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# Enhanced CEA production associated with aspirin in a culture of CW-2 cells on some polymeric films

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**Abstract**

Human colorectal adenocarcinoma tumor (CW2) cells were cultivated in RPMI 1640 media containing 0–7.5 mM aspirin and 10% fetal bovine serum for the production of carcinoembryonic antigen (CEA). By adding aspirin to the media, the production of CEA per cell increased by up to one hundred fold compared to cultivation in normal media containing no aspirin, even though the total cell concentration decreased with the increase in aspirin in the media. The production of CEA was also investigated for CW2 cells cultured on silk fibroin, poly($\gamma$-benzyl-L-glutamate) and poly($\gamma$-benzyl-L-glutamate)/poly(ethylene oxide) diblock copolymer films prepared by the Langmuir-Blodgett and casting methods. The highest production of CEA per cell was observed for the CW2 cells on poly($\gamma$-benzyl-L-glutamate) and its diblock copolymer films prepared by the Langmuir-Blodgett method in the medium containing 5 mM aspirin after 168 hr of inoculation. This originates from the fact that the cell density on the films in the medium containing 5 mM aspirin was the lowest under these conditions. It is suggested that CW2 cells produce CEA more effectively when the cell growth is suppressed by addition of toxic chemicals such as aspirin or by culture on unfavorable films for cell growth.

*Abbreviations:* CEA, carcinoembryonic antigen; PBLG, poly($\gamma$-benzyl-L-glutamate); PEO, polyethyleneoxide; PBGEO, block copolymer of PBLG and PEO; Pst, polystyrene; $LD_{50}$, chemical concentration allowing 50% cell survival; LB, Langmuir-Blodgett.

## Introduction

Mammalian cell culture is becoming important in the production of natural and recombinant bioproducts for analysis and therapy. Enhanced productivity of biological products by cultured animal cells has been the focus of several research studies due to economical and purity reasons.

Several methods of enhanced productivity of monoclonal antibody, which was achieved by increasing the cell concentration and/or the specific productivity of monoclonal antibody, were investigated by several researchers (Lee et al., 1994; Yamaguchi et al., 1997; Takahashi et al., 1994; Murakami et al., 1997). Productivity often depends on the components of culture media containing autocrine factors (Toyoda et al., 1990; Sugahara et al., 1991), extracts from plants and cells (Kim et al., 1993), and other components (Mochizuki et al., 1993).

Cells can be specialized to produce a specific protein preferably when the cell division is suppressed. Several suppressing proteins and reagents on cell growth were reported, i.e., interleukin-6, TGF-$\beta$, sodium n-butyrate (Suzuki and Ollis, 1990), potas-

sium acetate (Somenshein and Brawerman, 1976; Suzuki and Ollis, 1990; Fong et al., 1997), thymidine (Gentry et al., 1965; Takahashi et al., 1994), hydroxyurea (Suzuki and Ollis, 1990), caffeine, c-AMP, cycloheximide and actinomycin D (Takahashi et al., 1994). It is known that maximum antibody production took place during the G/S phase of the cell cycle (Al-Rubeai and Emery, 1990). Therefore, various reagents which inhibit DNA synthesis (e.g., thymidine, hydroxyurea and sodium butyrate) or inhibit the synthesis of proteins other than antibodies (e.g., potassium acetate) were used for the stimulation of antibody synthesis by cells.

The detection and quantitation of carcinoembryonic antigen (CEA) are currently used extensively in basic research as well as in clinically oriented situations (David et al., 1978). Therefore, a reference standard of CEA must be produced by some cell lines (David et al., 1978; Rutzky et al., 1979; Quarles et al., 1980). In this study, the growth and production of CEA were investigated for human colorectal adenocarcinoma tumor (CW2) cells cultured with aspirin, which causes a toxic influence on the cells and suppresses the cell growth (Wakuri et al., 1993), because we observed aspirin enhances CEA production by CW2 cells during cytotoxicity test of aspirin, of which human actual oral lethal dose is reported (Wakuri et al., 1993). CW2 cells were also cultured on silk fibroin, poly($\gamma$-benzyl-L-glutamate) and poly($\gamma$-benzyl-L-glutamate)/poly(ethylene oxide) diblock copolymer films prepared by the Langmuir-Blodgett and casting methods. Whether the interaction of cells with a toxic chemical such as aspirin or with polymeric surfaces where cells are cultured enhances the productivity of CEA was examined.

## Materials and methods

### *Materials*

Poly($\gamma$-benzyl-L-glutamate), PBLG, was synthesized from $\gamma$-benzyl-L-glutamate N-carboxyanhydride using triethylamine as an initiator in 1,4-dioxane. Block copolymer consisting of poly($\gamma$-benzyl-L-glutamate) and poly(ethylene oxide), PBGEO, was synthesized by initiating the polymerization of $\gamma$-benzyl-L-glutamate N-carboxyanhydride with mono-amino-terminal PEO, as was described previously (Cho et al., 1990, 1996). PBGEO-1 and PBGEO-2 have 24 mol% and 44 mol% of PBLG content, respectively, which was determined by $^1$H-NMR.

