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Production of interferon-β in a culture of fibroblast cells on some polymeric films

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Abstract

Normal human skin (NB1-RGB) cells were cultured in the presence of polyinosinic and polycytidylic acids, diethylaminoethyl dextran, cycloheximide and actinomycin D, which induced human interferon-β. The simplest induction method, that required only polyinosinic and polycytidylic acids and diethylaminoethyl dextran was found to give the highest production of interferon-β by the cells. The cell growth and production of interferon-β were investigated for NB1-RGB cells cultured on silk fibroin, poly(γ-methyl-L-glutamate), poly(γ-benzyl-L-glutamate) and collagen films prepared by the Langmuir-Blodgett (LB) and casting methods. The cell density of NB1-RGB cells cultured on the LB films was found to be higher than that on the cast films made of the same polymer. This indicates that not only the chemical structure of the polymers used for the preparation of the films but the preparation methods of the films, i.e., casting and LB methods, are also a strong factor affecting the cell growth. The production of interferon-β per unit number of cells was found to be higher on the cast films than that on the LB films made of the same polymer. This is explained by the fact that the optimal suppressed growth of NB1-RGB cells on the cast films leads to the enhanced production of interferon-β on the cast films compared to those on the LB films prepared by the same polymer.

Abbreviations: poly I·poly C, polyinosinic and polycytidylic acids; DEAE, diethylaminoethyl; FBS, fetal bovine serum; PBLG, poly(γ-benzyl-L-glutamate); PMLG, poly(γ-methyl-L-glutamate); Pst, polystyrene; LB, Langmuir-Blodgett; CD, circular dichroism.

Introduction

Mammalian cell cuture 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 for economical and purity reasons.

In our previous study (Higuchi et al., 1999) human colorectal adenocarcinoma tumor (CW2) cells were cultivated on several polymeric films prepared by the Langmuir-Blodgett and casting methods for the production of carcinoembryonic antigen (CEA). The highest production of CEA per cell was observed for the CW2 cells on poly(γ-benzyl-L-glutamate) and its diblock copolymer films prepared by the Langmuir-Blodgett method. This research prompted us to think that cell attachment and cell communication between the cells and the film surface where the cells attached are also important for the enhanced production of bioproducts secreted by the anchor-dependent cells.

Interferon-β is a self-defensive protein produced by fibroblast cells, when they are infected with a virus or stimulated with a synthetic polynucleotide, polyinosinic and polycytidylic acids (poly I·poly C), which is an inducer of interferon (Tan et al., 1970; Yamada et al., 1991). Enhanced production of interferon was reported by pretreating the cells with interferon as known priming (Stewart et al., 1971; De Clercq, 1981), although the dose-dependent effect

of interferon pretreatment was confirmed (Friedman, 1966).

In this study, three cell lines of fibroblasts were used for the production of interferon-β, which was induced by poly I·poly C, diethylaminoethyl (DEAE) dextran and/or actinomycin D and cycloheximide. The fibroblast cells were cultured on two kinds of silk fibroin (from *Bombyx mori* and *Samia cynthia risini*), poly(γ-methyl-L-glutamate), poly(γ-benzyl-L-glutamate) and collagen films prepared by Langmuir-Blodgett and casting methods. The effect of polymeric films where the cells were cultivated in the production of interferon-β was examined.

Materials and methods

Materials

Poly(γ-methyl-L-glutamate), PMLG, was kindly supplied by Ajinomoto Co., Inc., and was purified by precipitation from 5 wt % dichloroethane in methanol. Poly(γ-methyl-L-glutamate), PBLG, was synthesized from benzyl-L-glutamate N-carboxyanhydride using triethylamine as an initiator in 1,4-dioxane. A 0.5 wt % collagen solution (Cellgen IPC-15, type I from calf skin, Koken Co., Ltd., Tokyo) was used as received and was diluted to 0.1 wt % collagen solution with ultrapure water adjusted to pH 3.0 with 0.1 N HCl.

Pure silk fibroin protein was obtained by removing the sericin from silkworm silk by boiling two types of the cocoons (*Bombyx mori* and *Samia cynthia risini*) in 100 °C water for 5 min and thereafter in 0.5 w/v % Marseilles soap solution for 30 min and then washing with ultrapure water. The pure silk fibroin protein was regenerated using 9.3 M LiBr solution and was dialyzed for three days in ultrapure water (Valluzzi et al., 1996; Asakura et al., A 0.5 wt % silk fibroin solution from *Bombyx mori* and *Samia cynthia risini* was obtained by adding ultrapure water to dialyzed silk fibroin solution.

