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Liquid-Phase Synthesis and Cell Attachment Activity of Bioactive Pentapeptide YIGSR

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ABSTRACT: Pentapeptide Tyr-Ile-Gly-Ser-Arg (YIGSR), an amino acid sequence existing in the cell attachment domain of the B1 chain of laminin, was synthesized by an improved liquid-phase procedure. Bioactivity of YIGSR as cell recognition determinant was investigated using L-929 fibroblast cell and A431 epidermoid cell. The YIGSR pentapeptide was immobilized to ethylene–acrylic acid copolymer (PEA) film, and the number of cells attached to the immobilized film was counted. In addition, the cell-attachment activity of YIGSR toward the cells was also evaluated by the cell inhibition test. These two tests led to a conclusion that YIGSR acts as a cell determinant toward L-929 and A431 cells.

KEY WORDS Tyr-Ile-Gly-Ser-Arg (YIGSR) Pentapeptide / Liquid-Phase Peptide Synthesis / Cell Attachment Activity / YIGSR-Immobilized Ethylene–Acrylic Acid Copolymer / L-929 Fibroblast Cell / A431 Epidermoid Cell /

Laminin, the major non-collagenous glycoprotein distributing only in the basement membrane, an extracellular matrix between epithelial cell and stroma, has various biological activities, such as adhesion, migration, differentiation, and growth, of cell.[1,2] Laminin is composed of three chains designated as A, B1, and B2, which are arranged in a cross-shaped structure.[3] These three chains of laminin have been cloned and sequenced, and several active sites in the molecule have been identified.[4-6] Iwamoto *et al.*[7] and Graf *et al.*[8,9] suggested that the peptide sequence Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg (CDPGYIGSR, in one letter amino acid code) in the B1 chain concerns with cell-attachment activity, and, in particular, pentapeptide sequence YIGSR plays important role for cell-attachment, cell-spreading and chemotaxis. In addition, Iwamoto and co-workers[7] have demonstrated that the synthetic pentapeptide YIGSR inhibited the formation of metastasis by malignant melanoma cells. In 1989, Murata and co-workers[10] reported that synthetic sequential polypeptide poly(YIGSR) drastically inhibited the metastatic formation of B16–BL16 melanoma cells.

The aim of this work is to establish a method to synthesize YIGSR pentapeptide in satisfactory yields by an improved liquid-phase procedure, and to elucidate the cell-attachment activity of the pentapeptide and of pentapeptide-immobilized polymer films. The reason why we use the liquid-phase procedure for peptide synthesis is that we can obtain much more amounts of pentapeptide

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in one cycle compared with the use of automatic peptide synthesizer. The polymer used for immobilization is an ethylene-acrylic acid copolymer (PEA) containing 8.9 mol% (20 wt%) acrylic acid. The cells used are fibroblast L-929 cell originating in mouse, and epidermoid A431 cell originating in human.

EXPERIMENTAL

Materials

t-Butoxycarbonyl-L-tyrosine (Boc-Tyr), *t*-butoxycarbonyl-*O*-benzyl-L-serine (Boc-Ser(Bzl)), glycine methyl ester hydrochloride (Gly-OMe·HCl), L-isoleucine methyl ester (Ile-OMe·HCl), were purchased from Kokusan Chemical Works, Ltd., and *t*-butoxycarbonyl-N^G-tosyl-L-arginine (Boc-Arg(Tos)) was from Peptide Institute, Inc. Dicyclohexylcarbodiimide (DCC) used as a dehydration agent, and *N*-hydroxysuccinimide (HOSu) were purchased from Peptide Institute, Inc. Triethylamine (TEA) used as a neutralization agent, and hydroxybenzotriazole (HOBt) were purchased from Katayama Chemicals, and Kokusan Chemical Works, respectively. Trifluoromethanesulfonic acid (TFMSA) (Kanto Chemicals), and trifluoroacetic acid (TFA) (Peptide Institute, Inc.) were used as deblocking agents. Laminin sample used for coating of cell culture dishes was purchased from Iwaki Glass, Co. All the solvents and chemicals used were of reagent grade.

