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2, 4-Thiazolidindion Induced Plasticity of Myoblast (C2C12) and Satellite Cells (Porcine) - A Comparative Study

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ABSTRACT : This study was conducted to determine the difference between satellite cells (porcine) and myoblasts (C2C12) in their differentiation under the influence of 2, 4-thiazolidindion. C2C12 myoblast cells and porcine satellite cells (isolated from 10 d old Landrace×Duroc piglets) were grown to absolute confluency. Post confluent cells (day 0) were further exposed to adipogenic induction medium along with 2, 4-thiazolidindion (8 μM) for 2 d. Thereafter, cells were exposed to 2, 4-thiazolidindion alone every 2 d till day 10 and analysed. The control was cultured in differentiation medium without any treatment. Increased ($p<0.05$) expression of transcriptional factors i.e. C/EBP-α and PPAR-γ and transition of cells to adipocyte morphology was noticed from 2 d and 4 d onwards in satellite cells (Porcine) and myoblasts (C2C12) respectively. Myogenesis was observed to be suppressed completely in case of satellite cells compared to myoblasts in response to 2, 4-thiazolidindion. Pax-7 (transcriptional factor) appeared as a sole entity to satellite cells only, as it was not identified in case of myoblasts. Although both the cells were converting to adipoblasts, the degree of their conversion was different in response to 2, 4-thiazolidindion. Therefore, the hypothesis that satellite cells contribute various domains to the growing myoblasts appeared obscured and found to be dependent on the proliferative energy/or degree of fusion. However, it revealed satellite cells as currency to myoblasts/muscle. (**Key Words :** Adipogenesis, Myoblast (C2C12), Satellite Cells (Porcine), Pax-7, 2, 4-Thiazolidindion)

INTRODUCTION

Satellite cells and myoblasts assist in skeletal muscle formation by differentiating to myotube and muscle fibres during growth and injury (Hawke and Garry, 2001). Skeletal muscle and adipose tissue are mesodermal in origin and has been reported that both satellite cells and myoblasts can differentiate to muscle and transdifferentiate to adipose tissue (Li et al., 2005).

The ability of C2C12 myoblasts to convert from myogenic to osteogenic or adipogenic cells following treatment with BMP2 or thiazolidinediones respectively are well established (Katagiri et al., 1994; Teboul et al., 1995; Singh et al., 2007). Furthermore, myoblasts derived from single-fiber cultures are also capable of converting to osteogenic or adipogenic cells following BMP or adipogenic induction or culture in Matrigel (Asakura et al., 2001; Wada et al., 2002). However, it has only recently been demonstrated that adult muscle satellite cells are capable of converting to non-myogenic lineages. Treatment of myogenic satellite cells with rosiglitazone or adipogenic inducers/ciglitizone converts these cells into osteogenic or adipogenic cells (Jin et al., 2006; Kook et al., 2006; Singh et al., 2006; Choi et al., 2007). Though, the differentiation of myoblasts and satellite cells to adipoblast for intramuscular adipogenesis have been reported in mammals (Van Barneveld, 2003; Schoonmaker et al., 2004) but the potential inter-relationship of these cells in differentiating to another lineage have not been put forth.

Keeping all the above findings into consideration, the two sets of experiments were conducted to find out their comparative potential to transdifferentiate to adipoctye, and also to further confirm that whether the satellite cells really act as the currency to myoblast.

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MATERIALS AND METHODS

Materials

C2C12 myoblast cell line, Dulbecco's modified Eagle's Medium (DMEM), fetal bovine serum (FBS), horse serum, penicillin-streptomycin (PS) were obtained from American type culture collection (ATCC; Manassas; VA). Pig skin gelation, dexamethasone, insulin, isobutylmethylxanthine (IBMX), biotin, ascorbic acid, pantothenic acid, ascorbic acid, 2, 4-thiazolidindion (Fluka; Catlog #88405) and pronase were obtained from Sigma (Sigma Aldrich). Porcine satellite cells were isolated from the piglets under ethical guidelines of the institution.

Porcine satellite cells isolation

Three, d 10 old (Landrace×Duroc) piglets of either sex were selected for the study. Animal experimentation was approved by INSTITUTIONAL ANIMAL CARE AND USE COMMITTTE. Food and water was withheld for 12 h. Harvesting of muscle was done aseptically under general anaethesia. The right and left semitendinosus (ST) muscles were excised, denuded, and weighed. Satellite cells were isolated from ST muscles from d 10 old piglet as described by Doumit and Merkel (1992).

