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FAST TRACK

Identification and Initial Characterization of Spore-Like Cells in Adult Mammals

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Abstract We describe the identification and initial characterization of a novel cell type that seems to be present in all tissues. To date we have isolated what we term ''spore-like cells'' based on the characteristics described below. They are extremely small, in the range of less than 5 μm, and appear to lie dormant and to be dispersed throughout the parenchyma of virtually every tissue in the body. Being dormant, they survive in extremely low oxygen environments, as evidenced by their viability in tissues (even in metabolically very active tissues such as the brain or spinal cord) for several days after sacrifice of an animal without delivery of oxygen or nutrients. The spore-like cells described in this report have an exceptional ability to survive in hostile conditions, known to be detrimental to mammalian cells, including extremes of temperature. Spore-like cells remain viable in unprepared tissue, frozen at −86°C (using no special preservation techniques) and then thawed, or heated to 85°C for more than 30 min. Preliminary characterization of these cells utilizing basic and special stains, as well as scanning and transmission electron microscopy reveal very small undifferentiated cells, which contain predominantly nucleus within a small amount of cytoplasm and a few mitochondria. Focal periodic acid-Schiff and mucicarmine stains suggest a coating of glycolipid and mucopolysaccharide. In vitro, these structures have the capacity to enlarge, develop, and differentiate into cell types expressing characteristics appropriate to the tissue environment from which they were initially isolated. We believe that these unique cells lie dormant until activated by injury or disease, and that they have the potential to regenerate tissues lost to disease or damage. J. Cell. Biochem. 80:455–460, 2001. © 2001 Wiley-Liss, Inc.

Key words: stem cells; progenitor cells; cell biology; tissue engineering

The replacement of tissue lost to disease or damage represents a major medical challenge. Fields such as transplantation medicine, cell therapy, tissue engineering, and gene therapy are significantly dependent upon a cellular component. The ideal cell type would be readily available, able to survive harsh environments with low oxygen tensions, have a great replicative potential, and the capacity to differentiate into the all of the cell-types of the desired tissue.

Much effort has been focused on the promise of stem cells to meet this great need. Recently, adult mammalian stem cells have been studied with the hope that they might replace embryonic stem cells and their potential uses. Many feel they may be ideal candidates because of their undifferentiated state and their marked capacity to proliferate. Their medical applications are rapidly expanding. Although much is known about the basic biology of stem cells found in the central nervous system, the bone marrow, and the brain [McKay, 1997; Vogel, 1999] knowledge concerning their presence and function in other organ systems is quite limited, and currently being investigated [Hines, 1997].

Progenitor cells are also being studied extensively. They appear to have similar properties to stem cells in their undifferentiated nature and proliferative capacity, although they are generally believed to be somewhat committed to lineage. For example, a liver progenitor cell is believed to be committed to differentiate into a hepatocyte [Brille et al., 1993].

This preliminary report describes the identification and initial characterization of dormant, small and extremely simple cell-like structures, which we have termed "spore-like cells". These cells apparently have the ability to differentiate into mature cells of the tissue type from which they are isolated. Additionally, they are able to withstand harsh conditions such as

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hypoxia, extreme heat, and cold maintaining their capacity to proliferate and differentiate.

METHODS

Cells were isolated from 17 tissues, harvested from 60 animals and studied in four different environments (Group I = 17 tissues procured from 51 animals, Group II = 4 tissues procured from each of 3 animals, Group III = 4 tissues procured from each of 3 animals, Group IV = 4 tissues procured from each of 3 animals).

In Group I, 17 tissues, harvested from 51 animals within 2 h of sacrifice, were studied. Tissues from which cells were procured included the brain, spinal cord, retina, skin, spinal fluid, liver, pancreas, lung, small intestine, blood, adrenal glands, periosteum, perichondrium, fascia, kidney, and skeletal and cardiac muscle in the adult female Fisher rats after sacrifice.

In Groups II–IV, four tissues (liver, lung, fascia, and spinal cord), derived from nine animals (three animals in each group) were studied.

The four tissues in Group II were procured from three animals and kept in cold storage for 5 days after sacrifice. The tissues were harvested from animals within 2 h of sacrifice, placed in 50 cc centrifuge tubes (Fisher Scientific, Pittsburg, PA) filled with Phosphate buffered saline (PBS), and then capped. The tubes containing each tissue were then kept in cold storage at 4°C without supplemental oxygen for 5 days, at which time cells were isolated as described below.

