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# The Production of Tissue Type Plasminogen Activator from Normal Human Cell Line

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# 정상 인체 세포로부터 조직 플라스미노겐 활성인자의 대량생산

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**A method to produce tissue type Plasminogen Activator (tPA) from normal human fibroblast is developed by cultivating cells in serum free media containing heparin as an inducer. Optimal dose of this inducer was 30 ug/ml. The composition of serum free medium was also defined to fit to the industrial scale cultivation. 1.42 ug of tPA per $10^5$ viable cells per ml was produced. 1.1 gram of tPA can be produced every day from this cell line under normal perfusion chemostat operations assuming that same productivity is maintained when the process is scaled up. This method could reduce production costs and simplify purification processes by using serum free medium. Tissue type PA produced from this cell line has high ability of dissolving clots, based upon fibrin lysis test showing 50 $mm^2$ of clearing zones in agarose gel plate. These results were reproducible and in good agreement with results of ELISA assay. tPA from normal human cells will be safer than that from melanoma and recombinant cells in human clinical trials.**

Many efforts have been made to produce tissue Plasminogen Activator (tPA) from various sources of mammalian cells since its pharmaceutical activities on acute vascular diseases were discovered (1-17). Plasmingen Activator(PA) can dissolve fibrin clots in the blood stream without decreasing the blood's ability to form clots, and has higher activity to degrade fibrinogen than commercially available Urokinase (2). So far genetically engineered Chinese Hamster Ovary, mouse, and human melanoma cell lines have been studied to produce commercial amounts of tissue type PA from many companies (3,4). However, tPAs from cancer cells or recombinant DNA could might be contaminated with gene transforming factors in the blood stream and have side effects (6,7). Therefore, it is required to produce them from normal human cells. Unfortunately, human plasma contains only minute quantities of tPA (5). Several normal cell lines have been tried to produce tissue type PA in the laboratory scales (8,9). In this report, it is aimed to develop a suitable method of inducing commercial amounts of tPA from normal human cell line.

## Materials and Methods

### Cell culture

Human fibroblast (CCD-112CoN, Flow Lab., Japan) cells were cultivated for ten days with a confluency medium; mixture of Minimum Essential Medium Eagle (MEM) and Earl's balanced salt solution (Sigma, USA) with 10% of Fetal Bovine Serum (GIBCO, USA), 2.2 g/l of sodium bicarbonate and 40 ug/ml of gentamicin (Sigma, USA). The cells were maintained in a 75 T-flask (Falcon, USA) at 37°C incubator (MA012, UK) with 5% $CO_2$ enriched air by counting cell density and viability, to grow up to at least $1 \times 10^5$ cells/ml. Then,

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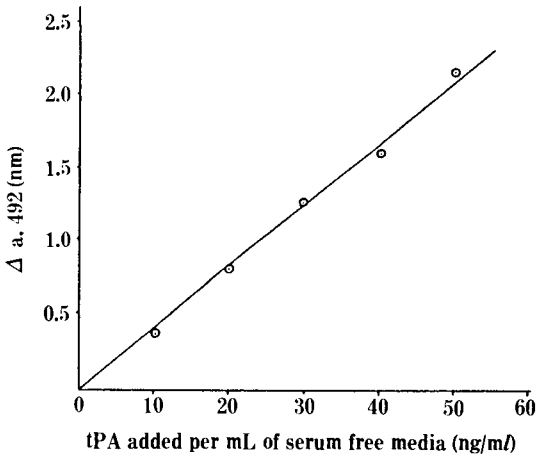


**Fig. 1. A standard curve of human tPA at the absorbance of 492 nm in serum free media.**

the culture was divided into three 75 T-flasks for testing the induction of tPA from three different media; confluency, serum free and inducer-containing serum free media. The composition of serum free media was MEM with Earl's BSS (1:1 v/v) (Sigma, USA), 10 ug/ml of mixture of Insulin and Bovine Transferrin (GIBCO, USA), 20 IU/ml of aprotinin and 40 ug/ml of gentamicin (Sigma, USA). 30 ug/ml of heparin (Sigma, USA) was added into the serum free media for inducing medium. Before changing media in each flask, cells were washed with $Ca^{+2}$ and $Mg^{+2}$ containing phosphate buffer solution (GIBCO, USA) four times. Three T-flasks were maintained in 37 °C water jacket incubator for five days, and viability and cell density were measured by Trypan blue dye exclusion method everyday (18).

