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Differential Control of Cellular Gene Expression by Diffusible and Non-Diffusible EGF[1]

Yoshihiro Ito,*,[2] Guoping Chen,† Yukio Imanishi,‡ Takaya Morooka,§ Eisuke Nishida,§ Yoshinori Okabayashi,‖ and Masato Kasuga‖

PRESTO, JST, Department of Biological Science and Technology, Faculty of Engineering, The University of Tokushima, Tokushima 770-8506; †Department of Material Chemistry, Graduate School of Engineering, Kyoto University, Kyoto 606-6501; ‡Graduate School of Materials Science, NAIST, Ikoma 630-0101; §Department of Biophysics, Graduate School of Science, Kyoto University, 606-8502; and ‖Second Department of Internal Medicine, Kobe University School of Medicine, Kobe 650-0017

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Cell gene expression is affected by both the kind and mode of growth factor stimulation (diffusive *vs.* non-diffusive). Epidermal growth factor (EGF) was pattern-immobilized on a polystyrene plate. Although the growth of the rat phaeochromocytoma cell line PC12 is stimulated by diffusible EGF, and differentiation is stimulated by diffusible nerve growth factor (NGF), immobilized (non-diffusible) EGF stimulated PC12 differentiation. The immobilized EGF caused a long-lasting stimulation of the intracellular signal protein mitogen-associated protein MAP kinase (MAPK, also known as ERK) and p38 (a subfamily of the MAPK superfamily) in cells, as did diffusible NGF. The switching between growth stimulation and differentiation is considered to be due to the duration of the stimulus. The function of the biosignal conjugate was regulated using conjugation methodology.

Key words: EGF, immobilized growth factor, juxtacrine, matricrine, PC12.

Cellular functions are in large part carried out by proteins such as growth factors and cytokines, and the discovery of new proteins is increasing due to expanded genomic information. Whereas proteins have many attractive clinical properties, it is difficult to derive their activities *in vivo*. Therefore, various types of sustained delivery formations have been developed (*1*, *2*). Polymer conjugation is also of increasing interest in pharmaceutical chemistry for delivering proteins (*3*, *4*). On the other hand, insoluble or biodegradable matrices conjugated with proteins are important in tissue engineering for the repair or replacement of damaged tissues (*5*). However, previously, it was generally believed that biosignal proteins stimulate cells only as diffusible proteins with paracrine, endocrine, or autocrine mechanisms. Recently, some researchers have found that immobilized growth factors work by a juxtacrine or matricrine mechanism (*6–9*). To visualize the effect of immobilized growth factors, growth factors have been micropattern-immobilized on matrices (*10*).

Growth factors immobilized on artificial matrices show mitogenic effects. However, this effect is just a multiplication of soluble growth factor. In the present study, the growth factor known as epidermal growth factor (EGF) was pattern-immobilized in a prescribed micropattern to provide alternate control of cell function. Cellular gene expression was differentially controlled by diffusible and non-diffusible EGF in the rat phaeochromocytoma cell line PC12, in which growth is normally stimulated by diffusible EGF and differentiation is normally stimulated by diffusible nerve growth factor (NGF) (*11*).

MATERIALS AND METHODS

Substratum Preparation—Micropattern immobilization was carried out as reported previously (*12*). First, the photo-reactive EGF conjugate was synthesized as follows. A phosphate-buffered solution (pH = 7.0, 10 ml) containing polyallylamine (MW = 60,000, 30 mg) was added to an *N,N*-dimethylformamide (DMF) solution (20 ml) of *N*-(4-azidobenzoyloxy)succinimide (25.8 mg) with stirring on ice. After stirring at 4°C for 24 h, the solution was concentrated by ultrafiltration (Millipore MoleCut II, filtration cut-off below 10 kDa). The product, the azidobenzoyl-derivatized polyallylamine, was purified by washing once with 5 ml of DMF/H_2O (2:1) solution and twice with 5 ml of distilled water. The azidobenzoyl-derivatized polyallylamine was further conjugated with EGF. To a 0.1 M 2-(*N*-morpholino)ethanesulfate-buffer (MES, pH = 4.5, 10 ml), water-soluble carbodiimide (WSC, 10 mg), EGF (300 μg), and azidobenzoyl-derivatized polyallylamine (600 μg) were added, and the reaction was allowed to proceed at 4°C for 72 h with stirring. Finally, the photoreactive EGF conjugate was purified by ultrafiltration.

