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Studies of Attachment of Fibroblasts on Microcarriers for the Production of Tissue Plasminogen Activator

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TPA 생산을 위한 섬유 세포의 미립 담체와의 접착성에 관한 연구

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ABSTRACT

Among commercially available five different types of micro-carriers, collagen coated Cytodex III supports the best cell growth and production of Tissue type Plasminogen Activator from fibroblasts for all three various kinds of media(FBS, horse serum containing and serum free medium). 95% of attachment yield is obtained with Cytodex III in FBS containing medium compared to about 70% in serum free medium. However, higher production of TPA can be observed in horse serum containing and serum free media as 1.2 μg / ml and 0.7 μg / ml, respectively with 5.5×10^6 cells / ml and 0.7×10^6 cells / ml under perfusion chemostat operations.

INTRODUCTION

Techniques of producing Tissue type Plasminogen Activator(TPA) *in vitro* have been intensively investigated since the clinical importance of TPA to treat heart attack was recognized in 1978(1). Many cell lines have been established to secret enough amouts of TPA in spent media in T-flasks since normal blood contains only 2-6 ng / ml(2) and secretion of TPA from cells heavily depends on cell lines and culture conditions(3-6)

Among those cell lines, recombinant cell lines such as SV-40 transformed Chinese Hamster Ovary(CHO) cells containing melanoma TPA genes can produce commercial amounts of TPA(about 20 μg / ml) in large scale cultivation(7). However, recently TPAs originated from recombinant cells have negative responses in clinical trails even though Genentech(USA) had FDA's approval for them(7).

It is worldwide trend to develop natural cell lines to secret large amounts of TPAs in industrial scale cultures. So far Human kidney, lung, ovary, uterus and heart cell lines have been established for this purpose (7-10, 20). It is very interesting that most natural cell lines producing TPA are anchorage-dependent type cells. This also supports hypothesis that the secretion of TPA has strongly correaltion with cell wall mechanism[11]. This implies that characteristics of the surface of culture vessel are critically important in growing cells and producing TPA in large scale cultivations. Most of research on secreting TPA from normal cell lines have been done in T- flask level, and several coating materials on T-flasks have been reported to enhance TPA productions (12). However, It has often caused problems in scaling up processes because microcarriers had to be used in cultivating cells in a suspension reactor.

Microcarriers are not ideal places for cells to grow since they do not have flat surfaces compared to T-flasks, resulting in that cell growth is not so fast as grown on T-flasks(13). It is absolutely necessary to carefully select types and concentrations of beads in cul-

turing cells in a bioreactor(7,14,15). Therefore, in this report, effects of several kinds of microcarriers on producing TPA from anchorage-dependent cells will be studied.

MATERIALS AND METHODS

Culture conditions

Human fibroblast(CCD-112 CoN, Flow Lab., Japan) cells were cultivated with Dulbecco Minimal Eagle's Media, DMEM(Sigma, USA) with 10% Fetal Bovine Serum, FBS(GIBCO, USA), 2.1g / l of sodium bicarbonate and 45μg / ml of gentamicin(Sigma, USA) in a 85 T-flask at 37℃ in a CO_2 incubator. Medium was changed every two days to keep cells in confluent phase by checking cell viability and density by Trypan blue dye exclusion method(16). 0.5% trypsin with EDTA solution(Sigma, USA) was used to detach cells from T-flasks and microcarriers. Collected cells from T-flasks were inoculated into spinner vessels and a 2L bioreactor(Celligen, USA) when cell density was reached to 1×10^5 viable cells / ml. Then, 10g / l of autoclaved microcarriers were added into the reactor. Beads were washed four times with Ca^{2+} and Mg^{2+} free phosphate buffer solution(Sigma, USA) before inoculation. After inoculating cells and beads, the reactor was filled up to 1.5L with 30℃ pre-warmed media by a peristaltic pump(Cole-Parlmer, USA) with gentle agitation(25 rpm), pH and dissolved oxygen concentration were set to 6.9 and 40%, respectively and automatically controlled by installed microprocessor through changing air flow rates(US patent No. 4727040). Initial air flow rate was 0.5L / min. A decantor was used to perfuse media by settling microcarriers in a filter system. Horse serum containing and serum free media were run through a perfusion reactor.

