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Using phosphatidylserine exposure on apoptotic cells to stimulate myoblast fusion

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Summary

The fusion of myoblasts, the skeletal muscle progenitors, is critical for skeletal muscle formation, function, and repair after muscle injury. Recognition of the phospholipid phosphatidylserine (PtdSer) exposed on certain myoblasts is required during fusion into multinuclear myofibers. Cell surface exposure of PtdSer is also a feature of cells dying through the process of apoptosis. Here, we describe the use of PtdSer exposing apoptotic cells as stimulators of myoblast fusion.

Keywords

myoblast fusion; BAI1; phosphatidylserine recognition; apoptotic cells

1. Introduction

Mammalian skeletal muscle is composed of single-celled, multinuclear myofibers that are bundled and surrounded by connective tissue. Myofibers form via the proliferation, differentiation and fusion of myogenic precursor cells known as myoblasts (1). In mature muscle, myoblasts residing along myofibers are referred to as satellite cells. Satellite cells comprise the muscle stem cell niche and, once activated, proliferate, migrate, and fuse to

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[2]In addition to optimizing myoblast growth/fusion conditions, optimization of the number of apoptotic targets for the rescue of caspase inhibitor-blocked fusion may be needed. We observed that adding too few or too many apoptotic targets yields a poor rescue of fusion, and that a 1:1 ratio of dying to viable cells produced the best results.

[3]Although we only assayed apoptotic myoblasts and mouse thymocytes, other apoptotic targets may also rescue zVAD-fmk/Q-VD-OPH-inhibited fusion, if they expose phosphatidylserine on their outer membrane.

[4]To stimulate myoblast fusion in GM, C2C12 low passage cells can be plated as described in 3.1.1. When the cells reach 80% confluency, apoptotic cells can be added in GM. Freshly prepared apoptotic cells in GM should be replaced every 24 hours, for 72 hours of total incubation. Optimization of the number of apoptotic targets may be required. To quantify fusion of C2C12 cells stimulated with apoptotic cells in GM, follow steps 3.1.6–3.1.19.

[5]The 72-hour time course detailed in this chapter was chosen, in part, because myoblasts in FM will eventually die even in the presence of a pan-caspase inhibitor. If you maintain your negative control (zVAD-fmk/Q-VD-OPH treated myoblasts) beyond 72 hours in FM, you will start to see floating/dead cells and the appearance of myotubes. Eventually, this may narrow the difference you see between control and treated samples.

[6]Many different software programs can be and are used to quantify myoblast fusion. We chose to count nuclei using the ImageJ/Cell counter program.

generate new myofibers, repair existing myofibers, or return to a quiescent state and repopulate the niche (2).

Previous studies have identified the signaling proteins and pathways necessary for myoblast fusion; however, much of the early characterization was carried out in the *Drosophila melanogaster* model system, primarily due to the ease of genetic manipulation and the ability to screen for mutations that affect muscle development (1). While many of the proteins identified in *Drosophila* have demonstrated conserved function in mammalian myoblast fusion, upstream regulators of this process have remained elusive (1, 3). Recently, the membrane protein, Brain-specific angiogenesis inhibitor 1 (BAI1), was shown to promote the fusion of myoblasts via signaling through the conserved Dock180/ELMO/Rac1 pathway (4).

BAI1 has been shown to recognize phosphatidylserine (PtdSer) on the surface of apoptotic cells and promote their engulfment via the Dock180/ELMO/Rac1 signaling pathway (5). Interestingly, a fraction of myoblasts undergo apoptosis, and expose PtdSer soon after the switch to fusion medium (4, 6). Recognition of PtdSer is required for efficient myoblast fusion, as preventing cell death or PtdSer recognition potently inhibits fusion (4). In this chapter, we detail the methods of adding apoptotic cells to myoblast cultures to stimulate myoblast fusion in the presence of caspase inhibitors. We have shown that the fusion of myoblasts in cultures in response to typical differentiation cues is potently inhibited when the cells are treated with the pan-caspase inhibitors, zVAD-fmk or Q-VD-OPH. However, these caspase inhibitors-treated myoblasts fuse if provided apoptotic cells (C2C12 or thymocytes) that expose PtdSer (4). Furthermore, we have also shown that in the absence of other fusion cues, myoblasts in growth medium also fuse if provided apoptotic cells, suggesting that PtdSer recognition by quiescent myoblasts can stimulate fusion (4).