Pure silk fibroin protein was obtained by removing the sericin from silkworm silk by boiling the cocoons in 100 °C water for 5 min and thereafter in 0.5 w/v% Marseilles soap solution for 30 min and then washing with distilled water. The pure silk fibroin protein was regenerated using 9.3 M LiBr solution and was dialyzed for three days in distilled water. A 0.5 wt% silk fibroin solution was obtained by adding distilled water to the dialyzed silk fibroin solution.

### *Preparation of cast polymeric films*

PBLG, PBGEO-1 and PBGEO-2 were dissolved to 0.5 wt% in dichloroethane, and the polymeric solutions were cast onto glass plates having a diameter of 15 mm inserted into flat Petri dishes. The 0.5 wt% silk fibroin solution was also cast on the flat Petri dishes. The films were finally prepared by drying at room temperature for 6 days and were subsequently dried under vacuum at room temperature for 24 hr.

### *Preparation of Langmuir-Blodgett films*

A polymer solution of PBLG, PBGEO-1, PBGEO-2 and silk fibroin having a concentration of 0.1 wt% was spread onto the surface of ultrapure water in a Langmuir-Blodgett (LB) trough (NL-LB200S-NWC, Nippon Laser & Electronics Lab., Nagoya) to form monolayers at 25 °C (Cho et al., 1996). The monolayer of polymer was transferred onto silicon-coated glass plates by a horizontal lifting method at 10 dyne/cm.

### *Cell line*

Cell line CW2 (Riken Cell Bank, Ibaraki, Japan) was maintained in RPMI 1640 media (JRH Bioscience, Lenexa, KS) supplemented with 25 mg/l streptomycin sulfate (Wako Pure Chemical Industry, Ltd.), 3.5 mg/l benzylpenicillin potassium (Wako Pure Chemical Industry, Ltd.) and 10% fetal bovine serum (FBS, JRH Bioscience, Lenexa, KS). CW2 cells were expanded by standard cell culture techniques in 75 cm$^2$ tissue culture flasks (3110–075, Iwaki Glass, Tokyo) containing 40 ml of serum-supplemented medium in a $CO_2$ incubator in 5% $CO_2$ atmosphere at 37 °C.

### *Cell growth and production of CEA on cast and LB Films*

After UV irradiation (30 cm distance, 10 W , GL10, Stanley Co., Tokyo) of the cast and LB films for 20

