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• *BRIEF REPORTS* •

Dissociation, culture and morphologic changes of interstitial cells of Cajal *in vitro*

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Abstract

AIM: To study the method of dissociation, culture and investigate its morphologic changes *in vitro* of interstitial cells of Cajal (ICC).

METHODS: Enzymatic digestion and Ficoll density centrifugation were used to dissociate ICC from the ileal segment of mice. Factors including contamination, Ca^{2+}, Mg^{2+} and collagenase, and stem cell factor, *etc.*, were investigated. ACK2, the antibody of c-kit, was used to identify the cultured ICC. Both light microscope and fluorescence microscope were used to observe the changes of ICC *in vitro*.

RESULTS: The method for dissociation and culture of ICC *in vitro* was successfully established. After 24 h, cultured ICC exhibited a few axis-cylinders, and longer axis-cylinders were observed to form synapse of each other after 3 d. More widespread connections formed within 7 d *in vitro*. The changes of its morphologic character were obvious within 7 d; however, there were no obvious morphologic changes after 30 d.

CONCLUSION: Many factors can influence the dissociation and culture of ICC.

Key words: Interstitial cells of Cajal; Culture; Morphologic change

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INTRODUCTION

Interstitial cells of Cajal (ICC) are the pacemakers in gastrointestinal (GI) muscles, and also transduce inputs from the enteric nervous system. In the intestine, slow wave generation has been linked to the presence of a layer of ICC within the Auerbach's plexus region[1-3]. ICC are mesenchymal cells that have precursor cells in common with smooth muscle cells, and possess the Kit tyrosine kinase membrane receptor as their special characteristics[4-6]. Although there are many researches on ICC now[5-7], the procedure in isolation and culture of ICC still has many difficulties that should be studied and discussed. Their morphologic changes during short-term culture *in vitro* have no clear explanation by now. In this study, the method of dissociation and culture of ICC were established and discussed. The process of their morphologic changes *in vitro* was observed at least for 30 d. The method of ICC culture should help us make deeper research of ICC in future.

MATERIALS AND METHODS

Experimental animals

The BALB/C mice (15-20 d old) of either sex were from the Experimental Animal Center of Daping Hospital, Chongqing. Animals were not given food for 24 h prior to the experiment. Mice were killed by cervical dislocation. The ileal segment about 10 cm proximal to the ileocecal junction was removed. The muscularis propria was gently peeled from the mucosa and placed in Ca-free Hank's balanced salt solution (Hyclone) with 1% antibiotic-antimycotic (Sigma). Subsequently, the segment was washed thrice through the duct with Ca^{2+}-free Hank's balanced solution (1% antibiotic-antimycotic). The segment was opened flat by cutting along the mesenteric line and pinned flat with the mucosa facing the dissecting wood board. The dissected muscle was carefully cut into small pieces (1-2 mm^3) for enzymatic digestion.

Enzymatic dissociation

The muscle pieces were incubated at 37 ℃ in collagenase-based dissociation solution containing 1.3 mg/mL collagenase (type II, Sigma), 2 mg/mL bovine serum albumin (BSA, Sigma), 2 mg/mL trypsin inhibitor (Sigma), and 0.27 mg/mL ATP (Sigma), 10 mL of calcium-containing Hank's balanced salt solution (Hyclone). The pH was adjusted to 7.0 with 0.1 mol/L NaOH. After 30 min at 37 ℃ without shaking the water bath, the tissue was bluntly triturated with pipette every 3 min until single cells were obtained for approximately 10 min. After passing through the sieve (size: #200), all cell suspension was layered on the surface of a 200 g/L Ficoll density cushion and spun at 15 r/min for 15 min. The cell band located at the interface was transferred to a new container

and resuspended with M199 medium with 10% fetal bovine serum (Hyclone), 1% antibiotic to the desired density (about 2×10^6). The suspension was plated into Falcon petri dishes (with collagen-coated coverslips) on the bottoms. The cells were maintained in 50 mL/L CO_2 at 37 ℃.

