three germ layers has been demonstrated in rodents[23] as well as in humans.[24] A full differentiation range can only be achieved by the generation of EB. These can be generated by either using "hanging drop" technology,[19] by culturing ESC colonies detached from cultures grown without a feeder cell layer,[25,26] or by the spontaneous aggregation of ESC in suspension culture without[22] or with spinning[27] or with a combination thereof.[28] Therefore, it seems that the formation of cystic EB may be a prerequisite for differentiation into cardiomyocytes. Applying a large scale approach, it is important that the variability in the differentiation state of the EB is minimal. Although, depending on the concentration of the ESC in the encapsulation matrix, the number of cells per microbead varies, we assume that at a reasonable high dilution almost all of the EB is derived from a single cell, which ensures a relatively narrow distribution in size and differentiation state of the EB. (This statement does not imply that a single cell colony is produced in the microcapsule.) Using microencapsulation, one ESC is assumed to give rise to one EB, which offers significant advantages over the hanging drop technology, where typically 500 cells are used for the formation of a single EB, or over spontaneous aggregation, where an unknown amount of ESC aggregate to EB. In addition, the monoclonality of bead-grown EB offers the possibility to test large scale gene-trap approaches[29] in ESC for the identification of tissue specific genes.

Out of an ESC line it is possible to generate *in vitro* a tissue-specific stem cell lineage.[30] Using our microencapsulation system it is possible to culture a large number of cells, well separated from each other, within a small volume or area. Therefore, microencapsulation of a single cell suspension of an EB containing beating structures might allow the isolation of a mesenchymal stem cell line with an increased potential to differentiate into the cardiomyocytic lineage.

The number of EB displaying spontaneously beating areas can vary (91%,[9] 70%,[31] or less). Factors potentially responsible for these differences might include (1) ESC line-specific differences, (2) differences in the medium composition, and (3) differences in the serum composition. This variability and the finding that only a part of an EB differentiates to cardiomyocytes points to the importance of the identification of cardiomyocyte stem cells. Since 80% of the myocardial volume is made up by cardiomyocytes but only 25% of all cells are cardiomyocytes, unequivocal identification of these cells within the myocardium has always been a controversial issue (Field, 1997). Therefore, reports describing the ability of bone marrow-derived mesenchymal stem cells to differentiate to cardiomyocytes[32] needs to be carefully revised.

We have tested the microencapsulation of ESC into alginate microbeads. Our results show that immobilization of ESC into hydrogel microbeads allows the mass production of EB. This process will need to be optimized in order to (1) increase the number of EB with beating areas, (2) increase the number of cardiomyocytes within a given EB, and (3) increase the survival of the EB-derived cardiomyocytes. Our data indicate that the bead matrix has an influence on the ability of the bead-grown ESC colonies to differentiate to EB and we believe that the influence of the matrix composition needs to be analyzed by a systematic testing of different alginate

preparations as well as by the use of other matrices like sulfocellulose.[33] Since a large number of factors and substances have been described that promote ESC differentiation towards cardiomyocytic lineage, evaluation of combinations of these is of great importance and might include: RA,[34] DMSO,[35] H_2O_2, menadione,[27] activin A,[36] and bone morphogenic protein 2 (BMP-2), together with fibroblast growth factor-4 (FGF-4),[37] insulin, fibroblast growth factor-2 (FGF-2),[38] insulin-like growth factor-1 (IGF-I)[38,39] serum-responsive factor (SRF),[40,41] and a high glucose content of the medium.[9]

In this paper we have shown the feasibility of a large scale production of equally developed embryonic bodies in a scale most likely suitable for tissue engineering approaches. Future work will need to optimize the condition to obtain the outmost number of differentiated cells.

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