2. Materials

2.1 C2C12 myoblast cell line culture

1. C2C12 murine skeletal muscle myoblast cell line (ATCC) (7,8).
2. Two-chamber LabTeK II Permanox chamber slides (Nunc).
3. zVAD-fmk (Enzo Life Sciences); Q-VD-OPH (SM Biochemicals).
4. C2C12 growth medium (GM): DMEM (4.5 g/L glucose and L-glutamine without sodium pyruvate) (Cellgro®) supplemented with 20% heat-inactivated FBS (Premium Select, Atlanta Biologicals) and 1X (final concentration) Penicillin-Streptomycin-Amphotericin B (Lonza).
5. Fusion medium (FM): DMEM (4.5 g/L glucose and L-glutamine without sodium pyruvate) (Cellgro®) supplemented with 2% heat-inactivated horse serum (Donor Equine Serum, HyClone), 1X (final concentration) Penicillin-Streptomycin-Amphotericin B (Lonza).
6. 1X Phosphate Buffered Saline (PBS) (Cellgro®).
7. 0.25% Trypsin/2.21 mM EDTA (Cellgro®).

2.2 Apoptotic C2C12 targets

1. Plan the experiment carefully to know how many apoptotic targets you will need, as the ratio of apoptotic targets to myoblasts can influence the extent of myoblast fusion. Apoptotic targets are added fresh daily during stimulation of myoblast fusion.
2. Plate enough C2C12 cells (in large enough vessels) so that they will reach 80% confluency in 24 hours. For example, we were able to acquire approximately $1x10^6$ apoptotic cells from one 15 cm^2 tissue culture dish (BD Falcon™) after 24 hours in FM.
3. When C2C12 cells are approximately 80% confluent, gently rinse the cells once with 1X PBS and add fusion medium (FM). Incubate the cells overnight at 37°C and 8.5% CO_2. Plate another vessel of C2C12 cells that will be at least 80% confluent in 24 hours (this will provide the next supply of apoptotic cells). Alternatively, plate several vessels (section 2.1.2) in stepwise density dilutions such that the cells will be reaching 80% confluency in 24 hour intervals. We have found the rate of C2C12 cell doubling to be about 24 hours, allowing the initial plating of the cells in 2-fold cell number plating steps.
4. Twenty-four hours after switching the C2C12 cells into FM, observe floating, apoptotic cells in the vessel. Collect the supernatant, gently wash the fusing cells with 1X PBS, and collect the PBS wash as well.
5. Centrifuge the cells at 1000 rpm for 5 minutes, and resuspend gently in a small volume (1 mL) of fresh FM.
6. Remove a sample of the cells to count and to measure viability via Trypan Blue exclusion, or perform an additional assay to measure viability, such as FITC-Annexin V viability test (BD Pharmingen).
7. Add apoptotic C2C12 cells to fusing cultures as described in 3.1.3.
8. Repeat steps 3–7 to generate apoptotic targets for the remainder of the experiment described in 3.1.

2.3 Apoptotic thymocyte targets

1. Using approved institutional protocols for the care of animals, isolate a thymus from a young (3–6 week-old) mouse under sterile conditions in a laminar flow hood.
2. In C2C12 fusion medium (FM), crush the thymus through a 70 μm cell strainer to achieve approximate single cell suspension.
3. Transfer cells into a 15 mL conical tube (BD Falcon™), and centrifuge at 1000 rpm for 5 minutes.
4. Wash the cells in 10 mL of FM, and centrifuge again at 1000 rpm for 5 minutes.
5. Resuspend the cells in 10 mL of FM and pour through a 70 μm cell strainer.

