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Isolation and Culture of Cardiac Progenitor and Cardiomyocytes from Adult Mouse

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Abstract.- Cardiomyocytes are principal cells in the heart. Earlier it was believed that cardiomyocytes cannot divide as these are terminally differentiated cells but latest research proved that these cells can divide and it has also been reported that there are also cardiac stem and progenitor cells in the heart. Cardiac stem cells and progenitor cells have a great potential for cellular therapy after myocardial infarction and other heart diseases. Primary culture of adult cardiomyocytes is also very important for different biochemical and physiological studies. We used explants culture, cold and warm tryptic digestion method for isolation of cardiac cells. We successfully isolated cardiac cells with a great proliferation potential by cardiac explant culture method and cold digestion method but not by warm digestion. The present study demonstrated that cardiac cells could be isolated by explants culture and mild enzymatic treatment and these cells retain good proliferation ability.

Keywords: Cardiomyocytes, progenitor cells, tryptic digestion, explant culture.

INTRODUCTION

The heart is a blood pumping organ of the body that changes its beating speed and contractility in response to changing conditions (Bers, 2001). Cardiac myocytes are principal cells in the heart, coordinate contractions and sense a large number of hormonal, neural, electrical and mechanical inputs through a variety of cell surface and nuclear receptors (Koch *et al*., 2000; Adams and Brown, 2001; Ross and Borg, 2001). Many physiological and pharmacological agents have been made to target cardiomyocytes, because of the need to regulate contraction strength and heart rate, additionally cardiomyocytes are involved in many cardiovascular diseases (Steinberg and Brunton, 2001; Cripps and Olson, 2000).

It was believed that after cardiac injury there is no myocardial regeneration because it was previously considered that in adult mammalian heart cardiomyocytes are terminally differentiated and are not capable for entering into a cell cycle (Soonpaa *et al*., 1996). By utilizing the latest techniques of cell isolation and analysis it has been shown that there is infact division in cardiomyocytes but at a comparatively low rate. Cardiomyocytes division was observed in adult mammals as well as human (Anversa *et al*., 2002). Evidences also suggested that immature cells reside in the heart tissue as they normally reside in other tissues of the body (skin, liver etc). These immature cells (progenitor cells) has the ability to proliferate and differentiate into cardiomyocytes (Young, 2004). The isolation and identification of adult cardiac stem cells offer the possibility of developing new treatment strategies for many heart diseases. Recently it has been reported that cardiac explants derived cells (EDC's) have cardiogenic potential (Messina *et al*., 2004).

Primary culture of cardiomyocytes has been widely used in basic cardiological research. Harary and Farley are the pioneers for mammalian myocyte culture (Harary and Farley, 1960). Adult myocytes are firmly connected to each other by intercalated discs and extracellular matrix and therefore are very difficult to isolate (Powell and Twist, 1976). Most of the long term studies on cultured myocytes were done on cells isolated from embryos or neonates (Simpson *et al*., 1982; Mitcheson *et al*., 1998). However it is preferable to study adult cardiomyocytes as compared to embryonic or neonatal cardiomyocytes because there is a different expression profile for certain proteins for example ion channels and contractile proteins during different development phases (Berger *et al*., 1994; Schackow *et al*., 1995).

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The present study describes convenient and time saving protocols for the isolation and culturing conditions of progenitor cells and adult cardiac myocytes from mice. This study will be helpful for further physiological and biochemical analysis of cardiac cells.

MATERIALS AND METHODS

Mouse dissection

For preparation of cardiac myocyte cultures, 6 week old BALB/c mouse was provided by animal house facility at School of Biological Sciences (SBS), University of the Punjab. Mouse was anesthetized by chloroform and sterilized by 70% ethanol and all dissection procedures were carried out in basic laminar flow cabinet. In short the protocol followed for dissection was as follows:

Mouse was killed by cervical dislocation. Then heart was immediately removed while it was still beating. Heart was gently squeezed in PBS with forceps to remove excessive blood from the heart followed by washing the heart several times by PBS. Afterwards the heart was placed in incomplete medium containing antibiotics (Penicillin and Streptomycin).