Cell culture

C2C12 myoblasts and porcine satellite cells were plated (on pig skin gelatin coated dishes) at a density of $2\times 10^3/cm^2$ and grown in Dulbecco's modified Eagle's Medium supplemented with 10% FBS and 1.1% of 100 IU/ml penicillin, and 100-mg/ml streptomycin (Proliferation media). Cells were kept at 37°C in a humified incubator with 5% CO_2. Media was changed every day and the confluence (almost 100%) was reached with in 5-10 days. Post confluent cells (d 0) were further cultured in DMEM supplemented with 2% horse serum and 1.1% Penicillin-Streptomycin with adipogenic mixture along with 2, 4-thiazolidindion (8 μM) for 48 h. From d 2 onwards cell in both experiments were cultured in differentiation medium with 2, 4-thiazolidindion (8 μM) alone every 48 h till d 10. Contents in adipogenic mixture were ascorbic acid (0.1 M), biotin, (33 mM), acetic acid (10 mM), pantothenic acid (34 mM), dexamethazone (5 mM), isobutylmethylxanthine (0.5 mM), insulin (10 μg/ml). The control was provided with differentiation medium without any treatment and the entire experiment was performed in triplicates.

Fusion index

Samples were collected at different intervals as committed in the methods. Cells were fixed with methanol and stained with hematoxlin stain (Sigma Aldrich) and was done as described by Dodson and Mathison (1988). The fusion index was calculated as the percentage of nuclei incorporated in the myotubes relative to the total number of nuclei. The structures containing at least three nuclei were scored as representing myotubes.

Adipocyte index

Oil-Red-O (Sigma Aldrich) staining was performed following the procedure described by Green and Kehinde (1974) with minor modifications. The adipocyte index was calculated in percentage as total number of adipocyte (Oil-Red-O positive) over the number of myonuclei at three randomly chosen microscopic fields.

Elution index

Cells were washed initially with PBS and fixed with 10% formalin. After washing all the formalin, the cells were stained with Oil-Red-O for 10min without touching the walls of the dishes. All the stain was removed using small transfer pipette and cells were washed with distill water 4-5 times. Dishes were allowed to air dry and further stain was eluted with 100% isopropananolol and optical density was measured at 500 nm over the 100% isopropanolol as blank (McNeil, 2005).

Immuno-histochemistry

The expression of myogenic and adipogenic proteins of differentiating myoblast and satellite cells were determined by indirect immuno-staining described by Michal et al (2002). Cells were washed with PBS, and incubated with anti-pax-7 (1:100) (Anti-mouse Monoclonal IgG1; R&D System; Minneapolis; MN), anti-C/EBP-α (1:200) (Rabbit Polyclonal IgG; Affinity BioReagents; CO), anti-myosin heavy chain (1:400) (Sigma Aldrich) and anti-PPAR-γ (1:200) (Sigma Aldrich) for 1 h and then exposed to biotinylated Goat Anti-mouse IgG antibody (1:500 dilution; Biomeda; foster City; CA). Finally, the cells were incubated with ABC peroxidase (Pierce; Rockford; IL) for 30 min at 37°C and then the peroxidase activity was developed using 3, 3'-diaminobenzidine stain (Sigma Aldrich) and cells were then examined by light microscopy.

Western blot

Equal amounts of protein extracts i.e. 30 μl from either cells were separated electrophoretically by 12% SDS-PAGE, blotted onto a PVDF membrane and probed with primary and secondary antibodies. Antibodies specific to adipocyte fatty acid-binding protein i.e. C/EBP-α and PPAR-γ diluted at 1:1,000 in PBST containing 1% Bovine Serum Albumin (BSA) were incubated with the membrane for 1 h at room temperature and detected as directed by the manufacturer's instructions (Bio rad) and subsequently photographed.

Statistical analysis

Statistical analysis of data was performed by one-way

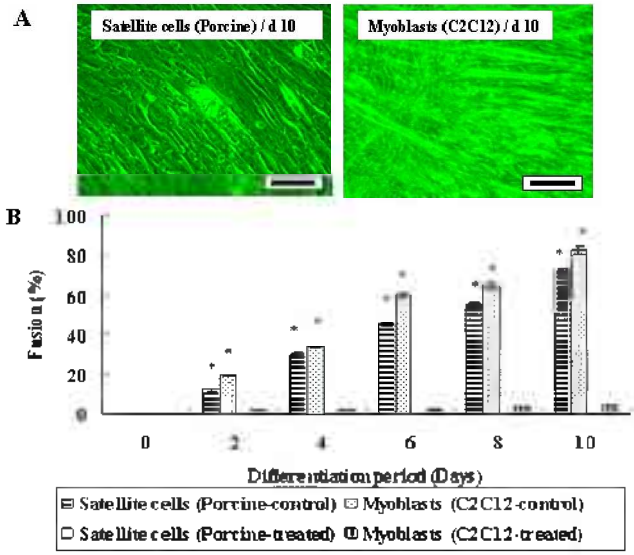
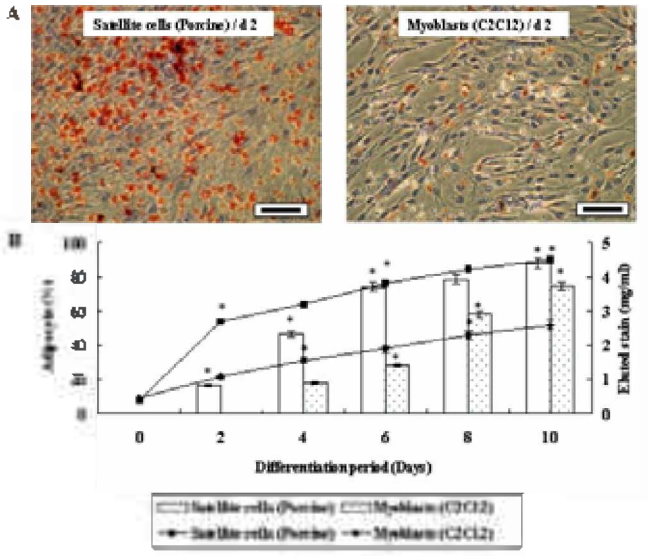