Attachment of cells on beads

Tests of attaching fibroblasts on different kinds of beads were performed with five 250mL spinner flasks(Belco, USA) by 25 rpm of agitation and 0.5 L /min of aeration at 37℃ and pH 7.1. Effects of media compositions on attaching cells were also checked by culturing cells with three different media, such as DMEM with 10% FBS, DMEM with 10% horse serum(Sigma, USA) and DMEM with 10μg / mL of mixture of Insulin and Bovine Transferrin(GIBCO, USA) and 20IU / mL of aprotinin(Sigma, USA). Each of the above media contains 40μg / mL of gentamicin. Cell density attached on beads was estimated by nuclei counting method(17), and unattached cells floating in supernatant were measured by Coulter couter(18). Then, the ratio of attachment of cells on beads was calculated as follows:

$$R = \frac{Xa}{Xt} \quad (1)$$

Where X_a is cell density attached on beads(cells / mL) and X_t is total cell density which is the summation of cell concentrations attached and unattached on beads in a vessel.

The amounts of TPA in media were measured by enzyme linked absorbant assay(ELISA) kit (Imubind, USA). Detail method is described elsewhere(7).

RESULTS AND DISCUSSION

Table I is the summary of characteristics of microcarriers used for growing fibroblast CCD-112 CoN. Five different brands of beads were selected because each of them was coated by different polymer and they have

Table 1. Characteristics of Commercially Available Microcarriers.

Product	Material	Average diameter (mm)	Surface area (Cm^2/g)	Specific gravity
Cytodex II	DEAE-sephadex	120	5500	1.04
Cytodex III	Collagen	150	4600	1.04
Biosilon	Polystyrene	200	2550	1.05
Biocarrier	Polyacrylamide	150	5000	1.04
Geli bead	Gelatin	180	3800	1.04

been most widely used for cell cultures. Average diameter of beads is 120-200 nm and specific gravity is 1.04-1.05. Biocarrier has the largest surface area as 5000 cm^2/g.

Results of growing cells on five different beads are Fig. 1-3 with three different kinds of media to observe the effect of media compositions on attachament of cells on microcarriers. For all three media, collagen coated Cytodex III shows the highest attachment yield followed by Cytodex II except for serum free medium. Biosilon shows the poorest yield for all cases possibly due to polystyrene since most fibroblasts did not grow well on this basic polymer(19). Cells did not favorably attach on any beads with Serum free medium by showing about 75% of attachment yield for cytodex III compared to 95% with DMEM plus 10% FBS.

Interestingly enough, Geli beads had better attachment of cells with serum free media than Cytodex II. Medium containing 10% horse serum had slight deterioration in attachment yield for all beads, compared to 10% FBS containing medium. Among them, Cytodex III showed higher attachment yield than any other bead for all three media. It could be safe to use horse serum rather than

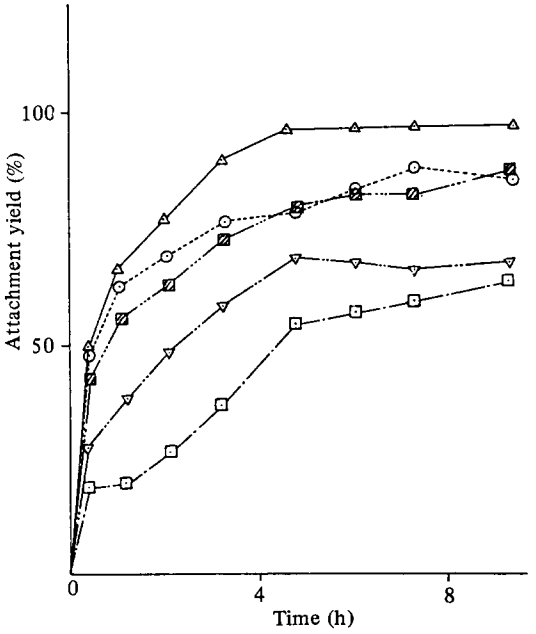

Fig. 1. Attachment of fibroblast cells on various types of microcarriers cultivated in DMEM with 10% FBS: ○,——, Cytodex II; △, ——, Cytodex III; □,—·—·—, Biosilon; ▽,—··—··— Biocarrier; ▨,—··—··—, Gelibead.

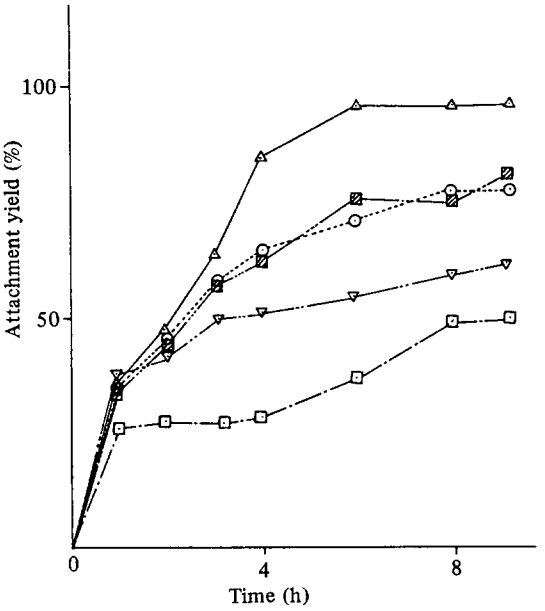

Fig. 2. Attachment of cells on microcarriers cultivated in DMEM with 10% horse serum.
Symbols are as defined in Fig. 1.

