Isolation and differentiation of Schwann progenitor cells

Isolation of Adult Stem Cells and Their Differentiation to Schwann Cells

Cristina Mantovani, Giorgio Terenghi, and Susan G. Shawcross

Abstract

Peripheral nerve injuries are an economic burden for society in general and despite advanced microsurgical reconstruction of the damaged nerves the functional result is unsatisfactory with poor sensory recovery and reduced motor functions (Wiberg and Terenghi, Surg Technol Int 11:303–310, 2003). In the treatment of nerve injuries transplantation of a nerve graft is often necessary, especially in nerve gap injuries.

Schwann cells (SC) are the key facilitators of peripheral nerve regeneration and are responsible for the formation and maintenance of the myelin sheath around axons in peripheral nerve fibers. They are essential for nerve regeneration after nerve injuries as they produce extracellular matrix molecules, integrins, and trophic factors providing guidance and trophic support for regenerating axons (Wiberg and Terenghi, Surg Technol Int 11:303–310, 2003; Bunge, J Neurol 242:S19–21, 1994; Ide, Neurosci Res 25:101–121, 1996; Mahanthappa et al. J Neurosci 16:4673–4683, 1996). However, the use of ex vivo cultured SC within conduits is limited in its clinical application because of the concomitant donor site morbidity and the slow growth of these cells in vitro (Tohill et al. Tissue Eng 10:1359–1367, 2004).

Mesenchymal stem cells (MSC or bone marrow stromal cells) and adipose-derived stem cells (ASC) are easily accessible non-hematopoietic stem cells that have proven essential for research purposes due to their plasticity and ability to differentiate into several functional cell types. This alternative source of cells is relatively simple to isolate and expand in culture. We have demonstrated that MSC and ASC can trans-differentiate along a SC lineage with functional properties and growth factor synthesis activities similar to those of native SC and could provide nerve fiber support and guidance during nerve regeneration.

Key words: Schwann cells, Differentiated mesenchymal stem cells, Adipose-derived stem cells

1. Introduction

In the last few decades a large group of publications described the use and the application of stem cells in a diverse range of injuries and diseases. The aim of our research is to utilize mesenchymal stem cells for repair and regeneration of damaged peripheral nerves (1–5). Stem cells can be identified as either embryonic or adult

stem cells. In the hierarchy of embryonic stem cells (ES), cells isolated from the fertilized oocyte are defined as *totipotent stem cells*; ES taken from the blastocyst are called *pluripotent* as these cells appear to be forming the three germ layers during embryogenesis. Fully developed adult tissues and organs contain niches of multipotent stem cells; these cells have been isolated from a wide range of adult tissues such as brain, heart, lungs, kidney, and spleen. However, the most well-characterized source of adult stem cells is the bone marrow. The bone marrow contains a mixed population of cells, including *hematopoietic stem cells* (HSC) and a subset of non-hematopoietic stem cells commonly called *marrow stromal cells* or *mesenchymal stem cells* (MSC).

The characterization of stem cells can lead to confusion as there is no universally accepted definition of the term "stem cell" and no unified theory describing their origin, plasticity, and function in the adult organism (6). The currently accepted characteristics of a stem cell are that the cells must be (1) undifferentiated (that is, lacking a tissue-specific differentiation markers), (2) capable of proliferation, (3) self-renewable, (4) able to produce a large number of differentiated functional progeny, and (5) able to regenerate tissue following injury (7).

MSC originate from the mesoderm germ layer; they give rise to connective tissue, skeletal muscle cells, and cells of the vascular system. Nowadays, there are still many unanswered questions about the true identity of the MSC, including location, origins, and multipotential capacity. Although isolation of MSC from many different tissues, such as adipose tissue, liver, amniotic fluid, umbilical cord blood, and dental pulp, has been described, the bone marrow remains the principal source of MSC with most potential and clinical application. Based upon recent knowledge, it is estimated that MSC constitute between approximately 0.001 and 0.01% of the nucleated cells isolated from the bone marrow (8, 9).