Synthesis of Tyr-Ile-Gly-Ser-Arg (YIGSR) by Liquid-Phase Procedure

N^G-Tosyl-L-arginine methyl ester (NH_2-Arg(Tos)-OMe) was synthesized[11] from Boc-Arg(Tos)-OH, and Boc-Tyr-OSu[12] was from Boc-Tyr-OH. Scheme of synthesis of YIGSR by liquid-phase procedure is shown in Figure 1. Fragment condensation method was used. First, Boc-Tyr-OSu and NH_2-Ile-OMe were coupled by *N*-succinimide procedure, secondly, Boc-Tyr-Ile-$NHNH_2$ was coupled with NH_2-Gly-OMe by azide procedure. On one hand, Boc-Ser(Bzl) was condensed with NH_2-Arg-(Tos)-OMe by DCC procedure. Finally, Boc-Tyr-Ile-Gly-$NHNH_2$ was coupled with NH_2-Ser(Bzl)-Arg(Tos)-OMe by azide procedure, and the product obtained was deblocked by saponification and TFMSA to obtain H-Tyr-Ile-Gly-Ser-Arg-OH.

Step 1: To 3.48 g (1.9×10^{-2} mol) NH_2-Ile-OMe·HCl dissolved in 35 ml tetrahydrofuran (THF), equimolar TEA was added. To the

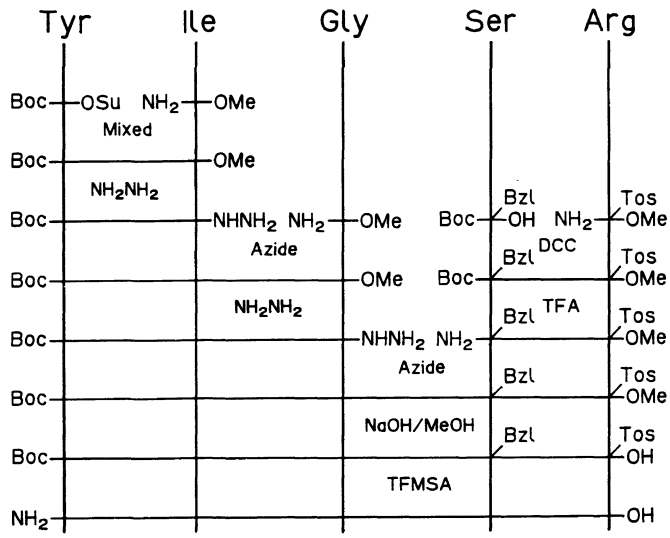

Figure 1. Scheme of Tyr-Ile-Gly-Ser-Arg synthesis by liquid phase-procedure.

solution obtained, 6.05 g (1.6×10^{-2} mol) Boc-Tyr-OSu was added, and then the mixture kept alkaline by TEA was stirred at 4°C for 24 h. After 24 h, the solution was filtered and the filtrate was evaporated in high vacuum. The resulting precipitate was extracted with ethyl acetate, and the extract was washed with 5% citric acid and 5% sodium hydrogencarbonate ($NaHCO_3$). Purity of the product was checked by using thin-layer chromatography (TLC; Merck) ($CHCl_3 : MeOH : H_2O = 8 : 3 : 1$). The extract was further washed with NaCl-saturated water, dried over Na_2SO_4 and then evaporated. Boc-Tyr-Ile-OMe obtained was dissolved in small amounts of MeOH, to which, 5 times the molar quantity of hydrazine hydrate was added, and the solution was kept at room temperature overnight. The gelatinous mass formed was collected by filtration and the filtrate was evaporated. The resulting solid was recrystallized from acetonitrile, and vacuum dried. Thus, Boc-Tyr-Ile-$NHNH_2$ (3.92 g; 0.96×10^{-2} mol) was obtained.