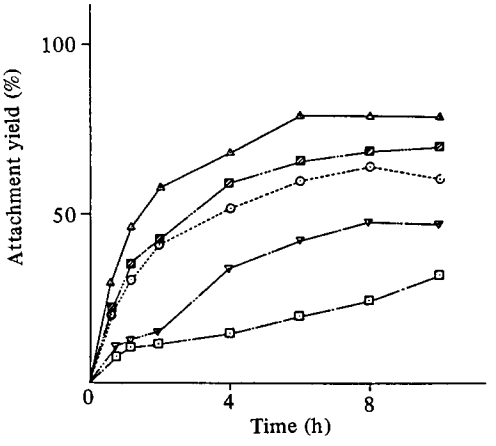

Fig. 3. Attachment of anchorage-dependent cells on several kinds of beads grown on serum free media (DMEM with 10 μg/mL of Insulin and Bovine Transferrin).
Symbols are as defined in Fig. 1.

FBS in case that horse serum can support cell growth and product formation. Regardless of any kinds of beads and media compositions, cells were attached mostly within 6-8 hours.

Cytodex III had the highest attachment rate for all three media, saturated within four hours, and showed fast attachment rate where medium has 10% fetal bovin serum.

Fig. 4 is the result of growing cells with two different media(medium with 10% FBS and with 10% horse serum) on Cytodex III under batch cultivation in a 500 mL spinner vessel. After reaching maximum cell density, both serum containing media were changed to serum free medium by stopping agitation and removing them. Arrows were starting points to be changed to serum free medium. For the case of cultivating cells with horse serum, cell growth and product secretion were not significantly affected compared to that for FBS when changed to serum free medium. Moreover, these cells can produce much higher amounts of TPAs in growing with horse serum and continuously secreting them than in FBS, even though cell growth and viability were slightly lower in growing with horse serum than in FBS. Therefore, it is obvious that serum free meduim with Cytodex III can be used to produce TPAs after precultivating fibroblasts with 10% horse serum by having about 1 μg of TPA per mL. Fig. 4 also illustrates that horse serum containing medium can enhance the producitivity of TPA production at least five times.

Fig. 5 is to show the cell growth and TPA productivity on Cytodex III in perfusing 10% horse serum containing medium with a 2L process controlled reactor(NBS) since Fig. 4 showed that horse serum can improve TPA production rate under batch cultivation. At each perfusion rate, cell density, TPA concentraton and attachment yield were estimated. As perfusion rates were increased, cell density and TPA production were also increased, noting that both of cell density and TPA pro-

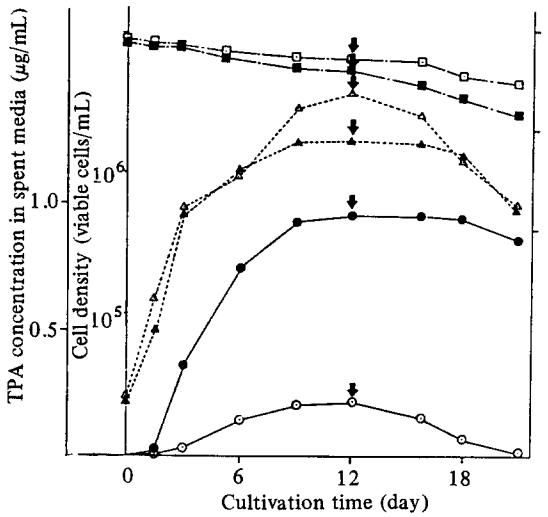

Fig. 4. The production of TPA from fibroblasts grown on Cytodex III beads with two different culture media: ○, ———, TPA conc. (μg/mL); △, - - - - - -, cell density (cells/mL); □, — — — —, cell viability (%). Open denotes results grown on medium of DMEM + 10% FBS and dark on DMEM + 10% horse serum. Arrows indicate changes of serum containing medium to serum free medium.