### Measurement of tPA in conditioned media

Commercially available enzyme linked immunosorbant Assay (ELISA) kit(Imubind, USA) was used to determine the concentrations of tPA in three different spent media (19). Figure 1 is the standard curve to measure the amount of tPA in each medium at the absorbance of 492 nm. The result showed very good linearity up to 0.1 ug of tPA/ml with a correlation factor of 0.98. Therefore, the samples were diluted with phosphate-Tris buffer whenever the sample concentrations were over this upper limit. Each sample was tested three times, and the data shown in Table 1 were average values of the triplicates.

**Table 1. The results of producing tPA by cultivating normal human fibroblast cells in several different media.**

| Conditions | Total crude tPA + (ug/ml) | Viable cell density ($\times 10^5$ cells/ml) |
|---|---|---|
| Confluency medium* | 0.02 | 1.40 |
| Serum free medium** | 0.75 | 1.10 |
| Inducing medium*** | 1.20 | 0.85 |

* MEM with EBSS and 10 % of FBS.
** Bovine Transferrin and Insulin were added into the mixture of MEM and EBSS (1:1 v/v).
*** 30 ug/ml of heparin was added into serum free media. The induction has been applied for 18 hours.
\+ Commercially available ELISA kit was used to determine the concentrations of tPA in spent media along with the standard curve of Fig. 1.

### Fibrin lysis test

2.0% of agarose (Sigma, USA) solution containing 10 mg/ml of fibrinogen (Sigma, USA) was boiled at 60 °C and poured into a 24 well plate (Corning, USA) and 15 ug/ml of plasminogen (Sigma, USA) was added into each well. A small hole was punctured in each well by a 200 ul size micro-pipette to drop 50 ul of each sample after agarose gel was solidified. The standard tPA solutions were prepared by mixing known amounts of tissue type PAs (Imubind, USA) with fresh serum free media. The plate was incubated in 37 °C humidified chamber for up to 40 hours. The diameters of clearing zones were measured every hour, which were caused by the action of tPA in spent media and standard solutions.

## Results and Discussion

Table 1 is to show the results of inducing tPA using three different media. These values are the averages of triplicate measurements. 1.42 ug of tPA per $10^5$ viable cells were produced by inducer-containing medium, which is equivalent to the production of 14.2 ug of tPA per $10^6$ cells under normal operating conditions because average cell density in suspension cultures is $1 \times 10^6$ cells/ml (10,11). This productivity can yield 1.1 g of tPA every day in a perfusion culture system, assuming 75 l of conditioned media per day whose flow rate is an average value in this system, as long as $1 \times 10^6$ viable cells

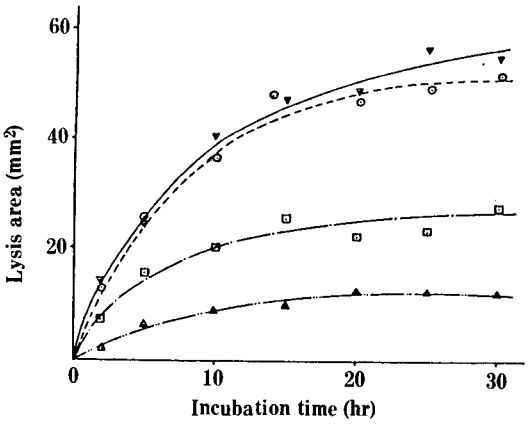


**Fig. 2. Kinetics of fibrinolytic lysis on agarose matrix between standard tPA solution and conditioned media from human fibroblast cultures;**
⊙, ----, standard tPA, 1 $\mu$g/mL; △, —·—, serum containing media; ⊡, —·—, serum free media; ▽, ——, serum free induction media.