Step 2: At a low temperature cooled down with ice and NaCl, 4*N* HCl-dioxane (5.8 ml) and isoamylnitrile (1.3 ml) were added successively to a solution of 3.92 g (0.96×10^{-2} mol) Boc-Tyr-Ile-$NHNH_2$ in 30 ml dimethylformamide (DMF), and the mixture was stirred for 30 min, where the hydrazine test was carried out. For hydrazine test, 1% $FeCl_3$/2M acetic acid and 1% K_3 [$Fe(CN)_6$] aqueous solution mixture (1 : 1) was used as a color-producing reagent. The solution was neutralized with TEA. 1.44 g (1.2×10^{-2} mol) NH_2-Gly-OMe·HCl was dissolved in 20 ml DMF, to which equimolar TEA was added. Now, the Boc-Tyr-Ile-$NHNH_2$ solution was mixed with the NH_2-Gly-OMe·HCl solution, and stirred at 0°C for 24 h, whereby, the pH of the mixture was kept alkaline. The mixture was neutralized with acetic acid, filtered and then the filtrate was evaporated in high vacuum. The resulting precipitate was extracted with ethyl acetate, and the extract was successively washed with 5% citric acid and 5% $NaHCO_3$ solution. Purity of the product was checked by TLC. Further, the extract was washed with NaCl-saturated water, dried over Na_2SO_4, and then evaporated. Boc-Tyr-Ile-Gly-OMe (2.45 g) obtained was dissolved in a small amount of MeOH, to which 5 times the molar quantity of hydrazine hydrate was added, and the solution was kept at room temperature overnight. The gelatinous mass formed was collected by filtration, and the filtrate was evaporated. The resulting solid was recrystallized from acetonitrile and vacuum dried. Thus, Boc-Tyr-Ile-Gly-$NHNH_2$ (2.14 g; 0.46×10^{-2} mol) was obtained.

Step 3: 2.95 g (1.0×10^{-2} mol) Boc-Ser(Bzl)-OH dissolved in 50 ml THF was mixed with 1.2 times the molar quantity of DCC and HOBt under stirring in an ice bath for 30 min. 4.53 g (1.2×10^{-2} mol) NH_2-Arg(Tos)-OMe·HCl was dissolved in 40 ml DMF and equimolar TEA was added. The Boc-Ser(Bzl)-OH solution was mixed with the NH_2-Arg(Tos)-OMe·HCl solution in a manner as mentioned above, and stirred at 4°C for 24 h. After 24 h, the Boc-Ser(Bzl)-Arg(Tos)-OMe obtained was purified by the same method mentioned in step 2. The resulting solid was dissolved in 5 ml anisole. TFA (10 ml) was added to remove Boc group at a low temperature cooled down in ice bath for 60 min. The solvent was evaporated, and the resulting solid was recrystallized from diethylether and vacuum dried. Thus, solid NH_2-Ser(Bzl)-Arg(Tos)-OMe (2.80 g; 0.54×10^{-2} mol) was obtained.

Step 4: At a low temperature cooled with ice and NaCl, 4*N* HCl-dioxane (2.8 ml) and isoamylnitrile (0.7 ml) were added successively to a solution of 2.14 g (0.46×10^{-2} mol) Boc-Tyr-Ile-Gly-$NHNH_2$ in 30 ml DMF, and the mixture was stirred for 30 min, while the hydrazine test was carried out. The solution was neutralized with TEA. 2.80 g (0.54×10^{-2} mol) NH_2-Ser(Bzl)-Arg(Tos)-OMe was dissolved in 20 ml DMF, to which equimolar TEA

was added. The Boc-Tyr-Ile-Gly-$NHNH_2$ solution was mixed with the NH_2-Ser(Bzl)-Arg(Tos)-OMe solution and stirred at 4°C for 24 h, whereby, the pH of the mixture was kept alkaline. The mixture was neutralized with acetic acid, and filtered then the filtrate was evaporated in high vacuum. The Boc-Tyr-Ile-Gly-Ser(Bzl)-Arg(Tos)-OMe was purified by the same method mentioned in step 2. The resulting solid was recrystallized three times from diethylether, purified by column chromatography, and vacuum dried. The protecting group at the C-terminal of Boc-Tyr-Ile-Gly-Ser(Bzl)-Arg(Tos)-OMe (2.64 g) was removed by saponification with 1*N*-NaOH/MeOH in an ice-bath for 4 h. The Boc-Tyr-Ile-Gly-Ser(Bzl)-Arg(Tos)-OH obtained was dissolved in a mixture, thioanisole (6.7 ml)/1,2-ethanedithiol (3.3 ml)/*m*-cresol (0.8 ml)/TFA (37.8 ml), at room temperature, to which TFMSA (5.0 ml) was added in an ice-bath for 1 h, and kept for 1 h at room temperature.[13] Diethylether was added in reaction system, and then the precipitate was filtrated, and washed with diethylether. The deblocked H-Tyr-Ile-Gly-Ser-Arg-OH (YIGSR) was dissolved in a small amount of H_2O and treated with CM-Sephadex C-25 ion exchange column chromatography (ammonium solution form; Pharmacia Fine Chemicals). The quantity of pentapeptide H-Tyr-Ile-Gly-Ser-Arg-OH finally obtained was 1.10 g. The eluates were lyophilized and dried over P_2O_5 in vacuum.