The four tissues in Group III were procured from three animals, frozen at −86°C for 2 months and then thawed. Animals in Group III were placed into plastic bags after being sacrificed, and then placed into a freezer at −86°C. After being frozen for 2 and 8 weeks, the animals were removed from the freezer and placed in a warm water bath at 37°C until they were thawed. Tissues were then harvested and cells isolated as described below.

The four tissues in Group IV, harvested from three animals, were heated to 85°C for 30 min prior to cell isolation. Tissues were again harvested within 2 h of sacrifice and placed in 50 cc centrifuge tubes filled with PBS. The tubes were then placed in a heated bath as the temperature of the bath was raised to 85°C. The temperature was monitored with sterile thermometers placed within the tubes holding each tissue. When the temperature had equilibrated to +85°C, it was held at that temperature for 30 min, then allowed to cool down to room temperature. Cells were then harvested as described above.

Samples of each tissue type, in Groups III and IV were saved for evaluation after exposure to the condition described, prior to cell harvest.

Cell Isolation

Using sterile conditions, the selected tissue was excised and then placed in cold PBS containing penicillin, 50 mU/ml and streptomycin 90 mg/ml (Gibco, Grand Island, NY). The tissue was then manually disassociated with a #11 scalpel and collected after centrifugation at 1200 rpm for 5 min. It was then resuspended in 10 ml of 0.05% trypsin (w/v) for 5 min at 37°C. Trypsin was inactivated by adding 10 ml of DMEM/F-12 (Gibco), containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco). The cells were then dispersed by trituration using progressively narrow fire polished reduced bore pasteur pipettes. Dispersed cells were collected by centrifugation at 1200 rpm for 5 min and removal of supernatant. The resulting pellet was resuspended in 10 ml of DMEM/F-12 medium containing 33 mM glucose (Sigma, St. Louis, MO), 10 mg/ml transferrin (Sigma), 20 mg/ml insulin (Sigma), 10 mM putrescine (Sigma), 100 nM selenium (Sigma), 10 nM progesterone (Sigma), 20 ng/ml EGF (Peprotech, Rocky Hill, NJ), and 20 ng/ml bFGF (Collaborative Biomedical, Raynham, MA). The primary cell suspension was incubated at 37°C in 5% CO_2, and the media changed every 3 days. Cells were passaged every 7–9 days by collecting the nonadherent cell aggregates, centrifuging them at 1200 rpm for 5 min and removing the media. Cells were resuspended in fresh media and triturated using narrow fire polished reduced bore pasteur pipettes. The cell suspension was then divided into two suspensions and placed into two new culture dishes.

The technique was somewhat modified as follows for isolation of cells from pancreatic and hepatic tissues. Pancreatic tissue was disassociated in DMEM with 10% Fetal Serum Albumin (FSA) to neutralize protease activity. Hepatic tissue was washed with cold PBS prior to disassociation. The procurement of spore-like cells from all tissues studied was otherwise the same.

Cells isolated from various tissues, which had been exposed to different environments, were characterized using the following approaches.

Microscopic studies using histology stain. Standard Hemotoxin and Eosin (H & E) sections were done on tissue fixed in 10% formalin. A simple Hall's stain was done on liver derived spore-like cells for the presence of bile. Standard stains for mucicarmine and periodic acid-Schiff were performed.

Cell replication. Doubling times were estimated using periodic phase microscopy field counts (10 fields counted and averaged at $100\times$) or viable cell counts using trypan blue with a hemocytometer.

Characterization of cells by scanning electron microscopy (SEM). Cells were suspended in PBS and fixed by adding 2.5% glutaraldehyde in 0.1 M Mollonig's phosphate buffer (MPB) (pH 7.2) to the culture tubes one drop at a time until the initial volume of media was doubled. They were allowed to stabilize in this solution for 10 min and then centrifuged at 3,000*g* for 10 min. The resulting pellet was resuspended in fresh 2.5% glutaraldehyde in 0.1 M MPB and allowed to fix for 30 min at room temperature. Following primary fixation, the specimens were again centrifuged at 3,000*g* and resuspended in fresh buffer for 10 min. The process was repeated three times. Following the final wash, the cells were resuspended in 10% ethanol and dehydrated through a graded series of ethanol to 100%; (2 changes), and then placed on Poretics (Livermore, CA) 0.08 μm PVDF discrete pore filters and allowed to air dry overnight in covered Petridishes. The dried filters containing specimens were then secured to a pure carbon sample bar (SPI #1687) with conductive colloidal graphite and sputter coated with 5 nm Au/Pd to stabilize the filters in the electron beam. The filters were then examined at $1000-7000\times$ using an ETEC autoscan SEM.