The chemical structure of the polymers used in this study is shown in Scheme 1.

Preparation of cast polymeric films

PMLG and PBLG 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 derived from *Bombyx mori* and *Samia cynthia risini* and the collagen solution of 0.1 wt% were also cast onto the glass plates inserted into 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 tenperature for 24 hr.

Preparation of Langmuir-Blodgett films

A polymer solution of PMLG, PBLG, collagen 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 (Higuchi et al., 1999; Cho et al., 1996). The monolayer of polymer was transferred onto silicon-coated glass plates by a horizontal lifting method at 10 dyne cm^{-1}.

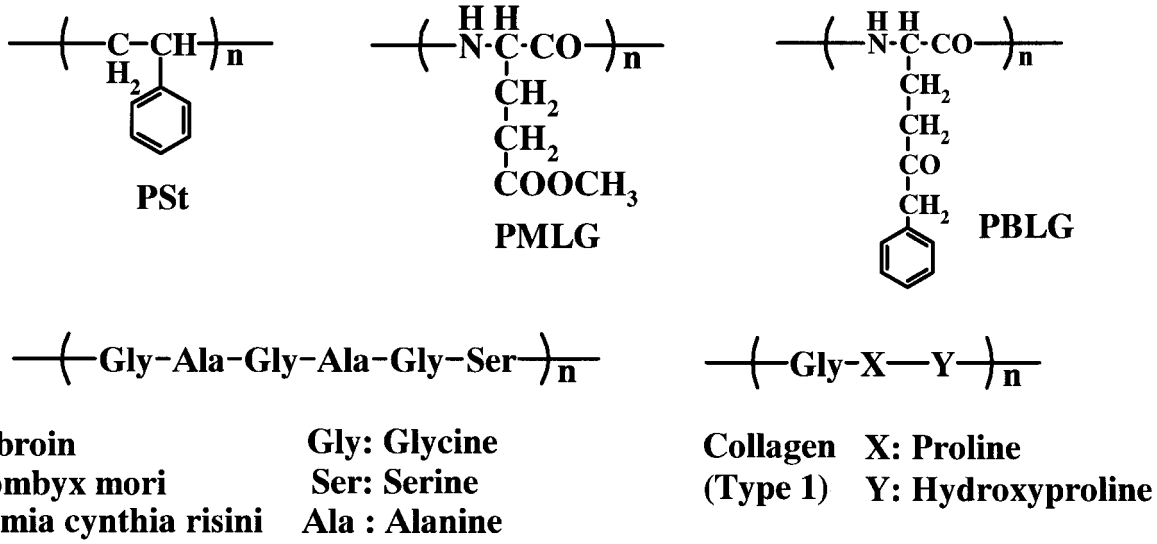
Cell lines

Three fibroblast cells, i.e., HUC-Fm (human umbilical cord cell), MRC-5 (normal human embryonic lung) and NB1-RGB (normal human skin), obtained from Riken Cell bank (Ibaraki, Japan) were maintained in MEM-α (JRH Bioscience, Lenexa, KS) supplemented with 25 mg l^{-1} streptomycin sulfate (Wako Pure Chemical Industry, Ltd.), 3.5 mg l^{-1} benzylpenicillin postassium (Wako Pure Chemical Industry, Ltd.) and 10% fetal bovine serum (FBS, JRH Bioscience, Lenexa, KS). The 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 interferon-β on cast and LB films

After UV irradiation (30 cm distance, 10 W, GL10, Stanley Co., Tokyo, Japan) on the cast and LB films for 20 min for sterilization, the films were inserted into 24-well tissue culture plates fitted with a lid (Iwaki Glass, Tokyo, well diameter = 16 mm). Three induction methods of interferon-β synthesis were used in this study.

Method A. (Stewart II et al., 1974; Harary et al., 1990). The cells in suspension (cell densities 1.0×10^5 ml^{-1} and 2 ml of medium) were inoculated into the tissue culture plates and were incubated in the CO_2 incubator in 5% CO_2 atmosphere for 18 h at 37 °C. The medium was then changed with 2 ml of 2% FBS/MEM-α containing 10 μg ml^{-1} of poly I·poly



Scheme 1.