Subsequently, an aqueous solution of the photo-reactive EGF conjugate (200 μg/ml, 100 μl) was coated on each of the six wells of a polystyrene plate and air-dried at room temperature. The plate was irradiated in the presence or absence of a 100-μm-stripe-patterned photomask for 10 s using a UV lamp from a distance of 5 cm. Thereafter, the

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[2] To whom correspondence should be addressed. E-mail: ito@bio.tokushima-u.ac.jp

plate was repeatedly washed with PBS at 4°C.

The plate pattern-immobilized with EGF was immersed in PBS containing 0.02% NaN_3 and 3% bovine serum albumin (BSA) at 4°C for 24 h, and subsequently incubated with anti-EGF IgG antibody diluted in PBS containing 0.02% NaN_3 and 3% bovine serum albumin (2 mg/ml) at 4°C for 12 h. After it was washed three times with PBS containing 0.02% NaN_3, the plate was incubated in PBS containing rhodamine-conjugated anti–mouse IgG antibody (2 μg/ml) and 0.02% NaN_3 at 4°C for 12 h. The stained plate was washed three times with PBS, briefly rinsed with distilled water, then mounted in Vectashield mounting medium and observed under a laser fluorescence microscope (Olympus, Tokyo).

Cell Culture—PC12 cells were cultured in RPMI 1640 medium containing 5% (v/v) FBS and 10% (v/v) horse serum under 5% CO_2 in air at 37°C. PC12 cells deprived of serum for 6 h were cultured on the plate pattern-immobilized with EGF in a medium mixture [Dulbecco's modified Eagle minimum essential medium (DMEM)/F12 medium = 1:1] under 5% CO_2 in air at 37°C for 6 h. Then, the cells were fixed for 30 min at 4°C with 3% paraformaldehyde in PBS. The fixed cells were washed three times with PBS containing 1 mM Na_3VO_4. Subsequently, the cells were permeabilized with PBS containing 0.25% Triton X-100 and 1 mM Na3VO4, and washed three times with 50 mM Tris-HCl buffer containing 150 mM NaCl and 0.1% Triton X-100 (TBST, pH = 7.4) and 1 mM Na_3VO_4. After an overnight incubation at 4°C in TBST containing 1.5% normal goat serum and 1 mM Na_3VO_4, the treated cells were incubated for 2 h room temperature with a solution of anti-phosphotyrosine mouse IgG diluted 1:100 with 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.01% Tween 20, 0.02% NaN_3, 1 mM Na_3VO_4 (TBS) containing 3% BSA. The cells were washed once with TBS, once with TBST and once with TBST containing 0.1% BSA. A solution of rhodamine-conjugated anti–mouse IgG antibody was diluted 1:200 with TBS containing 3% BSA and incubated with the cells for 2 h at RT. The cells were washed three times for 5 min each with TBST, three times with PBS, briefly rinsed with distilled water, and then mounted in Vectashield™ mounting medium and observed by fluorescence microscopy.

The subcultured PC12 cells were harvested by pipetting. After they were washed twice with the medium mixture (DMEM medium/F12 medium = 1:1) containing transferrin (5 μg/ml), insulin (5 μg/ml), and progesterone (0.8 μg/ml), the cells were suspended in the same medium (1×10^6 cells/ml). The plate pattern-immobilized with EGF was incubated in sterile PBS containing 50 μg/ml fibronectin at 37°C for 2 h, and washed twice with sterile PBS. The cell suspension (50 μl/well) was added onto the plate and cultured in medium mixture (DMEM medium/F12 medi-um = 1:1) containing transferrin (5 μg/ml), insulin (5 μg/ml), and progesterone (0.8 μg/ml) under 5% CO_2 in air at 37°C for three days. The cell number was counted using a cell counting kit (Dojin Chem. Kumamoto).