Immobilization of YIGSR onto Ethylene–Acrylic Acid Copolymer Film

The schema of surface activation of ethylene–acrylic acid copolymer (PEA) and oligopeptide immobilization are shown in Figure 2. The ethylene–acrylic acid copolymer pellet (acrylic acid content: 20 wt%, lot. No. EAA-A510W) contributed from Teijin Co. Ltd. was purified in a methanol Soxhlet extractor system. PEA films were prepared by casting from 5 wt% THF solution on glass plates. Finally the films were dried *in vacuo*

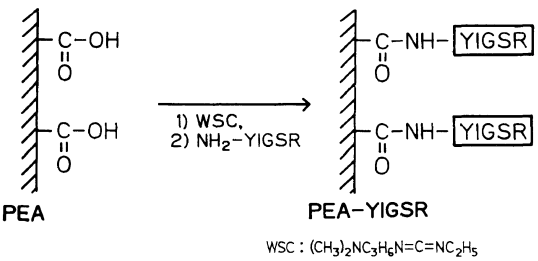

Figure 2. Scheme of oligopeptide immobilization to PEA films.

for 5 days at 40°C. The –COOH residues locating on the PEA film surface were activated at 4°C for 30 min with 4 g water-soluble carbodiimide, (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide), dissolved in 400 ml phosphate buffer solution (PBS). Then 80 mg YIGSR pentapeptide was dissolved in 80 ml PBS and reacted with the activated PEA films for 24 h at 4°C. The immobilized films were rinsed with PBS and dodecyl sulfate sodium salt solution (SDS) by ultrasonics cleaner and washed with pure water for 72 h. The immobilization condition mentioned above was obtained by examining the relation between the amount of YIGSR immobilized and activation time by WSC and reaction time with YIGSR, by means of ESCA measurements. The surface characterization of the YIGSR-immobilized films was carried out by means of C1s and N1s spectra measured with a Shimadzu 750 electron spectroscopy for chemical analysis (ESCA) spectrometer using Mg-$K_{\alpha 1,2}$ exciting radiation. The surface composition of the immobilized film was tested by the N/C ratio estimated from ESCA. From the N/C ratio obtained, it was confirmed that the YIGSR peptide was really immobilized onto PEA film surface. For cell-attachment tests, the films were stirred in 70% ethanol for overnight, and ethanol was displaced with PBS for 1 day. The test films were placed in 24-wells cell culture dish (FALCON).

Cell Attachment Activity Tests for YIGSR

A431 epidermoid carcinoma cells and L-929

fibroblast cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Nissui Seiyaku), and Eagle's MEM (Nissui Seiyaku), both containing antibiotics Kanamycin (60 $mg l^{-1}$) and 10% fetal bovine serum (FBS: M.A. Bioproducts, Maryland, U.S.A.), respectively. Cell lines used were A431[14] and L-929[15,16] cells (Riken Gene Bank, Tsukuba, Japan). A431 and L-929 were trypsinized from culture dish, washed once in a medium containing 10% FBS and once in a serum-free medium. The cell density was adjusted to 1.88×10^5 cells ml^{-1} for cell adhesion tests in serum-free medium. 1 ml cell suspensions were added to each of the polymer films placed in 24-wells in a cell culture dish and kept for predetermined periods in a humidified incubator conditioned to 37°C, 5% CO_2 and 95% air atmosphere. After incubation, the unattached cells were removed from the film surface by washing with PBS. Adhered cells were fixed by using glutaraldehyde, stained by Giemsa, and by glycerin. The number of cells attached to the films was counted.

For cell inhibition test, the starting cell density was adjusted to 1.88×10^5 cells ml^{-1} in a serum-free medium. 1 ml cell suspensions were added to each well, together with YIGSR pentapeptide of different concentrations, and kept in a humidified incubator conditioned to 37°C, 5% CO_2 and 95% air atmosphere for 1 h. Besides, 1 ml cell suspensions containing 2 mg of YIGSR pentapeptide were placed in 24-wells cell culture dishes, and kept for predetermined periods at the same conditions as mentioned above. The number of cells attached to the culture dish was counted as a function of incubation time. The cell culture dishes used for cell inhibition test were coated with laminin by adsorption method.

