Transactions of the Materials Research Society of Japan 25 [4] 911-914 (2000)

Noninvasive Method to Laminate Fibroblast Monolayers

Masayuki Hara*, Ayako Yamaki and Jun Miyake
National institute for Advanced Interdisciplinary Research (NAIR), Agency of Industrial Science and Technology (AIST), Ministry of International Trade and Industry (MITI), 1-1-4 Higashi, Tsukuba, Ibaraki 305-8562, Japan
*Corresponding author, Fax: 81-298-54-3009, e-mail: hara@nair.go.jp

We developed a novel method to detach a monolayer of cultured cells with a supporting collagen gel layer from a calcium alginate gel on a porous membrane in a culture dish by treatment with EDTA as a relatively noninvasive method without using protease. This method depends on the principle that hydrogel composed of calcium alginate is reversibly formed and dissolved in the presence and absence of calcium ions, respectively. We laminated several layers of cultured 3T3 fibroblast with spacer of collagen gel layers by this method.

Key words: calcium alginate, EDTA, fibroblast, noninvasive detachment, tissue engineering

1, INTRODUCTION

Along with the increasing interest for tissue engineering, manipulation technologies for cells and tissues are becoming more and more important. Especially noninvasive methods to detach cells which are cultured in confluent state on culture dishes without protease treatment.

Alginate is acidic natural polysaccharide extracted from marine brown algae, and a copolymer of glucuronic acid and mannuronic acid. It has characteristic property to form soft hydrogel containing cation-chelating complex called "egg-box junction" in the presence of divalent cations such as calcium ions. Many calcium ions can be sandwiched between long alginate molecules in which negatively charged carboxyl groups of the alginate chelate calcium ions [1]. Ethylenediaminetetraacetic acid (EDTA) is a strong chelator for calcium and other divalent cations. EDTA has ability to dissolve the calcium alginate gel by depriving calcium ions of egg box junction.

Calcium alginate gel is nontoxic biodegradative gel and therefore has been widely used for scaffold material for immobilization of enzymes or cells for bioreactors, and also for tissue engineering [2-6]. Chondrocytes [3], hepatocytes [4] and pancreatic Langerhans islets [5, 6] were immobilized in calcium alginate gel beads. Generally speaking, calcium alginate gel has attracted much attention of researchers in the field of tissue engineering because of its biocompatibility or implantability *in vivo*. However, we focused our interest on its calcium-dependent gel-forming ability, and applied combination of the gel and porous membrane filter for establishing a novel noninvasive detachment method of cultured fibroblast monolayer from culture dish.

2. MATERIALS AND METHODS

2.1 Cells

Mouse fibroblast cell (BALBc/3T3 clone A31, Cell No. RCB0005) was purchased from RIKEN CELL BANK (Tsukuba, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM) (D5796, Sigma, St. Louis, MO, USA) containing 10%(v/v) fetal bovine serum (FBS) (10082-147, Gibco BRL, N.Y., USA).

2.2 Preparation of gels

We prepared three-layers structure (TS) consisting of collagen gel, calcium alginate gel, and a membrane filter attached to the inner dish of Falcon Cell Culture Insert (3091, Becton Dickinson and Company, Franklin Lakes, NJ, USA) as follows. Cell Culture Insert is a set of inner and outer dish those are separated one another by a membrane filter made of porous material (pore size 3.0 μm) on the bottom of the inner dish. Aqueous 1%(w/v) solution of sodium alginate (Wako Pure Chemical Industry, Osaka, Japan) was poured into the inner dish. Then the inner dish was taken out and soaked in 0.1 M CaCl2 solution. When calcium chloride gradually diffused and permeated into the membrane filter from lower side, a thin layer of calcium alginate gel was formed on the membrane filter. Collagen solution containing 0.35%(w/v) type I collagen at pH 3.0 (IAC-05, KOKEN, Tokyo, Japan) was poured into the alginate-coated inner dish. Collagen gel layer was formed at room temperature. The thickness of the collagen gel was approximately 1 mm. Then we got inner dish of Cell Culture Insert with the TS.

