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Production of interferon-β by NB1-RGB cells cultured on peptide-lipid membranes

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Abstract

Cell growth and production of interferon-β (IFN-β) were investigated for normal human skin fibroblast cells (NB1-RGB) cultured on membranes prepared from peptide-lipids containing the arginine-glycine-aspartic acid [Arg-Gly-Asp] (RGD), tyrosine-isoleucine-glycine-serine-arginine [Tyr-Ile-Gly-Ser-Arg] (YIGSR) and arginine-glutamic acid-aspartic acid-valine [Arg-Glu-Asp-Val] (REDV) peptides. Cell density was found to be approximately the same on various peptide-lipid membranes, whereas production of IFN-β depended significantly on the peptide-lipid membranes on which NB1-RGB cells were cultured. The highest production of IFN-β was observed for NB1-RGB cells on REDV-lipid membranes prepared by a casting method (REDV-cast membranes) after 24 hr of cultivation. Specific binding between REDV of REDV-cast membranes and the receptor on the NB1-RGB cells may have caused the specific cell response for the production of IFN-β.

Abbreviations: LB – Langmuir-Blodgett, poly I : poly C – polyinosinic and polycytidylic acids, REDV – arginine-glutamic acid-aspartic acid-valine [Arg-Glu-Asp-Val], RGD – arginine-glycine-aspartic acid [Arg-Gly-Asp], YIGSR – tyrosine-isoleucine-glycine-serine-arginine [Tyr-Ile-Gly-Ser-Arg]

Introduction

The interaction between cells and biomaterials for cell culture plays a number of critical roles in regulating the morphology, proliferation, migration and differentiation of cells (Tamada and Ikada 1994; Park and Ito 2000; Ranucci and Moghe 2001; Nuttelman et al. 2001). Optimal communication and binding between the biomaterial surface and cells is one of the most important factors for cell adhesion and cell signaling (Ruoslahti and Pierschbacher 1987; Stedronsky et al. 1994; Bhadriraju and Hansen 2000; Woerly et al. 2001). The nature of cells is known to change and depend on the type of extracellular matrix proteins on which the cells grow (Ruoslahti and Pierschbacher 1987; Stedronsky et al. 1994; Gimond et al. 1996; Mercier et al. 1996; Bhadriraju and Hansen 2000).

Ishihara (1992) synthesized copolymers composed of an arginine-glycine-aspartic acid [Arg-Gly-Asp] (RGD) sequence as the adhesion molecule of cells for an insulinoma cell (MIN6) culture. In their study, MIN6 cells adhering to the poly(*N*-p-vinylbenzyl-D-maltonamide-co-6-(p-vinylbenzamido)-hexanoic acid-g-GRGDS) (p VMA-co-VB-GRGDS))-coated dishes were in a more aggregated form than on other polymer-coated surfaces. By interaction between the cells and the matrix, about 80% greater insulin secretion was observed from MIN6 cells on the p(VMA-co-VB-GRGDS) coated-dishes.

Tyrosine-isoleucine-glycine-serine-arginine [Tyr-Ile-Gly-Ser-Arg] (YIGSR), a peptide of the cell-binding domain sequence derived from laminin that is a major component of basement membranes, binds to both integrin and nonintegrin receptors. Kleinman et

al. (1989) found that cyclic YIGSR peptides were biologically more active than their linear counterparts. The orientation of the YIGSR peptide appeared to influence cell adhesion in his work. Arginine-glutamic acid-aspartic acid-valine [Arg-Glu-Asp-Val] (REDV) derived from fibronectin has been reported to support adhesion of endothelial cells while not allowing adhesion of other vascular or vessel wall-derived cells (Hubbell et al. 1991). Holt et al. (1994) attached a GREDVY sequence to Dacron vascular prosthetic material that was impregnated with polyethylene oxide to render it cell-nonadhesive. The attachment of GREDVY was found to increase endothelial cell adhesion while decreasing fibroblast adhesion in vitro (Holt et al. 1994).