RESULTS AND DISCUSSION

Pentapeptide Synthesis

We have examined condensations of Boc-Tyr with Ile-OMe, and of Boc-Tyr-Ile-OMe with Gly-OMe by DCC and active ester procedures, and found that the latter procedure is superior with regard to purity, purification procedure, and yield. Thus, we carried out the condensation of Boc-Tyr-OSu with NH_2-Ile-OMe by *N*-succinimide procedure. Further, Boc-Tyr-Ile-$NHNH_2$ derived from Boc-Tyr-Ile-OMe was condensed with Gly-OMe by azide procedure, not by DCC procedure, because of the same reason mentions above. By the liquid-phase procedure mentioned above, we succeeded in to synthesis YIGSR of the order of gram. However, if we use peptides synthesizer, pentapeptide obtained would be about 100 mg order in one cycle, and may contain impurities. The YIGSR obtained was hydrolyzed with 6*N*-HCl for 24 h at 110°C, and the product was being assayed by amino acid analysis. The elemental analyses were carried out with Boc-Tyr-Ile-Gly-Ser(Bzl)-Arg(Tos)-OMe and H-Tyr-Ile-Gly-Ser-Arg-OH. The results of amino acid analysis and of elemental analysis on YIGSR synthesized were given in Table I. The result of elemental analysis of protected YIGSR suggests that the

Table I. Amino acid analysis and elemental analysis of YIGSR pentapeptide

	Amino acid analysis	
	Count/nmol	Ratio
Tyr	18.8	1.00
Ile	19.4	1.03
Gly	19.9	1.06
Ser	18.0	0.96
Arg	20.3	1.08

	Protected YIGSR		Deblocked YIGSR	
	Elemental analysis/%		Elemental analysis/%	
	Calcd	Exptl	Calcd	Exptl
C	57.9	56.8	52.5	51.2
H	6.7	6.5	7.1	7.2
N	11.8	11.5	18.8	18.0

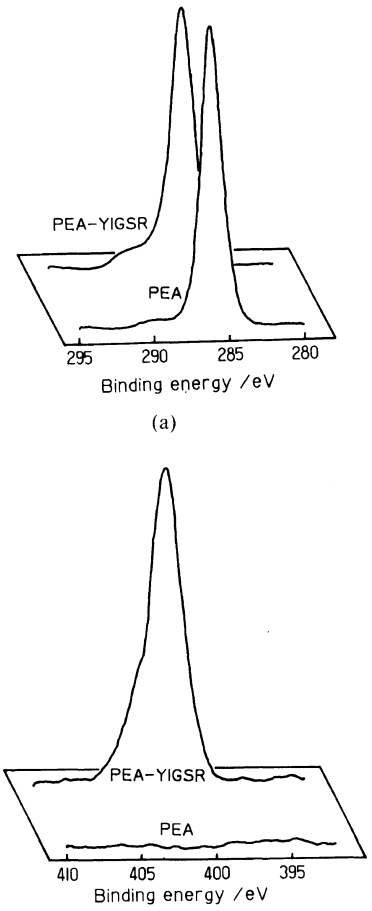

Figure 3. ESCA spectra of PEA and PEA-YIGSR films. (a), C1s spectra; (b), N1s spectra.

sample tested may contain 0.5 mol H_2O per mole pentapeptide as the water of crystallization, and deblocked YIGSR may contain 1 mol H_2O per mole pentapeptide. If we take into consideration the water of crystallization, the experimental values agree with the theoretical values. The results obtained from amino acid analysis and elemental analysis indicate that the YIGSR pentapeptide was really synthesized.

Characterization of YIGSR-Immobilized PEA-Film

Figures 3(a) and 3(b) illustrate C1s and N1s ESCA spectra, respectively, for PEA and PEA-YIGSR. Obviously, C1s of the PEA-YIGSR film exhibits a shoulder at 289—291 eV, characteristic to carbon atom of the amid bound. The N1s spectrum of PEA-YIGSR is remarkable, in contrast to the absence of peak for PEA. N/C ratios of PEA and PEA-YIGSR film estimated from ESCA spectra were shown in Table II. As mentioned earlier, the PEA sample is a copolymer composed of 91.1 mol% ethylene and 8.9 mol% acrylic acid. If we assume that the surface composition is almost the same as the bulk composition, then the elemental ratio O/C(%) of PEA surface is calculated as 8.0(%). Comparing this value with the experimental value 7.7(%), we may conclude that the surface composition is almost the same as the bulk composition.

