Research Article

Downregulation of Caspase-2 Expression in Somitic Cells following Coculture with Chicken Notochord

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1. Introduction

The body axis of early embryo is made up of repetitive series of segments called somites that develop from paraxial mesoderm flanking neural tube and notochord. They form epithelial blocks of mesodermal cells budding from the rostral end of bilaterally presomitic mesoderm which originates from primitive streak [1–3]. Upon formation, signals emanating from neighboring tissues promote the newly formed somitic cells differentiation into dorsal dermomyotome and ventral sclerotome. Then, the sclerotomal cells migrate toward the notochord and surround it to form axial skeletons from which the vertebrae and ribs will be shaped. The dermomyotomal cells are also differentiated to dorsomedial epaxial muscle precursor cells forming axial skeleton associated muscles and dorsolateral hypaxial muscle cells which give rise to body wall and limb muscles. The central dermal portion of dermomyotome also develops to the dermis [2, 4].

During gastrulation, the notochord, as an axial structure arising from primitive node, functions as an axis to support the early embryo and finally gives rise to intervertebral disc. Several studies indicate that the notochord also stimulates the developing neural tube to generate motoneurons and, hence, sonic hedgehog (Shh) as a signaling molecule secreting from the notochord and, then, floor plate of the neural tube has a key role in motoneuron generation [5–8]. Moreover, it has been shown that the notochord promotes the survival and differentiation of somites into sclerotomal cells and Shh mimics the effects of notochord to diminish cell death in the somitic cells and enhances their viability. On the other hand, ablation of the notochord in chick embryo also activates apoptotic pathway and promotes programmed cell death in somites [9, 10].

Apoptosis is a tightly regulated biological event which occurs in embryonic development and has a crucial role in the development of some organs including limb bud and
cardiovascular system [11, 12]. Signaling molecules including Fas ligand and tumor necrosis factor-α (TNFα) promote apoptosis via a family of cysteine proteases, called Caspases, which act as upstream initiators (e.g., Caspases-8 and -9) or downstream effectors (e.g., Caspases-3, -6, and -7) that are primarily responsible for the limited proteolysis. In addition, Caspase-2 is also one of the initiator caspases which is activated rapidly in response to extensive apoptotic signals, but its mechanism of activation is not known. It has been reported that Caspase-2 is activated particularly in developing tissues such as neural and retinal tissues and highly expressed in developing somites when embryonic axial organs are ablated [10]. Moreover, Bcl-2, as an antiapoptotic protein inhibits cell death by controlling mitochondrial membrane permeabilization and blocking the release of cytochrome C from the mitochondria and hence functions as a guard for mitochondrial integrity [13–16].

In vivo study of cellular signaling pathways is more complex. So, coculture systems can be applied as an in vitro strategy for study of different tissue interactions under controlled conditions. We have recently showed that the somites have a neural inducing ability upon coculturing with mouse embryonic stem cells (ESCs) and in association with notochord promote neural patterning of human ESCs and human ESCs-derived neural precursor cells [17–19]. Here, we demonstrate that the somitic cell survival in monolayer culture depends on the presence of notochord and reveals in vitro evidence for antiapoptotic role of notochord on somites through downregulation of caspase-2 expression. Our findings also indicate that the notochord concurrently promotes in vitro somitic cells differentiation into Pax-1 expressing sclerotomal cells which can be used as a cell model for further biological studies.

2. Materials and Methods

2.1. Coculturing of the Somites with Notochords. Fertile hen's eggs provided from commercial sources were incubated in humidified condition at 38°C. Chick embryos at stages 8–10, according to Hamburger and Hamilton's developmental table, were isolated from yolk surface and transferred to L15 medium (Gibco, 41300–021) (Figure 1(a)). After separating embryos from the area opaca, they were transferred to new L15 medium containing dispase (1 mg/mL, Gibco, 17105–041) for 3–5 min in order to loosen the chick embryo tissues. Subsequently, dispase was removed and the embryos were washed with L15 medium supplemented with 5% fetal bovine serum (FBS, Gibco, 10270). Then, notochord and somites were isolated from embryos under a dissecting microscope and transferred to L15 medium without FBS (Figures 1(b) and 1(c)). Finally, the notochords were encapsulated into alginate beads as previously described [20]. In brief, dissected notochord explants were transferred to 1.2% alginate solution (Sigma, A7003) in 0.9% saline solution and then 20 μL of the alginate solution containing 15 notochord explants as a single droplet were slowly released into 102 mM CaCl₂ (Sigma, C7902). After 10 min, beads containing notochord explants were washed twice by 0.9% saline solution followed by washing with DMEM/F12+Glutamax medium (Gibco, 31331). For coculturing of somites with notochord, 30 somites and 15 encapsulated notochords were transferred into 24-well culture dish coated with 0.1% gelatin (Sigma, G2500) in DMEM/F12+Glutamax medium supplemented with 10% FBS, 1% NEAA, (Gibco, I140050), 1% Pen/Strep (Gibco, I5140122), and 1 mM 2-Mercaptoethanol (Sigma, M7522) for 2, 4, 6, and 10 days.