In our previous study (Higuchi et al. 2000a, 2003a, 2003b), cell growth and production of interferon-β (IFN-β) were investigated for fibroblast (NB1-RGD) cells cultured on the Langmuir-Blodgett (LB) (Higuchi et al. 1999, 2000b) and cast membranes prepared from extracellular matrix proteins (e.g., collagen, fibronectin, laminin and vitronectin) (Higuchi et al. 2000a, 2003a). The cell density of the fibroblast 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 (Higuchi et al. 2000a, 2003a). This indicates that not only the primary structure of proteins but the preparation methods of membranes, i.e., casting and LB methods, are also a strong factor affecting cell growth. The concentration and production of IFN-β per cell on the LB membranes were found to be higher than those on the cast membranes made of the same proteins, except collagen (Higuchi et al. 2000a).

This research prompted us to think that cell attachment and cell communication between the cells and the membrane surface to which the cells attach are significantly important for the enhanced production of bioproducts secreted by the anchorage-dependent cells.

IFN-β is a self-defensive protein, an inducer of interferon, 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), (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 this study morphologies of normal human skin fibroblast (NB1-RGB) cells cultured on various peptide-lipid membranes were investigated. A peptide-lipid is a conjugate which has both peptide and lipid parts in one molecule. The cell growth and production of IFN-β were investigated for NB1-RGB cells cultured on the membranes prepared from peptide-lipid containing RGD, YIGSR and REDV peptide. We examined whether the productivity of IFN-β was enhanced by the interaction of cells with various peptides on the membranes where the cells are cultured.

Materials and methods

Synthesis of peptide-lipids

The lipid analogue [H_2N-$(CH_2)_6$-NHCO-$C_6H_3(OC_{12}H_{25})_2$, see Scheme 1] was efficiently synthesized from methyl 3,5-dihydroxybenzoate, in four steps: (i) alkylation with dodecylbromide; (ii) hydrolysis; (iii) condensation with 6-(tetr-butoxycarbamoylamino)hexyamine; and (iv) deprotection using trifluoroacetone (TFA) (Kawakami et al. 2002). The RGD peptide-lipid was synthesized by sequencial coupling of Fmoc amino acids with WSC-HOBt reagents and subsequent deprotection with piperidine in the solution phase. The crude products were purified by the use of LH-20 size exclusion gel chromatography. The final protected peptide-lipid was treated with TFA to remove all the side chain protecting groups. The peptide-lipids containing YIGSR and REDV sequences were also synthesized in a similar way using TFA (Kawakami et al. 2002). The chemical structures of the peptide-lipids are shown in Scheme 1.

Preparation of cast membranes

Peptide-lipids (i.e. RGD-lipid, YIGSR-lipid and REDV-lipid) were dissolved to 10 $\mu g\ ml^{-1}$ in chloroform. Peptide-lipid solutions were cast onto glass plates inserted into flat petri dishes. Peptide-lipid cast membranes (RGD-, YIGSR- and REDV-cast) were prepared by drying at room temperature for 6 d, followed by drying under vacuum at room temperature for 24 hr.

Surface pressure-area isotherm measurement of peptide-lipids

The peptide-lipid solution of 1 $mg\ ml^{-1}$ in chloroform

was spread on the surface of ultrapure water in a LB trough (NL-LB200S-NWC, Nippon Laser & Electrons Lab., Nagoya) to form monolayers at 25 °C. The spread peptide-lipid was compressed at the rate of 10 mm min^{-1}. The surface pressure-area isotherm (π-A isotherm) was recorded by using a Wilhelmy type film balance (Berndt et al. 1995; Pakalns et al. 1999). The limiting area of peptide-lipid molecules was estimated from the measurements of the π-A isotherm curves 3 times.

Preparation of Langmuir-Blodgett membranes

The peptide-lipid solution (1 mg ml^{-1}) was spread onto the surface of ultrapure water in a LB trough to form monolayers at 25 °C.

