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Production of interferon-β by fibroblast cells on membranes prepared by extracellular matrix proteins

Akon Higuchi[1,*], Yasunari Takanashi[1], Takeshi Ohno[1], Tetsuo Asakura[2], Chong-Su Cho[3], Toshihiro Akaike[4] and Mariko Hara[1]

[1]Department of Applied Chemistry, Seikei University, Musashino 180-8633, Japan; [2]Department of Biotechnology, Tokyo University of Agriculture and Technology, Koganei 184-8588, Japan; [3]School of Agricultural Biotechnology, Seoul National University, Suwon 441-744, Korea; [4]Department of Biomolecular Engineering, Tokyo Institute of Technology, Yokohama 226-8501, Japan; [*]Author for correspondence

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Abstract

The fibroblast cells from normal human skin were cultured on Langmuir-Blodgett (LB) and cast membranes prepared using extracellular matrix proteins (e.g., collagen, fibronectin, laminin and vitronectin). The cell density of the fibroblast cells cultured on the cast membranes was found to be higher than that on the cast membranes made of fibronectin, vitronectin and collagen-blended membranes. This indicates that not only the primary structure of proteins but the preparation methods of the membranes, i.e., casting and LB methods, are a strong factor affecting cell growth. The concentration and production of interferon-β per unit cell were found to be higher on the LB membranes than on the cast membranes made of the same proteins except in the case of collagen. However, the cell density on the cast membranes was higher than that on the LB membranes. These results appear to result from the suppressed growth of NB1-RGB cells on the LB membranes leading to the enhanced production of interferon-β on the LB membranes. The highest production of interferon-β per unit cell was observed for the NB1-RGB cells on the collagen-blended membranes with fibronectin and vitronectin. The collagen-blended membranes appear to offer a more natural and appropriate environment for NB1-RGB cells to produce interferon-β.

Abbreviations: poly I·poly C – polyinosinic and polycytidylic acids, DEAE – diethylaminoethyl, FBS – fetal bovine serum, PBS – phosphate buffered saline, LB – Langmuir-Blodgett

Introduction

Mammalian cell culture is increasingly important for 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 aimed at enhancing productivity and purity (Fong et al. 1997; Lee et al. 1994; Takahashi et al. 1994; Suzuki and Ollis 1990; Yamaguchi et al. 1997).

Interferon-β is a protein component of the immune system produced by fibroblast cells when they are infected with a virus or stimulated with a synthetic polynucleotide (Tan et al. 1970; Yamada et al. 1991). It has been used in therapeutic applications including treatment of hepatitis B, skin cancer (Ishihara 1992) and multiple sclerosis (Jacobs et al. 1996; Weyenbergh et al. 2001).

In our previous study (Higuchi et al. 2000), the cell growth and production of interferon-β were investigated for fibroblast (NB1-RGB) cells cultured on silk fibroin, poly(γ-methyl-L-glutamate), and poly(γ-

benzyl-L-glutamate) membranes prepared by the Langmuir-Blodgett (LB) and casting methods (Higuchi et al. 1999; Cho et al. 1996). The production of interferon-β per unit cell was found to be higher on the cast membranes than that on the LB membranes made of the same polymer. This was explained by the fact that the suppression of growth of NB1-RGB cells on the cast membranes leads to the enhanced production of interferon-β on the cast membranes compared to those on the LB membranes prepared with the same polymer. This prompted the conclusion that cell attachment and cell communication between the cells and the membrane surface where the cells attached are important for the enhanced production of bioproducts secreted by the anchor-dependent cells.

In this study, the fibroblast cells were cultured on the LB and cast membranes prepared with extracellular matrix proteins (e.g., collagen, fibronectin, laminin and vitronectin). The effect of natural protein membranes where the cells were intended for use in the production of interferon-β was examined.

Materials and methods

Materials

Solutions of 1 mg ml^{-1} laminin (T016, from human placenta, Takara Shuzo Co., Ltd., Kyoto), 0.5 mg ml^{-1} fibronectin (F0003, from human plasma, Asahi Technoglass Co., Ltd., Tokyo) and 0.5 mg ml^{-1} vitronectin (V0001, from human plasma, Asahi Technoglass Co., Ltd., Tokyo) were used as indicated by the manufacturer. A 5 mg ml^{-1} collagen solution (Cellgen IPC-15, type I from calf skin, Koken Co., Ltd., Tokyo) was diluted for use to 1 mg ml^{-1} collagen solution in ultrapure water adjusted to pH 3.0 with 0.1 mol l^{-1} HCl.

