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Immobilized Insulin Amyloid Enhances Cell Adhesion and Proliferation Due to Interaction with Fibronectin

Masafumi Sakono,*[1,4,#] Shigenori Akiyama,[2,#] Tamotsu Zako,*[1] Shujiro Sakaki,[3] Tomonori Waku,[2] Naoki Tanaka,*[2] and Mizuo Maeda[1]
[1]*Bioengineering Laboratory, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198*
[2]*Department of Biomolecular Engineering, Kyoto Institute of Technology, Goshokaido-cho, Matsugasaki, Sakyo-ku, Kyoto 606-8585*
[3]*Applied Chemistry Department, Akita National College of Technology, 1-1 Iijimabunkyo, Akita 011-8511*
[4]*PRESTO, Japan Science and Technology Agency, 3-5 Sanbancho, Chiyoda-ku, Tokyo 102-0075*

(Received January 5, 2011; CL-110006; E-mail: msakono@riken.jp, zako@riken.jp, tanaka@kit.ac.jp)

In this study, we investigated the possibility of employing an insulin amyloid without the cell binding motif sequence as a novel cell adhesion material. Immobilized insulin amyloid on cell culture plates shows higher cell adhesion properties compared with untreated plates. Interestingly, cells adhered to the insulin amyloid displayed marked cell proliferation. It was also demonstrated that the insulin amyloid interacted with fibronectin present within the serum.

Recently, investigations concerning the development of artificial tissue substitutes for impaired organs have attracted much attention.[1] The preparation of artificial tissues requires appropriate construction of a cell adhesion matrix.[2] Many kinds of biomolecules such as collagen,[3] alginate constructs,[4] cellulose,[5] and fibronectin[6] are known as major components of the extracellular matrix (ECM) which enhance and stabilize cell adhesion. ECM components, which function as cell adhesion factors, also play a fundamental role in maintaining cell involved cell anchorage, ultimate cell shape, and cell polarity.[7] Recent studies have shown that the Arg–Gly–Asp (RGD) sequence, which is known as a cell binding motif found in fibronectin,[8] is recognized by cell-surface receptors.[9] Interaction between RGD and receptors such as the integrin receptor mediates cell adhesion. Thus, there is great interest in the development of RGD-conjugated materials for use as novel ECM materials.[10]

The choice of supporting material for use with ECM components is important when preparing artificial tissues. For example, poly(*N*-isopropylacrylamide) (PIPAAm) is a representative supporting material.[11] PIPAAm grafted onto plate surfaces exhibits temperature-responsive cell adhesion and detachment properties. In addition, various materials such as poly(ethylene oxide),[12] and silicon[13] and carbon nanotubes[14] have been reported to be used as supporting materials to immobilize ECM components.

Recently, amyloid protein self-assemblies have been studied for application to cell adhesion material. Amyloids typically display rigid, unbranched structures with diameters of 10–20 nm and lengths of up to several micrometers. A study by Gras et al. showed that NIH3T3 cells can adhere effectively on plate surfaces possessing an amyloid comprising a chimeric transthyretin protein conjugated with RGD peptide,[15] suggesting that protein amyloids can be used as novel supporting materials for ECM components.

We previously reported that cultivation of HEK293 cells on insulin amyloid-coated plates is possible even if cell binding motif is not included in the immobilized amyloid,[16] which indicates that amyloids hold great potential in the preparation of many types of cell adhesion materials. Supporting materials for ECM immobilization could be prepared from a diverse range of protein or amino acid sequences because a variety of proteins and peptides have been shown to form amyloids.[17] In this study, cell cultivation on insulin amyloid-coated plates was investigated using two different cell lines to generalize our previous finding.[24] More importantly, the cell adhesion mechanism of insulin amyloid-immobilized plate was also investigated. We demonstrate that insulin amyloid immobilized on plates shows highly efficient cell adhesion for various cells even when cell binding motif sequences are not included in the insulin sequence. The number of cells adhered to plates increased with increasing amount of immobilized amyloid. More importantly, the cells adhered on insulin amyloid showed marked cell proliferation. It was also demonstrated that the insulin amyloid interacted with fibronectin present within the serum, suggesting that cell adhesion and proliferation on insulin amyloid is enhanced by the complexation of insulin amyloid and fibronectin.