The monolayer of peptide-lipid was compressed to the surface pressure of 10 mN m^{-1}. The peptide-lipid LB membranes were fabricated by depositing peptide-lipid monolayer onto silicon-coated glass plates by a horizontal lifting method. The peptide-lipid LB membranes (RGD-, YIGSR-, REDV-LB) were dried at 4 °C for 24 hr and were subsequently dried under vacuum at room temperature for 24 hr.

Cell lines

The fibroblast cells, NB1-RGB (normal human skin), obtained from the 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. Tokyo), 3.5 mg l^{-1} benzylpenicillin potassium (Wako Pure Chemical Industry, Ltd. Tokyo) 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(Higuchi et al. 1999, 2000a, 2000b, 2003a, 2003b).

Cell growth and production of IFN-β on various membranes

After UV irradiation (30 cm distance, 10 W, GL10, Stanley Co., Tokyo) of the peptide-lipid membranes for 20 min for sterilization, the membranes were inserted into 24-well tissue culture plates fitted with a lid (Iwaki Glass, Tokyo, well diameter = 16 mm). The non-specific binding sites of the membranes were blocked with 1 % bovine serum albumin of pH 7.4 at 37 °C for 2 hr and were subsequently rinsed with PBS 3 times.

Cells in suspension (cell densities $1.0\times10^5 ml^{-1}$ and 2 ml of medium) were inoculated into tissue culture plates containing serum-supplemented (10 % FBS) medium (MEM-α) and were incubated in the CO_2 incubator in 5 % CO_2 atmosphere for 18 hr at 37 °C. After 18 hr of incubation, the medium was changed with 2 ml of 2 % FBS/MEM-α containing 10 μg ml^{-1} of poly I : poly C ($S_{20,w}$=15, Yamasa Corp., Chiba) 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 hr (induction phase). After removing the medium, the cells were rinsed twice with phosphate buffered saline (PBS) pH 7.4 and then cultured for 24 hr in 2 % FBS/MEM-α (production phase).

The sampled medium was used for estimation of the concentration of IFN-β. The concentration of IFN-β was measured by the enzyme linked immunosorbent assay (ELISA) using a Human IFN-β ELISA kit (Fujirebio, Inc., Tokyo) (Higuchi et al. 1999, 2000a, 2000b, 2003a, 2003b).

The cell number was estimated by observation of the cells on the peptide-lipid 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 peptide-lipid membranes. These procedures were performed on each membrane using four independent membranes prepared from the same peptide-lipid (total, n=16), and the cell number was finally averaged to obtain reliable data.

Results and discussion

Surface pressure-area isotherms of peptide-lipids

The surface pressure-area isotherms of peptide-lipids (i.e., RGD-lipid, YIGSR-lipid and REDV-lipid) were measured to estimate the limiting area of the peptide lipid molecules. Figure 1 shows the surface pressure-area isotherms of each peptide lipid. When the surface membrane is compressed on water, the surface pres-

sure increases sharply in the area of 50–200 $Å^2$ molecule^{-1}, depending on the peptide-lipid molecules. With further compression, the monolayer membranes of RGD-lipid, YIGSR-lipid and REDV-lipid suddenly begin to collapse at 70, 22 and 12 $Å^2$ molecule^{-1}, respectively, and the surface pressure drops with the decrease in surface area. This indicates that the peptide-lipid LB membranes are very stiff films, and the solid LB membranes break into several membrane fragments at the broken surface area.

The limiting area was obtained by extrapolating the steepest line of the surface pressure-area isotherms to zero surface pressure. The limiting area of REDV-lipid, RGD-lipid and YIGSR-lipid was estimated to be 68, 122 and 163 $Å^2$ molecule^{-1}, respectively.