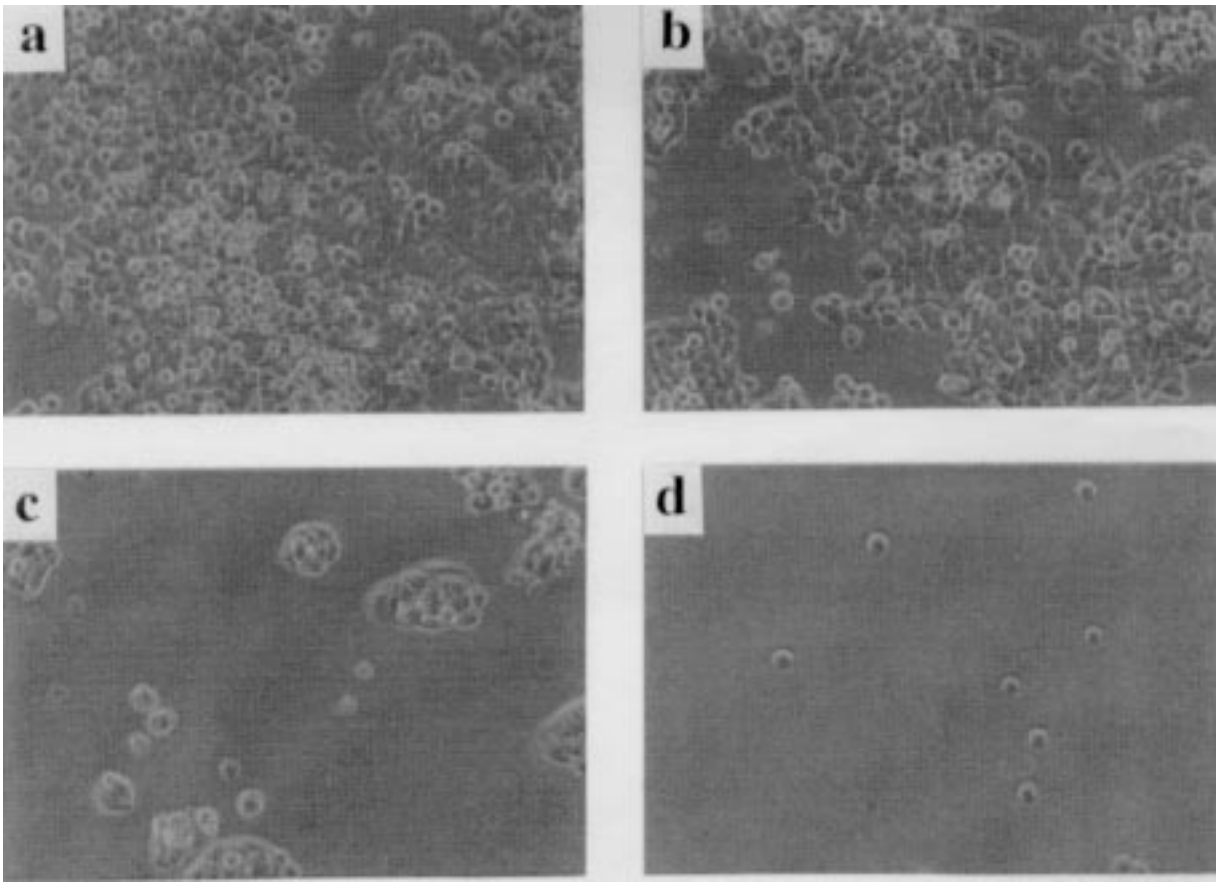


*Figure 1.* Micrographs of CW2 cells on culture dishes made of polystyrene after 24 hours of cultivation in RPMI 1640 medium containing 10% FBS and 0 mM (a), 2 mM (b), 5 mM (c) and 7.5 mM (d) aspirin using 2.0 x $10^4$ cells/$cm^2$ initially at 37 °C.

min for sterilization, the films were inserted into 24-well tissue culture plates fitted with a lid (Iwaki Glass, Tokyo, well diameter = 16 mm). CW2 cells in suspension (cell densities $2x10^4/cm^2$ and 2 ml of medium containing 0, 1, 2, 3, 5 or 7.5 mM aspirin) were inoculated into the tissue culture plates and were incubated in the $CO_2$ incubator in 5% $CO_2$ atmosphere at 37 °C. Half of the medium (1 ml) in each hole of the 24-well tissue culture plates was exchanged with fresh serum-supplemented medium containing 0–7.5 mM aspirin every two days. The sampled medium was used for estimation of the concentration of CEA (Higuchi et al., 1998). The concentration of CEA was estimated from an enzyme immunoassay using an Imzyme CEA kit (Fujirebio, Inc., Tokyo) and UV-VIS spectroscopy (JASCO Corporation, Tokyo).

The cell number was estimated by observation of the cells on the cast and LB films in the tissue culture plates maintained at a constant temperature of 37 °C using an inverted microscope (Diaphoto TMD300, Nikon Co., Tokyo) equipped with a CCD video camera, ARGUS 20 (Hamamatsu Photonics K.K., Hamamatsu) and a temperature-regulated box. The cell number was calculated from 4 pictures observed from different places on the same films. These procedures were performed on each film using four independent films prepared from the same polymers (totally n=16), and the cell number was finally averaged to obtain reliable data.

## Results and discussion

### *Cell growth and morphologies in the culture containing aspirin*

Cell growth and morphology, which are indexes indicating cell behavior and function, were investigated by phase-contrast micrographies of CW2 cells. Figure 1 shows the micrographies of CW2 cells on a culture

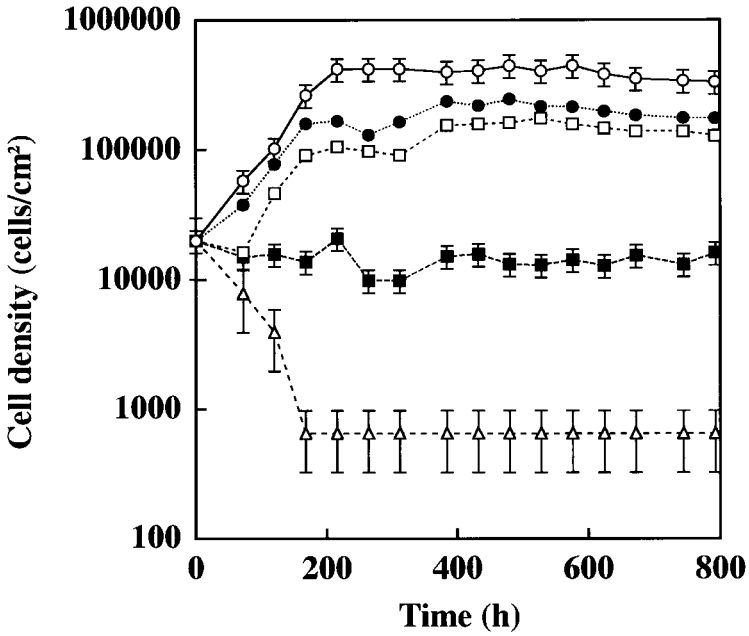


*Figure 2.* Growth curves of CW2 cells on culture dishes made of polystyrene in RPMI 1640 medium containing 10% FBS and 0 mM (○), 2 mM (●), 3 mM (□), 5 mM (■) and 7.5 mM (△) aspirin using 2.0 x $10^4$ cells/$cm^2$ initially at 37 °C. Data in the medium containing 0, 5 mM and 7.5 mM aspirin are expressed as the means S.D. of four independent measurements.

