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Biochemical and Biophysical Research Communications 345 (2006) 255–259
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Effect of photo-immobilization of epidermal growth factor on the cellular behaviors

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Received 17 March 2006
Available online 19 April 2006

Abstract

We constructed photo-reactive epidermal growth factor (EGF) bearing *p*-azido phenylalanine at the C-terminal (HEGFP) by genetic engineering to investigate the possibility of immobilized EGF as a novel artificial extracellular matrix (ECM). The constructed recombinant protein was immobilized to glass surface by ultraviolet irradiation. A431 cells adhered both to HEGFP-immobilized and collagen-coated surfaces. Interaction between immobilized HEGFP and EGF receptors in the A431 cells was independent of Mg^{2+} although integrin-mediated cell adhesion to natural ECMs is dependent on Mg^{2+}. Phosphorylation of EGF receptors in A431 cells was induced by immobilized HEGFP as same as soluble EGF. DNA uptake of hepatocytes decreased by immobilized HEGFP whereas it increased by soluble EGF. Liver-specific functions of hepatocytes were maintained for 3 days by immobilized HEGFP whereas they were not maintained by soluble EGF, indicating that immobilized HEGFP follows different signal transduction pathway from soluble EGF.

Keywords: Epidermal growth factor; Immobilization; Genetic engineering; Hepatocytes

Artificial extracellular matrices (ECMs) have been designed to stimulate cell adhesion and cellular functions because the cell adhesion to them has been a concern in tissue engineering as well as in cell biology research. For example, Arg-Gly-Asp (RGD) peptide that binds to adhesion receptors such as integrin was incorporated into biomaterials to control cell adhesion, cell morphology, and cellular specific functions [1,2]. Recently, it has been reported that growth factors and cytokines such as epidermal growth factor (EGF) [3] and erythropoietin [4] play important roles in cell adhesion, survival, and morphological changes in the cell cytoskeleton. In this adhesion mechanism, cells recognize them through a receptor-mediated mechanism rather than integrin-mediated adhesion. Also, it has already been reported that an immobilized growth factor induced a different signal transduction compared to a soluble growth factor. In the case of heparin-binding EGF-like growth factor (HB-EGF) [5], membrane-anchored HB-EGF as the model of immobilized HB-EGF showed growth-inhibitory activity whereas soluble HB-EGF was growth-stimulatory. Furthermore, immobilization of growth factors such as EGF [3,6,7] and insulin [8,9] transduced a signal for longer time than soluble ones did. Immobilized EGF continuously activated mitogen-activated protein kinase (MAPK) whereas the MAPK activation induced by soluble EGF decreased rapidly with time [3,6]. Also, the immobilization of EGF to a solid substrate is an important tool to provide a long retention of biological activity of mitogen and motogen by permitting greater control of temporal and spatial availability in the extracellular environment [10]. The most commonly used methods for photo-immobilization of EGF include photo-irradiation of azidobenzoyl derived EGF obtained by chemical reaction [6,7]. However, these common photo-immobilized biomolecules are of limited utility in aqueous environments and the active intermediates derived from photosensitive moieties can react with the surrounding chemicals by non-specific insertion into chemical bonds.

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0006-291X/$ - see front matter
doi:10.1016/j.bbrc.2006.04.001

In this study, we prepared genetically engineered-recombinant photo-reactive EGF (HEGFP) not reported elsewhere, to produce a novel artificial ECM which will be applied for tissue engineering because the HEGFP can be immobilized stably to glass surface by ultraviolet (UV) irradiation, and we investigated the cell adhesion, the phosphorylation of EGF receptors of A431 cells, and DNA uptake and liver-specific functions of hepatocytes by the immobilized EGF compared with soluble EGF because the hepatocytes have many functions such as growth, detoxication, and metabolism. The stimulation of an immobilized growth factor will induce different functions of hepatocytes compared to that of a soluble growth factor.