6. Count the thymocytes and prepare a 2X suspension of $10x10^6$ thymocytes per mL.
7. Transfer 500 μL of this 2X suspension into each well of a 12-well plate (BD Falcon™).
8. Add 1μL per well of 2 μg/μL Pro-G (Sigma).
9. Add 5 μL per well of 1 μg/μL of Jo2 anti-Fas (CD95) antibody (BD Biosciences).
10. Add 500 μL of FM to each well and pipette up and down to mix.
11. Incubate at 37°C for 2 hours.
12. Each well contains $5x10^6$ apoptotic thymocytes in 1 mL of FM (the correct amount to add per well of the two-well Permanox chamber containing C2C12s). Wash step of the apoptotic thymocytes to remove anti-Fas antibody is optional (we found that C2C12 cells do not react to anti-Fas antibody treatment), and apoptotic targets can be added directly to C2C12 cells without a wash step.
13. Reserve a well of apoptotic thymocytes to perform viability analysis. Usually >95% of thymocytes will bind Annexin V (apoptotic) after 2 hours of anti-Fas treatment.
14. Each day, repeat steps 1–13 to generate apoptotic thymocytes for the remainder of the experiment described in 3.1.

2.3 Immunofluorescent analysis of fusion

1. 4% paraformaldehyde solution: 32% paraformaldehyde, methanol free (Electron Microscopy Sciences) diluted to 4% final concentration in 1X PBS pH 7.0. Store unused 4% paraformaldehyde solution at 4°C for up to one month.
2. Permeabilization solution: 1X PBS pH 7.0 with 0.2% Triton-X100 (Sigma) and 0.1% sodium citrate (Sigma).
3. Blocking solution: 2% BSA (Bovine Serum Albumin, Fraction V, heat shock, Roche) in 1X PBS pH 7.0.
4. Primary antibody: anti-myosin MY-32 (Sigma).
5. Secondary antibody: Alexa Fluor® 647 goat anti-mouse IgG antibody (Invitrogen). Alternatively, any fluorescent conjugate of anti-mouse IgG antibody can be used that does not overlap with Hoechst 33342 fluorescence.
6. Hoechst 33342 (Invitrogen).
7. ProLong Gold (Invitrogen).
8. Fisherbrand microscope cover glass 22×22 mm.

3. Methods

3.1 Rescue of caspase inhibitor blocked myoblast fusion with apoptotic targets

Unless specified otherwise, the total volume per chamber is 1 mL. Apoptotic C2C12 myoblasts are added at an approximate 1:1 ratio to the plated, viable myoblasts at the time of switch into fusion medium. Apoptotic thymocytes are added at an approximate 10:1 ratio. Appropriate controls to include are C2C12 cells maintained in fusion medium with no further treatment (positive control) and C2C12 cells maintained in fusion medium containing zVAD-fmk (or Q-VD-OPH) only (negative control).

1. Using two-chamber Permanox slides, plate low passage C2C12 myoblasts in growth medium (GM) at $1x10^5$ cells/chamber (see [Notes]). Incubate cells at 8.5% CO_2 37°C for 24 hours.
2. C2C12 myoblasts in chamber slides should be approximately 30–50% confluent. Add zVAD-fmk or Q-VD-OPH (100 μM) to inhibit caspase-mediated apoptosis directly to the growth medium. Incubate cells at 8.5% CO_2 37°C for 24 hours.
3. C2C12 myoblasts in chamber slides should be approximately 80% confluent. Gently aspirate medium, rinse cells with 1X PBS, gently aspirate PBS and add fusion medium (FM) containing zVAD-fmk or Q-VD-OPH (100 μM) and $5x10^5$ apoptotic C2C12 myoblasts or $5x10^6$ apoptotic thymocytes.
4. Gently centrifuge the chamber slides for 2 min at 500 rpm to bring the apoptotic cells in contact with the viable myoblasts. Incubate cells at 8.5% CO_2 37°C for 24 hours.
5. Repeat steps 3 and 4 in 24-hour intervals for a combined total of 72 hours in FM with apoptotic cells.
6. To quantify fusion, gently aspirate FM/dying cells, rinse cells with 1X PBS, gently aspirate PBS and apply 1 mL of 4% paraformaldehyde in 1X PBS pH 7.0 for 20 minutes at room temperature.
7. Gently remove 4% paraformaldehyde and dispose as per institutional regulations. Wash fixed cells with 1X PBS for 5 minutes at room temperature.
8. Gently aspirate PBS and apply permeabilization solution for 5 minutes at room temperature.
9. Gently aspirate permeabilization solution and wash fixed cells with 1X PBS for 5 minutes at room temperature.
10. Aspirate PBS and apply blocking solution for 20 minutes at room temperature.