2.3 Cell culture

Fibroblast cells (10^4 cells in 0.5 ml medium) were inoculated on the inner dish with the TS. Culture medium was added to both inner and outer dish. Cells were cultured at 37°C under atmosphere with 5%(v/v) CO2 in CO2 incubator. Culture medium was changed in the next day from starting the culture, and then changed once in three days after that. Growth of the cells was observed using an optical microscope (IX 70, Olympus, Co. LTD., Tokyo, Japan).

Fig. 1 Detachment of the cultured cell from the Cell Culture Insert and lamination..

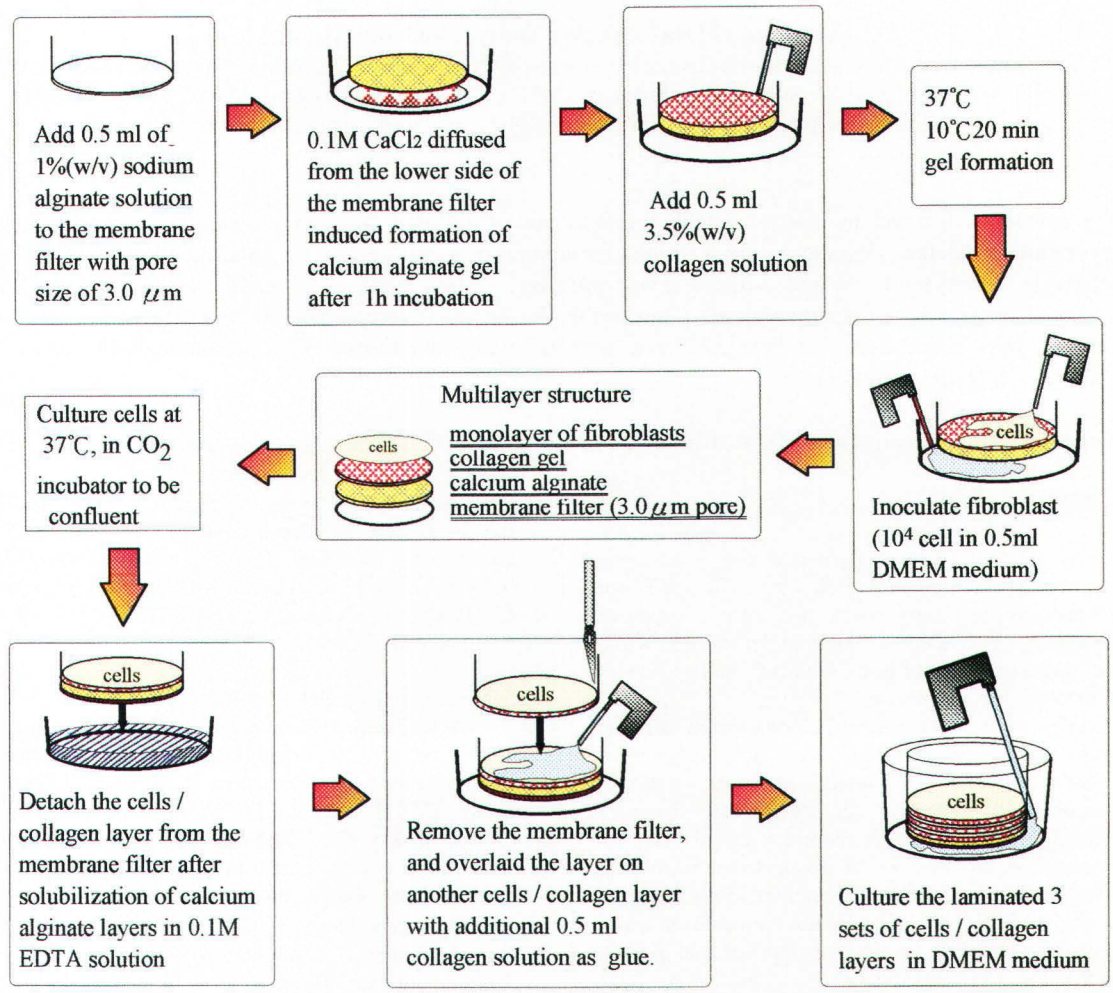
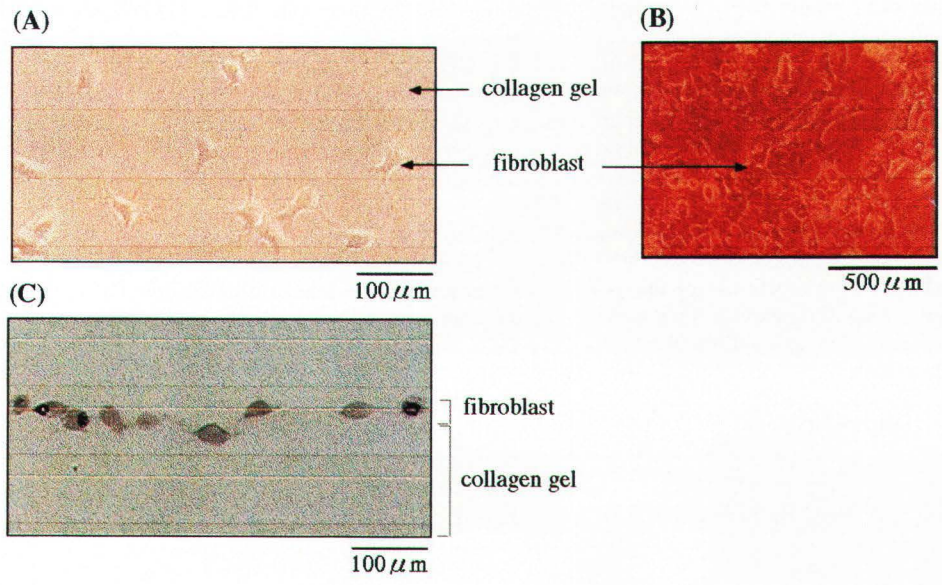