Materials and methods

Construction of HEGFP. Expression plasmids, pQE-9 encoding a mutant aminoacyl-tRNA synthetase and an auxotrophic *Escherichia coli* (*E. coli*) cell line, AF-IQ, were kindly provided from Prof. Tirrell at California Institute of Technology [11–13]. The pQE-9 contains an open-reading frame of Histidine-tag (His-tag), followed by multicloning site. Human EGF cDNA was cloned by PCR using 5′-GGA TCC ATG AAT TCC GCA AGC-3′ and 5′-GGA TCC **TTA** AAA ACG CAG TTC CCA CCA TTT CAG-3′ primers. Terminal codon is shown in boldface, and phenylalanine codon is underlined. Phenylalanine codon was incorporated into the C-terminal of EGF fragment, followed by termination codon. This EGF gene fragment was introduced into *Bam*HI–*Bam*HI site of expression plasmids pQE-9. This plasmid was named as a pQE-9-EGF.

Expression of HEGFP. Expression and refolding of the HEGFP were performed by the same method previously reported [12,14]. Briefly, *E. coli* cell line, AF-IQ carrying plasmid pQE-9-EGF, was grown in M9 minimal medium supplemented with 0.2% glucose, 5 mg/L thiamine, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 20 amino acids (20 mg/L for phenylalanine and 40 mg/L for other amino acids), and antibiotics (ampicillin and chloramphenicol). At an optical density of 0.8–1.0 at 600 nm, the culture medium was sedimented by centrifugation for 10 min at 4 °C and the cell pellets were washed twice with 0.9% NaCl. The cell pellets were resuspended in M9 minimal medium without chloramphenicol and phenylalanine. Aliquots were transferred to culture flask after 0.5 g/L *p*-azido phenylalanine (Chem-Impex International Inc., IL) was added. After 10 min incubation, 1.0 mM isopropyl-1-thio-β-D-galactoside (IPTG) was added to induce protein expression, HEGFP. And then, the cells were harvested by centrifugation and washed by wash buffer A (20 mM ethylenediaminetetraacetic acid (EDTA), 0.1 M Na_2HPO_4 and pH 7.8) for 4 h incubation. The washed cells were disrupted by freeze/thaw cycles. HEGFP inclusion body pellets were obtained by centrifuging the cell lysate and then washed by wash buffer B (10 mM EDTA, 0.2 M Na_2HPO_4 and pH 7.5). Sodium dodecyl sulfate (SDS) was used to dissolve the inclusion body. An aliquot of the washed inclusion body was added to dissolution buffer containing 1.0% SDS and 46 mM Na_2CO_3 of pH 9.8 at room temperature for 24 h. Approximately two-thirds of the SDS were precipitated by cooling to 4 °C, and the precipitate was removed by centrifugation. The residual SDS in the supernatant was removed by dialysis. To the elute 1 mM L-cysteine was added for reduction. After 1 h, 5 mM L-cystine was added for oxidative refolding for 16 h at room temperature. The permeate was fed to Ni^{2+}-HiTrap affinity column (Amersham Biosciences Corp., NJ). The bound HEGFP was eluted with carbonate buffer containing 250 mM imidazole. The HEGFP fractions were dialyzed against PBS (−).

Immobilization of HEGFP onto aminosilane-treated glass. Glass coverslip (24 × 24 mm; MATUNAMI, Japan) was soaked in isopropyl alcohol containing supersaturated KOH overnight, rinsed in deionized water, and dried at 50 °C overnight. The glass coverslip was immersed in 0.2% 3-aminopropyltrimethoxysilane dissolved in anhydrous acetone and placed at room temperature. The silane-treated glass coverslip was rinsed with acetone to remove non-reacted silane residues and dried at room temperature. An aqueous solution of the photo-reactive HEGFP at 100 pmol/cm^2 was placed on the silane-treated glass and air-dried at room temperature. The glass was irradiated for 125 s using a UV lamp from a distance of 5 cm. And then, the glass was washed with PBS (−) at 4 °C. Anti-EGF antibody (Santa Cruz Biotechnology Inc., CA) was used to detect immobilized HEGFP.

Cell culture. A431 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), and antibiotics (50 μg/mL penicillin, 50 μg/mL streptomycin, and 100 μg/mL neomycin) under 5% CO_2 at 37 °C.