Culture

The pericardium was carefully removed. The ventricle was sliced into approx. 1–2 mm^3 pieces with a fine scissor. Subsequently in order to isolate progenitor cells and cardiomyocytes, heart tissue was processed in three different ways.

Explant culture

Ventricle pieces were washed with Ca^{2+} and Mg^{2+}-free PBS buffer twice to remove the blood cells and placed into cell culture dish (Corning), (10 pieces in one cell culture dish). The pieces were allowed to adhere to the surface for 15 min and then DMEM/F12 supplemented with glutamine, 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin was added. The dish was incubated at 37°C with 5% CO_2 in humidified environment for 3 days and the medium was changed after 3 days. Cells were observed each day. The explants were removed when cells were observed to be migrating out from the explants. Cells were then trypsinized by trypsin-EDTA. Cells were counted and subcultured into 25cm^2 flask.

Warm digestion

Ventricle pieces were washed twice with Ca^{2+} and Mg^{2+}-free PBS buffer containing antibiotics (penicillin and streptomycin). Five pieces were plced into microfuge tube containing 0.5 ml (IX) rypsin-EDTA (PAA) and incubated at 37°C for 1 hour. The trypsin digested pieces were resuspended by 10cc syringe by passing the tissue through syringe several times. The cells were collected by spinning the resuspended cells at 200g for 5 minutes. The resultant pellet was resuspended, counted the number of cells and finally resuspended the cells in complete medium and were added in tissue culture dish (Corning). The cells were incubated at 37°C with 5% CO_2 in humidified environment for 3 days. The medium was changed with fresh medium on third day.

Cold digestion

The procedure for cold digestion was mostly similar to warm digestion. With the only difference of incubation of ventricle pieces containing 0.5ml trypsin/EDTA (1X, PAA) at 4°C for 12 h. The cells were resuspended and plated exactly in the same way as described in the protocol for warm digestion.

The medium was changed after three days which allowed removal of dead and un-adhered cells. The cells were allowed to grow till they were 70-80% confluent. After which the cells were trypsinized and sub-cultured in 25cm^2 tissue culture flask.

RESULTS

Explant culture

After about 5 days in complete medium cells started migrating out from the tissue explants as shown in Figure 1. Most of the cells are of spindle shape showing morphology similar to cardiomyocytes progenitor cells.

When enough cells were migrated out of the explants, the explant was removed and cells were sub-cultured. The cells retained their shape after sub-culturing as shown in Figure 2.

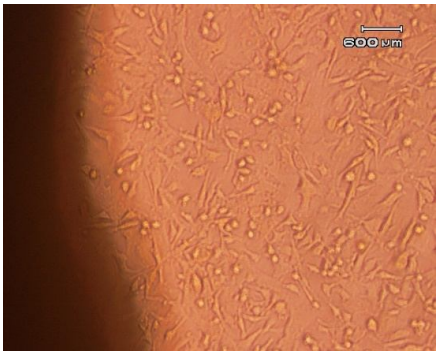

Fig. 1. Explant culture of cardiac tissue. Explant visible on left side of the image and on right side spindle shaped cells are visible which have migrated out of explant. Most of these cells are spindle shaped but cells with firbroblasts like morphology can also present.

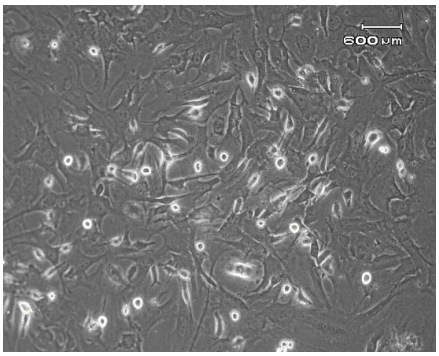

Fig. 2. Cardiac progenitor cells growth after sub-culturing from explant culture. Round cells are the dividing cells in the culture.