Protein Kinase Assays—After disinfection with 70% ethanol, the EGF-immobilized plates and the control plates were incubated in sterile PBS containing 50 μg/ml fibronectin at 37°C for 2 h, and then washed twice with sterile PBS. The EGF-immobilized plates and the control plates were then used for the following experiments. PC12 cells deprived of serum for 6 h were incubated at 37°C for different periods of time in medium mixture (DMEM medium/F12 medium = 1:1) containing transferrin (5 μg/ml), insulin (5 μg/ml), and progesterone (0.8 μg/ml) in 6-well plates preadsorbed with fibronectin in the presence of NGF (50 ng/ml, 5 ml), diffusible EGF (100 ng/ml, 5 ml) or immobilized EGF. The cells were then washed twice with PBS, and lysed in 500 μl of a solution containing 25 mM Tris-HCl (pH = 7.4), 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 nM okadaic acid, 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), and 1 mM phe-

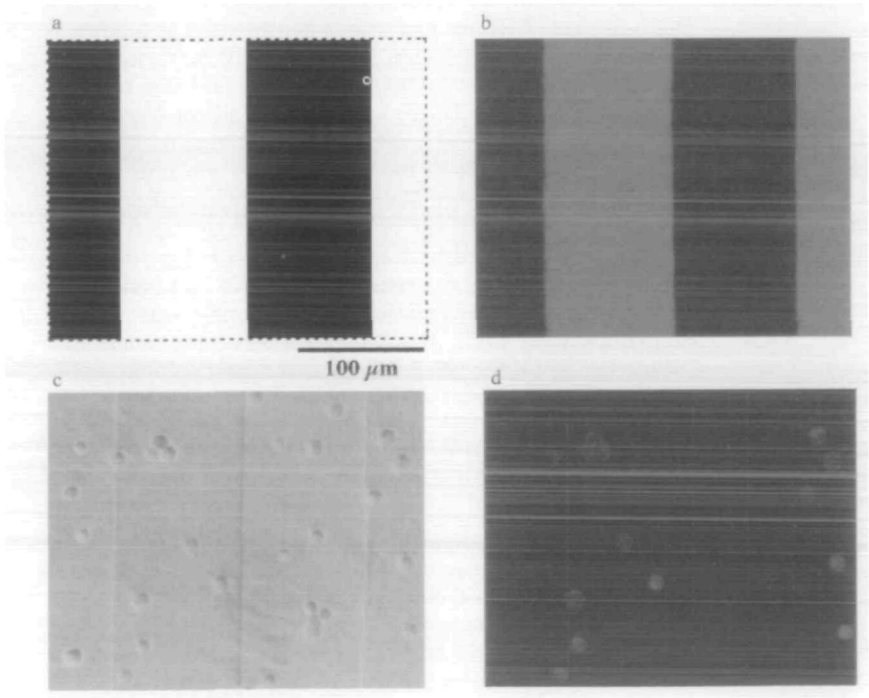

Fig. 1. **Photomask pattern (a), fluorescence micrograph of a polystyrene plate with pattern-immobilized EGF stained by anti-EGF antibody (b), phase-contrast micrograph of PC12 cells after 6-h culture on a polystyrene plate with immobilized EGF in a stripe pattern (c), and fluorescent micrograph of PC12 cells stained by anti-phosphotyrosine antibody on the pattern-immobilized EGF (d).**

nylmethylsulfonyl fluoride (PMSF), and the lysate was centrifuged at 15,000 rpm for 15 min at 4°C. Immunoprecipitation and immune complex kinase assays were performed as described previously (*13*, *14*). ERK/MAPK and p38 MAPK activities were assayed with His-tagged activating transcription factor 2 and myelin basic protein, respectively, as substrates. After electrophoresis, radioactivity was analyzed with an image analyzer (Bio-Rad). Immunoblotting was performed as described previously (*13*, *14*).

RESULTS AND DISCUSSION

EGF was pattern-immobilized on a cell-culture polystyrene plate, and PC12 cells were cultured on the substrate. Figure 1 shows cell activation, as assessed by staining with anti-phosphotyrosine antibody, only on the regions containing immobilized EGF, although the cells adhered to the plate independently of immobilized EGF. This finding indicates that the immobilized EGF did not enhance cell adhesion but transduced a signal to the cells through the phosphorylation of tyrosine residues on signal proteins.