Adhesion assay. A431 cells were seeded onto HEGFP immobilized- or collagen-coated surface pretreated with 0.5% bovine serum albumin (BSA) at 37 °C for 2 h. The cells suspended in PBS (−) were seeded onto each surface for Mg^{2+}- and Ca^{2+}-free condition. After 30 min of incubation in DMEM containing 0.5% FBS at 37 °C, the cells were washed with PBS (−) to remove the non-adherent cells. Adhered cells were quantified by colorimetric MTT assay as previously described [15].

Effect of pretreatment by EGF on cell adhesion. A431 cells were seeded onto HEGFP immobilized- or collagen-coated surface treated with 0.5% BSA at 37 °C for 2 h after the cells were pretreated with 100 ng/mL EGF. After 30 min of incubation in DMEM containing 0.5% FBS at 37 °C, the cells were washed with PBS (−) to remove the non-adherent cells. Adhered cells were quantified by MTT assay.

Phosphorylation of EGF receptor by Western blot assay. A431 cells were lysed in Nonidet P-40 lysis buffer [1% Nonidet P-40, 150 mM EDTA, 50 mM Tris–HCl (pH 8.0), 1 mM phenylmethylsulfonylfluoride, 1 mM leupeptin, 2 mM aprotinin, 0.25% sodium deoxycholate, and 1 mM Na_3VO_4]. Equal amounts of proteins were separated by SDS–PAGE at reduced condition. Anti-activated EGF receptor antibody (BD Transduction Laboratories, CA), anti-EGF receptor antibody (Santa Cruz Biotechnology Inc., CA) and anti-β actin antibody (Sigma, MO) were used to detect each protein.

Isolation and culture of hepatocytes. Hepatocytes were isolated from livers of male ICR mouse obtained from Japan SLC Inc. (5–7 weeks old) by a modification of the in situ collagenase perfusion method including perfusion with ethylene glycol-bis [β-amino ethyl ether]-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) before collagenase treatment as described previously [16]. Briefly, the liver was perfused by perfusion buffer and collagenase buffer through a needle aligned along the inferior vena cava. The collagenase-perfused liver was dissected, suspended in Hanks solution (30 ml), and filtered through cheesecloth and 100 μm nylon membrane to remove connective tissue debris and cell clumps. Hepatocytes were purified by a density-gradient centrifugation (42*g* force, 10 min) using 45% Percoll solution at 4 °C. Cell viability measured by Trypan blue exclusion was more than 90%.

RT-PCR analysis. Hepatocytes were seeded onto HEGFP-immobilized, collagen-coated or poly [*N*-*p*-vinylbenzyl-*O*-β-D-galactopyranosyl-(1 → 4)-D-gluconamide] (PVLA)-coated surface and cultured in Williams' Medium E (WE) for 3 days. Cells cultured on collagen were stimulated with or without 100 ng/mL EGF. Total RNA was extracted with TRIzol reagent (Invitrogen, CA) by following the manufacturer's instruction and cDNA was synthesized from 1 μg total RNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen). For PCR amplification, following primer pairs were used: for CYP1A2: 5′-TGTTCTGGATGGTCAGAGCC-3′ and 5′-CCTCATGGATCTTCCTCTGC-3′; for HNF-4α: 5′-GGCTTAAGAAGTGCTTCCGG-3′ and 5′-AGCAGGTTGTCAATCTTGGC-3′; for GAPDH: 5′-CTCTTGCTCAGTGTCCTTGC-3′ and 5′-CTCATGACCACAGTCCATGC-3′. PCR products were analyzed by 1.5% agarose gel electrophoresis.

Analysis of DNA synthesis. Hepatocytes were seeded onto HEGFP-immobilized, collagen-coated or PVLA-coated surface and cultured in WE for 2 h. Cells cultured on collagen were stimulated with or without 100 ng/mL EGF. The DNA synthesizing activity was evaluated as the incorporation of 5-bromo-2-deoxyuridine (BrdU) using BrdU Labeling and Detection kit I (Roche Diagnostics Co., Mannheim) by following the manufacturer's instruction. BrdU was added to each dish after 1 day of incubation. BrdU uptake of hepatocytes was detected after 2 days of incubation.

