Review

A Novel Tool of Temperature-Responsive Cell Culture Surfaces and Its Application to Matrix Biology

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Received, 12 January 1999; accepted, 20 January 1999.

Abstract: Matrix biology has one characteristic feature which is not covered by traditional biochemistry or molecular biology. Matrix biology inevitably includes solid materials for the alignment of cells in tissue architectures. One critical problem to overcome for elucidation of the consolidated structure and function is limitation in solubilization of the extracellular matrix (ECM) components. In this mini-review, we introduce a unique novel cell culture dish developed by us. The dish surface shows similar hydrophobicity as normal polystyrene tissue culture dishes at 37°C. When the temperature is reduced below 32°C, the surface rapidly hydrates and becomes hydrophilic. Therefore, by only reducing temperature viable cultured cells and intact deposited ECM are recovered from the temperature-responsive culture dish surface with no requirement of digestive enzymes or denaturing treatments. The possibility that cell culture system utilizing this unique novel culture surface provides a useful tool to investigate solid ECM is discussed.

Key words: temperature-responsive, poly(N-isopropylacrylamide), extracellular matrix, endothelial cell, fibronectin

INTRODUCTION

Gene analysis of extracellular matrix (ECM) components has enormously progressed. For example, collagen superfamily has enlarged to more than thirty different genes. Nonetheless, examination of the secreted and deposited molecules should be indispensable for understanding its posttranslational modification and supramolecular structure. Up to now, isolation of ECM components from tissues is highly limited, because they are noncovalently interacted and/or covalently cross-linked. Pepsin has been widely used for solubilization of collagens, since collagen triple helices are resistant to the enzymatic digestion. However, proteoglycan core proteins as well as glycoproteins such as fibronectin, laminin, and fibrillin are highly susceptible to enzymatic digestion. Furthermore, it has been revealed that many types of collagen have non-collagenous domains. Such digestive enzymatic treatments often destroy the molecular structure. Detergents have been also utilized to solubilize ECM, although the molecular and/or supramolecular structure can be lost by the treatments. Solubilization of ECM is a critical point in matrix biology. Recently, it has been revealed that many cell types deposit and construct the characteristic ECM during the culture1. These findings imply that cell culture system can provide another promising source for ECM components.

ECM Deposited by Cultured Cells

ECM deposited during cell culture would provide a model system for the investigation of matrix formation including collagen5-9, fibronectin8-11, laminin8-13, proteoglycans14-15, and elastin16. They are not only synthesized and secreted by cultured cells, but also organized into the specific supramolecular structures as observed in vivo. For example, type VIII collagen deposited by cultured corneal endothelial cells shows its characteristic hexagonal lattices seen in cornea Descemet's membrane17-18. Type X collagen is also known to assemble to hexagonal supramolecular structure in ECM deposited by cultured hypertrophic chondrocytes as observed in vivo19. And type VI collagen multimerization and deposition have been analyzed in cell culture20,21. Potent advantage of cell culture system is that it is easy to decrease crosslinks by the use of inhibitors such as β-aminopropionitrile (BAPN) for lysyl oxidase22 and putrescine for transglutaminase23. In fact, complete solubilization of ECM deposited by fibroblasts with BAPN-supplemented culture was achieved after its scraping24. By biochemical analysis of the ECM solubilized without pepsin, type VI collagen, that is labile to enzymatic digestion, was revealed to be a major component of deposited ECM and comparable to type I collagen in
amount. This finding suggests that crosslink-inhibited culture is useful. However, the deposited matrix could not be intactly recovered from culture dishes because of its tight adsorption to dish surfaces in general. Physical procedure such as scraping as well as chemicals including detergents can destroy the supramolecular structure. Therefore, a novel technique to recover deposited ECM noninvasively is required.

Temperature-Responsive Culture Surface

We have developed a new cell culture dish surface that responds reversibly and dynamically to temperature changes\textsuperscript{25}). The temperature-responsive polymer, poly-(N-isopropylacrylamide) (PIPAAm)\textsuperscript{26,27}, was covalently attached to solid surfaces by electron beam irradiation\textsuperscript{25} or specific chemical immobilization reactions\textsuperscript{28}). This surface shows similar hydrophobicity as normal polystyrene tissue culture dishes above PIPAAm's lower critical solution temperature (LCST) of 32°C, where PIPAAm becomes hydrophobic according to the dehydrated and compact chain conformation on the surface. When the temperature is reduced below the LCST, grafted PIPAAm chain rapidly hydrates and becomes hydrophilic.