dish made of polystyrene 24 hr after inoculation in the medium containing 0–7.5 mM aspirin at 37 °C using 2.0x$10^4$ cells/$cm^2$ initially. The cell density decreased with the increase in concentration of aspirin in the medium, and the cells exhibited more spherical morphology in the medium containing a higher concentration of aspirin. The growth kinetics of CW2 cells cultured in the medium containing 0–7.5 mM aspirin were investigated. The growth kinetics are shown in Figure 2. The cell density was found to decrease with the increase in concentration of aspirin in the medium at any incubation time investigated. In Figure 2, the cell density increases up to 240 hrs and shows approximately constant cell density after 240 hrs of inoculation, when the cells are cultured in the medium containing less than 3 mM aspirin. On the other hand, cell density remains constant or decreases up to 200 hrs and is constant after 200 hrs of inoculation for the cells cultured in the medium containing more than 3 mM aspirin.

$LD_{50}$, the chemical concentration allowing 50% cell survival, of aspirin is estimated to be 2.0 ± 0.5 mM from a dose-response plot for aspirin based on the cell density after inoculation for 168 hr in Figure 2.

*Effect of aspirin in production of CEA*

Production of CEA was investigated in CW2 cells cultured with 0–7.5 mM aspirin, which is the concentration around the $LD_{50}$ values of aspirin obtained in this study. Figure 3 shows the effects of different concentrations of aspirin on the CEA concentration after inoculation for 168 hr. It was found that CEA concentration in the medium increased with the increase in aspirin concentration in the medium up to 5 mM where CW2 cells were cultured, although aspirin suppressed the cell growth and decreased the cell density as shown in Figures 1 and 2. The CEA concentration in the medium of the cells cultured with 7.5 mM aspirin, on the other hand, was approximately 1/4 of that in the control experiments. This is explained by the fact that the cell density decreased with the concentration of aspirin in the medium, and the cell density in the medium containing 7.5 mM aspirin decreased to approximately 1/400 of that in the control experiments (see Figure 2).

The specific CEA productivity 168 hr after inoculation was also studied to investigate the effect of aspirin on CEA productivity per cell and is shown in Figure 4. The specific CEA productivity increased with the increase in aspirin concentration in the medium.

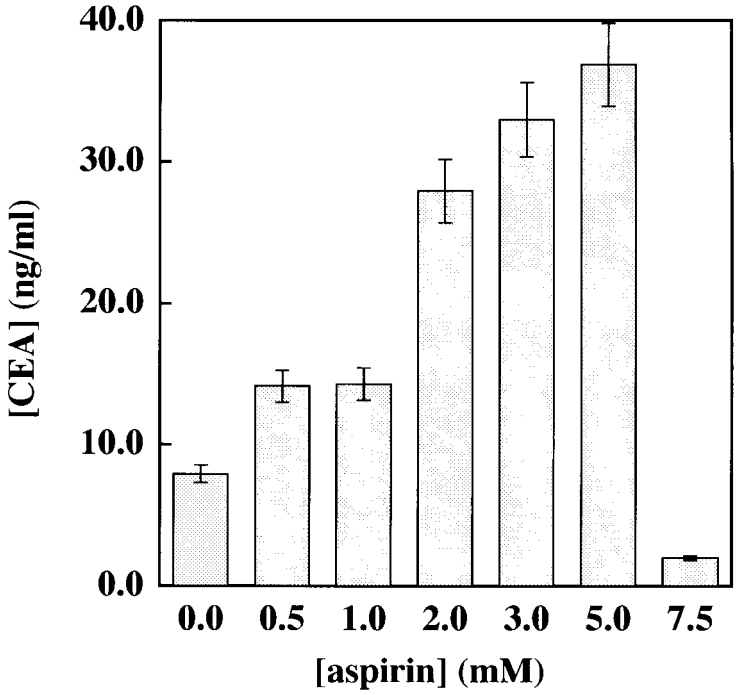


*Figure 3.* CEA production by CW2 cells on culture dishes after 168 hours of cultivation in RPMI 1640 medium containing 10% FBS and 0–7.5 mM aspirin using 2.0 x $10^4$ cells/$cm^2$ initially at 37 °C. Data are expressed as the means ± S.D. of four independent measurements.