Results and discussion

Construction of HEGFP

The expression vector for HEGFP was obtained by fusion of EGF bearing phenylalanine codon in the 3′-terminal. The HEGFP containing *p*-azido phenylalanine at the C-terminal was expressed in *E. coli* expression system established previously [11–13]. Zhang et al. [17] prepared photo-reactive protein containing *p*-azido phenylalanine by the similar system and immobilized it to substrate. The HEGFP was purified by His-tag protein purification system because HEGFP contains His-tag at N-terminal of this recombinant protein. The purified EGF was detected by Western blotting using anti-EGF antibody (Fig. 1A). No other major bands were seen by Coomassie brilliant blue staining (data not shown), indicating that the protein was almost completely purified. Azido-modified HEGFP was immobilized to glass surface by UV irradiation (Fig. 1B). On the contrary, the HEGFP containing only normal phenylalanine was not immobilized to the surface (data not shown).

Adhesion of A431 cells on immobilized HEGFP

Adhesion of human epidermoidcarcinoma A431 cells was performed to verify whether the cells could adhere to the immobilized HEGFP. As shown in Fig. 2A, almost same amounts of cells adhered to HEGFP-immobilized surface compared with collagen-coated surface. To investigate whether this cell adhesion is EGF receptor-mediated mechanism, A431 cells pretreated with 100 ng/mL soluble EGF were seeded onto each surface. As shown in Fig. 2B, these cells adhered onto HEGFP-immobilized surface remarkably decreased whereas the cells adhered onto collagen-coated surface were not much changed, indication of receptor-mediated adhesion. We also investigated whether Mg^{2+} in the media is necessary or not at this EGF receptor-mediated adhesion. It is already reported that Mg^{2+} is essential for the cells to adhere onto natural ECMs such as collagen and fibronectin in the integrin-mediated adhesion [18]. As shown in Fig. 2C, the cells adhered

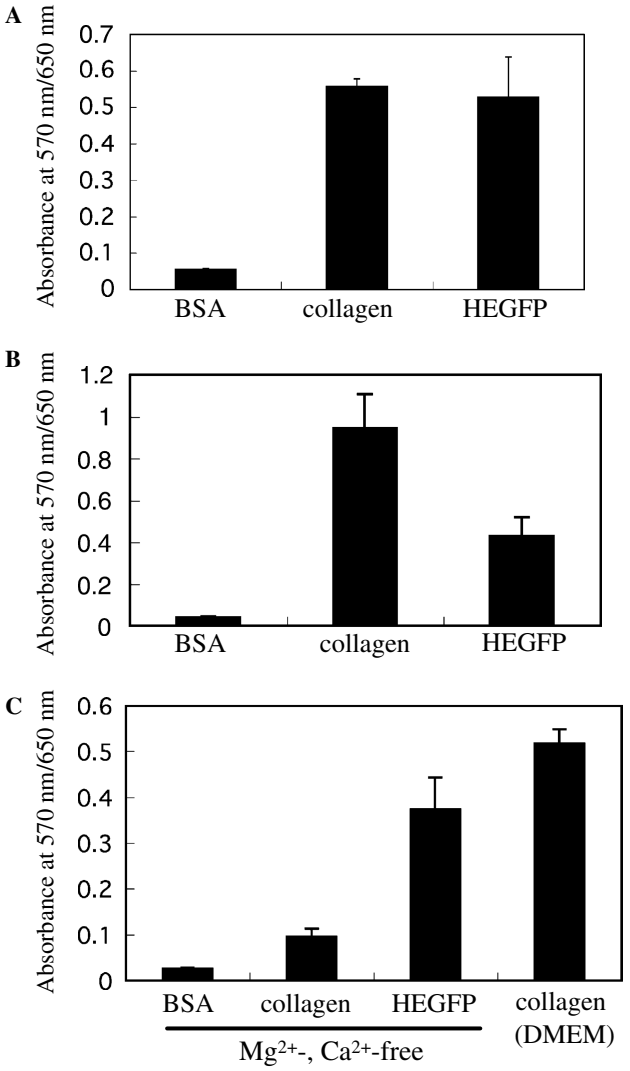

Fig. 2. Cell adhesion to HEGFP: (A) A431 cells adhered to the HEGFP-immobilized and collagen-coated surfaces after 30 min of incubation. The data represent means ± SD of experiments ($n = 3$); (B) A431 cells pretreated with 100 ng/mL soluble EGF were seeded onto each surface. Adhesion ratio of the cells was measured after 30 min of incubation; (C) A431 cells suspended in PBS (−) were seeded onto each surface. As a positive control, the cells suspended in DMEM containing 0.5% FBS were seeded on collagen-coated surface.