Observation of ICC under light microscope

ICC were observed under Olympus inverted microscope, with 100×, 200× or 400× power. The images were captured with Olympus color video camera and then directly recorded in the computer.

Immunofluorescence labeling of ICC

Cultured cells were prepared for immunofluorescence labeling by fixation in acetone (4 ℃, 10 min). After fixation, the cells were incubated in normal goat serum for 1 h (10% in PBS), and then at 4 ℃ with a rat monoclonal antibody specific for Kit protein (ACK2, 5 mg/mL) in PBS overnight. Immunoreactivity was detected using fluorescein isothiocyanate (FITC)-conjugated secondary antibody (FITC-anti-rat, Zhongshan, China; diluted 1∶100 in PBS, 1 h, room temperature). Control cultures were prepared in a similar manner, but ACK2 was omitted from the incubation solution. The cells were examined with a LEICA TCS SP2 (Germany) confocal microscope with an excitation wavelength appropriate for FITC (488 nm). All images were captured and recorded in the computer.

RESULTS

Identification of ICC cultured in vitro

We performed immunofluorescence analysis to determine the morphology of cells that displayed Kit immunoreactivity. The cells showed fusiform cell bodies, prominent nuclei, and multiple thin processes extending from the nuclear region on light micrograph. ICC, with this morphology expressed Kit-like immunoreactivity, could form network of each other as shown on the fluorescence micrograph (Figure 1).

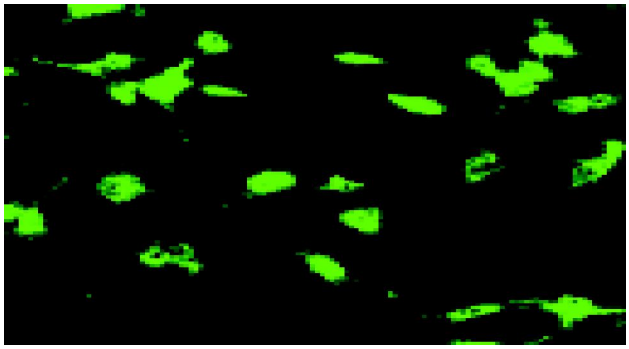

Figure 1 C-kit immunoreactivity on fluorescence micrographs of ICC cultured *in vitro* (400×).

Morphologic changes of ICC

After 24 h in culture, most cells showed fusiform cell bodies, large and prominent nuclei, and multiple, short and thin processes extending from the nuclear region (Figure 2A). Cells with this morphology were easily distinguished from the smooth muscle cells.

After 72 h in culture, cells with more and longer processes extending from the nuclear region could be observed (Figure 2B). Some cells began to form a characteristic network of each other.

After 7 d in culture, cells with more multiple and longer processes could be observed under phase-contrast microscope (Figure 2C). More characteristic networks could be found and the morphologic changes could be observed even after 30 d.

DISCUSSION

In GI tract, ICC are intimately connected with smooth muscle cells, fibrocytes and nerve fasciculi. Some methods can isolate and culture ICC[5-7]. Many difficulties should be overcome in the procedure of culturing ICC. However, no detailed research on the method of ICC culture is available before this study. In this study, we found that following factors should be discussed in the procedure of ICC culture.

Preventing contamination

If the culture medium is contaminated, few cells can be adhered to the coverslips at first, and no cells could be obtained after 3 d. The sources of infection mainly include *E. coli*, staphylococci, eumycetes, etc. In the study, no food was given for at least 12-24 h prior to the experiment. It is helpful to make the small intestine clean. In the procedure of isolating ICC, sufficient washing of the small intestine should be equally important to reduce contamination. Moreover, antibiotics (1% antibiotic-antimycotic was used in the study) should be added in the washing solution and culture medium. At last, strict aseptic manipulation should be mentioned in the process of cell culture.