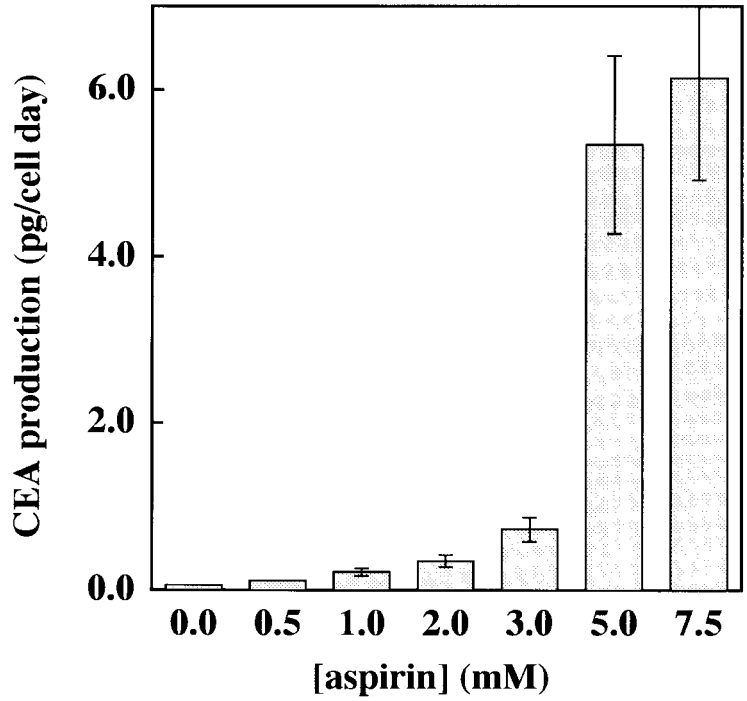


*Figure 4.* Specific CEA productivity by CW2 cells on culture dishes after 168 hours of cultivation in RPMI 1640 medium containing 10% FBS and 0–7.5 mM aspirin using 2.0 x $10^4$ cells/$cm^2$ initially at 37 °C. Data are expressed as the means ± S.D. of four independent measurements.

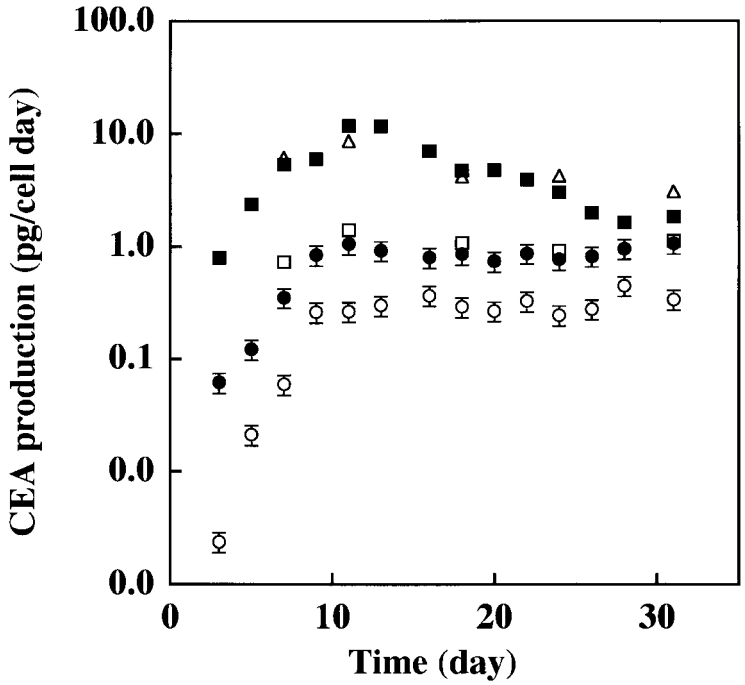


*Figure 5.* Time dependency of specific CEA productivity by CW2 cells on culture dishes in RPMI 1640 medium containing 10% FBS and 0 mM (○), 2 mM (●), 3 mM (□), 5 mM (■) and 7.5 mM (△) aspirin using 2.0 x $10^4$ cells/$cm^2$ initially at 37 °C. Data in the medium containing 0 and 2 mM aspirin are expressed as the means ± S.D. of four independent measurements.

The CEA productivity in the cell-culture medium containing 2 mM aspirin was found to be approximately 6 times the CEA productivity in the medium containing no aspirin. CW2 cells in the medium containing 5 mM and 7.5 mM aspirin were found to be produced by a factor of more than 100. Moreover, the long-term effect of aspirin on CEA production of CW2 cells was investigated.