Characterization of cells by transmission electron microscopy (TEM). Cells were suspended in PBS, fixed and washed as described above for SEM. Following the last wash the cells were resuspended in 1.0% osmium tetroxide in MPB for 30 min at 25°C to postfix, then washed three times in MPB as described above, dehydrated through a graded series of ethanol (10% to 100%; 2 changes), transferred through two changes of propylene oxide (10 min each), and put into propylene oxide: Embed 812/Arildite 502 epoxy resin (50:50/V:V) for 12 h at 25°C. Following infiltration in the resin, they were pelleted by centrifugation and resuspended in pure Embed 812/Arildite 502 epoxy resin (2 changes; 30 min each), and pelleted again, transferred to Beem capsules containing the final embedding resin mixture. The samples were polymerized overnight at 70°C, and ultrathin sections were cut and stained with uranyl acetate and lead citrate and examined on a Philips CMIO at 80 kV.

DAPI staining. Cells (500,000/ml) were incubated with poly-lysine coated coverslips. The coverslips were incubated at room temperature for 30 min and washed twice with PBS. They were then fixed with a 1:1 solution of acetone and methanol for 2 min, stained with DAPI (4′, 6-Diamidine-2-phenylindole), and washed three times with PBS. The coverslips were fixed with the gel mount and processed for microscopic examination with a blue filter.

Fluorescein-conjugated probes for insulin. Cells were harvested from the tissue culture flask. To stain cells with antibodies against internal antigen(s) insulin, adhered cells were washed twice with ice cold PBS and then fixed with acetone/methanol mixture (1:1) and kept at 4°C. Cells were rinsed twice with PBS/Tween-20 solution, blocked, and incubated with primary monoclonal antibody (Human insulin—1:1000 dilution) for 2 h at room temperature, washed three times with PBS/Tween-20 buffer and incubated with biotinylated goat antimouse secondary antibody (1:10,000 dilution) for 1 h at room temperature. Bound antibodies were revealed by fluorescein conjugated streptavidin (1:1000 dilution) for 30 min at room temperature. Cells were analyzed using fluorescence microscopy, as well as phase contrast after thorough washing.

BrdU uptake. Cells were seeded on chamber slides at a 50% confluence until they attached (usually several hours). BrdU (25 mM, final concentration) was diluted in sterile PBS, added to the culture, and incubated overnight at 37°C. After labeling, cells were rinsed with PBS and fixed in acetone/methanol (1:1) or in 4% paraformaldehyde. Cells were permeabilized with 500 mM HCl for 10 min. Blocking was done with 3% bovine serum albumin (BSA)/0.3% NP-40/PBS for 1 h at room temperature or overnight/N at 4°C. Mouse anti-BrdU antibody (1:1000, Sigma, St. Louis, MO) was added and

incubated for 1 h at room temperature. After removal of unbound first antibody with PBS/ 0.3% NP-40, FITC-conjugated goat antimouse second antibody was added. The fluorescence was visualized by a fluorescence microscope.

RESULTS

In all cases (Groups I–IV), immediately after isolation; small, viable structures with smooth contours having diameters up to 5 microns were visualized by phase contrast, and in group I, using SEM and TEM (Fig. 1A). Structures isolated from different tissues and conditions were morphologically indistinguishable at day 0. Microscopic examination using a DAPI stain was positive for DNA within the structures. Spore-like cells from all tissues multiplied in vitro in defined media and formed clusters that grew in size. Evaluations using focal periodic acid-Schiff and mucicarmine stains were suggestive of external material containing glycolipid and mucopolysaccharide. Spore-like cells isolated from various tissues demonstrated a doubling time ranging from 12 to 36 h. The approximate doubling time for both pancreatic and spinal cord derived spore-like cells was 36 h. Doubling times for cells derived from