Fig. 2 Top view (A, B) and side view (C) of 3T3 fibroblast cultured on the collagen gel. Backgraound region in (A) is a surface of collagen gel with the membrane filter. Sample in (C) was a section stained with hematoxyline and eosin after Bouin's fixative. Cells in (A) and (B) were before confluent whereas cells in (B) were almost confluent.



2.4 Lamination of the cultured cell

Experimental procedure for lamination of the cultured cells is summarized in Fig. 1. Detachment of cells on the collagen gel layer from the membrane filter was carried out with EDTA as follows. After culture medium was removed, the inner dish was soaked in 0.1 M EDTA solution in neutral pH. When EDTA gradually diffused and permeated into the membrane filter from lower side, boundary between the membrane filter and calcium alginate gel was dissolved. Then the collagen layer with confluent cells was removed from the inner dish. If the lateral surface of the collagen layer still bound to the plastic wall of inner dish, a blade of scalpel was gently inserted between the wall and collagen gel to help the detachment. Residual amount of calcium alginate gel remained on the bottom surface of collagen gel layer. Detached collagen gel layer with cells were rinsed with DMEM to remove EDTA and then overlaid onto another detached layer. Three collagen layers with cells were overlaid and collagen solution was added as glue between each layer. After additional collagen was hardened at 37°C under atmosphere with 5%(v/v) CO_2, we got the laminated sample consisting of fibroblast monolayer, collagen gel and calcium alginate gel. It was dipped in DMEM containing 5%(v/v) FBS and cultured for 3 days at 37°C with 5%(v/v) CO_2.

2.5 Histology

Overlaid sample was fixed in the mixture of acetic acid, formalin (35%(w/v) formaldehyde) and aqueous saturated solution of picrylsulfonic acid (15 : 5 : 1 (v/v)). It was dehydrated by incubation in the mixture of butanol and ethanol, and embedded in paraffin. Thin sections of the sample with the thickness of 4–5 μ m were prepared using a microtome. After extraction of paraffin, those sections were stained with hematoxylin and eosin according to the standard procedure.