2.2. Survival Assay by MTT. Viability of somitic cells was evaluated by using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. For this purpose, the somitic cells were washed with PBS after removing the notochord and culture medium. Then, 20 μL of MTT solution (Sigma, M2128) were mixed with 180 μL medium and added to somitic cells. The cells were incubated for 4 h at 37°C in CO₂-incubator. Finally, MTT solution was completely replaced with the same volume (200 μL) of DMSO and the somitic cells were shaked at 150 rpm for 10 min, and optical density at 540 nm was registered by the plate reader.

2.3. Semiquantitative Reverse Transcriptase—PCR. Total RNA was isolated from somitic cells immediately after isolation (on day 0, as a control group) and the cells cultured with (Som + Not) or without (Som) notochord for 6 days by using Trizol reagent (Gibco, 15996-026) as described by the manufacturer's instructions. Then, 1 μg extracted RNA was used for cDNA synthesis and subjected for PCR amplification by 2-step RT-PCR Kit (Vivantis, RTREPL2). The amounts of total mRNA in the amplification mixtures were made equivalent according to their GAPDH content. Primer sequences, annealing temperature, number of cycles, and the length of amplified products were presented in Table 1. PCR products were electrophoresed in 1.2% agarose gels containing ethidium bromide and bands were visualized with UV light (Uvidoc, UK). Finally, to analyze semiquantitative expression of different mRNAs, the amount of cDNA was normalized based on the GAPDH mRNA expression using ImageJ software. Gels of three independent repeats were analyzed.

2.4. Spectrophotometry for Caspase-2. Enzyme activity for Caspase-2 was determined by spectrophotometric methods by using Caspase-2 kit according to the manufacturer's protocol (Biovision, K117-25). The rate of hydrolysis p-nitroanilide from the labeled substrate was measured at 405 nm at 37°C and the results were presented as fold increase for somitic cells in Som and Som + Not groups after 6-day coculture.

2.5. Statistical Analysis. Quantification of MTT assay and spectrophotometry test for Caspase-2 activity was performed based on five and three independent repeats, respectively, and the data normalized with control group on day 0 (upon somite isolation) were presented as a mean percentage or fold increase ± SEM and analyzed by one-way/two-way ANOVA followed by Tukey's post hoc multiple group comparison test or independent Student's t-test. Statistically, a difference between groups was considered as significant if P < 0.05.
Figure 1: Photomicrographs of a chick embryo at stage 8 (a), an isolated notochord (b), and the somites (c) from the chick embryo. Encapsulated notochord in alginate bead was cultured for 2 (d), 4 (e), and 6 days (f). The survival rate of the notochordal cells was quantified by MTT assay during different days in culture and presented as fold increase of control after normalizing with the same cells on day 0 (Control group upon isolation) (*P < 0.05) (bar = 50 μm).
Table 1: Primer sequences, annealing temperature (AT), the length of amplified products (bp), and number of cycles for different genes.

| Gene   | Primer Sequence (5' → 3') | AT (°C) | Length (bp) | Cycle |
|--------|---------------------------|---------|-------------|-------|
| GAPDH  | F: AGTCATCCCCTGAGCTGAATG R: AGGATCAAGTCCACAAACAG | 50      | 330         | 28    |
| Pax3   | F: GCCTGCAGCAGCGGCAACCT R: GCCTGGGTTGAAAACCCCTCC | 61.5    | 218         | 32    |
| BMP4   | F: GACCGCCAGGAAAGACTGG R: GCACGCTGCTGAGGTTGAAG | 59      | 352         | 32    |
| Bcl2   | F: TACCTGCTACATCTAGGAA R: ATGACTATGATGGAGTGGCA | 54.2    | 330         | 32    |
| Caspase2 | F: GTGGATTCTGTATTTGACG R: ATCCACCGTGGTGTTGTC | 49.2    | 302         | 35    |
| Pax1   | F: GCCTGCCGTCTGGTGCTAGAC R: ACTGTTAACGGCTTGGT | 55      | 451         | 35    |
| MyoD   | F: ACTACACGGAATCACCAGATGGAC | 55      | 200         | 35    |

3. Results

3.1. Survival Assay for Encapsulated Notochord. To prevent notochord from mixing with the somites, it was encapsulated in alginate beads and studied 2, 4, and 6 days after culturing. To assess survival of notochordal cells in culture condition, MTT test was also done. Microscopic observations showed that the notochord became thick and rolled on its axis (Figures 1(d)–1(f)) and the survival rate of its cells increased from 1.35 ± 0.4 folds on day 2 to 3.9 ± 1.2 and 4.4 ± 1.4 times on 4 and 6 days after culturing, respectively (Figure 1(g), P < 0.05).