Warm digestion

After the warm digestion of heart tissue pieces no cell growth was observed. We changed the medium after 3 days and allowed the cells to grow for a week but there were no cells attached to the surface of culture dish and all the cells were remained in suspension.

Cold digestion

After cold digestion, cells started to adhere to the plastic surface and on the second day of growth cells were completely attached to the surface. There are two type of growth of cells on the basis of morphology. Some cells were spindle shaped and other showed flattened morphology like fibroblasts as shown in Figure 3.

These cells were grown until they became 80% confluent and then these were subcultured and grown under the same culture condition until they became 90% confluent as shown in Figure 4.

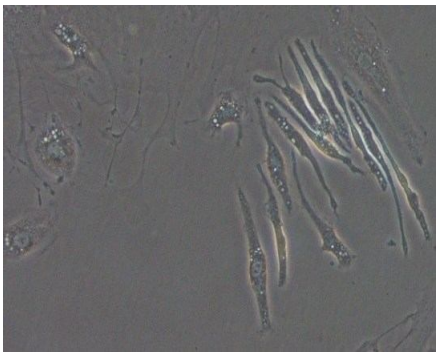

Fig. 3. Cold tryptic digestion cardiac cells culture. The image showing the cardiac cells grown after two days of culture. Spindle shaped cells are clearly visualized in this picture.

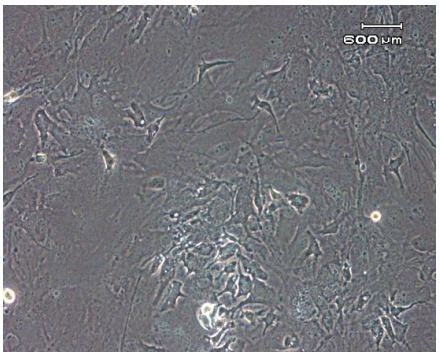

Fig. 4. 1st passage of cells isolated by cold digestion procedure. (Image at 20X magnification by IX51 Olympus microscope).

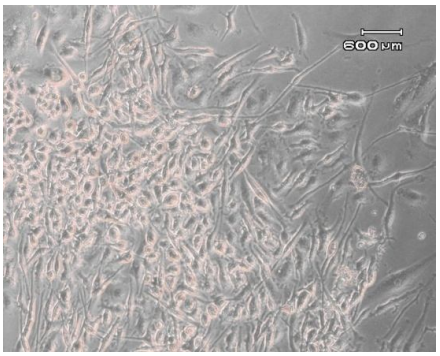

Fig. 5. Culture of cardiac cells isolated by cold tryptic digestion procedure after 5th passage. Some cells are dividing indicated by round shape, other cells have spindle shape of cardiomyocytes.

Cells were grown under the same culture condition until 5 passages and they retained their shape and spindle shaped morphology. At the end of 5th passage mostly cells were spindle shaped so it mean spindle shaped cells showed better proliferation as compared to flattened cells and dominate in culture as shown in Figure 5.

DISCUSSION

Primary culture of cardiac cells is a valuable tool for many pharmacological and toxicological studies. In the past two decades a lot of advancement has been seen in the area of culturing of cardiac cells. This has resulted in better management, cure and treatment of many cardiovascular diseases (Fu *et al*., 2005). Therefore it is very important to develop a convenient and time saving protocol for culturing of cardiac cells.