Preparation of cast membranes

The laminin, fibronectin and vitronectin solutions were diluted to 10 μg ml^{-1} with phosphate buffered saline (PBS) of pH 7.4. The collagen solution was diluted to 0.5 mg ml^{-1} and 10 μg ml^{-1} with ultrapure water adjusted to pH 3.0 with 0.1 M HCl.

The laminin, fibronectin and vitronectin solutions of 10 μg ml^{-1} and the collagen solution of 0.5 mg ml^{-1} were cast onto glass coverslips inserted into 24-well tissue culture plates. The protein solutions cast into 24-well tissue culture plates were allowed to stand for 24 h at 37 °C for preparation of the protein membranes.

An equivalent amount of collagen solution of 10 μg ml^{-1} was added to the laminin, fibronectin and vitronectin solutions of 10 μg ml^{-1}. The collagen-blended solutions were cast onto the glass coverslips inserted into 24-well tissue culture plates. The protein solutions cast into 24-well tissue culture plates were allowed to stand for 24 h at 37 °C for the preparation of collagen-blended membranes.

After discarding the protein solutions, the protein membranes were washed with PBS solution of pH 7.4 three times and were blocked with 10 mg ml^{-1} bovine serum albumin (BSA)-PBS of pH 7.4 at 37 °C for 2 h. The protein membranes were finally prepared by drying at room temperature for 24 h and subsequently drying under vacuum at room temperature for 24 h.

Preparation of Langmuir-Blodgett membranes

A protein solution of laminin (1 mg ml^{-1}), fibronectin (0.5 mg ml^{-1}), vitronectin (0.5 mg) and collagen (1 mg ml^{-1}) 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).

An equivalent amount of collagen solution of 1 mg ml^{-1} or 0.5 mg ml^{-1} was added to the laminin (1 mg ml^{-1}), fibronectin (0.5 mg ml^{-1}) and vitronectin (0.5 mg ml^{-1}) solutions. The collagen-blended solutions were also 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 protein was transferred onto silicon-coated glass plates by a horizontal lifting method at 10 dyne cm^{-1}. The protein membranes were blocked with 10 mg ml^{-1} BSA-PBS of pH 7.4 at 37 °C for 2 h, dried at room temperature for 24 h and subsequently dried under vacuum at room temperature for 24 h.

Cell lines

The fibroblast cells, 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 potassium (Wako Pure Chemical Industry, Ltd.) and 10 vol% 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 membranes

After UV irradiation (30 cm distance, 10 W, GL10, Stanley Co., Tokyo) on the cast and LB membranes for 20 min for sterilization, the protein membranes were inserted into 24-well tissue culture plates fitted with a lid (Iwaki Glass, Tokyo, well diameter = 16 mm).

The cells in suspension (cell densities 1.0×10^5 ml^{-1} and 2 ml of medium) were inoculated into the tissue culture plates containing the protein membranes 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 vol% FBS/MEM-α containing 10 mg ml^{-1} of poly I·poly C ($S_{20,w}=15$, Yamasa Corp., Chiba, Japan) and 300 mg ml^{-1} of diethylaminoethyl dextran (DEAE-dextran, 500,000 dalton, Sigma Chemical Co., St. Louis, MO) and the cells were incubated on the protein membranes for 4 h (induction phase). After removing the medium, the cells were rinsed twice with PBS of pH 7.4 and then cultured for 24 h in 2 vol% FBS/MEM-α (production phase).

The sampled medium was used for estimation of the concentration of interferon-β (IFN-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) and an ELISA reader (Bio-Rad Laboratories, Inc., CA). The concentration of interferon-β was expressed as IU ml^{-1} in accordance with the calibration method adopted by the manufacturer.

The cell number was estimated by observation of the cells on the cast and LB membranes 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 membranes. These procedures were performed on each membranes type using four replicate membranes of the same proteins (totally n=16), and the cell number was finally averaged.

Results and discussion

Cell growth and morphology on cast and LB membranes

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 membranes prepared by extracellular matrix proteins (i.e., collagen, laminin, fibronectin and vitronectin). Figure 1 shows the micrographs of NB1-RGB cells on the cast and LB membranes after 24 h of induction at 37 °C using 1.0×10^5cell cm^{-2} initially.

The cells adherent to both cast and LB membranes were found to show high filopodia and flattered morphology except for the cells on laminin-cast membranes. No morphological difference was observed for the cells cultured on the cast and LB membranes prepared by fibronectin (FN), vitronectin (VN), collagen (COL), collagen+laminin (COL+LN), collagen+fibronectin (COL+FN) and collagen+vitronectin (COL+VN). NB1-RGB cells on the laminin-cast membranes showed different morphologies and considerably spread shapes compared to the cells on other membranes investigated in this study. The specific reason for strong morphological changes of NB1-RGB cells on the laminin-cast membranes was not clear. However, the cell-binding sequences in laminin could play an important role on the morphological changes of NB1-RGB cells.