C ($S_{20,w}$ = 15, Yamasa Corp., Chiba, Japan) and 300 μg ml^{-1} of diethylaminoethyl dextran (DEAE-dextran, 500,000 dalton, Sigma Chemical Co., St. Louis, MO) and the cells were incubated for 4 h (induction phase). After removing the medium, the cells were rinsed twice with phosphate buffered saline of pH 7.4 and then cultured for 12–48 h in 2% FBS/MEM-α (production phase).

Method B. (Yamada et al., 1991; Tan et al., 1970). The cells in suspension (cell densities 1.0 $\times$ 10^5 ml^{-1} and 2 ml of medium) were inoculated into the tissue culture plates and were incubated in the CO_2 incubator in 5% CO_2 atmosphere for 18 h at 37 °C. The medium was then changed with 2 ml of 2% FBS/MEM-α containing 10 μg ml^{-1} of poly I·poly C and 5 μg ml^{-1} of cycloheximide. After a 4-h incubation at 37 °C, actinomycin D (final conc., 4 μg ml^{-1}) was added to the medium and the cells cultured for 1 h (induction phase). After removing the medium, the cells were rinsed twice with phosphate buffered saline of pH 7.4 and then cultured for 12–48 h in 2% FBS/MEM-α (production phase).

Method C. The cells in suspension (cell densities 1.0 $\times$ 10^5 ml^{-1} and 2 ml of medium) were inoculated into the tissue culture plates and were incubated in the CO_2 incubator in 5% CO_2 atmosphere for 18 h at 37 °C. The medium was then changed with 2 ml of 2% FBS/MEM-α containing 10 μg ml^{-1} of poly I·poly C, 300 μg of DEAE-dextran and 5 μg ml^{-1} of cycloheximide. After a 4-h incubation at 37 °C, actinomycin D (final conc., 4 μg ml^{-1}) was added to the medium and the cells cultured for 1 h (induction phase). After removing the medium, the cells were rinsed twice with phosphate buffered saline of pH 7.4 and then cultured for 12–48 h in 2% FBS/MEM-α (production phase).

The sampled medium was used for estimation of the concentration of interferon-β. The concentration of interferon-β was measured by the enzyme linked immunosorbent assay (ELISA) using a Human IFN-β ELISA kit (Fujirebio, Inc., Tokyo, Japan) and an ELISA reader (Bio-Rad Laboratories, Inc., CA, USA). IFN-β was measured using an O.D. value obtained at 450 nm.

The cell number was estimated by observation of the cells on the cast and LB films in the tissue culture plates maintained as 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.

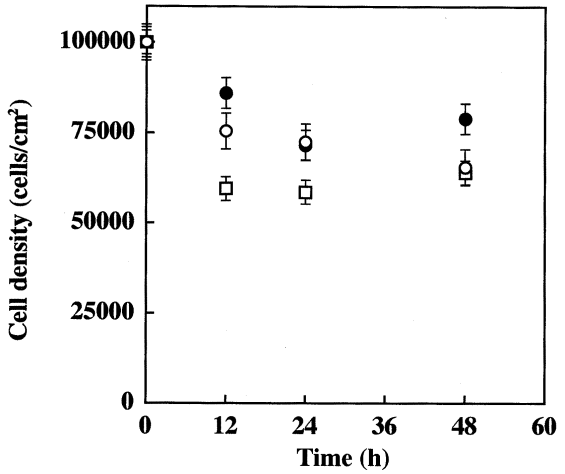


Figure 1. Culture of NB1-RGB cells on PSt plates after three induction methods (method A (●), method B (○) and method C (□)) of interferon-β. Data are expressed as the means ± S.D. of four independent experiments.