The advantage of MSC as therapeutic tools is that they can be easily isolated from the bone marrow and expanded in vitro, used in allogeneic transplantation, and show paracrine-mediated effects and migratory behavior to site of injury. There is evidence that MSC are capable of neuronal antigen expression in vitro (10, 11) and in vivo (12, 13). They have been shown to differentiate into astrocytes following direct transplantation into the rodent brain (14). Recent studies described remyelination of spinal cord lesions and showed that local delivery of MSC at the site of spinal cord injury was associated with the formation of neurofilament bundles at the interface between scar tissue and graft (15, 16). It is not clear what mechanisms govern the in vivo differentiation and migration of MSC within zones of injury; however, it is likely that the local milieu of growth factors, cytokines, and local stem cells has some influence.

Adipose-derived stem cells (ASC) are isolated from the stromal vascular fraction (SVF) of homogenized adipose tissue. ASC can be easily isolated from liposuction waste and exhibit the potential for chondrogenic, osteogenic, adipogenic, and myogenic differentiation (17, 18) and some aspects of neurogenesis (19). Although ASC show some similarities to MSC, they have a number of distinct features in terms of cells surface markers, differentiation potential, and abundance in the body. Up to 300-fold more stem cells can be harvested from 100 g of adipose tissue compared to 100 ml of bone marrow aspirate (8, 20). ASC are generally defined as CD34-positive CD31-negative (CD34$^+$ CD31$^-$). Many factors can influence the cellular composition of ASC cultures, such as species of origin, donor age, tissue location, isolation procedures, culture conditions, and cell storage. Moreover, the choice of experimental methods and reagents may also affect the outcome of any given study concerning ASC expression profile, differentiation potential, and therapeutic capacity. ASC and MSC share more than 90% of phenotypic markers; however, differences in surface protein expression have been reported (17). Furthermore, ASC are easier to culture for long periods and showed faster growth rates than MSC (21). Finally, ASC differentiated into a SC-like phenotype were recently shown to improve axonal regeneration across gaps repaired with fibrin conduits seeded with these differentiated ASC cells (22). Taken together these observations suggest that ASC are ideal candidates for tissue engineering-based injury repair and for future clinical applications.

Standard techniques such as histological, immunohistological, biochemical, and mechanical assays have been used to characterize the differentiation of both animal and human MSC. Cell lineage analysis has shown that the differentiation of cells is stimulated by cell-specific transcription factors that act as gene expression switches (23).

In vitro osteogenic differentiation can be induced using ascorbic acid, β-glycerophosphate, and dexamethasone: the differentiation of the cells is observed as increased expression of alkaline phosphatase (AP) and calcium accumulation with time (24).

In vitro chondrogenic differentiation is stimulated by transforming growth factor β (TGF-β), which results in an induction of protein kinases and is observed as an increase in the proteoglycan extracellular matrix (25).

In vitro adipogenic differentiation is promoted by the addition to the MSC cell culture of dexamethasone, indomethacin, and isobutyl methyl xanthine, which inhibits the enzymatic conversion of cyclic AMP to 5′ AMP by phopshodiesterase. This results in the upregulation of protein kinase A, which in turn upregulates hormone-sensitive lipase; this lipase converts triacylglycerides to glycerol and free fatty acids and is observed as an accumulation of lipid-rich vesicles (8).

2. Materials

2.1. Mesenchymal Stem Cell Isolation and Expansion

1. α-MEM (M8042; Sigma-Aldrich UK).

2. L-glutamine 200 mM (M11-004; PAA, UK).

3. Fetal Bovine Serum (FBS) (10270-106; Invitrogen Life Technologies, UK: Origin: South American—EU Approved).

4. Penicillin–Streptomycin (P11-010; PAA).

5. Hank's balanced salt solution (HBSS) (H9394; Sigma-Aldrich).

6. Trypsin, 0.25% (1×) with EDTA•4Na, liquid (25200-056; Invitrogen Life Technologies).

7. *Chlorhexidine in Spirit*, 70% (D549, Williams Medical Supplies).

8. A class II microbiological safety cabinet for cell culture and a vertical laminar flow cabinet for tissue dissection: both should be equipped with UV light for decontamination purposes.