Figure 2 shows the cell density of NB1-RGB cells cultured on the cast and LB membranes and a glass plate 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 cast membranes was found to be higher than that on the LB membranes made of the same proteins except laminin and collagen (i.e., the cell density on the cast membranes vs. that on the LB membranes; $p < 0.05$ for fibronectin, vitronectin, collagen+laminin, collagen+fibronectin and collagen+vitronectin membranes and no significant difference for collagen membranes [$p > 0.05$]). Lower cell density of NB1-RGB cells on the laminin-

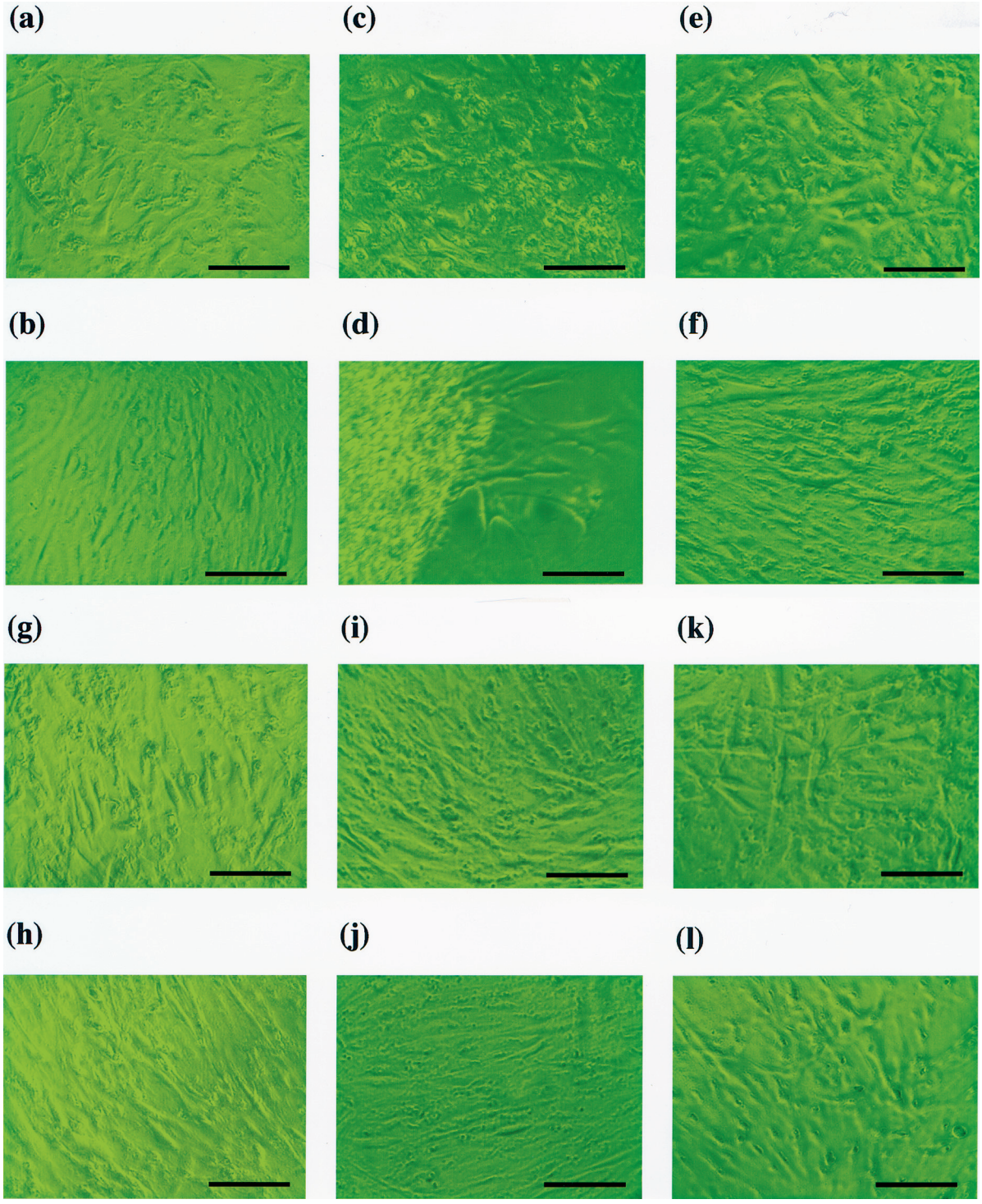

Figure 1. Micrographs of NB1-RGB cells cultured on collagen-cast membranes (a), collagen-LB membranes (b), laminin-cast membranes (c), laminin-LB membranes (d), fibronectin-cast membranes (e), fibronectin-LB membranes (f), collagen+laminin-cast membranes (g), collagen+laminin-LB membranes (h), collagen-fibronectin-cast membranes (i), collagen-fibronectin-LB membranes (j), collagen-vitronectin-cast membranes (k) and collagen+vitronectin-LB membranes (l) after 24 h of induction (original magnification x200; scale bar: 100 μm)

cast membranes is explained by the different morphology of the cells as seen in Figure 1. These results indicate that not only the primary structure of the proteins used for the preparation of the membranes but also the methods of membrane preparation, i.e., cast and LB methods, are a strong factor affecting cell growth (Higuchi et al. 1999). The authors have shown that membranes prepared with the same polymer by cast and LB methods had different surface roughness and contact angle of water which is an index of hydrophilicity/hydrophobility of membranes (Higuchi et al. 1999). These physical properties also generated different cell growth on the cast and LB membranes (Higuchi et al. 2000).

Interferon-β production on cast and LB membranes