3.RESULTS AND DISCUSSION

3.1 Microscopic observation

Figure 2(A) shows the micrograph of fibroblast on collagen gel before confluent. Fibroblast cells attached to the surface of collagen gel and expand their pseudopodia as a typical shape of those cells. Cells grew well to be confluent from this stage in our experiment as shown in Fig. 2(B). Figure 2(C) shows the side view of the sectioned sample after fixation and hematoxyline-eosin staining. Their shape look like viable at 3 days after another set of collagen gel after another set of cells on a collagen gel was overlaid on it.

3.2 Comparison with other methods

Usually detachment of cultured cells from a culture dish needs protease (trypsin etc.) treatment or EDTA treatment to digest extracellular matrix or to weaken the cell-dish adhesion. Recently another method to detach cell sheet from the culture dish which had been grafted with poly (N-isopropylacrylamide) (PIPAAm) was reported [7]. It was also reported the cell sheet detached from culture dish and overlaid on another cell sheet by using that temperature-responsive gel without digesting extracellular matrix proteins. Therefore that method is a noninvasive method for construction of artificial tissues although PIPAAm itself is toxic for various cells. The toxicity of the material will cause problems it is implanted *in vivo*. We started to develop another alternative noninvasive method to detach cells from culture dish by using non-toxic ion sensitive gels and then chose calcium alginate for this purpose.

3.3 Problems remained to be solved

A present shortcoming to our method is that it is technically difficult to prepare a thin (thickness < 0.5 mm) collagen layer. Therefore each monolayer of cells apart from each other with the thick spacer layer of collagen gel. We used collagen gel layer on the calcium alginate gel to improve adhesion of 3T3 in our experiment. However collagen gel layer is not absolutely necessary if the cells attach well to the surface of calcium alginate gels. Therefore it will be possible that several sets of cultured cells on a thin layer of calcium alginate gel will be laminated in such case. Not only calcium alginate gel but also any other ion-sensitive gels can be used for noninvasive detachment method if they are combined with porous membrane filters along with the same principle.

3.4 Perspective

Tissue engineering is an expanding research field in a recent decade [8]. Methods of noninvasive detachment and lomination of cultured cells are useful for constructing artificial tissues and tissue-like structures of various organs.

As a conclusion, we showed the noninvasive method to laminate monolayers of cultured cell with an example of 3T3 fibroblast in this paper. This method will be applicable also to other types of cells in future.

5. ACKNOWLEDGEMENT

This research is supported by Biomolecular Mechanism and Design Project in NAIR, AIST, MITI. We thank Dr. M. Kato in NAIR for kind instruction of preparation of tissue sections.

5. REFERENCES

[1] Gel Handbook, Ed. by Y. Osada andK. Kajiwara, NTS, Tokyo (1997) pp. 738, in Japanese.

[2] M. Ibrahim, M. Decokin, M.-M. Batt, E. Dellacherie and G. Siest, *Applied Biochemistry and Biotechnology*, **12**, 199-213 (1986).

[3] D.A. Lee, T. Reisler, M.Akmal and D.L. Bader, in Proceedings of the 4th International Conference on Cellular Engineering, Nara, Japan, Nov. 30-Dec. 3, 1999, Ed. by M.

Aizawa, 1-S-15.
[4] K. Yagi, K. Tsuda, M. Serada, C. Yamada, A. Kondoh and Y. Miura, *Artificial Organs*, **17**, 929-934 (1993).
[5] R.P. Lanza, W. Ecker, W.M. Kuhtreiber, J.E. Staruk, J. Marsh and W.L. Chick, *Transplantation*, **27**, 1485-1487 (1995).
[6] J. Zimmerberg, F.S. Cohen and A. Finkelstein, *Science* 210, 908-910 (1980).
[7] A. Kushida, M. Yamato, C. Konnno, A. Kikuchi, Y. Sakurai and T. Okano, *J. Biomed. Mat. Res.*, **45**, 355-362 (1999)
[8] Principle of Tissue Enginnering, Ed. by R.P. Lanza, R. Langer and W.L. Chick, Acdemic Press, London, (1997)

(Received December 16, 1999; Accepted September 30, 2000)