9. A static water bath with temperature control.

10. Centrifuge U-320/U-320R (swing out rotor 45°, 138 radius) BOECO-Germany.

11. Sterile 15 ml Falcon-type conical bottom polypropylene centrifuge tubes (for example, 62.554.002; Sarstedt, UK).

12. Incubator with temperature and gas composition controls.

13. Inverted microscope with phase-contrast ability (for example, the Olympus IX51).

14. Sterile 10 ml syringes with 21-gauge needles.

15. Pipettes—sterile Pasteur-type, sterile serological, and Gilson-type autopipettes.

16. 70 μm mesh filters (1520012; BD-Falcon).

17. 75 cm^2 vent-cap tissue culture flasks (Corning 430641 or similar).

18. Sterile surgical scissors, surgical forceps, scalpel, bone nibblers, and forceps (Fine Science Tools, Germany).

19. Dissecting stereomicroscope.

2.2. Adipose-Derived Stem Cells Isolation and Expansion

1. α-MEM with (M8042; Sigma-Aldrich).

2. L-Glutamine 200 mM (M11-004; PAA).

3. Fetal bovine serum (FBS) (10270-106; Invitrogen: Origin: South American—EU Approved).

4. Penicillin–Streptomycin (P11-010; PAA).

5. HBSS (H9394; Sigma-Aldrich).

6. Trypsin, 0.25% (1×) with EDTA•4Na, liquid (25200-056; Invitrogen).

7. Sterile serological pipettes (5, 10 and 25 ml).

8. Sterile 15 ml and 50 ml Falcon-type conical bottom polypropylene centrifuge tubes (for example, 62.554.002 (15 ml) and 62.547.004 (50 ml); Sarstedt).

9. Sterile razor blades.

10. Sterile plastic Petri dish, 75 mm diameter.

11. Type I Collagenase (LS004197; Worthington Biochemical Products, UK).

12. Some 0.22 μm low protein-binding sterilization/filter units (for example, SLGP033RS Millex GP 0.22 μm unit; Millipore, UK).

13. 75 cm² vent-cap tissue culture flasks (Corning 430641 or similar).

14. Water bath with shaker facility.

2.3. MSC and ASC Differentiation to SC-Like Cell

1. Sigmacote® (SL2-25 ml; Sigma-Aldrich). *Important:* see siliconizing method (see Subheading 3.1) before proceeding with preparation of growth factor solutions.

2. Liquid nitrogen for snap-freezing growth factor/reagent aliquots.

3. Stem cell growth medium (SCGM): 45 ml α-MEM, 5 ml FBS, and 0.5 ml penicillin-streptomycin solution (10,000 U/ml and 10,000 μg/ml, respectively) and 0.5 ml of L-glutamine (2 mM).

4. Harvest buffer preparation: 10 ml HBSS medium containing 100 μl of penicillin-streptomycin; store on ice during the harvesting process.

5. Collagenase solution preparation: this should be freshly prepared just before starting the isolation process. Dissolve 30 mg collagenase type I in 15 ml of HBSS and filter sterilize using a 0.22 μm Millex® filter unit.

6. β-mercaptoethanol (βME) (M3148; Sigma): the final concentration of βME is 1 mM in the cell growth media without growth factors. Add 3.905 μl in 50 ml of SCGM.

7. All-*trans*-retinoic acid (RA) (R2625-100MG; Sigma-Aldrich) preparation: To 50 mg All-*trans*-RA solid, as supplied by Sigma-Aldrich, add 1.43 ml dimethyl sulfoxide (DMSO) to obtain a stock solution of 35 mg/ml (w/v). Dilute 2 μl stock in 20 ml of medium to a final concentration of 3.5 μg/ml. Dilute this stock solution of RA into SCGM to obtain a 350 ng/ml solution.

8. Basic Fibroblast Growth Factor (bFGF) (RCYT-218B; Sera Laboratories International (SLI, UK) is supplied lyophilized at 50 μg per vial and the final concentration of bFGF in differentiation medium is 10 ng/ml. First prepare the 5 mM Tris

(pH 7.6) solution by dissolving 0.6057 g Tris base (mwt 121.14) in total a volume of 1,000 ml (adjusted to pH 7.6). Next, dissolve the supplied 50 μg bFGF in 0.5 ml of 5 mM Tris (pH 7.6) solution to make a stock solution of concentration 0.1 mg/ml. Add 5 μl of bFGF stock solution to 50 ml SCGM to obtain a concentration of 10 ng/ml. Aliquot into small volumes, snap freeze in liquid nitrogen, and store at –40°C.