Cell growth was investigated in the presence of soluble EGF and immobilized EGF, and the results are shown in Fig. 2a. Although soluble EGF enhanced cell growth, immobilized EGF did not. The morphology of cells cultured on the pattern-immobilized EGF after 3 days of culture is shown in Fig. 2b. Surprisingly, the cells on the regions with immobilized EGF formed neurites. It is known that NGF induces neurite formation in PC12. The immobilized EGF critically switched the cells from growth to neural differentiation, as does NGF. No concentration of soluble EGF induced neural differentiation.

Because it has been reported that the switching between differentiation and growth is related to the time course of activation of both ERK/MAPK and p38 MAPK in PC12 cells (*15–17*), the time course of MAPK activation was investigated using native (diffusible) EGF, immobilized EGF, and native (diffusible) NGF. Diffusible EGF transiently activated ERK/MAPK and p38 MAPK (Fig. 3). Diffusible NGF quickly activated ERK/MAPK and p38 MAPK, and the effects continued for a longer time after a slight decrease in activation. Immobilized EGF also activated ERK/MAPK and p38 MAPK for a longer time after a delayed activation caused by cell adhesion to the plate. The immobilized EGF activated ERK/MAPK and p38 MAPK as much as or more than NGF.

It has been demonstrated that immobilized growth factors are not released from the matrix and are not internalized into cells (*8*, *18–21*). Previously, in order to explain the growth enhancement caused by immobilized growth factor, several possibilities were discussed (*8*, *18*); (i) inhibition of down-regulation without internalization of growth factor; (ii) induction of simultaneous multiple stimulation of growth factors by a highly local concentration of growth factor; (iii) prevention of lateral diffusion of the activated receptor in the plane of the cell membrane; (iv) difference of stimulation sites between the cell-medium interface and