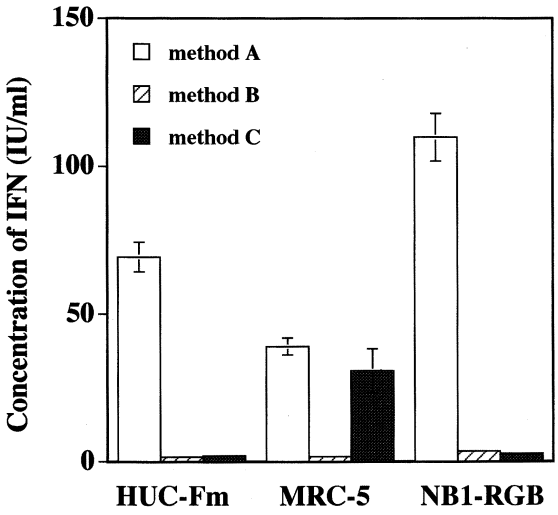


Figure 2. Productivity of interferon-β by HUC-Fm, MRC-5 and NB1-RGB cells on PSt plates after 24 h of induction. Data are expressed as the means ± S.D. of four independent measurements.

Results and discussion

Optimal induction method and cell line

Before the examination of the effect of polymeric films where the fibroblast cells were cultivated for the production of interferon-β, we examined the optimal induction method of interferon-β from three induction methods, which were described in the experimental section.

Figure 1 shows cell survival of NB1-RGB cells cultured on polystyrene dish after induction by three induction methods of interferon-β. 70 ± 10% survival was observed in any cases induced by three methods for 48 h after induction. We also found exactly the same tendency for the cell survival of other cell lines used, i.e., HUC-Fm and MRC-5 after the induction of interferon-β (data not shown). The toxic effect of chemicals on the cells is found to be almost the same among three induction methods for three cell lines used in this study during 48 h after the induction.

The production of interferon-β by three cell lines was examined in the cells induced by three induction methods. Figure 2 shows the concentration of interferon-β in the media of three fibroblasts cultured on polystyrene dish after 24 h of induction. It is found that method A that used only DEAE-dextran and poly I·poly C for the induction gave the highest production of interferon-β in HUC-Fm and NB1-RGB cells. HUC-Fm and NB1-RGB cells induced by methods B and C, which used cycloheximide and actynomycin D, produced only a trace amount of interferon-β. Cycloheximide is known as an inhibitor of peptide chain elongation (Somenshein and Brawerman, 1976; Takahashi et al., 1994), and actinomycin D is an inhibitor of RNA synthesis (Darzynkiewicz et al., 1981). Although both of the reagents led to suppression of cell growth by inhibition protein synthesis, they did not induce any interferon-β production by methods B and C except for MRC-5 cells induced by method C.

Yamada et al. reported stimulation of interferon-β production of human foreskin diploid fibroblasts by α- and β-caseins (Yamada et al., 1991). They used method B as the induction method of interferon-β, by which no production of interferon-β was observed in our cell lines. Their fibroblasts, on the other hand, produced 36–274 IU ml^{-1} of interferon-β. No production by method B in our cell lines probably originated from the difference in sensitivity and the origin of the cells compared to the foreskin diploid fibroblasts reported by Yamada et al. (Yamada et al., 1991).

MRC-5 cells showed another specific characteristic in which only MRC-5 cells induced by method C produced interferon-β significantly and the production of interferon-β by method C was almost the same as that by method A. DEAE-dextran was used for the induction of interferon-β in both methods A and C, although cycloheximide and actynomycin D were additionally used in method C. This observation suggests that MRC-5 cells are rather strong cells for cell survival compared to HUC-Fm and NB1-RGB cells and