Table II. Surface composition of YIGSR-immobilized PEA film compared to PEA film

Designation	Elemental ratio/%	
	O/C	N/C
PEA	7.7	0
PEA-YIGSR	13.2	5.0

On the other hand, for PEA-YIGSR, the experimental values are: O/C = 13.2% and N/C = 5.0%. If we designate the mol% immobilization of YIGSR to the whole film surface as x, then the experimental values O/C = 13.2% and N/C = 5.0 led to $x = ca.$ 3% and $x = ca.$ 2%, respectively. Namely, YIGSR pentapeptide is immobilized to about 2.5% of the film surface. This figure indicates that 28 mol% of the whole acrylic acid units is reacted with YIGSR.

Cell Attachment Activity of YIGSR-Immobilized PEA Film

Figures 4 and 5, illustrate the % cell attachment to PEA and PEA-YIGSR films for A431 and L-929, respectively, at the concentration of 1.0×10^5 cells/cm^2. The error bars represent

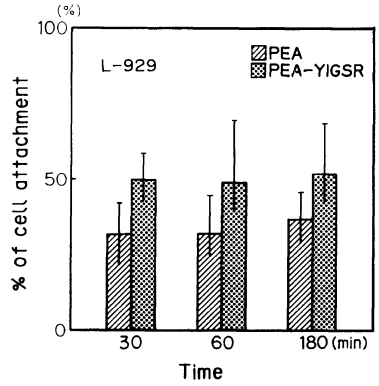

Figure 4. Percent of L-929 cells adhered onto PEA and PEA-YIGSR films, in the absence of serum.

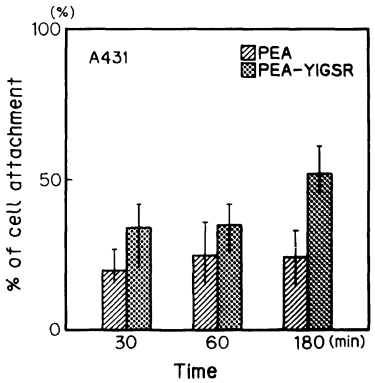

Figure 5. Percent of A431 cells adhered onto PEA and PEA-YIGSR films, in the absence of serum.

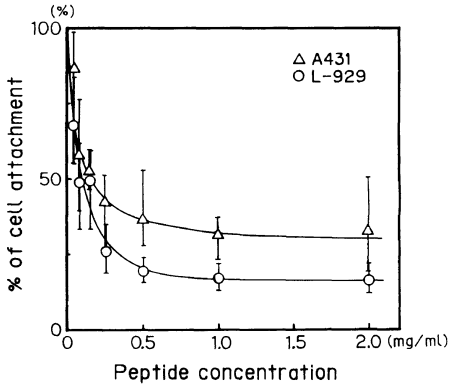

Figure 6. Inhibition effects of YIGSR pentapeptide toward L-929 and A431 as functions of YIGSR concentration. Incubation time, 1 h.

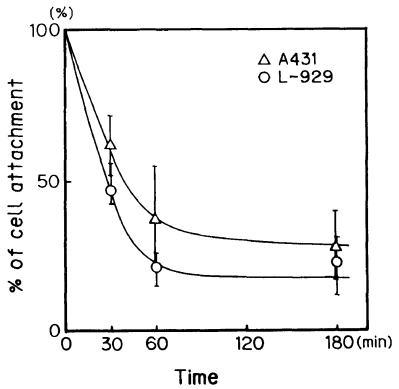

Figure 7. Inhibition effects of YIGSR pentapeptide toward L-929 and A431 cells as functions of time. YIGSR concentration, 2 mg ml^{-1}.

the standard errors for five different experiments ($N=5$), each containing 20 measuring points. As a whole, numerical values of % cell attachment are rather uniform, and the number of points showing the maximum and minimum values of error bar are only 10%. So the results are regarded as significant. For L-929 cell, about 50% of the cell was adhered to PEA-YIGSR, almost independent of incubation time (30—180 min), compared with the figure 30% to PEA. For A431 cell, % cell adhesion to both PEA-YIGSR and PEA were lower than those for L-929, and somewhat depended on the incubation time, and at 180 min, the % of cell adhesion to PEA-YIGSR (*ca.* 50%) was about twice that to PEA. Such rather low values of % cell attachment for PEA-YIGSR may originate from that the degree of YIGSR immobilization is low as 2.5 mol% of the film surface.