[1]The successful rescue of zVAD-fmk- or Q-VD-OPH-inhibited myoblast fusion will require optimization. Initially, spend some time optimizing myoblast fusion on chamber slides without treatment. Determine the best growth/fusion conditions for the myoblasts in your lab. For example, we found that C2C12 myoblasts grow/fuse best at 8.5% CO2 concentration in our lab; however, other labs report the appropriate conditions to be anywhere between 5–10% CO2. In general, we detect 20–25% fusion in our cultures after 72 hours in fusion medium (FM), as assessed by calculating the fusion index (percent of nuclei within myotubes among total nuclei per field of view). Fusion can be enhanced greatly with the addition of several culture supplements, if necessary; however, abundant fusion may complicate the quantification.

11. Gently aspirate blocking solution and apply anti-Myosin primary antibody diluted 1:1000 in blocking solution. Incubate overnight at 4°C on a standard platform rocker.
12. Gently aspirate primary antibody and wash stained cells with 1X PBS for 5 minutes at room temperature.
13. Repeat step 12 a total of three times.
14. After final wash, gently aspirate PBS and apply fluorescently-conjugated secondary antibody diluted 1:400 in blocking solution. Incubate for 1 hour at room temperature in the dark.
15. Gently aspirate secondary antibody and wash stained cells with 1X PBS for 5 minutes at room temperature in the dark.
16. Repeat step 15.
17. Gently aspirate PBS and apply 1X PBS containing 1 μg/mL Hoechst 33342 in order to stain cell nuclei. Incubate for 2 minutes at room temperature in the dark.
18. Gently remove the nuclear staining solution and briefly rinse slide with 1X PBS. Add 1 drop of ProLong Gold per chamber and apply coverslip taking care to avoid bubbles.
19. Allow the mounting ProLong Gold to set (approximately 24 hours), and observe myoblast fusion using appropriate filter sets on a fluorescent microscope.

References

1. Abmayr SM, Pavlath GK. Myoblast fusion: lessons from flies and mice. Development. 2012; 139:641–656. [PubMed: 22274696]
2. Cornelison DD. Context matters: in vivo and in vitro influences on muscle satellite cell activity. J Cell Biochem. 2008; 105:663–669. [PubMed: 18759329]
3. Krauss RS. Regulation of promyogenic signal transduction by cell-cell contact and adhesion. Exp Cell Res. 2010; 316:3042–3049. [PubMed: 20471976]
4. Hochreiter-Hufford AE, et al. Phosphatidylserine receptor BAI1 and apoptotic cells as new promoters of myoblast fusion. Nature. 2013; 497(7448):263–267. [PubMed: 23615608]
5. Park D, et al. BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module. Nature. 2007; 450:430–434. [PubMed: 17960134]
6. van den Eijnde SM, et al. Transient expression of phosphatidylserine at cell-cell contact areas is required for myotube formation. J Cell Sci. 2001; 114:3631–3642. [PubMed: 11707515]
7. Yaffe D, Saxel O. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. Nature. 1977; 270:725–727. [PubMed: 563524]
8. Blau HM, et al. Plasticity of the differentiated state. Science. 1985; 230:758–766. [PubMed: 2414846]