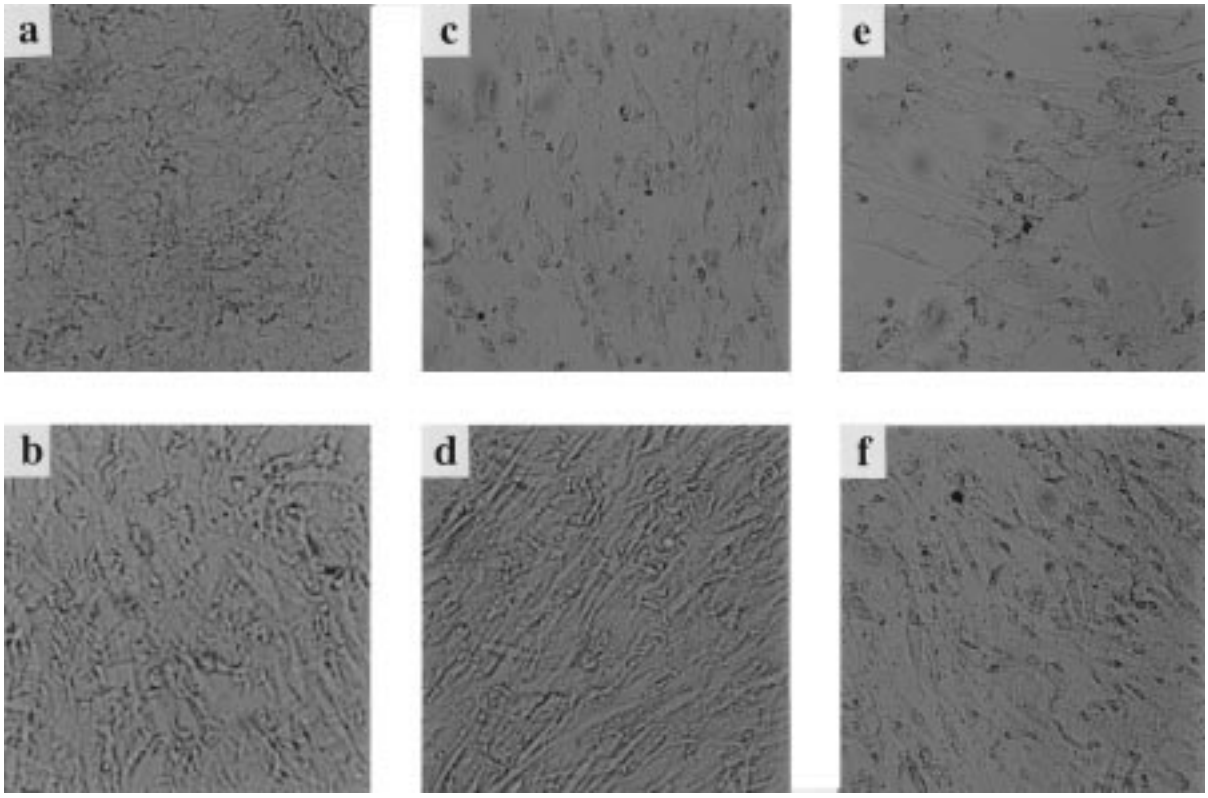


Figure 3. (a–j): Micrographs of NB1-RGB cells cultured on PMLG cast films (a), PMLG LB films (b), PBLG cast films (c), PBLG LB films (d), collagen cast films (e), collagen LB films (f), fibroin (from *Bombyx mori*) cast films (g), fibroin (from *Bombyx mori*) LB films (h), fibroin (from *Samia cynthia risini*) cast films (i), fibroin (from *Samia cynthia risini*) LB films (j) and PSt plates (k) after 24 h of induction.

can tolerate toxic reagents such as cycloheximide and actynomycin D. MRC-5 cells can, therefore, produce interferon-β after the induction of poly I·poly C and DEAE-dextran with and without the toxic reagents.

NB1-RGB cells were selected for the examination of the effect of polymeric films on the cell growth and production of interferon-β in the following sections, because the cells induced by method A produced the highest amount of interferon-β in this study.

Cell growth and morphology on cast and LB films

Cell growth and morphology, which are indexes indicating cell behavior and function, were investigated by micrographs of NB1-RGB cells cultured on cast and LB films. Synthetic poly(α-amino acid)s, i.e., PMLG and PBLG, and natural proteins, i.e., collagen and fibroins (from *Bombyx mori* and *Samia cynthia risini*), were used for the preparation of cast and LB films. The chemical structure of the polymers used in this study is shown in Scheme 1. The main chemical structure of fibroins (from *Bombyx mori* and *Samia cynthia risini*) has the amino acid sequence shown in Scheme 1. However it is known that the amino acid sequences of fibroins are much complicated and have different sequences depending on the species of silkworms such as *Bombyx mori* and *Samia cynthia risini* (Minoura et al., 1995). NB1-RGB cells were cultured on the cast and LB films and were examined for the effect of polymeric films on the cell growth and production of interferon-β. Figure 3 shows the micrographs of NB1-RGB cells on the cast and LB films and a culture dish made of polystyrene after 24 h of induction at 37 °C using 1.0×10^5 cell/cm^2 initially.

