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Influences of the recombinant artificial cell adhesive proteins on the behavior of human umbilical vein endothelial cells in serum-free culture

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Abstract

To improve the safety of cellular therapy products, it is necessary to establish a serum-free cell culture method that can exclude animal-derived materials in order to avoid contamination with transmissible agents. It would be optimal if the proteins necessary to a serum-free culture could be provided as recombinant proteins. In this study, the influences of recombinant artificial cell adhesive proteins on the behavior of human umbilical vein endothelial cells (HUVECs) in serum-free culture were examined in comparison with the influence of plasma fibronectin (FN). The recombinant proteins used were Pronectin F (PF), Pronectin F PLUS (PFP), Pronectin L (PL), Retronectin (RN), and Attachin (AN). HUVECs adhered more efficiently on PF or PFP than on FN. No cells adhered on PL. Regarding the VEGF or bFGF-induced cell growth, the cells on PF and PFP proliferated at a similar rate to the cells on FN. RN and AN were less effective in supporting cell growth. Since cell adhesion on PF and PFP induced phosphorylation of focal adhesion kinase, they are thought to activate integrin-mediated intracellular signaling. The cells cultured on PF or PFP were able to produce prostaglandin I_2 or tissue-plasminogen activator in response to thrombin. However, thrombin caused detachment of the cells from PF but not from PFP or FN, meaning that the cells were able to adhere more tightly on PFP or FN than on PF. These data indicate that PFP could be applicable as a substitute for plasma FN.

Keywords: Cell adhesive protein; Recombinant protein; Fibronectin; Endothelial cells

1. Introduction

The widespread development of cellular therapy products has advanced to the stage of non-clinical and clinical testing [1–3]. Regulatory documents for human somatic cell therapy including instructions for investigational new drug applications have been published [4–6]. To guarantee the safety of both product recipients and the public at large, it is crucial to prevent contamination of cellular therapy products by infectious agents [7].

Serum-free culture is one of the desired methods for manufacturing cellular therapy products when safety issues are a concern [8,9]. Although serum is a very effective additive for a culture medium that can support cell adhesion, survival, growth, and functions, animal-derived materials such as serum may contain transmissible agents or human allergens [10–13]. Since serum is composed of proteins, sugars, lipids, vitamins, and other ingredients, its quality is affected by the genetic and environmental circumstances of the animal used, which means there are lot-to-lot variations in composition and potency. This variability could reduce the consistency of cellular therapy products, and a serum-free culture that could contribute to improving the consistency of the cell features is thus needed.

Protein factors often need to be added to serum-free culture to substitute for the functions of serum. The proteins used for this purpose must also pose no risk of infection. Because recombinant proteins can be produced without using animal-derived materials, and pose little risk of contaminating human pathogens, they are a useful biomaterial for culturing cells

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when safety issues are a concern. In addition, the functions of recombinant proteins can be improved by modifying their amino acid sequences, which is also an advantage. In this study, using a serum-free culture of human umbilical vein endothelial cells (HUVECs), the usefulness of recombinant artificial cell adhesive proteins as a plasma fibronectin substitute was evaluated.

The extracellular matrix glycoprotein fibronectin consists of two similar polypeptide chains, each with a molecular mass of approximately 250 kDa joined at their respective C termini by disulfide bonds [14,15]. The RGD (arginine–glycine–aspartic acid) recognition sequence located within the molecule was the first amino acid motif shown to mediate cell adhesion [16,17]. Intracellular signaling induced by cell adhesion on fibronectin plays a critical role in cytoskeletal reorganization, cell cycle progression, and cell survival [18,19].

This study defines the influences of five different kinds of recombinant artificial cell adhesive proteins on endothelial adhesion, proliferation, and antithrombotic function in serum-free culture for which plasma fibronectin is needed. The recombinant proteins used were Pronectin F, Pronectin F PLUS, Pronectin L, Retronectin, and Attachin (Table 1).

2. Materials and methods

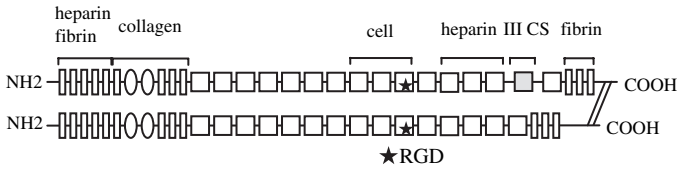
2.1. Recombinant cell adhesive proteins

Pronectin F, Pronectin F PLUS, and Pronectin L were purchased from Sanyo Chemical Industry (Kyoto, Japan), Retronectin from TaKaRa (Shiga, Japan), and Attachin from Bio999 (Taipei, Taiwan). All of these proteins were produced via bacterial fermentation. Pronectin F is a genetically engineered protein containing repeating units of the RGD sequence interspersed with a β-silk peptide for structural stability [20]. It is comprised of 980 amino acids. Based on its sequence, the molecular weight is estimated to be 72,728. The amino acid sequence is fMDPVVLQRRDWENPGVTQLNRLAA HPPFASDPMGAGS(GAGAGS)$_6$GAAVTGRGDSPASAAGY-[(GAGAGS)$_9$GAAVTGRGDSPASAAGY]$_{12}$-(GAGAGS)$_2$GA GAMDPGRYQLSAGRYHYQLVWCQK. Pronectin F PLUS is a positively charged water-soluble variant of Pronectin F that is produced by chemical modification [21,22]. Pronectin L is a protein polymer that exhibits IKVAV epitopes from the laminin alpha chain with a similar backbone as Pronectin F [21]. It is comprised of 1019 amino acids. The molecular weight is estimated to be 75,639. The amino acid sequence is fMDPVVLQRRDWENPGVTQLNRLAAHPPFASDPMGA GS(GAGAGS)$_6$GAAPGASIKVAVSAGPSAGY-[(GAGAGS)$_9$ GAAPGAIKVAVSAGPSAGY]$_{12}$-(GAGAGS)$_2$GAGAMDPG RYQLSAGRYHYQLVWCQK. Retronectin consists of a central cell-binding domain, a high affinity heparin-binding domain II, and a CS1 site within an alternatively spliced type III connecting segment region of human fibronectin [23]. It is comprised of 574 amino acids. The molecular weight is estimated to be 62,631. Attachin is an artificial fusion protein with the molecular weight of 30 kDa that has several functional domains, including a fibronectin-like cell attachment domain [24]. It has reportedly been used to promote the adhesion of several kinds of cell lines, including CHO-K1, MDBK, PK-15, L929, Vero, COS, U373, Swiss 3T3, and MRC-5 [25].

2.2. Cells and materials

Human umbilical vein endothelial cells (HUVECs) were purchased from Sanko Junyaku (Tokyo, Japan) and maintained

Table 1
Fibronectin and recombinant cell adhesive proteins used in this study

Proteins	Structure	Molecular Weight
Fibronectin		250K x 2
Pronectin F	Head-[(GAGAGS)9GAAVTGRGDSPASAAGY]12-Tail ★	73K
Pronectin F Plus	Positively charged, water-soluble variant of Pronectin F	73K
Pronectin L	Head-[(GAGAGS)9GAAPGASIKVAVSAGPSAGY]12-Tail ◆	76K
Retronectin	Chimeric protein of human fibronectin fragment 	63K
Attachin	A fusion protein constructed by molecular biotechnology	30K

Fibronectin.
▯ Type I module.
⬯ Type II module.
☐ Type III module.
★: cell attachment sequence derived from fibronectin.
◆: cell attachment sequence derived from laminin.