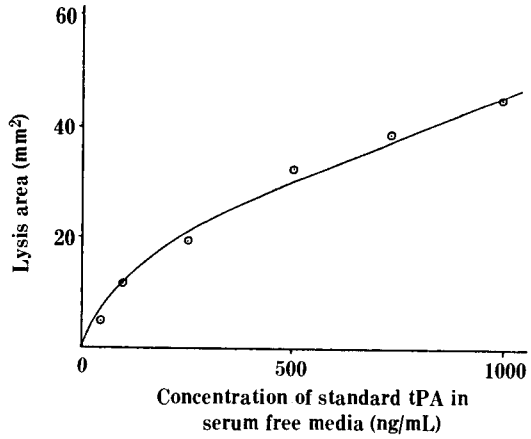


**Fig. 3. The effect of tPA concentrations on fibrinolytic lysis activity; ⊙ is experimental data points.**

are maintained (12-14). Only 20 ng of PA was produced from a confluency medium in this experiment. It indicates that the natural production of tissue type PA in blood stream is quite minute and that an induction method needs to be developed for commercial production. Interestingly, serum free medium itself can induce PA as about 650 ng/$10^5$ viable cells mainly due to the changes of catabolic processes within the cell (15).

It should be pointed out that the cell density was decreased when an inducer was added because this inducer can affect formation of the cell wall and these phenomena are somewhat critical in scaling-up processes since this cell line requires a microcarrier to grow up. Therefore, It is absolutely necessary to design a special type of bead by coating collagen on the surface of them. Characteristics of tPA produced from fibroblast cells under this condition were not examined except for testing activities of activating plasminogen in the treatment and confirming the results of ELISA assay by fibrin lysis test as described in Materials and Methods. The purpose of this work is to develop a method of producing pharmaceutically active tissue type PA from normal cell line. Moreover, it is believed that the PA produced from this cell would be the mixture of single and double chain tPAs having a molecular weight of 60,000 to 70,000 daltons which is similar to that of most PAs from other normal tissues (16). This tPA could be better and safer than those from melanoma and recombinant cells (16).

Fig. 2 shows the result of testing kinetics of lysing fibrin by PAs from three different media. Circles are the diameters of clearing zones by treating 1 ug of standard tPA/m*l* as a function of incubation time to compare with tPA from experimental media. The lysing areas by inducing-medium are similar to them by 1 ug/m*l* of standard tPA for overall incubation times, which implies that the results of ELISA assay in Table 1 are reliable, since serum free induction medium contains 1.2 ug/m*l* of tPA that is approximately same amount of tPA for standard solution. All lysing diameters were no longer increased after 15 hour incubation possibly because of the loss of activities. The life time of this PA is exceptionally longer than other sources of tPA (17), which is another excellent merit for clinical treatment.

Fig. 3 is to show the effect of various concentrations of tPA on fibrinolytic activities. The activities linearly increase as the doses of tPA are increased after 250 ng of tPA/m*l*. At 1 ug of standard tPA/m*l* the lysis area is about 45 $mm^2$ whose value is similar to that in inducer-containing medium in Fig. 2. This result also confirms accurate quantitation of tPAs in conditioned media.

## Conclusions

It is proved that normal human fibroblast cell line can produce commercial amounts of tissue type PA with high ability of dissolving clots by adding an effective inducer, heparin into serum free media. This induction method will reduce tPA production

cost, which is the one of the major bottle-necks in the commercialization of mammalian cell derived products, since this process needs only serum free medium except for scaling-up period for ten days. However, it must be stressed that it is necessary to develop a proper type of bead to attach cells on the microcarriers in the suspension culture with using serum free induction medium. Further biochemical analysis of this tPA should be carried out to define specific characteristics besides the pharmaceutical activities.

## 요 약

무혈청 배지에 생산촉진제로 30 μg/ml의 Heparin을 첨가해 정상인의 섬유 세포로부터 상업적으로 tPA를 생산할 수 있는 방법의 개발과, 효과적인 tPA 생산을 위해 대량 배양에 적합한 무혈청 배지의 조성을 확립했다. 이 방법으로 연속배양 공법하에서 매일 1.1 gram의 tPA가 생산될 수 있으며, 이 생산성은 tPA 생산 단가를 크게 낮출 뿐만 아니라 무혈청 배지의 사용으로 tPA의 순수 정제 과정을 크게 단축 시킬 수 있다. 또한 이 세포에서 생산되는 tPA는 fibrin lysis 시험결과 섬유질 분해능력이 높음이 입증되었으며, ELISA 결과와도 상충했다.

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