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Establishment of a microcarrier culture system with serial sub-cultivation for functionally active human endothelial cells

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ABSTRACT

A microcarrier culture system was established for a large-scale production of functional human endothelial cells. It has been difficult to cultivate human endothelial cells in large quantities for the reasons that specific growth factor and extracellular matrix are required for the survival and proliferation of the cells and the life span of the primary cells are limited. A lot of studies have reported that the shear stress gives significant influences on the structure, growth rate and biological functions of endothelial cells. We aimed to develop a convenient microcarrier culture system for human endothelial cells which can reproduce the flow effects experienced in vivo or in vitro. In 200 mL volume culture, human umbilical vein endothelial cells (HUVEC) could be serially sub-cultivated by optimizing the culture conditions such as shear strength, growth factor, beads and seeding cell concentration, serum concentration, and passage timing. The growth rate was enhanced depending on the shear strength and the life span of the cells was elongated until over 43 PDL which is much longer than those of monolayer cultures. The cells maintained the diploidy of over 80% without obvious abnormal changes in the chromosomes. The serially sub-cultured microcarrier cells maintained various endothelial cell functions such as the syntheses of von Willebrand factor (vWf), prostacyclin and other biological substances, the expression of CD31, and the $VEGF_{165}$ dependent growth characteristic. The synthesis of biological products was affected by shear strength. In the case of prostacyclin, a different synthesis response was observed between steady flow and transiently reduced shear strength. The synthesis of endothelin-1 (ET-1) was down-regulated by increase of shear strength different from those of other products. The culture system was scaled up until 2 L volume under the optimum DO control. The cells synthesized IL-6 in response to shear strength. These results indicate that the established microcarrier system might be able to contribute to the supply of functional human endothelial cells for various medical applications such as the reconstruction of injured blood vessels caused by atherosclerosis or restenosis of coronary arteries after angioplasty, and the construction of an anti-coagulable artificial blood vessel or an artificial skin with good transplant-ability.

1. Introduction

The vascular endothelial cells line the inner surface of blood vessels and are constantly subjected to hemodynamic shear stress resulting from blood flow. There has been speculation for many years that fluid dynamics plays an important role in vessel pathology in diseases such as atherosclerosis (Glagov et al., 1988; Nerem and Cornhill, 1980). It was demonstrated that endothelial cell morphology, structures and growth were influenced by shear stress (Potter et al., 2011; Nakadate et al., 2010; Dewey et al., 1981; Kim et al., 1989; Ando et al., 1987, 1990). It was also shown that endothelial progenitor cell adhesion from blood can occur under physiological levels of shear stress (Angelos et al., 2010) suggesting the flow effect to the restoration of injured blood vessels. Recently, the secretome of endothelial cells under shear stress was comprehensively analyzed (Burghoff and Schrader, 2011). The synthesis of prostanoids is an important endothelial cell function to keep the homeostasis of vascular vessels. Particularly, prostacyclin (PGI2) has been documented to have some important biological functions such as anti-platelet (Moncada et al., 1976), vasodilating (Bunting et al., 1976) and cyto-protective (Sakai et al., 1990) activities. Prostacyclin production was increased several-fold with the onset of laminar shear stress (Frangos et al., 1985; Bhagyalakshmi and Frangos, 1989) and pulsatile shear stress stimulated an even greater production of prostacyclin than steady shear stress (Frangos et al., 1985; Grabowski et al., 1985). Endothelium derived relaxing factor (Rubanyl et al., 1986), tissue plasminogen activator (tPA;

Abbreviations: HUVEC, human umbilical vein endothelial cells; vWf, von Willebrand factor; ET-1, endothelin-1; VEGF, vascular endothelial cell growth factor; DO, dissolved oxygen; PGI2, prostaglandin-I2; PGF2α, prostaglandin-F2α; TBX2, thromboxane-B2; Bfgf, basic fibroblast growth factor; tPA, tissue plasminogen activator; PDL, population doubling level.

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Diamond et al., 1989), intercellular adhesion molecule-1 (ICAM-1; Nagel et al., 1994; Tsuboi et al., 1995), nitric oxide synthetase (Nishida et al., 1992), transforming growth factor beta-1 (TGFβ; Ohno et al., 1995), basic fibroblast growth factor (bFGF; Malek et al., 1993), platelet-derived growth factor (Mitsumata et al., 1993; Malek et al., 1993), monocyte chemotactic protein-1 (Shyy et al., 1994) and heparin-binding epidermal growth factor-like growth factor (Morita et al., 1993) were also up-regulated by shear stress. On the contrary, the expression of vascular adhesion molecule-1 (VACM-1; Ohtsuka et al., 1993), the leukocyte-endothelial cell adhesion (Ling et al., 2003) and the monocytic cell adhesion to endothelium (Urschel et al., 2010) were down-regulated by the stimulation of shear force. About endothelin (ET) production, both the cases were reported (Yoshizumi et al., 1989; Sharefkin et al., 1991). Recently, it was reported that human stem cells are differentiated to endothelial cells by fluid shear stress (Tan et al., 2010) and mouse ES cells are primed for differentiation through shear stress (Toh and Voldman, 2011). These reports demonstrate that fluid dynamics gives an important influence to endothelial cell functions that are critical to normal vessel wall function.