Shortening the time in vitro and minimizing zero Ca^{2+} exposure

Solution containing no Ca^{2+} solution is very important to

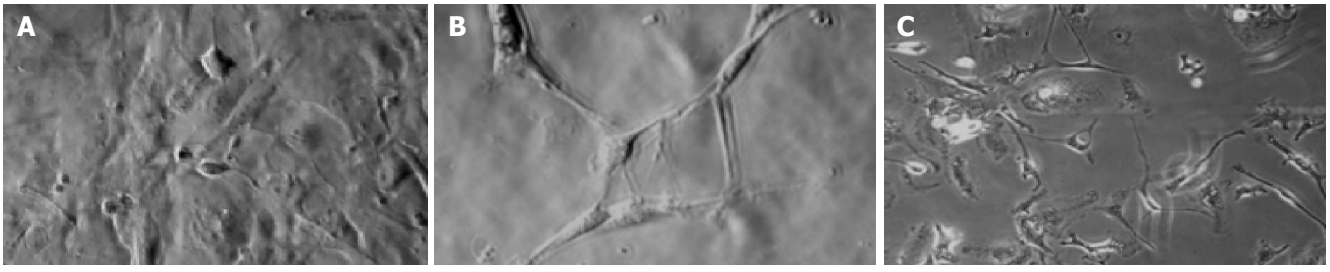

Figure 2 Morphologic changes of cultured ICC after 24 h (**A**), 72 h (**B**), and 7 d (**C**).

decrease the connection of ICC to other cells and matrix. For good dissociation of tissue, it is unavoidable to expose ICC to solution containing no Ca^{2+}[5-8]. However, when it is re-exposed to normal Ca^{2+} levels, overshoot of cellular Ca^{2+} levels would occur. We suggest that tissues exposed to low Ca^{2+} levels before placement in medium M199 (about 2.5 mmol/L Ca^{2+}) should be helpful to reduce the overshoot of Ca^{2+}. To get the desired quantity of ICC, shortening the time *in vitro* and minimizing exposure to solution containing no Ca^{2+} should be mentioned.

Appropriate enzyme digestion

Enzyme digestion is necessary in releasing ICC from tissues. Collagenase was used to isolate ICC in this study, while some other enzymes have been used either alone or in combination in other researches[5]. BSA and inhibitor of trypsin can be added in the solution to retain collagenase activity. We found that collagenase worked better in the presence of Ca^{2+}. Besides the component of enzyme solution, appropriate time of the enzyme digestion should also be mentioned. We found that insufficient digestion would get few single cells, while sufficient digestion would not get desired quantity of ICC as well. In this study, 30-min digestion at 37 ℃ was conducted to isolate ICC.

Changing the culture medium

In the study, the culture medium was changed after 24 h in culture. Then the medium was changed every 3-4 d. Twenty-four hours would be enough for ICC to adhere to the coverslips. Changing the culture medium in time can provide nutrient for ICC.

Stem cell factor is essential to maintain ICC in culture

Stem cell factor (SCF) is the ligand of the c-kit (CD117). The signal stimulated by c-kit/SCF is essential to maintain the phenotype of ICC[8-10]. In the culture medium M199, murine recombinant SCF was added at the concentration of 5 ng/mL in our study. Both the results in our study and other works showed that SCF is essential to obtain and maintain ICC in good condition[8,11-13]. In the study, we found that the c-kit with SCF was expressed on ICC in a dose-dependent manner.

In the study, the isolated ICC could contact each other. Multiple networks of ICC could also be found after about 7 d in culture, suggesting that the specific network of ICC is essential to maintain and realize its phenotype function.

It has been confirmed that c-kit only emerges on ICC and mast cells (-5%) in GI[10]. We consider that almost all c-kit-stained cells should be ICC. In the study, ICC were identified by immunofluorescence with c-kit-specific antibody ACK2.

After ICC were plated in M199 medium, the cells became round and could be adhered to the coverslips within 24 h in culture. The cell body was generally triangular or stellar and with a large nucleus surrounded by little cytoplasm. In the first 3 d, there were generally 3-5 primary processes, which could branch into secondary and tertiary processes. In the study, the isolated ICC could contact each other and form multiple networks within 7 d. These specific networks of ICC are the same as *in vivo*, suggesting that it is essential to maintain and realize its phenotype and function.

The detailed method of ICC culture can contribute to its deeper research.

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