The cells adherent to both cast and LB films were found to show high filopodia and flat shapes except the cells on PMLG-cast films. No morphological difference was observed for the cells cultured on cast and LB films prepared by PBLG, collagen and fibroins (from *Bombyx mori* and *Samia cynthia risini*). NB1-RGB cells on PMLG-cast films showed different morphologies and considerably spread shapes compared to the cells on other films investigated in this study. The reason of strong morphological changes of NB1-RGB

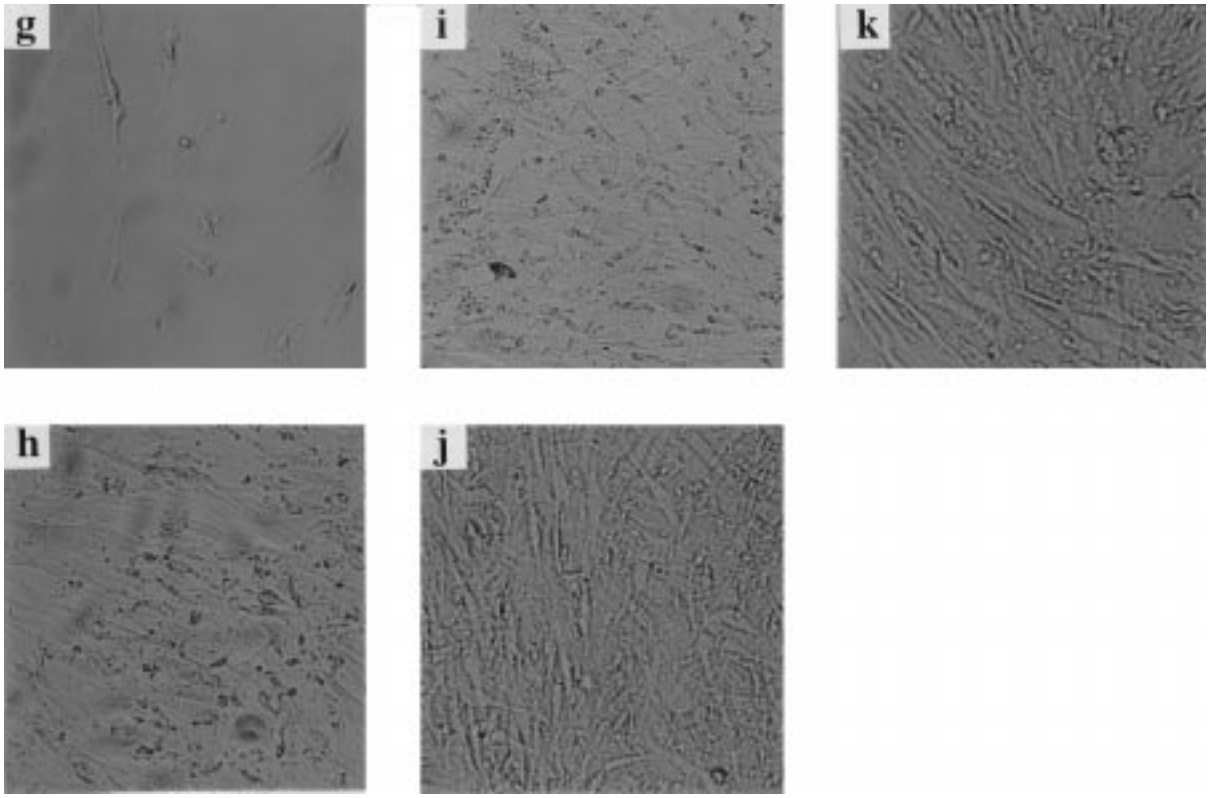


Figure 3. Continued.

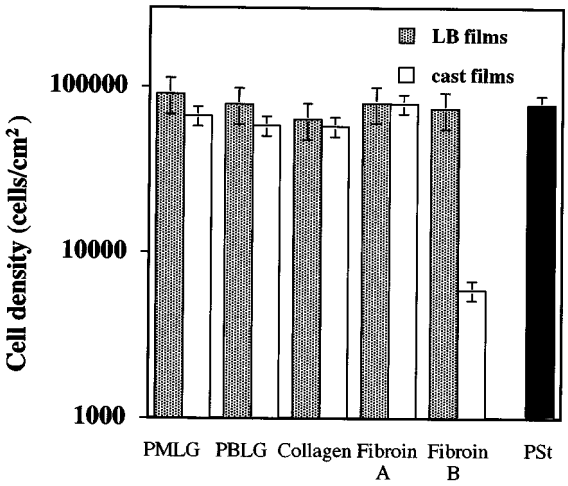


Figure 4. Cell density of NB1-RGB cells on representative films after 24 h of induction. Fibroins A and B indicate fibroins from *Samia cynthia risini* and *Bombyx mori*, respectively. Data are expressed as the means ± S.D. of four independent measurements.

cells on PMLG-cast films is unknown at this moment.