Inhibition Effect of YIGSR Pentapeptide for L-929 and A431 Cells

More distinct means to evaluate the interactions between the pentapeptide and cells is the cell inhibition test. In Figure 6, the % cell attachments of L-929 and A431 are plotted against the YIGSR concentration. The % cell attachment of L-929 dramatically decreases with increasing peptide concentration and

levels off to *ca.* 20% at 1.0 mg ml^{-1}. With respect to A431 cell, the % cell attachment at 1.0 mg ml^{-1} is 30%. Such results indicate that YIGSR pentapeptide inhibits 80 and 70% cell attachment of L-929 and A431, respectively, to the laminin-coated culture dish at 2.0 mg ml^{-1} YIGSR concentration.

Figure 7 shows the % cell attachments of L-929 and A431 against the incubation time at 2 mg ml^{-1} peptide concentration. As obvious from the figure, % cell attachment of L-929 drastically decreases at initial stage and leveled off to 70% at 60 min, however, that of A431 gradually decreases with incubation time and reaches to 30% (*i.e.*, 70% inhibition) at 180 min.

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REFERENCES

1. J. R. Couchman, M. Höök, D. A. Rees, and R. Timpl, *J. Cell Biol.*, **96**, 177 (1983).
2. H. K. Kleinman, F. B. Cannon, G. W. Laurie, J. R. Hassell, M. Aumailley, V. P. Terranova, G. R. Martin, and M. Dubois-Dalcq, *J. Cell. Biochem.*, **27**, 317 (1985).
3. J. E. Engel, E. Odermatt, A. Engel, J. Madri, H. Rhode, and R. Timpl, *J. Mol. Biol.*, **150**, 97 (1981).
4. M. Sasaki, H. K. Kleinman, H. Huber, R. Dutzmann, and Y. Yamada, *J. Biol. Chem.*, **263**, 16536 (1988).
5. M. Sasaki, S. Kato, K. Kohno, G. R. Martin, and Y. Yamada, *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 935 (1987).
6. M. Sasaki and Y. Yamada, *J. Biol. Chem.*, **262**, 17111 (1987).
7. Y. Iwamoto, F. A. Robey, J. Graf, M. Sasaki, H. K. Kleinman, Y. Yamada, and G. R. Martin, *Science*, **238**, 1132 (1987).
8. J. Graf, R. C. Ogel, F. A. Robey, M. Sasaki, G. R. Martin, Y. Yamada, and H. K. Kleinman, *Biochemistry*, **26**, 6896 (1987).
9. J. Graf, Y. Iwamoto, M. Sasaki, G. R. Martin, H. K. Kleinman, F. A. Robey, and Y. Yamada, *Cell*, **48**, 989 (1987).
10. J. Murata, I. Saiki, I. Azuma, and N. Nishi, *Int. Biol. Macromol.*, **11**, 97 (1998).
11. N. Izumiya, T. Kato, H. Aoyanagi, and M. Waki, "Basis and Experiments for Peptide Synthesis" (in Japanese), Maruzen, Tokyo, 1987, p 50.
12. N. Izumiya, T. Kato, H. Aoyanagi, and M. Waki, "Basis and Experiments for Peptide Synthesis" (in Japanese), Maruzen, Tokyo, 1987, p 97.
13. H. Yajima, N. Fujii, H. Ogawa, and H. Kawatani, *J. Chem. Soc., Chem. Commun.*, 107 (1974).
14. D. J. Giard, S. A. Aaronson, G. J. Todaro, P. Arnstein, J. H. Kersey, H. Dosik, and W. P. Parks, *J. Natl. Cancer Inst.*, **51**, 1417 (1973).
15. H. Katsuta, T. Takaoka, K. Mitamura, I. Kawada, H. Kuwabara, and S. Kawabara, *Jpn. J. Exp. Med.*, **29**, 191 (1959).
16. H. Katsuta and T. Takaoka, "Nutritional Requirements of Cultured Cells," Japan Scientific Societies Press, Tokyo, Japan, 1978, p 257.