3.2. Morphology of Somitic Cells. The somites were cultured with (Som + Not group) and without notochord (Som group) for 2–10 days. After 24 h, isolated somites started to spread onto the culture dish (Figures 2(a) and 2(b)) and after 2 days many somitic cells expanded (Figures 2(c) and 2(d)). Our results also indicated that morphology of somitic cell in Som + Not group changed to slender shape accompanying with short and thin processes (Figures 2(e) and 2(g)). However, the same morphology in a few somitic cells of Som group was seen after 6-day culture (Figure 2(f)). Finally, many somitic cells in both groups underwent death and apoptotic bodies appeared on day 10 after culturing (Figure 2(h)).

3.3. Survival of Somites Cocultured with Notochord. To evaluate in vitro somitic cell survival, the somites were cocultured with alginate-encapsulated notochord. After 2 days, 59 ± 12.8% of somitic cells were alive in the presence of notochord (Som + Not) compared to 51.3 ± 6.9% of the cells in Som group (P > 0.05, Figure 3). After 4 and 6 days coculture with notochord, 74.4 ± 16.4% and 89.7 ± 17.9% of the somitic cells in Som + Not group significantly appeared to be alive compared to 28.2 ± 5.1% and 53.8 ± 10.3% of the cells in Som group, respectively (P < 0.05, Figure 3). Ten days after culturing, the somitic cells started to die and only 38.5 ± 7.7% of them were alive in Som + Not group in comparison with the cells in Som group (17.9 ± 5.1%, P < 0.05, Figure 3).

3.4. Analysis of Gene Expression. RT-PCR was used for analyzing genes expression involved in apoptosis pathway and those have a role in somitic differentiation into sclerotome and dermomyotome. Since 6 days coculturing of somites with notochord indicated better results, we used this coculturing time in the rest of experiments. Semiquantitative RT-PCR analysis confirmed our morphological results and showed somewhat upregulation of BMP4 expression in somitic cells in Som + Not group. Bcl2 as a marker for survival cells was also upregulated in Som + Not group. In contrast, Caspase-2 expression was significantly upregulated in Som group on day 6 compared to the cells in Som group on day 0 (Control group) and Som + Not group on day 6 (P < 0.05) (Figure 4). As stated, six days after coculturing with notochord, morphology of the somitic cells group changed to slender shape (Figure 2(e)). These cells also expressed Pax1, a sclerotomal cell marker and downregulated myotomal cell marker, MyoD and Pax3, an early somitic cell marker compared to cells in Som (day 0) group and the cells of Som group (day 6) (P < 0.05) (Figure 4). Taken together, these findings showed diminishes of apoptosis in somitic cells and their direction to sclerotome differentiation following coculture with notochord.

3.5. Caspase-2 Activity in Somites. Spectrophotometry assay was used to evaluate Caspase-2 enzyme activity in somitic cells. Our results indicated 1.78 ± 0.18-fold increase activity of Caspase-2 in Som group, but it significantly decreased to 1.27 ± 0.16-fold increase in Som + Not group (P < 0.05, Figure 5).

4. Discussion

Our results present another evidence for survival effect of the notochord on somites by using alginate-based coculture system, a strategy that provides an appropriate in vitro study method for cell-cell interactions without direct mixing.
Figure 2: Photomicrographs of the somite upon isolation from chick embryo (a) and twenty-four hours after plating (b). The somitic cells were grown with notochord (Som + Not group) ((c) and (e)), and without it (Som group) ((d) and (f)) for 2 and 6 days, respectively. These cells indicated morphology of mesenchymal-like cells with thin and short processes, especially in Som + Not group (g). After 10 days, a large variety of somitic cells in both groups showed apoptosis (h) (bar = 50 μm).

Figure 3: Percentage of viable somitic cells normalized with the same cells on day 0 (Control group upon isolation) was quantified by MTT test in Som + Not and Som groups during different days in culture (*P < 0.05, **P < 0.02).