Several peptide-lipids that have two long alkyl tails have been reported (Berndt et al. 1995; Pakalns et al. 1999; Winger and Chaikof 1997; Abe et al. 1995). The shape of their pressure-area isotherms was reported to depend on the structure of both peptide and lipid portions. At low surface pressure, the isotherm depended more on the peptide sequence. At higher surface pressure, the peptide portion became tightly packed and the surface area approached a common value of 40–50 $Å^2$ molecule^{-1}. This value was contributed by the lipid portion. The general trends of previous works (Berndt et al. 1995; Pakalns et al. 1999; Winger and Chaikof 1997; Abe et al. 1995) also hold in our results. Since the N-terminal amine is free and the net charges of REDV, RGD and YIGSR on the peptide-lipids are 0, +1 and +2, respectively (see scheme 1), the surface area becomes larger, in this order, at low surface pressure. It seems that the electrostatic repulsion, rather than the amino acid sequence or the sequence length, is the main determinant for the limiting area of peptide-lipids. At surface pressure higher than about 50 mN m^{-1}, the surface area of REDV and YIGSR peptide-lipids approaches the common value, but the change is rather drastic compared with other reported peptide-lipids, and in the case of RGD peptide-lipid the collapse comes before the surface area approaches the limiting area of the lipid portion. This unique feature may arise from our lipid structure. The lipid part is stiff and cannot accommodate the packing of the peptide portion of our peptide-lipid at high surface pressures. At the air-water interface, it is expected that the peptide portion is oriented toward the water and the lipid portion is toward the air, based on the hydrophilicity-hydrophobicity of peptide-lipid. Thus, the LB method must provide the peptide-presenting surface of the peptide-lipid LB membranes. Using a similar method of casting, we have shown that the peptide portion was presented on a hydrophobic surface by specific interaction between a peptide and its antibody (Kawakami et al. 2002). Therefore, the molecular axis of the peptide-lipid is assumed to be not parallel but perpendicular to the water surface, which should be tilted to some degree. This indicates that the cell-signaling peptides (i.e., RGD, YIGSR and REDV) appear on the surface of the peptide-lipid LB membranes.

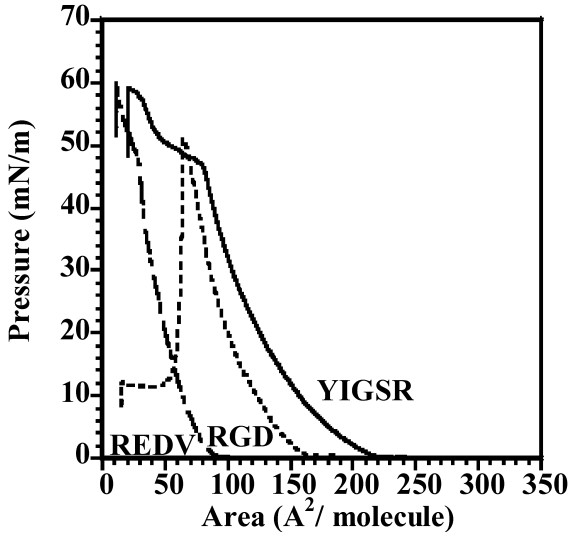

Figure 1. Surface pressure-area isotherms of peptide-lipids (RGD-, YIGSR- and REDV-lipids).

Cell growth and morphology on the peptide-lipid membranes

NB1-RGB cells were cultured on peptide-lipid membranes prepared from RGD-lipid, YIGSR-lipid, and REDV-lipid.

Cell growth and morphology, which are indexes of cell behavior and function, were investigated by phase-contrast micrographs of NB1-RGB cells on the cast and LB membranes prepared using the peptide-lipids. Figure 2 shows photomicrographs of NB1-RGB cells after 24 hr of culture on the peptide-lipid cast and LB membranes.

We found that NB1-RGB cells adhered very well on any peptide-lipid membrane. No morphological difference was observed for the cells cultured on the peptide-lipid cast and LB membranes in this study.