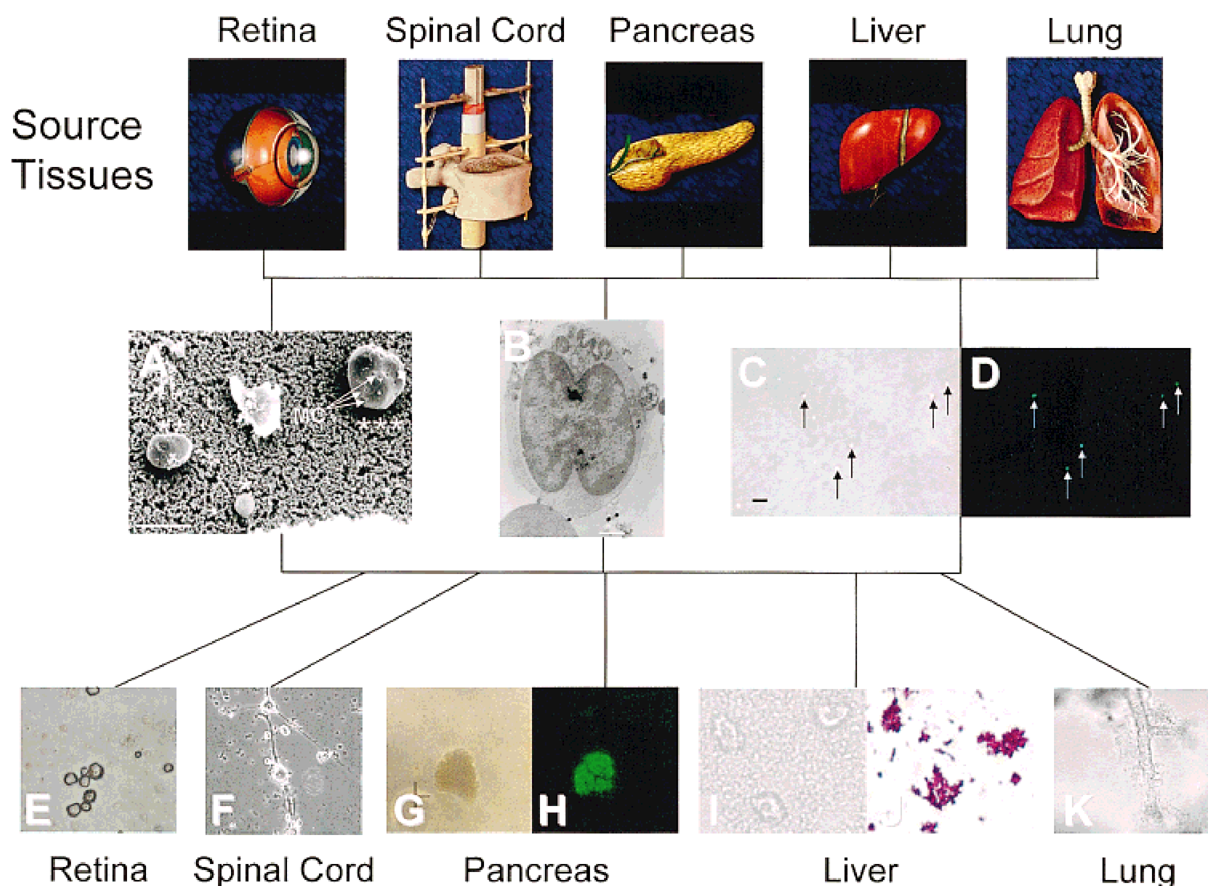

Fig. 1. (**A**) SEM of spore-like cells (original mag = 7, 000 × ; bar = 10 µm) showing a single cell (*) and clusters of multiple cells (** and ***) demonstrating mitotic clefts (MC) characteristic of cell division. (**B**) TEM of a single spore-like cell in the process of cell division (original mag = 38,000 × ; bar = 1µm). (**C**) Phase contrast microscopy of multiple spore-like cells, a subset of which (arrows) demonstrates DNA synthesis indicated by BrDu uptake using FITC-conjugated antibody detection scheme (**D**) (original mag = 400 × ; bar = 10 µm). Cells derived from 6 retinas developed morphology similar to that of rods and cones (**E**) (400 ×). Cells isolated from the spinal cords of 6 animals exhibited the morphology of neurons, astrocytes and oligodendrocytes (**F**). Pancreas-derived cells, isolated from 6 animals, developed islet-like structures (**G**) that stained positive for insulin using a monoclonal antibody (**H**) (both 400 ×). Liver-derived cells, acquired from 6 animals, developed the morphology of hepatocytes (**I**) and stained for bile using Hall's stain (**J**) (both 400 ×). Cells isolated from pulmonary tissues, harvested from 6 animals, developed bronchiolar structure similar to that of lung tissue (E) (400 ×).

skeletal muscle and liver were 12 and 24 h respectively. TEM demonstrated the development of primitive structures containing a large nucleus and several mitochondria (Fig. 1B). Multi-cell clumps, containing what appeared to be mitotic clefts were observed using SEM (Fig. 1A). This was, as supported by phase contrast and epifluorescence microscopy with BrDu uptake (Fig. 1C,D), suggestive of cell division.