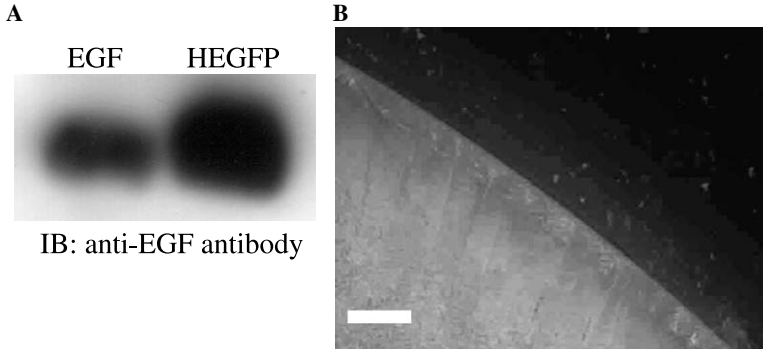

Fig. 1. Expression and immobilization of HEGFP: (A) purified HEGFP was detected by Western blotting using an anti-EGF antibody; (B) immobilized HEGFP to the glass surface was detected using an anti-EGF antibody. Scale bar indicates 250 μm.

onto HEGFP-immobilized surface, but not onto collagen-coated surface in the absence of Mg^{2+}, suggesting that Mg^{2+} does not play a prominent role in the EGF receptor-mediated interaction. In our previous studies, we already reported the receptor-mediated adhesions such as asialoglycoprotein receptors of hepatocytes to PVLA [19,20] and EGF receptor of A431 cells to EGF-Fc [3]. Ito et al. [4] also reported that human leukemia cell line adhered to immobilized erythropoietin. The results suggest that this interaction is receptor-mediated interaction rather than integrin-mediated adhesion.

Phosphorylation of EGF receptor and MAPK

Phosphorylation of EGF receptors was detected by Western blotting using anti-activated EGF receptor antibody. As shown in Fig. 3, phosphorylation of EGF receptors was induced by immobilized HEGFP. Moreover, amounts of total EGF receptors on the immobilized EGF were almost same as that of collagen-coated surface in the absence of EGF, indicating that phosphorylation of EGF receptor was induced by the stimulation of immobilized EGF and these activated receptors stayed on the cell membrane. Activation of EGF receptors by immobilized EGF was weaker than that of soluble one, suggesting that activity of HEGFP was decreased by immobilization of EGF to substrate.

MAPK is one of the most important proteins in growth and differentiation in Ras-MAPK pathway. Also, the activation of MAPK is induced by the binding of EGF to EGF receptors. Our results indicated that the MAPK was activated after 24 h by the stimulation of immobilized HEGFP whereas activation of MAPK was rapidly decreased by soluble EGF (data not shown). This result coincided with our previous result [3] and the other's result [6].

Maintenance of liver-specific functions by the immobilized EGF

Growth and differentiation of the cells are closely related to activation of growth factor receptors. Previous reports have been already found that DNA uptake of hepatocytes was increased and liver-specific functions of them were decreased by the stimulation of soluble EGF [21,22]. In our previous study, liver-specific functions of hepatocytes were maintained on the culture of PVLA-coated surface [23]. Hepatocytes cultured on immobilized HEGFP formed spheroids after 3 days (data not shown). Moreover, as shown in Figs. 4A and B, DNA uptake of hepatocytes cultured on the immobilized HEGFP was decreased and liver-specific functions, CYP1A2 and hepatocyte nuclear factor (HNF)-4α, of them were maintained as same level as hepatocytes cultured on PVLA. The cytochrome P450 enzyme CYP1A2 mediates the rate-limiting step in the metabolism of many drugs and HNF-4α regulates the expression of many genes preferentially in the liver. The results suggested that immobilized HEGFP follows different signal transduction pathway from soluble EGF.