Figure 1. Myogenic potential of satellite cells (porcine) and myoblasts (C2C12). A. Myogenic differentiation in DMEM+2% Horse serum+1.1% Penicillin/streptomycin. The original magnification was 20×. B. Fusion index. Bars are mean±SE and represent a total of three piglets. * Indicates a significant difference ($p<0.05$) with an item compared with the undifferentiated (d 0) cells.



Figrue 2. Adipogenic potential of satellite cells (porcine) and myoblasts (C2C12) in response to 2, 4-thiazolidindion. A. Adipogenic differentiation in DMEM+2% Horse serum+1.1% 1.1% Penicillin/streptomycin with adipogenic mixture plus 2, 4-thiazolidindion for 48 h and subsequently with 2, 4-thiazolidindion alone. Fixed cells were stained with Oil-Red-O stain and adipocytes were identified as Oil-Red-O positive cells. Counter staining was done with Gill's hematoxylin. The original magnification was 20×. B. Adipocyte index and Eluted stain index. Bars are mean±SE and represent a total of three piglets. * Indicates a significant difference ($p<0.05$) with an item compared with the undifferentiated (d 0) cells.

analysis of variation (ANOVA). Significant differences were detected ($p<0.05$) by Duncan's multiple range tests using a PC statistical package (SAS, release 8.01. SAS Institute. Inc., Cary, NC).

RESULTS

Myotube formation was observed in both porcine satellite cells and C2C12 myoblasts when postconfluent (day 0) cells were maintained in Dulbecco's medium with 2% Horse Serum (HS)/1.1% Penicillin-Streptomycin (PS). Rate of fusion increased ($p<0.05$) from d 2 onwards in satellite cells and myoblasts compared to d 0. However, the rate of fusion appeared higher in myoblasts compared to the satellite cells (Figure 1).

Oil-red-O staining was performed to confirm 2, 4-thiazolidindion, induced conversion of satellite cells/ myoblasts to adipoblasts. Completely round and intensely red cells were considered adipoblasts and were counted in three randomly chosen fields at 400×. Total number of adipoblasts were counted over the nuclei and expressed as percentage. Adipoblast count was significantly ($p<0.05$) higher in satellite cells than myoblasts (Figure 2). Pattern of elution index followed adipocyte index and was remained significantly ($p<0.05$) higher for satellite cells which reflected higher rate of adipoblast formation (Figure 2).

Satellite cells were found to be positive when incubated with anti pax-7 on d 0 as compared to myoblasts (Figure 3). Expression of C/EBP-alpha and PPAR-gamma was noticed during adipogenic differentiation whereas myosine heavy chain was noticed during myogenic differentiation in both cells. Antibodies to cellular proteins used for immunohistochemistry were also tested on electrophoresed lysates of cells of both experiments during differentiation and the results shown in (Figure 3).

DISCUSSION

Plasticity of a cell in other words is transdifferentiation, which is known to be associated with a change in cellular morphology with a change in the gene expression (Slack and Tosh, 2001; Tosh and Slack, 2002) and more specifically due to posttranslational modification of transcriptional factors during the cell cycle. Satellite cells showed higher adipogenic index and elution index compared to myoblasts. Adipocyte index in the present study revealed adipogenesis in both cells. However, some degree of myogenesis was invariably apparent with myoblasts even during the transdifferentiation. It was therefore, assumed that myoblasts being a subsequent stage of satellite cells might have acquired stronger myogenic genotype while developing into myotube or myofibre. Because of this reason, 2, 4-thiazolidindion (PPAR-gamma