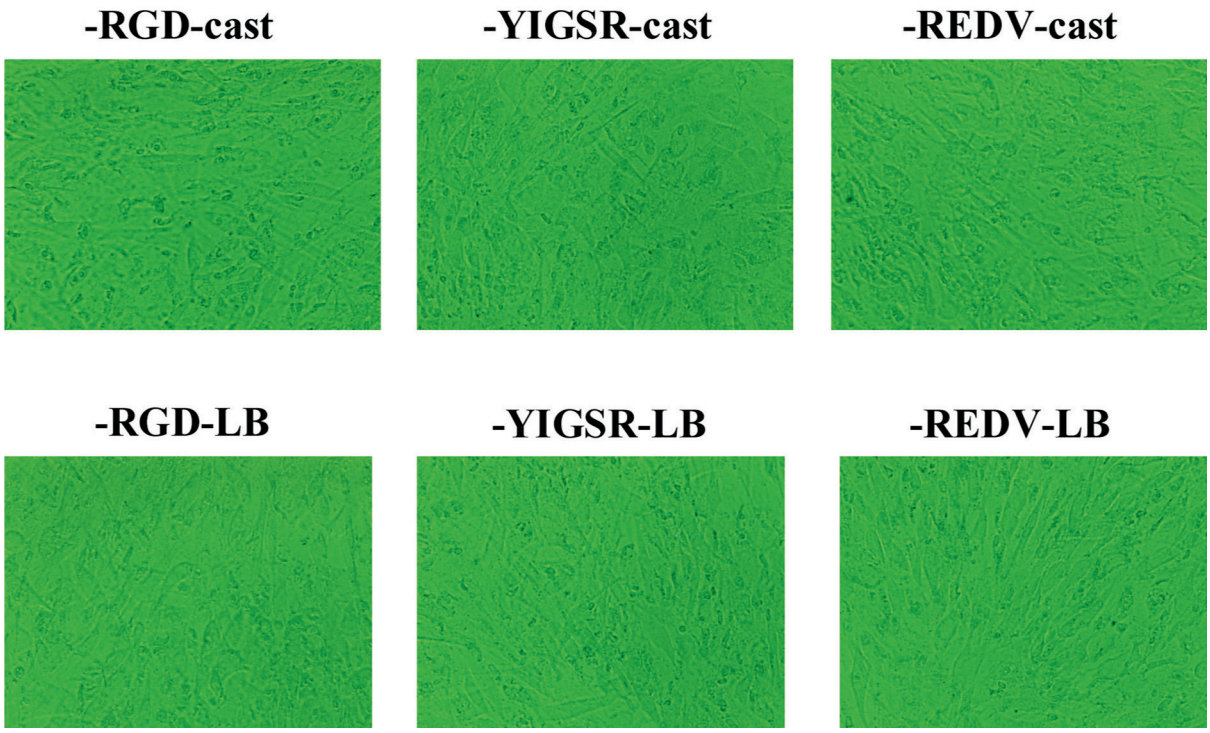

Figure 2. Micrographs of NB1-RGB cells cultures on peptide-lipid membranes after 24 hr of incubation in MEM-α medium containing 10 % FBS using 1.0×10^5 cells cm^{-2} initially.

Cell growth on the peptide-lipid membranes

We investigated the effect of the peptide-lipid membranes on the NB1-RGB cell density. Figure 3 shows the cell density after 24 hr of induction of NB1-RGB cells cultured on peptide-lipid cast membranes (RGD-cast, YIGSR-cast and REDV-cast), peptide-lipid LB membranes (RGD-LB, YIGSR-LB and REDV-LB), as well as on glass plates. The density of NB1-RGB cells on RGD-lipid, YIGSR-lipid and REDV-lipid membranes and a glass plate was found to be very similar, and no significant effect of peptide-lipids on cell density was observed.