Figure 5 shows the time dependence of specific CEA productivity in CW2 cells. The specific productivity of CEA was found to increase up to 10 days for CW2 cells cultured with 0–7.5 mM aspirin. This phenomena was generally observed in the production of antibodies by hybridoma cells and was explained by the fact that cells can produce proteins more efficiently in the late exponential growth and stationary phases of batch culture (Suzuki et al., 1997). The addition of aspirin to the culture medium was observed to effectively enhance CEA production and reduce the time lag of production in the early exponential growth phase. Constant specific productivity was observed after approximately 10 days of cell culture with 0, 2 and 3 mM aspirin. On the other hand, maxima of specific productivity of CEA were observed and the specific productivity gradually decreased after 10 days of cell culture with 5 and 7.5 mM aspirin. The high concentration of aspirin in the culture medium probably induces damages in the CW2 cells over the long term, and the CW2 cells cultured with a high concentration of aspirin can therefore produce CEA less efficiently in the stationary phases, although CW2 cells cultured with 7.5 mM aspirin still showed 9 times higher production of CEA per cell 31 days after inoculation of CW2 cells.

We found an extraordinary phenomenon in which CW2 cells produced CEA by more than 100-fold by addition of 5–7.5 mM aspirin to the culture medium after inoculation for 168 hr in this study. The enhanced production of CEA in CW2 cells will be explained by the growth-suppression induced by aspirin, which might arrest cells at the G/S phase of the cell cycle, because cells can be specialized to produce a specific protein preferably when cell division is suppressed (Takahashi et al., 1994; Fong et al., 1997; Al-Rubeai and Emery, 1990).

### *Cell growth on cast and LB films*

Cell growth and production of bioproducts will also be affected by the environment of cell culture, espe-

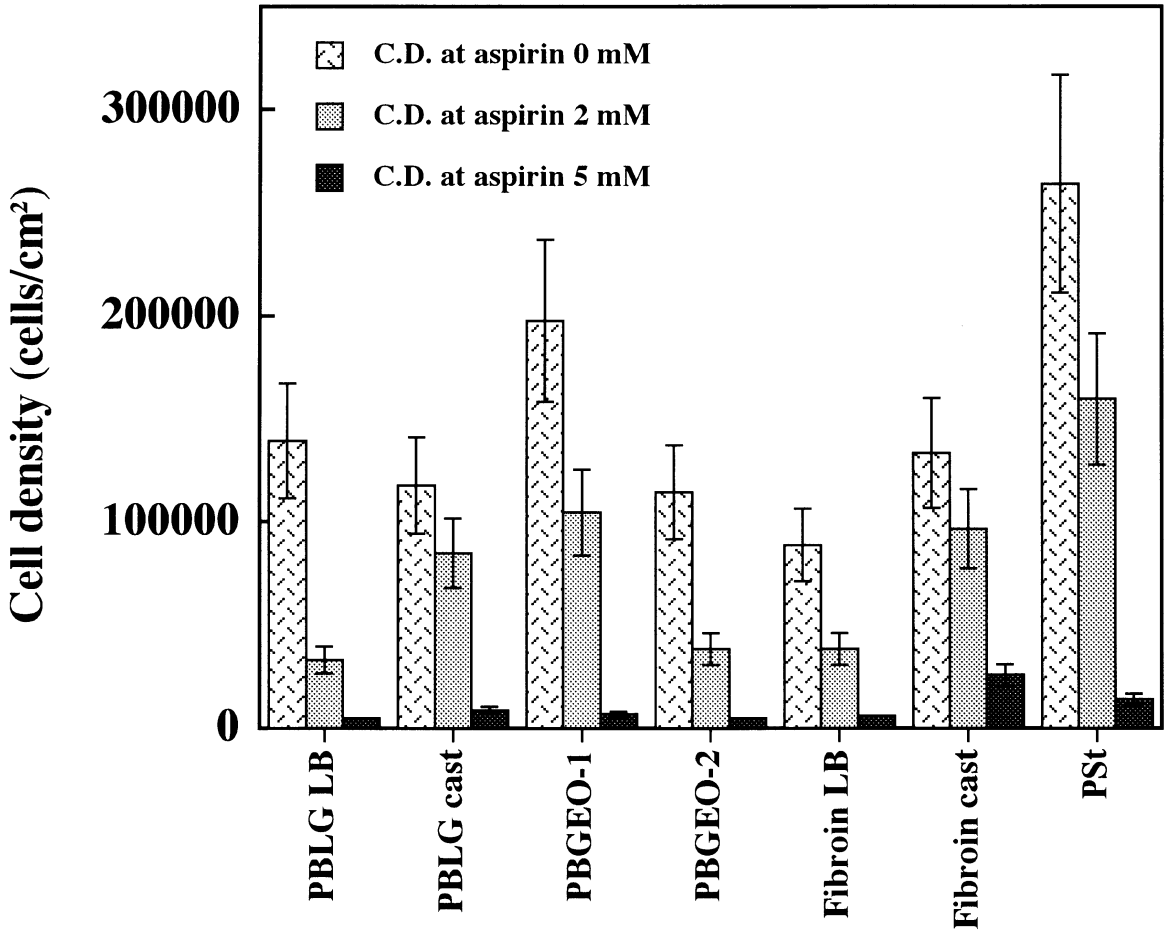


*Figure 6.* Cell density of CW2 cells on representative films after 168 hours of cultivation in RPMI 1640 medium containing 10% FBS and 0, 2 and 5 mM aspirin using 2.0 x $10^4$ cells/$cm^2$ initially at 37 °C. Data are expressed as the means ± S.D. of four independent measurements.

cially polymeric surfaces where the cells are attached. Cell growth on the films prepared by the cast and LB methods was investigated, and it was found that the cells attached well on both the LB and cast films prepared from some polymers in this study, and no significant differences were found in the morphologies of the cells attached on different films by phase-contrast micrography (data not shown).