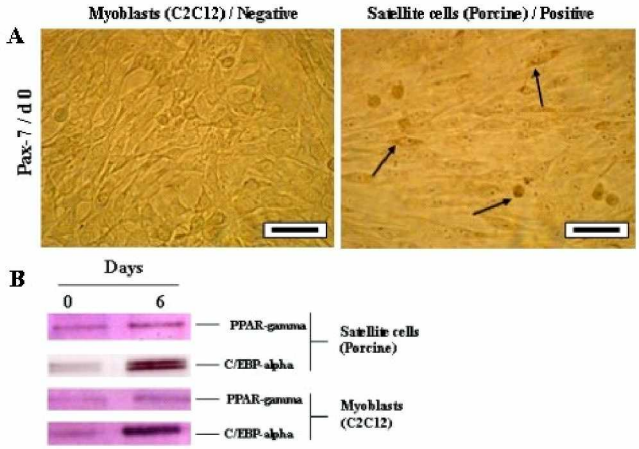


Figure 3. Expression of myogenic and adipogenic transcription factors during differentiation of satellite cells (porcine) and myoblasts (C2C12). A. Immunohistochemical analysis for pax-7 (arrows) transcriptional factor. The Original magnification (20×). B. Immunoblot analysis.

ligand) used in our study might have not been able to suppress myogenesis absolutely in case of C2C12 myoblasts. Although, it has been well established that C2C12 myoblasts can convert to osteocytes (Katagiri et al., 1994) and adipocytes in response to bone morphogenetic proteins (BMP) and thiazolidinediones and fatty acids (Teboul et al., 1995; Singh et al., 2007) respectively but their degree of conversion was never reported.

Pax-7 was observed immunohistochemically in porcine satellite cells in the present study confirmed that the cells harvested from porcine muscle were satellite cells as C2C12 myoblasts were found devoid of pax-7. Statetment that satellite cells could be considered as the currency to myoblasts as per cell stages (Anderson, 2006) supports our finding that satellite cells are distinct from myoblasts. This distinction of satellite cells are also in agreement with Kook et al. (2006) who stated that satellite cells are stem cell like with an identity distinct from that of myoblasts. A recent study of Pax-7-null mouse further defends the above finding as it revealed that paired box transcription factor is essential for satellite cell formation (Seale et al., 2000). Furthermore, pax-7 mutant mice showed severe muscle deficiencies at birth and premature lethality and were completely devoid of satellite cells (Seale et al., 2000). However, above observations demonstrates the requirement for pax-7 in satellite cell formation, it remains to be elucidated whether the satellite cells arises from a pre-determined myoblasts in the dermomyotome, a fetal myoblast, or from a non-somatic progenitor. It is emphasized that satellite cells might have originated from specified pax-7 positive cells prior to activation of muscle specific transcriptional factors, and thus represents a true precursor to the myogenic lineage. Alternatively, it can also be proposed that satellite cells might be originating from determined myoblasts which, instead of differentiating, continue to proliferate until withdrawing from the cell cycle and taking up residence beneath the basal lamina of myofibers (Chen and Goldhamer, 2003).

PPAR-γ and C/EBP-α expression was noticed in porcine satellite cells and C2C12 myoblast during transdifferentiation as early as on day 2 and 4 in our experiments respectively. Expression of adipogenic transcription factors i.e. PPAR-γ and C/EBP-α brings about change in the myogenic lineage of either satellite cell (Kook et al., 2006) or myoblast (Singh et al., 2007), and begins to acquire adipogenic lineage as demonstrated by their adipogenic differentiation in response to 2, 4-thiazolidindion in our study. Response of both cells to 2, 4-thiazolidindion in our study was in corroboration with Cowherd et al. (1999) who demonstrated the coordinated regulation of transcriptional factors C/EBP-α and PPAR-γ in *in vitro* models of adipogenesis. Both PPAR-γ and the C/EBPs are as the direct transcriptional activators of several fat cell genes, and the best characterized adipocyte-specific regulatory sequences have been shown to contain binding sites for both factors (Tontonoz et al., 1994; Tontonoz et al., 1995).

Minor myogenesis in myoblast during transdifferentiation could be attributed to the stronger acquisition of myogenic lineage by myoblast while coming from satellite cells and hence the environmental cue for adipogenesis used in our study appeared weaker in suppressing myogenesis absolutely in myoblasts. Although, transdifferentiation phenomenon with 2, 4-thiazolidindion was noticed in both satellite cells (porcine) and myoblasts (C2C12) but the degree of transdifferentiation in myoblasts appeared lower compared to satellite cells. Furthermore, the presence of pax-7 transcription factor in the satellite cells confirms the obvious difference between them in context to their cell cycle stage during development. With certain difference observed in their conversion ability in response to 2, 4-thiazolidindion, myoblasts could be proposed as the traveled phase of satellite cells. However, further studies are required to develop deep insight in their relationship.

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