Production of interferon-β was investigated for NB1-RGB cells cultured on the cast and LB membranes prepared by extracellular matrix proteins. Figure 3 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 culture supernatant occurred where the cells were cultured on blended membranes with collagen (i.e., COL+LN, COL+FN and COL+VN membranes). The cells in tissues are usually in contact with a complex network of secreted extracellular macromolecules in the extracellular matrix and the extracellular matrix is composed of various proteins (e.g., collagen, fibronectin, laminin and vitronectin) and polysaccharides. Variations in the relative amounts of the different types of matrix macromolecules and the way they are organized in the extracellular matrix are known to give rise to the diversity of forms and the controlling of cell behavior (Alberts et al. 1994). Thus, the collagen-blended membranes with fibronectin, laminin and vitronectin might be a more natural and appropriate environment for NB1-RGB cells to produce interferon-β, because a structural protein (i.e., collagen) is mixed with one of the cell adhesive proteins (i.e., fibronectin, laminin and vitronectin) in the collagen-blended membranes.

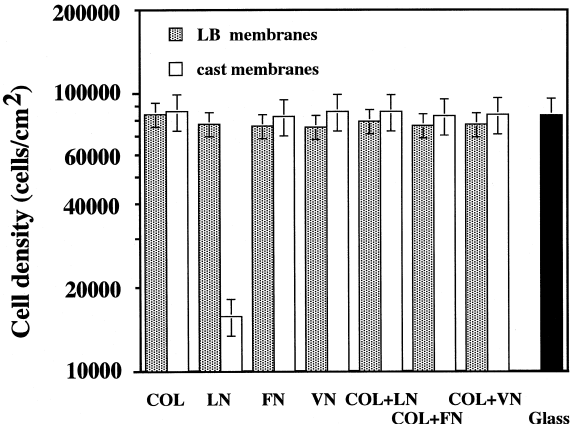

Figure 2. Cell density of NB1-RGB cells on representative membranes after 24 h of induction. Data are expressed as the means ±S.D. of four independent measurements.

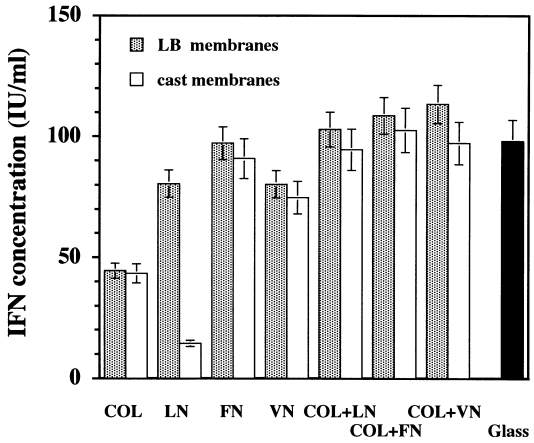

Figure 3. Concentration of interferon-β secreted by NB1-RGB cells on representative membranes after 24 h of induction. Data are expressed as the means ±S.D. of four independent measurements.

The lowest concentration of interferon-β in culture supernatant was found where the cells were cultured on the laminin-cast membranes. This was probably the consequence of relatively lower cell density on the laminin-cast membranes and possibly due to the abnormal morphologies of these cells.