CW2 cells were also cultured on the cast and LB films in the medium containing 0–7.5 mM aspirin at 37 °C using 2.0x$10^4$ cells/$cm^2$ initially. Figure 6 shows the cell density of CW2 cells cultured on the cast and LB films and a culture dish made of polystyrene as control experiments in the medium containing 0, 2 and 5 mM aspirin after inoculation for 168 hr at 37 °C using 2.0x$10^4$ cells/$cm^2$ initially. The cell density of CW2 cells cultured on the culture dish made of polystyrene was found to be the highest in the medium containing 0 or 2 mM aspirin among the cell densities investigated in this study. The cell density cultured on the cast films made of PBLG and silk fibroin was found to be higher than that on the LB films made of the same polymer compared to the cast films in the medium containing 2 and 5 mM aspirin. The cell survival due to aspirin having cell toxicity on the cast films was therefore found to be higher than that on the LB films.

*CEA production on cast and LB films*

Production of CEA was investigated for CW2 cells cultured on the cast and LB films and a cultured dish made of polystyrene in the medium containing 0, 2 and 5 mM aspirin after inoculation for 168 hr at 37

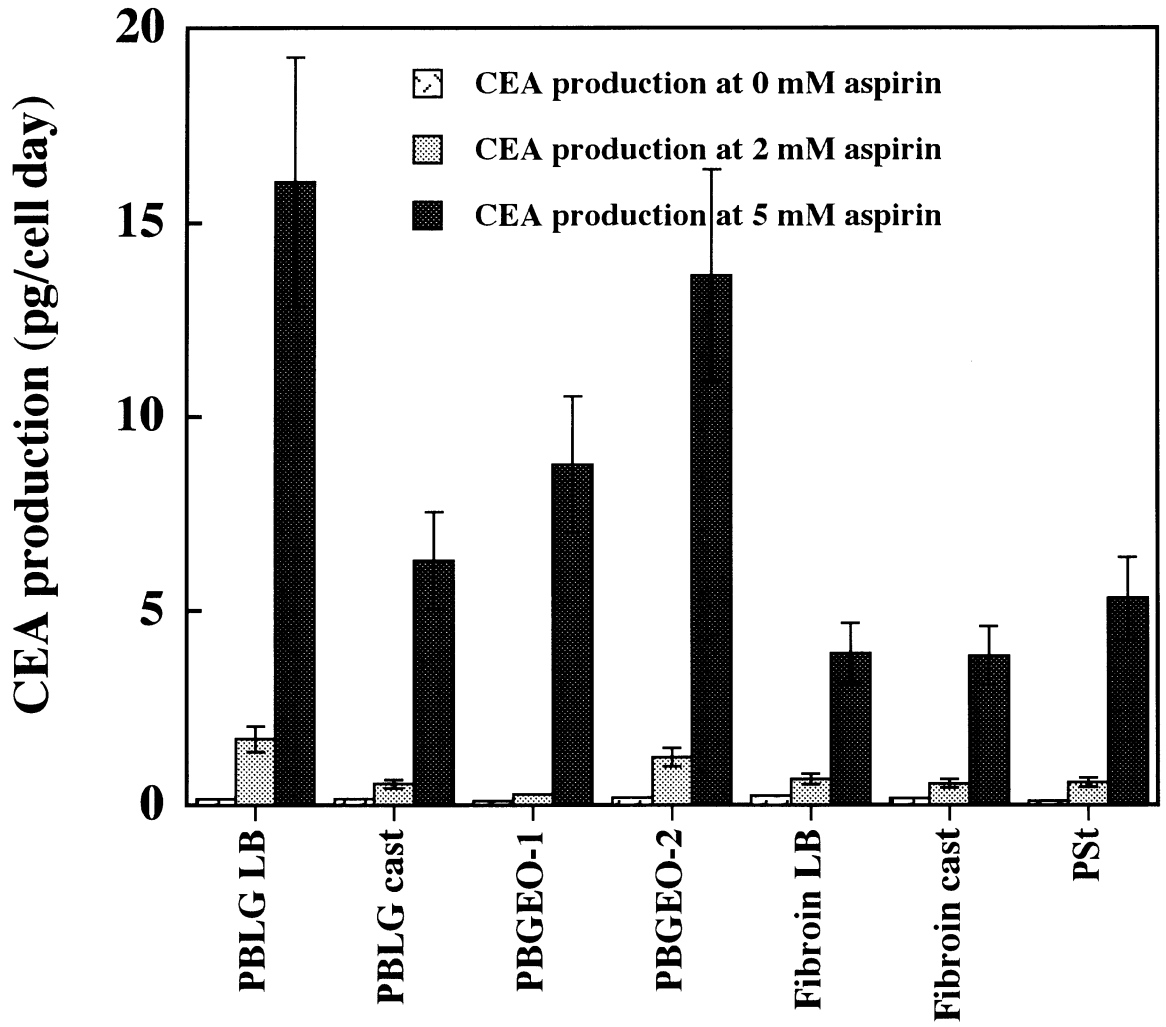


*Figure 7.* Specific CEA productivity by CW2 cells on representative films after 168 hours of cultivation in RPMI 1640 medium containing 10% FBS and 0, 2 and 5 mM aspirin using 2.0 x $10^4$ cells/$cm^2$ initially at 37 °C. Data are expressed as the means ± S.D. of four independent measurements.

°C using 2.0x$10^4$ cells/$cm^2$ initially. The production of CEA on the cast and LB films and a cultured dish made of polystyrene is shown in Figure 7.

The highest production of CEA per cell was observed for the CW2 cells on PBLG and PBGEO-2 films prepared by the LB method in the medium containing 5 mM aspirin in this study. This originates from the fact that the cell density on the PBLG and PBGEO-2 films in the medium containing 5 mM aspirin was the lowest in this study (see Fig. 6).