9. Platelet-Derived Growth Factor (PDGF) (RCYT-568B; SLI) is supplied as a lyophilized powder of 10 μg per vial and the final concentration of PDGF in differentiation medium is 5 ng/ml. Prepare a stock of 10 mM acetic acid by adding 60.24 μl acetic acid (99.5%) to 100 ml distilled water. Dissolve the 10 μg PDGF powder, as supplied, in 100 μl 10 mM acetic acid, which will produce a final concentration of 0.1 mg/ml. Aliquot into small volumes, snap freeze in liquid nitrogen, and store at –80°C. Add 2.5 μl of the 0.1 mg/ml PDGF stock to 50 ml SCGM to make a stock concentration of 5 ng/ml.

10. Forskolin (catalogue number F3917, SIGMA-Aldrich) is supplied as a powder at 10 mg per vial. Dissolve 10 mg Forskolin, as supplied, in 2.436 ml in DMSO to produce a 10 mM stock solution, Add 70 μl of the 10 mM stock solution to 50 ml SCGM. Aliquot into small volumes, snap freeze, and store at –80°C.

11. Neuregulin NRG1-1 (377-HB; R & D Systems, Abingdon, UK) is used to differentiate stem cells to a Schwann cell phenotype. NRG1-1 is supplied as a lyophilized powder at 50 μg per vial. The NRG-1 should be reconstituted in sterile PBS containing at least 0.1% (v/v) human or bovine serum albumin and to a concentration of 100 μg/ml. Store the reagent at –20°C. Add 10 μl stock solution to 50 ml SCGM.

12. Stem cell differentiation medium (SCDM) is prepared as follows: 50 ml SCGM plus 70 μl forskolin solution, 5 μl bFGF, 2.5 μl PDGF-AA solution, 5 μl or 10 μl NRG1-1 solution (for bone marrow or adipose stem cells, respectively). Preparation of the growth factors and forskolin stocks as detailed above (see Note 6).

3. Methods

3.1. Siliconizing Plasticware

Treat plasticware (tips and centrifuge tubes) with a solution of silicone to reduce or eliminate cell and growth factor attachment: proteins adhere to plastics and in the case of growth factors this reduces the amount available to the cultured cells. From our experience, we recommend using Sigmacote®. Start with clean

plasticware and apply the Sigmacote® at about 5 ml Sigmacote® per 50 ml volume of the item to be coated; thoroughly coat the entire inner surface of the tube(s) by swirling the Sigmacote® around within the tube(s). Drain excess Sigmacote® from the plasticware and allow it to air-dry for about 10 h: the excess solution can be retained and reused for future applications—store in a dark-colored, capped bottle or tube at 4°C. Thoroughly rinse the plasticware with sterile distilled water; then leave to air-dry in a class II tissue culture cabinet. The coated plasticware should be stored at 4°C and used within a couple of months of coating. Do not autoclave the plasticware after the coating with Sigmacote®.

3.2. Isolation and Culture of Mesenchymal Stem Cells from Rat Bone Marrow

1. Bone marrow is harvested from the long bones of one adult Sprague-Dawley (or other strain of laboratory *Rattus norvegicus*) rat. Following terminal anesthesia with CO_2, the hindquarters of the rat are shaved and washed with chlorhexidine solution. After incising the skin, the muscles are split along the lines of the femur and tibia and the bones dissected free of muscle. The bones are then dislocated at the hip and ankle joints and placed in chilled HBSS with 1% (v/v) penicillin–streptomycin. The bones are then transferred to the tissue culture laboratory for marrow harvesting.

2. Harvest the BM in a class II microbiological safety cabinet or laminar flow cabinet using aseptic technique. Cut the proximal and distal ends from the tibia and femur—just below the end of marrow cavity—using a pair of sterile, sharp bone nibblers. Insert a 21-gauge needle attached to a 10 ml syringe containing SCGM into the spongy bone. Flush out the marrow plug through the cut end of the bone with 5 ml of complete medium and collect it in a sterile 15 ml tube on ice. This procedure is repeated for each bone and the resulting cell suspension is twice triturated through a 21-gauge needle (see Note 1).