The concentration of interferon-β in the culture supernatant from cells cultured on the LB membranes was found to be higher than that on the cast membranes made of the same proteins except in the case of collagen ($p < 0.05$ for laminin, fibronectin, vitronectin, collagen+laminin, and collagen+vitronectin membranes and $p < 0.06$ for collagen+fibronectin membranes).

Contradictory results were observed from the data shown in Figures 2 and 3 in which the cell density of NB1-RGB cells on the cast membranes was higher than that on the LB membranes made of the same proteins and the concentration of interferon-β where the cells on the cast membranes was lower than that

on the LB membranes made of the same proteins. It is known that the suppressed growth of hybridoma cells results in enhanced production of antibody (Suzuki and Ollis 1990; Fong et al. 1997). Several growth-suppressing reagents such as interleukin-6, TGF-β, sodium *n*-butyrate (Suzuki and Ollis 1990), potassium acetate (Somenshein and Brawerman 1976; Fong et al. 1997), caffeine, thymidine (Takahashi et al. 1994), cycloheximide, actinomycin D and aspirin (Higuchi et al. 1999) were reported for enhanced production of antibody and antigen. Therefore, the NB1-RGB cells on the LB membranes may suppress growth whilst also leading to the enhanced production of interferon-β compared to cells cultured on the cast membranes prepared with the same proteins.

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 membranes but the production of interferon-β per unit number of cells cultured on the cast and LB membranes is also important for the high production of interferon-β.

Figure 4 shows the production of interferon-β per unit cell by NB1-RGB cells cultured on the cast and LB membranes and a glass plate after 24 h of induction at 37 °C using 1.0×10^5cells cm^{-2} initially.

The highest production of interferon-β per unit cell was observed for the NB1-RGB cells on the collagen-blended membranes with fibronectin and vitronectin. This may be due to the collagen-blended membranes providing a more natural and appropriate environment for NB1-RGB cells to produce interferon-β.

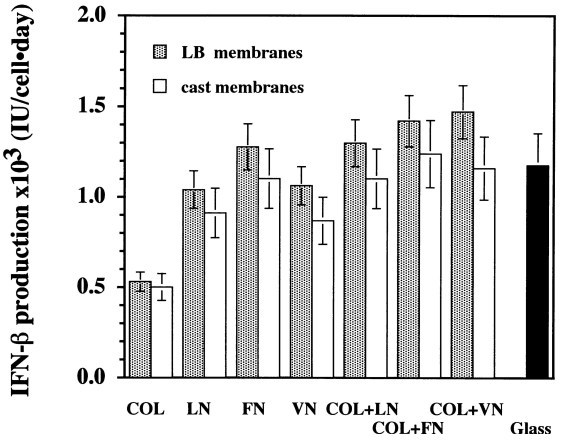

Figure 4. Specific productivity of interferon-β by NB1-RGB cells on representative membranes after 24 h of induction. Data are expressed as the means ±S.D. of four independent measurements.

The production of NB1-RGB cells cultured on the LB membranes was found to be higher than that on the cast membranes made of the same proteins except for collagen membranes (i.e., $p < 0.05$ for laminin, fibronectin, vitronectin, collagen+laminin, collagen+fibronectin and collagen+vitronectin membranes). No significant difference of the production of interferon-β by NB1-RGB cells on LB membranes and cast membranes made of collagen was observed in this study ($p > 0.05$).

Conclusions

The cell density of NB1-RGB cells cultured on the cast membranes was found to be higher than that on the LB membranes made of fibronectin, vitronectin and collagen-blended membranes. This indicates that not only the primary structure of proteins but the preparation methods of the membranes, i.e., casting and LB methods, are also a strong factor affecting cell growth.

The highest concentration of interferon-β in the culture supernatant was found where the cells were cultured on the collagen-blended membranes prepared by the LB method (i.e., COL+LN, COL+FN and COL+VN membranes). However, for the same membrane proteins the cell density on cast membranes was higher than that achieved on the LB membranes whilst the concentration of interferon-β was lower on the cast membranes than on LB membranes. These results appear to result from the suppressed growth of NB1-RGB cells on the LB membranes leading to the enhanced production of interferon-β. The production of interferon-β per unit cell was also found to be higher on the LB membranes than those on the cast membranes made of the same proteins except collagen. The highest production of interferon-β per unit cell was observed for the NB1-RGB cells on the collagen-blended membranes with fibronectin and vitronectin. The collagen-blended membranes appeared to offer a more natural and appropriate environment for NB1-RGB cells to produce interferon-β.

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