The relationship between CEA production per cell and cell density after inoculation for 168 hr was investigated for CW2 cells cultured on the cast and LB films and a cultured dish made of polystyrene in the medium containing 0, 2 and 5 mM aspirin and is shown in Figure 8. The following equation was obtained from the results shown in Figure 8, where the logarithm of CEA production ($P_{CEA}$/pg $cell^{-1}day^{-1}$) is correlated with the logarithm of cell density (CD/$cm^{-2}$) after 168 hr of inoculation;

$$\log(P_{CEA}/\text{pg cell}^{-1}\text{day}^{-1}) =$$

$$-1.194 \times \log(\text{CD/cm}^{-2}) + 5.488 \qquad (1)$$

The correlation coefficient of eq. (1) is calculated to be 0.920. Good correlation between CEA production per cell and cell density is therefore observed for CW2 cells in this study.

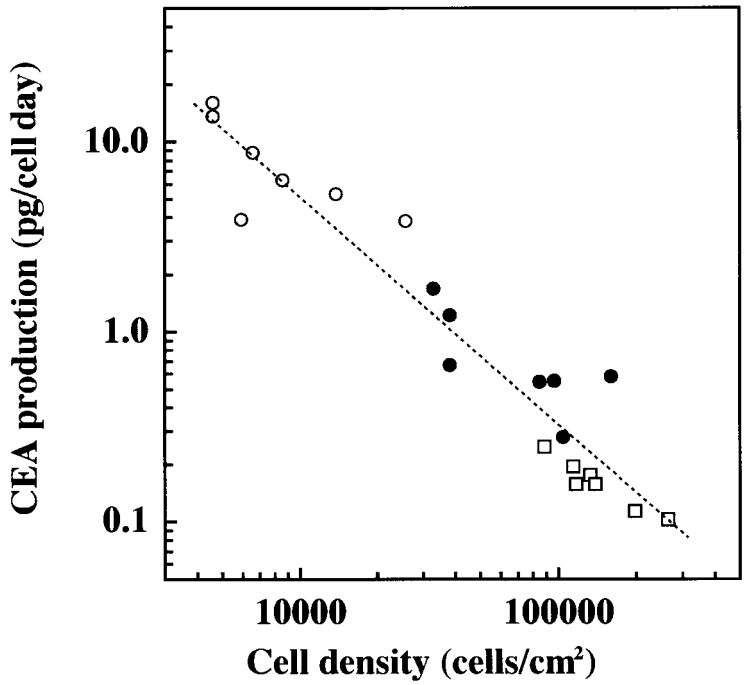


*Figure 8.* The relationship between specific CEA productivity and cell density of CW2 cells on representative films after 168 hours of cultivation in RPMI 1640 medium containing 10% FBS and 0 mM (□), 2 mM (●) and 5 mM (○) aspirin using 2.0 x $10^4$ cells/$cm^2$ initially at 37 °C.

It is generalized that CW2 cells produce CEA more effectively when the cell growth is suppressed by addition of toxic chemicals such as aspirin or by culture on unfavorable films for the cell growth. Addition of the aspirin to the media might be a promising and effective method for large-scale production of CEA by CW2 cells in bioreactors, although the aspirin is rather expensive chemicals and the procedure of aspirin removal in purification processes of CEA is necessary for mass production of CEA in the present method.

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