IFN-β production on peptide-lipid membranes

Production of IFN-β was investigated for NB1-RGB cells cultured on the peptide-lipid membranes and a glass plate. IFN-β levels released into cell-growth media were further examined to determine the effect of NB1-RGB cell binding of the specific binding site of ECM proteins on IFN-β production. Figure 4 shows the concentration of IFN-β in the media where NB1-RGB cells were cultured, after 24 hr of induc-

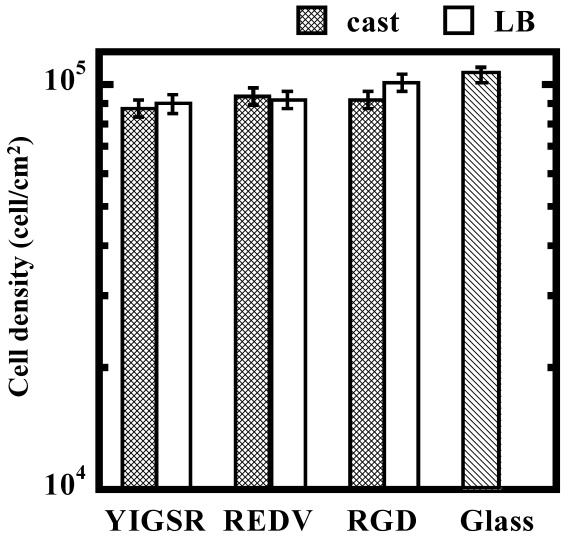

Figure 3. Cell density of NB1-RGB cells cultured on cast and LB membranes containing various peptide-lipid as well as on glass plates after 24 hr of induction. Data are expressed as mean ± S.D. of four independent measurements.

tion. The highest concentration of IFN-β in the medium was observed where NB1-RGB cells were cultured on the REDV-cast membranes (i.e., the concentration of IFN-β in the medium where NB1-RGB cells cultured on REDV membranes vs. that on RGD-cast, RGD-LB, YIGSR-cast, YIGSR-LB and REDV-LB membranes). The concentration, 150 IU/ml, in the medium where NB1-RGB cells on REDV-cast membranes is also higher than that where NB1-RGB cells cultured on membranes prepared using silk fibroin, poly(γ-methyl-L-glutamate), poly(γ-benzyl-L-glutamate) and extracellular matrix proteins (e.g., collagen, fibronectin, laminin and vitronectin) as reported in our previous studies (Higuchi et al. 2000a, 2003a). These results indicate that the NB1-RGB cells on the peptide membranes containing the cell-binding peptide REDV produce a higher amount of IFN-β when the peptide-lipid membranes are prepared by the casting method. In contrast, the lowest concentration of IFN-β in the medium was found in the media where the cells were cultured on YIGSR-LB membranes.

The concentration of secreted bioproducts by cells in the media was directly related to the cell density (number) and the quantity of bioproducts produced per unit number of cells. Therefore, not only the cell density on the membranes but also the IFN-β produced per cell cultured on the membranes was an important factor for high production of IFN-β.

Figure 5 shows the production of IFN-β per cell by NB1-RGB cells cultured on the peptide-lipid membranes and a glass plate after 24 hr of induction. The cell densities of NB1-RGB cells on RGD-lipid, YIGSR-lipid and REDV-lipid membranes, and a glass plate were found to be approximately the same, while the production of IFN-β per cell on peptide-lipid LB membranes was slightly lower than on peptide-lipid cast membranes. The highest production of IFN-β per cell was observed for NB1-RGB cells on the REDV-cast membranes. It was found that cells on the peptide-lipid membranes containing REDV produced higher amounts of IFN-β per cell when the membranes were prepared by the casting method.

The reduced production of IFN-β by NB1-RGB cells on the peptide-lipid LB membranes compared to that on the peptide-lipid cast membranes can be explained by the high density of peptide on the surface of the peptide-lipid LB membranes in this study. This high density of peptide gives rise to a lack of flexibility in peptide mobility due to the compact packing of peptide in the peptide-lipid LB membranes from the surface pressure isotherms of peptide-lipid as shown in Figure 1.