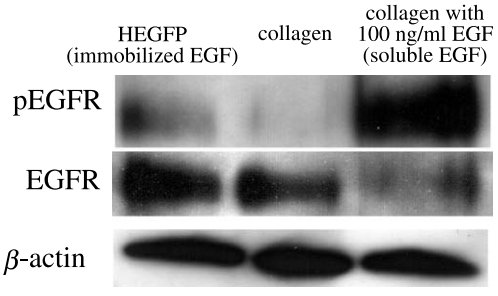

Fig. 3. Adhesion to HEGFP leads to the phosphorylation of EGF receptors in A431 cells. A431 cells were seeded onto HEGFP-immobilized or collagen-coated surface and cultured in DMEM containing 0.5% FBS for 2 h. Cells cultured on collagen were stimulated with or without 100 ng/mL EGF. Lysates were subjected to Western blotting with anti-activated EGF receptor antibody, anti-EGF receptor antibody or anti-β-actin antibody.

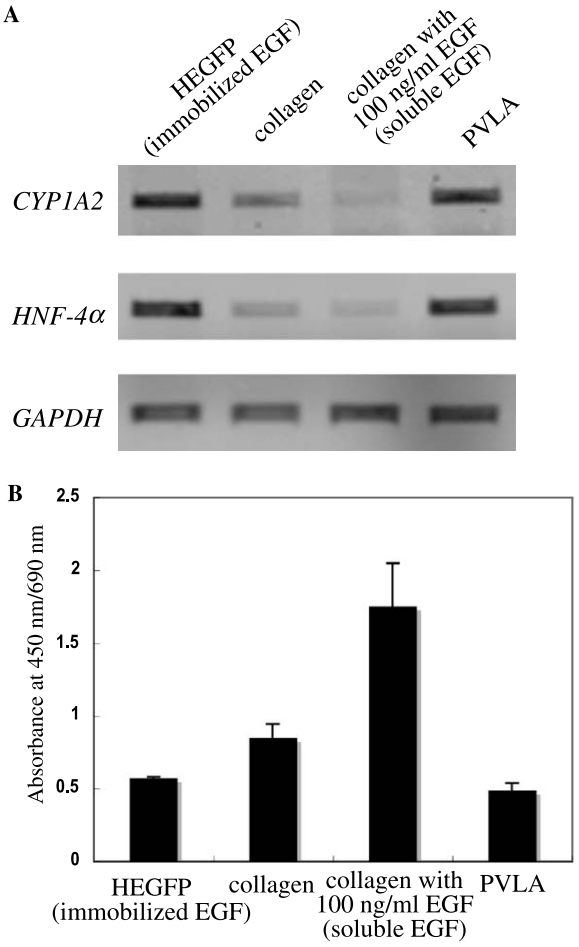

Fig. 4. Liver-specific functions and DNA uptake of hepatocytes: (A) hepatocytes were seeded onto HEGFP-immobilized, collagen-coated or PVLA-coated surface and cultured in WE for 3 days. Cells cultured on collagen were stimulated with or without 100 ng/mL EGF. *CYP1A2* and *HNF-4α* mRNA were detected by RT-PCR analysis; (B) hepatocytes were seeded onto HEGFP-immobilized, collagen-coated or PVLA-coated surface and cultured in WE. Cells cultured on collagen-coated surface were stimulated with or without 100 ng/mL EGF. BrdU was added to each dish after 1 day of incubation. BrdU uptake of hepatocytes was detected after 2 days of incubation.

In conclusion, A431 cells adhered to immobilized HEGFP through EGF receptor-mediated interaction but not intergrin-mediated adhesion. Moreover, liver-specific functions of hepatocytes were maintained for 3 days by the stimulation of immobilized HEGFP.

Acknowledgment

This research is supported by grants by from the Ministry of Education, Sports, Science and Technology of Japan.

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