3. Filter the cell suspension through a 70 μm mesh filter to remove any bone fragments and other debris.

4. Centrifuge the cell suspension at $600 \times g$ for 5 min, then gently aspirate the supernatant, and resuspend the pellet in 10 ml of fresh SCGM.

5. Seed the BM cells in a 75 cm² vent-cap tissue culture flask and incubate at 37°C in 5% CO_2 in a humidified chamber. The flasks must remain undisturbed for 24 h (see Note 2).

6. Next day: the cells present at this stage are a mixture of adherent marrow stem cells, non-adherent haematopoietic cells, dead cells, and debris. Remove the non-adherent, free-floating cells (hematopoietic cell lineage) by washing the culture twice with 5 ml HBSS. Add fresh SCGM. Repeat this step for the first 3 days of culture (see Note 3).

7. On the third day of culture the adherent cells appear isolated when viewed under a phase-contrast microscope. With proliferation, the number and size of the cell colonies appear to increase gradually on days 4–6. During the following days the culture becomes more confluent and reaches 65–70% confluence (=passage 0).

8. When the cells reach confluence, they should be trypsinized and counted as described below. Aspirate the medium and wash the cells with HBSS to remove all traces of FBS as this interferes with the trypsinization process. Add 3 ml of 0.25% Trypsin–EDTA (ready to use from the supplier) to the cells and incubate for 5 min at 37°C (incubator). Neutralize the trypsin by adding 7 ml of SCGM and transfer the cell suspension into a new 15 ml tube. Centrifuge at $600 \times g$ for 5 min. After centrifugation, resuspend the pellet in 10 ml of fresh medium and count the cells using a hemocytometer slide. Replate the MSC at a concentration of 3.75×10^5 cells per 75 cm² flask. The cells can also be stored in liquid nitrogen at each passage stage to build up cell stocks (see Note 4).

9. Change the culture medium (SCGM) every 3 days (10 ml per 75 cm² flask). Typically, cell confluence is reached in 7 days. Figure 1a shows uMSC in culture.

3.3. Adipose-Derived Stem Cell Harvesting

1. Dissect out the visceral fat encasing the stomach and intestines of adult rats and chop to a fine consistency using a sterile razor blade in a clean, sterile Petri dish.

2. Transfer the tissue into a sterile 50 ml tube containing 15 ml freshly made collagenase type I solution and place in a static water bath at 37°C for 1–2 h.

3. Neutralize the enzymes by the addition of an equal volume of SCGM and centrifuge the solution at $600 \times g$ for 5 min—at this stage an upper layer of floating adipose cells should be observed and a pellet of cells constituting the stromal fraction, which contains the stem cells, will have formed. Carefully aspirate and discard the upper layer and the remaining medium to leave behind the pellet of stromal cells.

4. Resuspend the pellet in 10 ml SCGM and pass the suspension through a 70 μm filter to remove any large pieces of undissociated tissue; then transfer the filtrate to a fresh 15 ml tube.