After 7–10 days in culture, cells developed tissue-specific morphology. Retina-derived cells (Group I) developed into larger structures having morphology of rods and cones (Fig. 1E). Cells derived from the spinal cord (Groups I–IV) obtained morphology of neurons with connections between cells that resembled synapses (Fig. 1F). Pancreas-derived cells (Group I) differentiated into large clusters that resembled islets (Fig. 1G) and produced insulin (Fig. 1H). Cells derived from the liver (Groups I–IV) obtained hepatocyte-like morphology (Fig. 1I) and contained bile (Fig. 1J). Lung cells (Group I) developed complex morphology resembling bronchioles (Fig. 1K).

Spore-like cells with approximately 50% viability, as indicated by the trypan blue exclusion, were isolated from each of the four tissues exposed to one of the three extreme (Groups II–IV: cold storage, heat, or a freeze/thaw cycle) conditions. Cells isolated from these tissues retained the ability to proliferate in vitro and to differentiate into tissue-specific structures. Histological evaluation of the frozen and heated source tissues showed necrosis and corresponding freezing and heating artifacts.

DISCUSSION

This report describes the initial characterization of small, rounded structures, up to 5 μm in diameter, containing DNA, and covered with a material suggestive of both mucopolysaccharides and glycolipids. These structures are apparently ubiquitous having virtually been isolated in most of the organ systems of the body. Transmission electron microscopy of these structures corroborates these observations.

Upon initial isolation, these cells apparently initially have a very low demand for oxygen. They demonstrate the capacity to differentiate into the cellular components of the organ system from which they were isolated. The degree of tissue specificity exhibited by these cells is under investigation. Their characteristics differ from reports concerning immature cells, progenitor cells, and stem cells in their extremely small size, simple structure, and ability to withstand oxygen deprivation. Concerning the potential for a cell less than 5 μ in diameter to contain the entire genome, the concept of a minimal genome is beginning to emerge. This is exemplified by the description of a mycoplasma organism that contains 517 genes but requires only 265–350 of these genes for survival [Hutchison et al., 1999]. If one considers the exquisite simplicity of the DNA code, comprising 4 nucleotides with the pattern of adenine with thymine, and guanine with cytosine complementing each other, it seems plausible that the complex information stored in this simple code could be compressed to require less nucleotide material.

Spore-like structures, described above, might explain reported observations that seem to be conflicting. For example, two recent papers report the isolation of neural stem cells from the adult mammalian brain. One paper states that they arise from ependymal cells [Johansson et al., 1999], while the other reports their origin to be from subventricular zone (SVZ) astrocytes [Doetsch et al., 1999]. These reports seem to be contradictory. Our data suggest that small spore-like structures, present in both areas, may have been the source of the neural stem cells. Another paradox that may be explained by these structures is the tremendous regenerative capacity of the liver, while scientists are unable to stimulate hepatocyte replication in vitro.

Although these cells appear to have the capacity to differentiate into cells specific to the organ from which they were isolated, microenvironmental cues of the tissue from which they were isolated may be directing their lineage, or these structures may inherently be committed to tissue-specific lineage. Further studies are needed to clarify this issue.

In conclusion, we provide an initial description of a simple structure isolated from many organs of the body in the adult mammal. Early characterization shows them to be small, rounded structures containing DNA surrounded by mucopolysaccharides and possibly glycolipids. They possess the ability to replicate and differentiate into the cell-type present in the tissue from which they were isolated. They have the potential to survive extremes of temp-

erature and oxygen deprivation to a degree not previously described, or even considered. Much work needs to be done to further characterize these structures. There may be many potential clinical uses of these spore-like cells.

Greek mythology describes a magnificent bird called the Phoenix whose rebirth was described as arising from its fiery destruction. A phenomenon representing a recycling mechanism that allows individual organisms to maintain structure and function seems not only possible, but probable.

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REFERENCES

Brille S, Holstp, Seagel S, Zvibel I, Fiorino A, Ochs A, Somasundaran U, Reid LM. 1993. Hepatic progenitor populations in embryonic neonatal and adult liver. Proceed Soc Exp Biol Med 204(3):261–269.

Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Averez-Builla A. 1999. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell 97(6):703–716.

Hines PJ. 1997. Frontiers in medicine: regeneration. Science 276:59.

Hutchison CA, Peterson SN, Gill SR, Klein RT, White O, Fraser CM, Smith HO, Venter JC. 1999. Global transposon muted genesis and a minimal mycoplasma genome. Science 286(5447):2165–2169.

Johansson CB, Momma S, Clarke DL, Risling M, Lendahl U, Frisen J. 1999. Identification of a neural stem cell in the adult mammalian central nervous system. Cell 96(1):25–34.

McKay R. 1997. Stem cells in the central nervous system. Science 276:66–71.

Vogel G. 1999. Science 238–239.