Despite of the success story for isolating the cardicmyocytes, there is still a need to improve protocol so that maximum number of cardiac myocytes could be isolated in short span of time and in convenient way. A common problem faced by many researchers during the isolation of cardiomyocytes is the loss of proliferative ability so that there is a continuous need to isolate cardiac cells on repetitive basis. Additionally as heart cells are strongly attached to each other so the dissociation process is hard and takes more time. It has also been reported that repeated digestion with trypsin for short period of time is preferable over long digestion and this might be the reason that we could not isolate cells by warm digestion protocol. For isolation of cardiomyocytes excessive digestion is also one of the main problems as this could result in non-adherence of cardiomyocytes in the coated plastic surface while digestion for short time results in low yield of cardiac cells. Typically five to eight incubations (10-20 min each) with trypsin are required for cardiac cell isolation (Gorelik *et al*., 2004), so there is a need of an optimized procedure by which cells can be isolated in a convenient way.

In the present study cells were observed to migrate out from the explants of cardiac tissues after about 5 days of culture. For this reason it is very important that tissue explants should be firmly attached with the surface of the dish. Most of the cells observed were spindle shaped but other cell shapes although less in number were also observed as shown in Figure 1. It is reported in literature that the cells that migrate out from the explants cardiac tissue expresses cardiac stem cell marker (c-Kit, Sca-1 etc), so cardiac stem cells and progenitor cells for cardiomyocytes migrated out in cardiac explants culture. These cells can be confirmed by immunostaining for cardiac stem cells and progenitor cells markers (Kondo *et al*., 2003). Myocardial infarction results in death of cardiomyocytes. Cellular therapy is one of the option to restore cardiac function so trials was done using progenitor cells from muscles (Hutchesin *et al*., 2000), bone marrow and embryos and they showed promising results (Orlic *et al*., 2003; Nir *et al*., 2003). An alternative approach could be to use the progenitor cells from the heart and proliferate and differentiate them into functional cardiomyocytes. In our results we found that cells migrate out from the explants tissue and as it is reported (Kondo *et al*., 2003) that these are the stem cells and progenitor cells so conditions should be optimized for their proliferation and differentiation so that they could be used as best cellular therapy.

We could not obtain the cardiomyocyte after warm digestion by trypsin. The reason could be effect of trypsin on cell surface proteins. Later we can try to optimize the exposure of trypsin to cardiac tissue pieces and mostly it is reported to expose cardiac tissue to trypsin in intervals (10-20 min) for 5-8 times for better isolation of cardiac cells (Gorelik *et al*., 2004). From the adult animal cells are isolated by perfusion of heart and mechanical agitation (Stemmer *et al*., 1989; Tytgat, 1994). This is the most common method in use now days for isolation of mature cardiomyocytes from adult animal.

As sometime trypsin treatment for cells is harsh and cell can't grow after tryptic digestion so we applied a gentle trypsin treatment for cardiac tissue. This approach worked very well and we could isolate cardiomyocytes as shown in figure 3. These cells had the same morphology as that of cells isolated by explants culture. The yield of cells was not very high after cold tryptic treatment as the number of cells attached to the surface were very less than the actual number of cells cultured.

The life of cardiac cells in culture is short and they become quiescent, one of the reason could be harsh enzymatic treatment during the isolation process, that damage the cell surface receptors and proteins responsible for cellular attachment (Kruppenbacher *et al*., 1993; Wolska and Solaro, 1996). In our study we grew the cells for 5 passages and till 5 passages the cells were growing fine under the given culturing condition as shown in figure 5. So I think we should isolate the cardiac myocytes not by harsh tryptic or other enzymatic treatment but by mild treatment so that their proliferation potential should not be lost in addition the yield of explants culture was very good and in this case there is no use of any enzyme so this method can also be used to isolate cells but it take more time as compared to tryptic digestion procedure.

CONCLUSIONS

This study demonstrate that cardiac stem, progenitor cells and mature cardiomyocytes can be isolated from adult heart using explants culture and they have good proliferation potential but further investigation will be done to study their differential potential. Additionally we can increase the proliferation potential of cardiomyocytes and other cardiac cells if we isolate the cells by gentle enzymatic treatment or by explants culture.

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