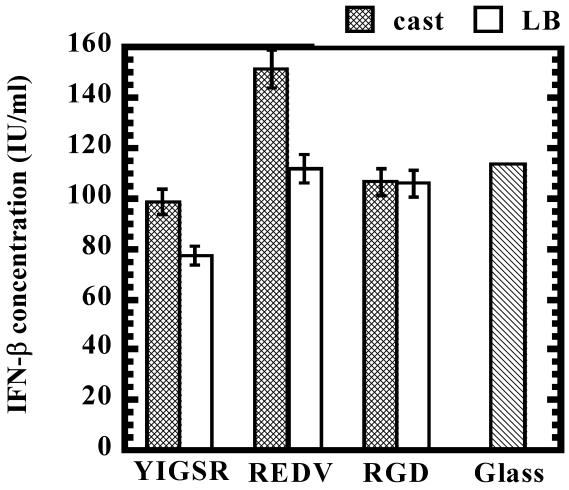

Figure 4. IFN-β concentration by NB1-RGB cells cultured on various peptide-lipid membranes and glass plates after 24 hr of induction. Data are expressed as mean ± S.D. of four independent measurements.

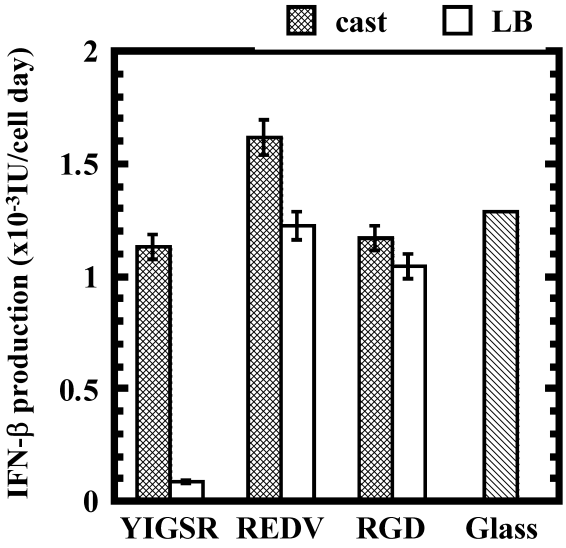

Figure 5. IFN-β production per cell by NB1-RGB cells cultured on various peptide-lipid membranes and glass plates after 24 hr of induction. Data are expressed as mean ± S.D. of four independent measurements.

Conclusion

The cell growth and production of IFN-β were investigated for normal human skin fibroblast cells

(NB1-RGB) cultured on LB and cast membranes prepared from peptide-lipid containing the RGD, YIGSR and REDV peptides. The limiting area of REDV-lipid, RGD-lipid and YIGSR-lipid was estimated from the surface pressure-area isotherms to be 68, 122 and 163 $Å^2$ molecule^{-1}, respectively. The size of limiting area was determined by the electrostatic repulsion, rather than the amino acid sequence or the sequence length, because the net charges of REDV, RGD and YIGSR on the peptide-lipids were 0, +1 and +2, respectively. From the relationship of hydrophilicity and hydrophobicity in the peptide-lipid and hydrophobic silicon-coated glass plate, it is assumed that the peptide portion of the peptide-lipid LB membranes is not directed toward the glass surface but toward the cell surface.

The cell density was found to be approximately the same on various peptide-lipid membranes prepared from casting and LB methods, although the production of IFN-β depended significantly on the peptide-lipid membranes where the NB1-RGB cells were cultured. The highest production of IFN-β was observed for the NB1-RGB cells on the REDV-lipid membranes prepared by a casting method (REDV-cast membranes) after 24 hr of cultivation. The specific binding between the REDV on the REDV-cast membranes and the receptor on the NB1-RGB cells may have caused the specific cell response for the production of IFN-β. A reduced production of IFN-β by NB1-RGB cells on the peptide-lipid LB membranes was found in this study when compared with that observed for cells on the peptide-lipid cast membranes. This can be explained by the high density and compact packing of peptide on the surface of the peptide-lipid LB membranes. The present study suggests that the flexible mobility of the cell binding domain sequence of peptides is also an important factor for cell signaling resulting from the interaction between cells and biomaterials containing the cell binding domain sequence of peptides.

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