Figure 4 shows the cell density of NB1-RGB cells cultured on the cast and LB films and a culture dish made of polystyrene after 24 h of induction at 37 °C using 1.0×10^5 cells/cm^2 initially. The cell density of NB1-RGB cells cultured on the LB films was found to be higher than that on the cast films made of the same polymer except fibroin from *Samia cynthia risini* (i.e., the cell density on the LB films vs. that on the cast films; $p < 0.05$ for PMLG, and fibroin from *Bombyx mori* films, $p < 0.08$ for collagen films and no significant difference for fibroin from *Samia cynthia risini* films). This indicates that not only the chemical structure of the polymers used for the preparation of the films but the preparation methods of the films, i.e., cast and LB methods, are also strong factors affecting the cell growth (Higuchi et al., 1999). We already found that the films prepared with the same polymer by cast and LB methods had a different contact angle of water and surface roughness (Higuchi et al., 1999). These physical properties may generate different cell growth on the cast and LB films.

Interferon-β production of cast and LB films

Production of interferon-β by method A was investigated for NB1-RGB cells cultured on the cast and LB

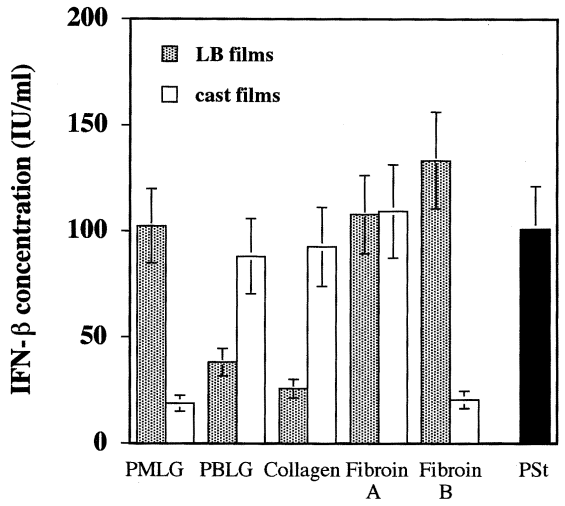


Figure 5. Concentration of interferon-β by NB1-RGB cells on representative films after 24 h of induction. Fibroins A and B indicate fibroins from *Samia cynthia risini* and *Bombyx mori*. Data are expressed as the means ± S.D. of four independent measurements.

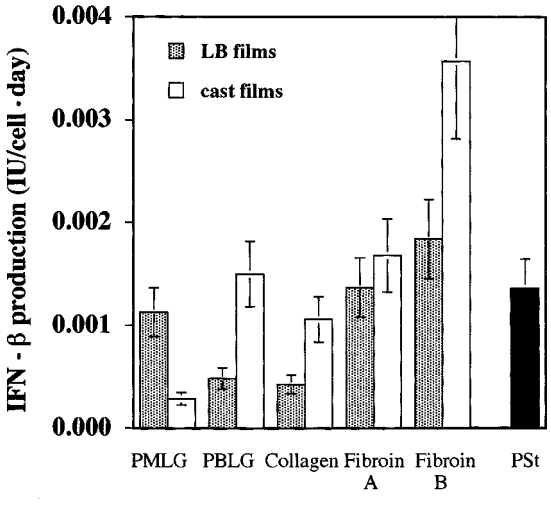


Figure 6. Specific productivity of interferon-β by NB1-RGB cells on representative films after 24 h of induction. Fibroins A and B indicate fibroins from *Samia cynthia risini* and *Bombyx mori*. Data are expressed as the means ± S.D. of four independent measurements.

films and a culture dish made of polystyrene. Figure 5 shows the concentration of interferon-β in the media where NB1-RGB cells were cultured after 24 h of induction at 37 °C using 1.0×10^5 cells cm^2 initially. The highest concentration of interferon-β in the media was found in the media where the cells were cultured on fibroin (from *Bombyx mori*)-LB films. On the other hand, the lowest concentration of interferon-β in the media was found in the media where the cells were cultured on fibroin (from *Bombyx mori*)-cast and PMLG-cast films. These results originate from the relatively lower cell density on the fibroin (from *Bombyx mori*)-cast films and the abnormal morphologies of the cells on PMLG-cast films.