Several studies have shown that the presence of tissues surrounding the somites is required for somitic cells maintenance and patterning in the mouse and chick embryos and in the absence of midline structures of the embryo, the somite-derived cells undergo apoptosis. However, the somites appeared to have a greater size and significantly enhanced viability when the neural tube was located adjacent to the notochord with somites. Alginate acid is a widely used component for cell encapsulation in which entrapped cells are immobilized but allowed to release their secretion into medium due to porosity in alginate bead [21–24]. Previous studies revealed that alginate can promote proliferation or apoptosis in encapsulated cells but has no effect on plated cells when added to culture media without entrapped cells. Moreover, another investigation showed that the notochord cocultured with embryonic stem cells began to proliferate enrolled in alginate bead after four-day encapsulation [19, 20, 22, 25]. Since in coculture system, the culture condition should support both tissues or cell types, we studied viability of notochordal cells in culture condition. Our findings showed the encapsulated notochordal cells after 6 days culture had about 4.4 times more survival rate than the same cells on day 0. These results clearly indicate that the alginate doesn’t have any apoptotic effect on notochordal cells but in contrast, it allows the notochordal cells to be alive and possibly proliferate in culture condition.
somites [9, 26]. Moreover, it has been revealed that Shh secreted from the midline structures acts as a survival and proliferative factor for somitic cells and mimics the effect of notochord on them to differentiate into sclerotome. This signaling molecule also maintains myotomal and dermomyotomal cell survival, since Shh mutation in mouse embryo resulted in impairment of the maintenance of cell survival in the sclerotome [9, 27, 28]. In our study, the encapsulated notochord increased somitic cells viability, so that, 6 days after coculturing, about 90% of somitic cells in Som + Not group significantly were alive compared to 50% in Som group. It is possible that the somitic cells are proliferated in presence of the notochord. However, after 10 days, the somitic cells underwent apoptosis in both groups. The presence of exogenous survival signals such as Shh may be needed in culture medium, since after 10 days the notochordal cells started to die and they could not support somitic cell survival.

Previous work indicated that the somites isolated from axial structures of the early embryo undergo programmed cell death and Caspase-2, as an upstream initiator enzyme involved in developing tissues apoptosis such as somite, is overexpressed, while Bcl-2 expression, as an antiapoptotic marker, is diminished [10]. Our results are consistent with recent findings, since 6 days after coculturing, gene expression profile clearly showed high Bcl-2 and low Caspase-2 expression in somitic cells in Som + Not group compared to the cells in Som group in which the rate of apoptotic somitic cells was high. These results were also confirmed by Caspase-2 enzyme activity assay by which the activity of Caspase-2 in somitic cells was significantly lower in Som + Not group than the cells in Som group.

It has been shown that the fate of somitic cells is controlled by extrinsic factors emanating from the surrounding tissues including axial structures [26, 29, 30]. Based on misexpression experiments in chick embryo, Pax3 transcription factor is expressed in the early developing somites and then it is restricted to dermomyotome. Mutation in Pax3...
results in apoptosis in presomitic mesoderm and developing somites [31]. In addition, BMP4 expression in lateral plate mesoderm leads to differentiation in lateral side of the somites, but a BMP antagonist, noggin, expressed in medial side of somite, broadly expands myogenic lineage regulator, MyoD and Myf5 expression in epaxial muscle precursor cells [32, 33]. Expression of these myogenic regulator genes in somites will be upregulated by combinatorial signaling factors from notochord and neural tube/roof plate and localized expression of Shh also activates MyoD and Pax1 expression but inhibits Pax3 expression [27, 34, 35]. These findings are in contrast with using notochord alone that is not sufficient for MyoD and Myf-5 expression in somite [26]. Our RT-PCR analysis was consistent with these studies and confirmed that Pax3 is expressed in somite and that the notochord slightly decreased Pax3 expression in somitic cells but increased Pax1 expression after 6-day coculturing. These results also showed that the cultured somitic cells in Som+ Not group morphologically changed to mesenchymal-like cells with short and thin process represented differentiation into Pax1 expressing sclerotomal cells, but MyoD expression was rigorously downregulated compared to the cells in Som groups on day 0 and 6. The information presented here supports the idea that the notochord enhances the viability of somitic cell in in vitro through decreasing the apoptosis and induces somitic cell differentiation.

5. Conclusions

Taken together, this study clearly shows that the presence of the notochord cocultured with somite enhances in vitro somitic cell survival and inhibits apoptosis through Caspase-2 downregulation and Bcl-2 upregulation and likely promotes in vitro differentiation of somitic cells to sclerotomal cells by Pax1 overexpression and MyoD downregulation.

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