Figure 1 shows phase-contrast microscopic images of NIH3T3 cells grown on each plate 24 h after cell seeding. The plate coated with insulin amyloid (Figure 1a) showed a higher number of adhered cells compared with the untreated plate (Figure 1c), suggesting that insulin amyloid possesses high cell adhesion properties. Although the plate treated with native insulin showed modest cell adhesion (Figure 1b), cell aggregations were observed on this plate, suggesting that amyloid formation is required to facilitate homogeneous cell adhesion to the plate.

To confirm the cell adhesion properties of insulin amyloid-coated surfaces, the adhesion of NIH3T3 cells to plates coated with different amounts of insulin amyloid was investigated. As shown in Figure 2, the adhesion of NIH3T3 cell increased in a dose-dependent manner, possibly due to the increase in surface coverage by the amyloid. The plate covered with 100 $\mu g\,mL^{-1}$ insulin amyloid showed about 10 times higher adherent efficiency compared with the untreated plate in terms of live cell adhesion. This suggests that insulin amyloid plays an important role in cell adhesion.

High toxicity of amyloid aggregates has been widely documented.[18] It has also been reported that insulin amyloid possesses dose-dependent toxicity.[19] Therefore, we attempted to ascertain the viability of NIH3T3 cells on insulin amyloid-immobilized plates using propidium iodide (PI). PI can enter

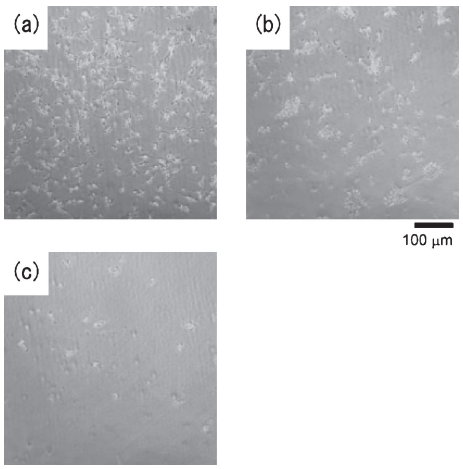

Figure 1. Morphology of NIH3T3 cell binding on 96-well polystyrene plates with or without pretreatments observed by phase contrast microscopy. (a) Insulin amyloid-immobilized plate, (b) native insulin-immobilized plate, and (c) untreated plate.

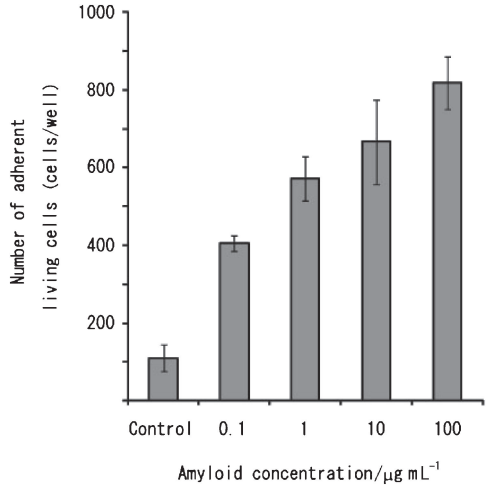

Figure 2. Effect of immobilized amyloid amount on adherent NIH3T3 cell number. The number of living cells adhered onto amyloid-coated plates was estimated using a LDH-cytotoxic test kit.

cells through damaged cellular membranes such as those resulting from cell death and can be detected by the fluorescence resulting through interaction with DNA.[20] PI fluorescence was not detected for cells on insulin amyloid-immobilized plates, indicating that cells on amyloid-immobilized plates remain intact and viable (data not shown).