5. Centrifuge cell suspension at $600 \times g$ for 5 min and resuspend the resulting pellet in 10 ml SCGM before transferring into 75 cm² tissue culture flasks. Maintain cultures at sub-confluent levels in a 37°C incubator with 5% CO_2; trypsinize and split as required (see Note 5). Figure 1b shows uASC in culture.
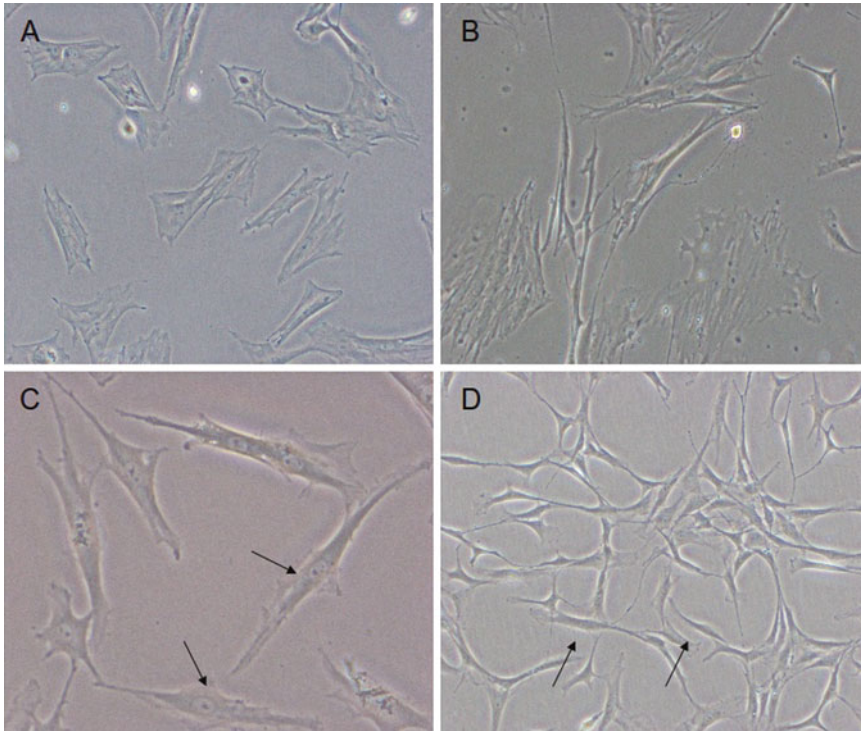
Fig. 1. *Morphology of the undifferentiated stem cells and the differentiated Schwann cell-like cells*. Phase-contrast images of various types of cell in culture were obtained using an Olympus IX51 inverted microscope and Olympus DP12 camera. (**a**) uMSC at passage 1 and (**b**) uASC at passage 2 display star-like morphology. Cultures of MSC (**c**) and ASC (**d**) undergoing differentiation and displaying the spindle-shaped (*arrows*) morphology similar to that of Schwann cells: the dMSC were at passage 5 and the dASC at passage 3. Magnification: Images **a** through **c** were taken at ×20 magnification and image **d** at ×10 magnification.

3.4. MSC and ASC Differentiation Procedure

1. Prior to differentiation of the MSC and ASC it is advisable to freeze cells from a few flasks to create stocks for future analysis and experiments.

2. Aspirate the SCGM from sub-confluent cultures of passage 2–3 undifferentiated MSC or ASC and replace with SCGM supplemented with 0.8 μl βME per 10 ml of medium and incubate for 24 h at 37°C in 5% CO_2.

3. Next day, wash the cells with HBSS and replace with fresh SCGM containing 350 ng/ml all-*trans*-retinoic acid and incubate at 37°C with 5% CO_2.

4. Following 3 days of incubation, wash the cells with HBSS and replace with 10 ml stem cell *differentiation* medium (SCDM).

5. Incubate the cells (3) for 2 weeks and with medium (SCDM) changes approximately every 72 h (see Note 7). Figure 1c, d shows dMSC and dASC, respectively, in culture.

4. Notes

1. Avoid bubble formation. The cells may become trapped within the bubbles and lost from the preparation.

2. Ensure that the incubation conditions are exactly as described in the method. Hypoxia, high CO_2 level and low humidity are highly detrimental to MSC growth and survival.

3. At this stage remove the culture medium very gently because rapid aspiration may cause the MSC to lift and detach from the bottom surface of the flask with the consequent loss of the adherent (MSC) cells. Bacterial contamination is always a possibility at this stage so particular vigilance is required.

4. The *time* and *temperature* of the trypsin incubation are very important. A longer incubation in this solution may cause cell death. It is important to prepare the fresh medium in advance so that it is ready to stop action of the trypsin.

5. A similar protocol for isolation of mouse adipose-derived stem cells from inguinal fat pads has been reported (26).

6. It is helpful to treat the polypropylene tubes in which the stock growth factors are stored and used with Sigmacote® (SL2; Sigma) to minimize the adhesion of the growth factors to plasticware.

7. Prepare only the required quantity of differentiation medium: upon storage at 4°C there is a loss of growth factor activity.

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