The concentration of secreted bioproducts by cells in the media is directly related to the cell density (number) and the production amount of bioproducts per unit number of cells. Therefore, not only the cell density on the films but the production of interferon-β per unit number of cells cultured on the cast and LB films is also important for the high production of interferon-β.

Figure 6 shows the production of interferon-β per unit number of cells by NB1-RGB cells cultured on the cast and LB films and a culture dish made of polystyrene after 24 h of induction at 37 °C using 1.0×10^5 cells/cm^2 initially. The highest production of interferon-β per unit number of cells was observed for the NB1-RGB cells on the fibroin (from *Bombyx mori*)-cast films. The production of NB1-RGB cells cultured on the cast films was found to be higher than that on the LB films made of the same polymer except for PMLG films. NB1-RGB cells on the PMLG-cast films were observed to have abnormal morphologies compared to those on other films examined in this study; therefore, the production of NB1-RGB cells cultured on the PMLG-cast films was regarded as an exceptional case in this study.

Contradictory results were observed from Figures 4 and 6 in which the cell density of NB1-RGB cells on the LB films is higher than that on the cast films made of the same polymer and the production of interferon-β per unit number of cells on the LB films is lower than that on the cast films made of the same polymer. It is known that the suppressed growth of hybridoma cells results in enhances production of antibody (Suzuki and Ollis, 1990; Fong et al., 1997). Several growth-suppressing reagents such as inerleukin-6, TGF-β, sodium n-butyrate (Suzuki and Ollis, 1990), potassium acetate (Somenshein and Brawerman, 1976; Fong et al., 1997), caffeine, tymidine (Takahashi et al., 1994), cycloheximide, actinomycin D and aspirin (Higuchi et al., 1999) were reported for enhanced production of antibody and antigen. Therefore, the optimal suppressed growth of NB1-RGB cells on the cast films may lead to the enhanced production of interferon-β per unit number of cells on the cast films compared to those on the LB films prepared by the same polymer in this study.

Cycloheximide and actinomycin D, which were the growth-suppressing reagents, were added to the media of the cell culture, and the production of interferon-β was investigated in this study (i.e., method C in Figure 2). However, no effect for MRC-5 cells or a worse effect for HUC-Fm and NB1-RGB cells was observed in this study.

We also added 0–20 mM of other growth-suppressing reagents such as thymidine, hydroxyurea, caffeine and aspirin to the media of NB1-RGB cells (data not shown). The concentration of interferon-β in the media decreased with the increase in the growth-suppressing reagent in any cases. Therefore, the reason that the higher production of interferon-β per unit number of cells is observed on the cast films compared to the LB films, which are made of the same polymer, originates from the adequate growth-suppressing effect of NB1-RGB cells on the cast films compared to those on the LB films.

The highest concentration of interferon-β was observed in the media where NB1-RGB cells were cultured on fibroin (from *Bombyx mori*)-LB films. A relatively high concentration of interferon-β was also observed in the media where NB1-RGB cells were cultured on fibroin (from *Samia synthia risini*)-LB and -cast films (see Figure 5). Therefore, it is suggested that fibroin (from *Samia cynthia risini*) films and fibroin (from *Bombyx mori*)-LB films are suitable for the cell culture matrix for the purpose of interferon-β production.

Conclusions

The simplest induction method that required only polyinosinic and polycytidylic acids and diethylaminoethyl dextran was found to give the highest production of interferon-β by HUC-Fm, MRC-5 and NB1-RGB cells. The cell density of NB1-RGB cells cultured on the LB films was found to be higher than that on the cast films made of the silk fibroin poly(γ-methyl-L-glutamate), poly(γ-benzyl-L-glutamate) and collagen. This indicates that not only the chemical structure of the polymers used for the preparation of the films but the preparation methods of the films, i.e., casting and LB methods, are also a strong factor affecting the cell growth. The production of interferon-β per unit number of cells on the cast films was found to be higher than that on the LB films made of the same polymer. This is explained by the fact that the optimal suppressed growth of NB1-RGB cells on the cast films leads to be enhanced production of interferon-β on the cast films prepared by the same polymer.

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