Cell proliferation on insulin amyloid-coated plates was examined using two different cell lines (Figure 3). NIH3T3 or HepG2 cells adjusted to 5×10^3 cells/well were seeded in commercially available cell culture plates (Nunclon Delta), amyloid-immobilized plates, and untreated plates for cell culture. As shown in the figure, the number of NIH3T3 and HepG2 cells seeded on each plate increased with increasing incubation time. Cell growth on insulin amyloid-coated plates was similar to that on commercial Nunclon Delta cell culture

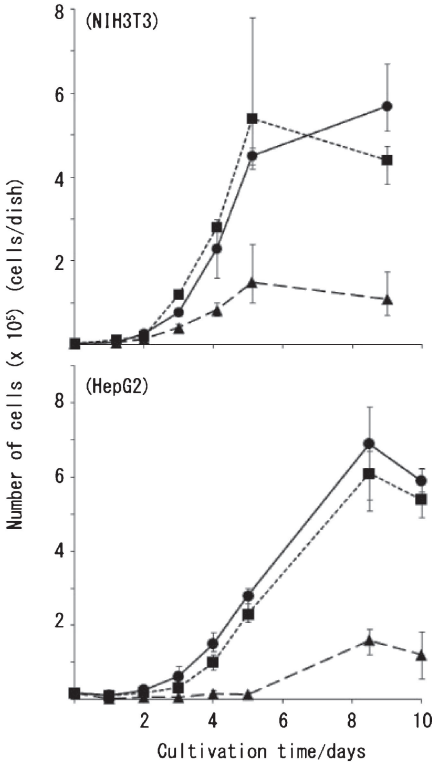

Figure 3. Time course of NIH3T3 and HepG2 cell proliferation adhered on plates (square: amyloid-immobilized plate, circle: commercially available cell culture plate (Nunclon Delta), triangle: untreated plate). Cells were trypsinized at various periods of incubation time and then counted using a hemocytometer chamber.

plates, and three to four times higher than that on untreated plates. This indicates that insulin amyloid immobilization on cell culture plates is effective not only for cell adhesion but also for cell proliferation.

In an effort to investigate the mechanism of cell adhesion enhancement by amyloid immobilization, we hypothesized that insulin amyloids immobilized on plates form complexes with cell adhesion factors contained in serum. To confirm our hypothesis, the interaction between insulin amyloid and fibronectin, a representative cell adhesion factor contained in serum, was examined by dot-blot analysis. The insulin amyloid used for immobilization was incubated in DMEM culture medium containing fetal bovine serum (FBS) and then collected by centrifugation. The blot containing fibronectin on the membrane was detected using a fibronectin antibody. As shown in Figure 4, a strong signal appeared for the amyloid sample incubated in DMEM medium containing FBS. No signal was detected for control samples incubated without FBS. This indicates that fibronectin present in FBS forms a complex with the insulin amyloids. Since fibronectin is known to bind cell surface receptors,[21] it is suggested that the enhanced cell adhesion observed on insulin amyloid-coated surfaces is mediated by interactions between fibronectin and insulin amyloids. Since both fibronectin and amyloid are known to possess hydrophobic properties,[22] it is plausible that the complex is formed by hydrophobic interactions.

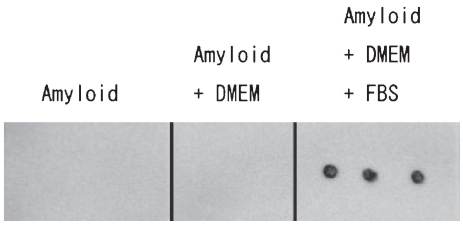

Figure 4. Detection of fibronectin by dot-blot analysis using electrochemical luminescence. Spotted amyloid samples were treated with pure water, DMEM medium, and DMEM medium containing FBS. Each sample was spotted at three points.

In conclusion, we reported that immobilized amyloid made from bovine insulin, which does not possess cell binding motif sequences, could enhance cell adhesion and proliferation for two different cells. The effectiveness of insulin amyloid-immobilized culture cell plates is similar to that of commercially available cell culture plates. Cell adhesion and proliferation on amyloid-immobilized plates is suggested to result from complexation of amyloid and fibronectin present in the serum and possibly involve hydrophobic interactions. Our results suggest that amyloids could be utilized as effective supporting materials for cell culture and tissue engineering. Amyloids are known to form gel structures,[23] suggesting that they could be utilized as scaffolds in 3D cell culture. Further studies are necessary to confirm this idea.

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References and Notes

# Equal contribution

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