Various cell types including fibroblasts, endothelial cells, hepatocytes, macrophages, and retinal pigmented epithelial cells are cultured on the temperature-responsive surfaces\textsuperscript{25-28,30}). Stress fibers, peripheral bands, as well as focal contacts were established\textsuperscript{31}). However, already spread cells were prompted to detach simply by reducing the medium temperature without any enzymatic digestion or divalent cation chelators. This cell detachment is inhibited by various chemical inhibitors of cell functions, such as sodium azide, genistein, phalloidin, and cytochalasin\textsuperscript{32}). This finding suggests a requirement of cellular structural and metabolic activity in the detachment process. Cells recovered by low temperature treatment consistently maintain their differentiated functions more highly than cells recovered by trypsinization\textsuperscript{29}). When culture temperature is decreased after cells proliferate to confluency, cells detach

![Fig. 1. Detachment of BAEC as a single contiguous sheet from a temperature-responsive culture surface by reducing culture temperature. BAEC were plated on temperature-responsive culture surfaces at 37°C. They adhered, spread, and proliferated to confluency (a). Upon reducing culture temperature to 20°C, BAEC spontaneously detached from the dish periphery. They retained cell-cell junctions and made a single contiguous sheet. The cell sheet folded and wrinkled (b). Rhodamine-phalloidin staining revealed that stress fibers and peripheral rings composed of F-actin were maintained in the detached cells (c).](image)

![Fig. 2. Double fluorescence microscopy of detaching BAEC sheets. Detaching BAEC sheets were fixed and double-stained with anti-fibronectin antibody (upper) and a fluorescent dye for DNA (lower) simultaneously. Fibronectin matrix was recovered with endothelial cell sheets and no remnant was observed on the surfaces from which cell sheets detached (right bottom corner).](image)
from the PIPAAm-grafted surface as a single contiguous cell sheet maintaining intact cell-cell junctions32(Fig. 1). Cytoskeleton and junctional complexes remained organized even in detached cell sheets. We also developed techniques to transfer cell sheets intact onto other surfaces33. Cell sheet transplantation would be a promising technique in tissue engineering, extending capabilities already achieved with cultured keratinocyte sheets34.

**Recovery of ECM by Reducing Temperature**

In addition to such an application for tissue engineering, this temperature-responsive culture surface can be utilized in matrix biology, since deposited ECM is easily detached from the surface by low temperature treatment. For example, we show the recovery of fibronectin matrix33, Bovine aortic endothelial cells (BAEC) were cultured on temperature-responsive surfaces in the presence of fetal bovine serum. Immunofluorescence microscopy revealed that fibronectin was deposited and accumulated on the grafted surfaces during the culture. Only by reducing culture temperature below 32°C, all the BAEC were detached as a contiguous cell sheet (Fig. 2). Detaching cell sheets are likely to fold and wrinkle without scaffold. By immunofluorescence microscopy with anti-fibronectin antibody, no fluorescence was detected on dish surfaces where cells had detached. The fluorescence intensity of the cell sheets showed no change between before and after detachment from the temperature-responsive cell culture surfaces. Immunoblotting with anti-bovine fibronectin antibody revealed that typical trypsin treatment completely digested fibronectin (Fig. 3). To the contrary, deposited fibronectin was intactly recovered by low temperature treatment. The recovery was essentially the same in amount as that by physical scraping with silicone rubber. The amount of fibronectin deposited during four-week culture was estimated as 4 μg per 35 mm dish and 4 pg per cell. By transmission electron microscopy, thick and electron-dense ECM was observed to adhere only to the basal side of the recovered cell sheets. Thus, deposited ECM together with cells can be noninvasively recovered and transferred to a test tube. It is not easy to handle materials in typical culture dishes because of the shape and capacity. However, once they are transferred to test tubes, various treatments including heat, chemicals, and sonication are pretty easy. In addition to cell sheet transfer, the intelligent culture surfaces also enable us to examine cell surfaces and deposited ECM from the basal side that were contacted with culture dish surfaces before detachment. Cell sheets can be easily detached from the dish surfaces together with deposited ECM by reducing temperature. Appropriate scaffold prevents folding and wrinkling. Then the cell sheets are turned upside down and are subject to examination such as atomic force microscopy and scanning electron microscopy.

**CONCLUSION**

We'd like to propose that combination of cell culture system supplemented with crosslink-inhibitors and the temperature-responsive culture surfaces can open a novel way to utilize deposited ECM. We believe that the unique culture surfaces will become a novel tool in matrix biology as well as in tissue engineering.

**Acknowledgments:** We are thankful to Prof. T. Hayashi (Univ. Tokyo) for the opportunity to introduce our intelligent culture dish. The present work was supported by The Japan Society for the Promotion of Science, ‘Research for the Future’ Program (JSPS-RFTF96l00201).

**References**

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J. Clin. Invest. 83, 1505–1511