Our objective in this study is to establish an in vitro large-scale culture system for human endothelial cells which can reproduce the above various flow effects. Although recent progress in culture technique has made possible in vitro cultivation of normal human endothelial cells, there are some difficulties in the culture. At first, the specific growth factors such as vascular endothelial cell growth factor (VEGF; Leung et al., 1989) and bFGF (Gospodarowicz et al., 1986; Weinstein and Wenc, 1986) are essential for the survival and proliferation of the cells. Some endothelium functions are known to be affected by these growth factors although the effects on the growth and angiogenesis are common (Hoshi et al., 1988). The extracellular matrix such as collagen is also necessary for the adhesion and proliferation of the cells (Thornton et al., 1983). Then, the life span of normal human cells is limited and the growth and biological functions of senescent cells go down (Shimada et al., 1990). Therefore, most of the experiments for shear stress have been carried out using animal tissue or cells because these preparations are relatively easy and the cells can be cultured stably for a long time. The endothelial cells is usually identified by confirming the squamous epithelium like morphology, the growth factor dependent growth, the synthesis abilities of vWf or prostacyclin and the expression of specific molecules such as CD31 (Jaffe et al., 1973; DeLisser et al., 1993).

Up to now, some reliable methods for endothelial cell culture with shear stress have been developed. A disk-type apparatus (Nomura et al., 1988) and an apparatus utilizing the geometry of a cone-plate viscometer (Bussolari et al., 1982) were devised. Further, a parallel plate chamber in a flow system (Koslow et al., 1986) and an apparatus with controlled laminar flow through a rectangular tube (Viggers et al., 1986) have been designed. Recently, a parallel plate flow chamber with microstructured polymer substrates was devised as a model of shear stress (Brown et al., 2011). These have been developed only to study the effects of fluid shear stress. Microcarrier culture has been expected as a simplified model in vitro for the study of several aspects of endothelial cell biology (Busch et al., 1982; Ryan et al., 1980; McArthur et al., 1986). Ryan et al. (1980) and Sanford et al. (2002) attempted to cultivate the bovine cells using porous polyacrylamide beads or collagen-coated sephadex beads in microgravity-based rotating wall vessel bioreactor. The cells could grow until tissue-like aggregate formation, however, it is not clear whether the proliferated cells are real endothelial cells. The large-scale culture of human endothelial cells was very difficult although McArthur et al. (1986) and Busch et al. (1982) tried the cultivation of HUVEC.

Previously, we have developed effective microcarrier culture systems for human diploid fibroblasts (Sano et al., 1987) and recombinant CHO or C127 cells (Sano et al., 1988). These systems are acceptable to give well-controlled shear stress. By applying these systems, we could establish a microcarrier culture system for human endothelial cells with serial sub-cultivation.

2. Materials and methods

2.1. Cell and monolayer culture

Human umbilical vein endothelial cells (HUVEC) were routinely isolated from human umbilical cord veins according to the method introduced by Jaffe et al. (1973). HUVEC were cultured in collagen-coated flasks (Iwaki, Chiba, Japan) using M199 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% of fetal calf serum (FCS; Life Technologies, Grand Island, NY) and $VEGF_{165}$ prepared in our laboratory. $VEGF_{165}$ was purified from the culture supernatant of human fibroblasts using Heparin-sepharose (Pharmacia, Uppsala, Sweden) and Cu^{2+}-chelate affinity chromatography (AF-Chelate TOYOPEARL 650M, Tosoh, Minato, Tokyo). Basic-FGF (bFGF, Pepro Tech, Rocky Hill, NJ) was also used for control experiments. The cell number was analyzed by coulter counter (Coulter counter Z1, Beckman Coulter, Fullerton, CA). The population doubling level (PDL) was determined by calculating the total number of population doublings of the cells since their primary isolation in vitro.

2.2. Identification of endothelial cell

The endothelial cells were identified by confirming the characteristic squamous epithelium like morphology, the synthesis abilities of vWf and prostacyclin, the expression of CD31 and the growth factor dependent growth. The morphology was observed under a microscope (Olympus CK-40, Tokyo, Japan). The vWf was analyzed by an enzyme-linked immunosorbent assay (ELISA) as previously introduced by Rennard et al. (1980). Rabbit anti-human vWf polyclonal antibody and peroxidase conjugated rabbit anti-human vWf antibody were purchased from Daco, UK (Cambridge, UK). The purified vWf for standard sample was obtained from human plasma in our laboratories. The prostacyclin was assessed as 6-keto-PGF1α which is a stable metabolite of prostacyclin using ELISA kit purchased from Cayman Chemical (Ann Arbor, MI). The expression of CD31 was examined by flow cytometry using a CD31–FITC conjugated antibody obtained from Beckman Coulter (Fullerton, CA). The analysis was performed using FACS-Calibur (Becton Dickinson, Franklin Lakes, NJ) and Flowjo software (BioLegend, San Diego, CA). The FITC labeled cells were excited at 488 nm and the fluorescence of CD31–FITC was analyzed on FL1 (525 nm). The growth factor dependency of the growth was examined using $VEGF_{165}$.

2.3. Microcarrier culture

The small-scale 200 mL culture was carried out using the same spinner flasks according to the method previously described by Sano et al. (1987,1988). Gelatin beads (Gelibeads, Hazleton/KC, Lenexa, KS) were used as microcarrier. The beads suspended in 200 mL PBS(−) were put into a 500 mL spinner flask (Iwaki, Chiba, Japan) and were autoclaved. The sterilized beads were stirred at 37 °C for about 1 h in culture medium before cell seeding. The cells were cultured in M199 medium supplemented with 10% FCS and $VEGF_{165}$ and the culture medium was replenished with fresh medium every other day. The confluent microcarrier cells were passaged using 0.1% trypsin solution (Sigma–Aldrich, St. Louis, MO) after rinsing twice with PBS(−). 2 L scale culture was carried out under DO control. The spinner bottles for 2 L scale culture were also devised in our laboratory. The dissolved oxygen (DO) was