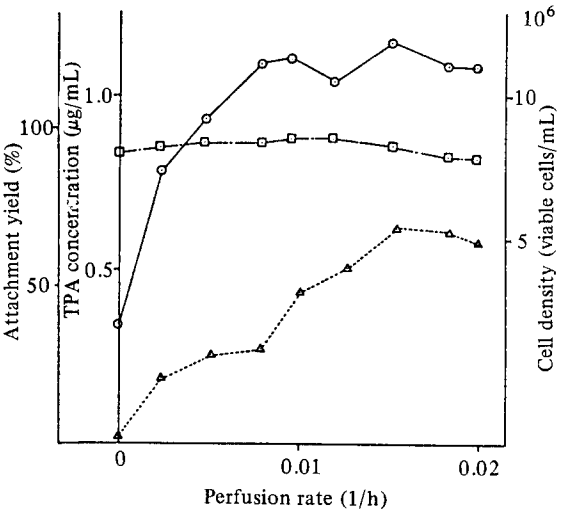

Fig. 5. Effects of perfusing fresh media on TPA production, cell growth and microcarrier attachment for fibroblast cells under Perfusion chemostat operations with DMEM + 10% horse serum: ○, ———, TPA conc. (μg/mL); △, - - - - - -, cell density (cells/mL); □, — — — —, attachment yield (%).

duction under perfusion operation were higher than under batch cultivaton in Fig. 4. And 95% of cells were attached on cytodex III under perfusion chemostat cultivation, compared with 90% of attachment yield for batch process as shown in Fig. 2.

Fig. 6 is to illustrate the behavior of cell growth and TPA production grown on serum free medium with Cytodex III at 0.015(1 / h) of perfusion rate for about 25 days because it is absolutely necessary to develop a culture process with serum free medium for a large scale cultivation to significantly reduce production costs. However, cell growth and viability were gradually decreased in growing with serum free medium in Fig. 4. Therefore, it is important to know the length of culturing fibroblasts in serum free medium for applying the process to a industrial system. Cell density was decreased very fast from 7×10^6 to 5×10^5 cells / mL within 25 days along with attachment yield from 90% to 55%; however, the production of TPA was not greatly decreased compared to cell growth (from 1.2 to 0.56 μg / mL). It is recommended that the culture system should be operated under batch mode with 10% horse serum containing medium for at least 14 days after perfusing serum free medium for about 20 days. It allows cells to maintain continuous growth and product formation.

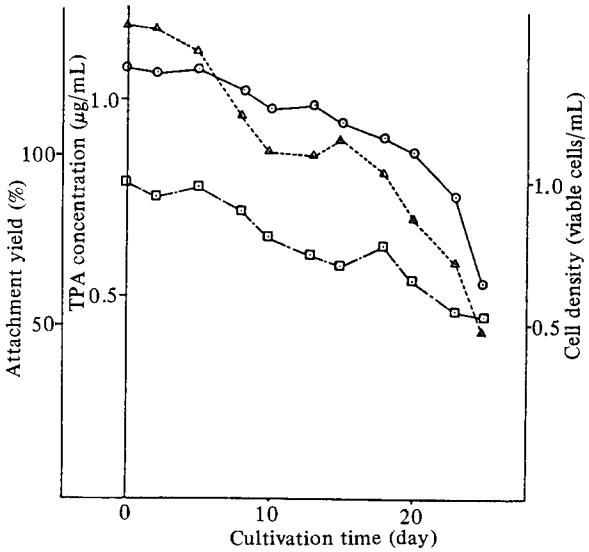

Fig. 6. Results of cell growth, TPA production and attachment on beads in cultivating fibroblasts by perfusing serum free media after adapting with 10% horse serum media; ○, ——— , TPA conc. (μg/ml); □, —·—·— , attachment yield (%); △, - - - - - - - - - , cell density (viable cells/mL).

요 약

접착 세포용으로 가장 많이 쓰이고 있는 미립 담체들의 다섯가지 종류를 소 혈청, 말 혈청 및 무 혈청 배지에 섬유 세포를 배양한 결과 collagen으로 싸여진 Cytodex III이 가장 좋은 접착성을 나타냈으며, 세포 성장 및 TPA 생산성도 우수했다. 소 혈청을 함유한 배지가 95%의 가장 좋은 접착율을 나타냈으며 무 혈청 배지가 75%로 낮은 접착도를 보였다. 하지만 연속 배양(Perfusion Chemostat)시 TPA의 생산성은 말 혈청을 함유한 배지 및 무 혈청 배지가 각각 1.2 μg / mL, 0.7 μg / mL으로 더 높았으며, 이때 세포의 농도는 각각 5.5×10^6cells / mL와 0.7×10^6cells / mL였다.

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