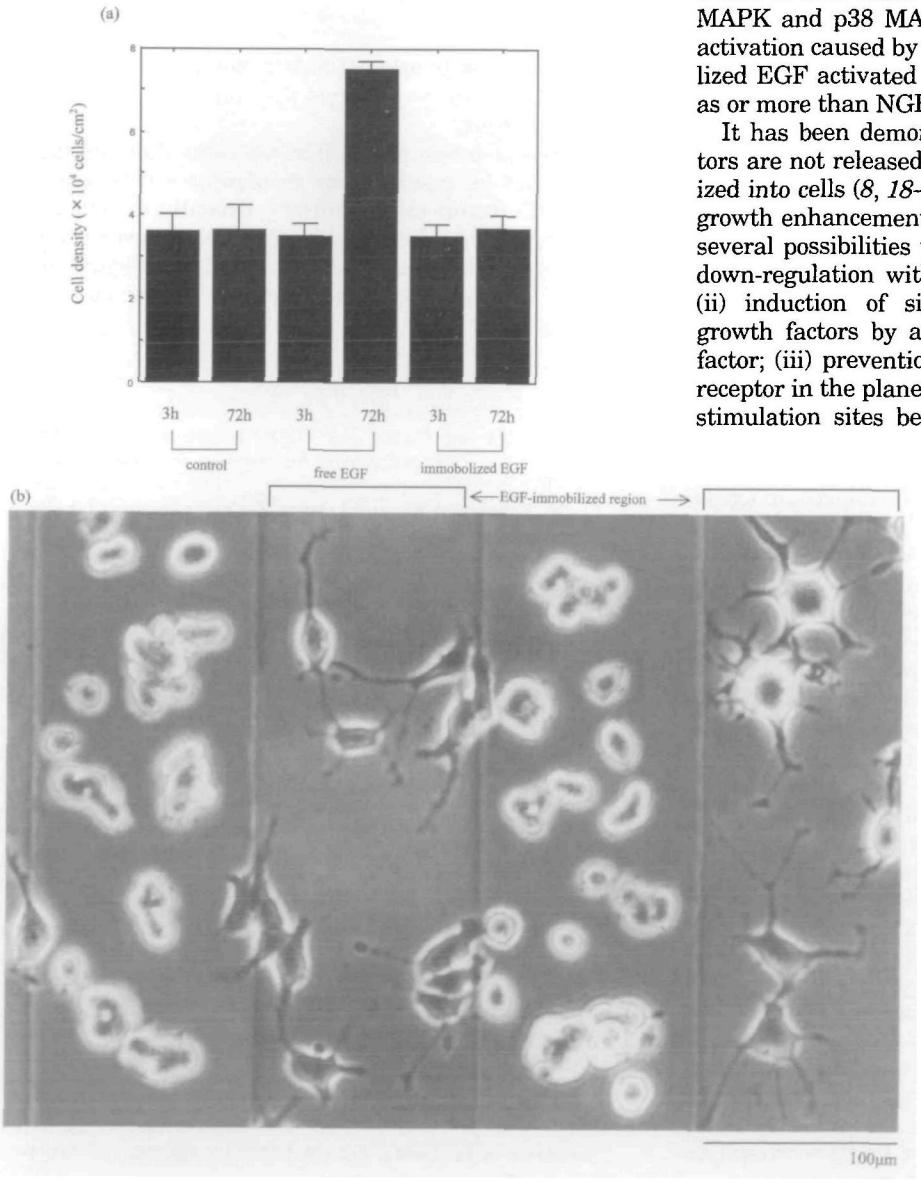

Fig. 2. **(a) Number of PC12 cells in the presence of soluble EGF (100 ng/ml), immobilized EGF (100 ng/cm²), or no addition (control). (b) Phase contrast micrograph of PC12 cells cultured on pattern-immobilized EGF (100 ng/cm²) in the presence of soluble EGF (100 ng/ml) for 72 h.**

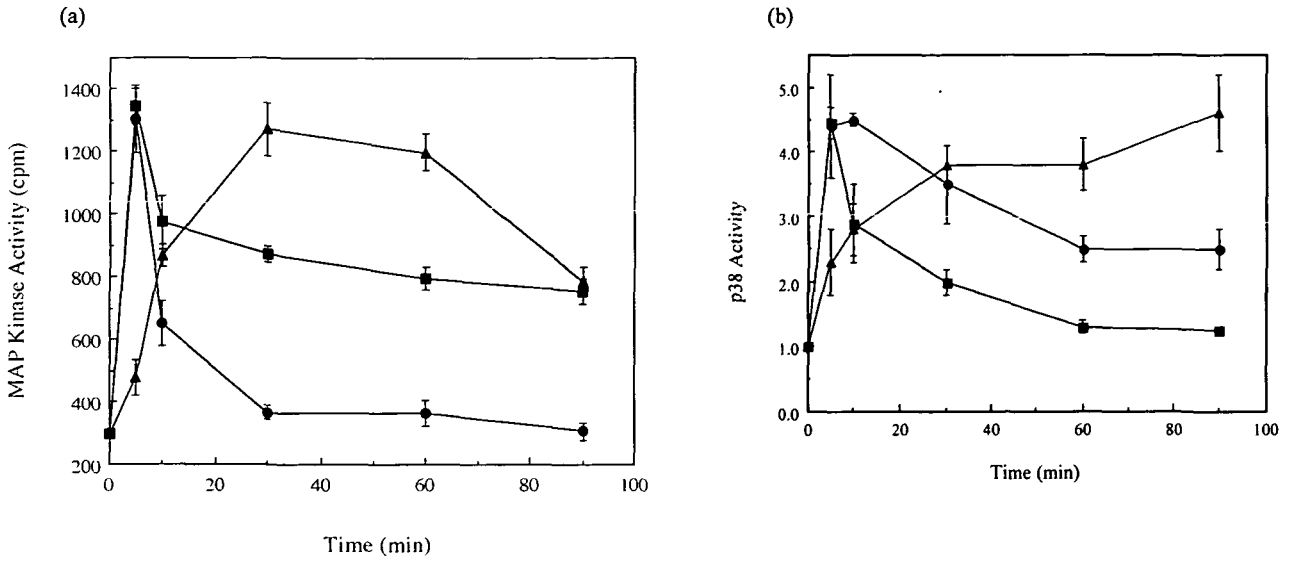

Fig. 3. **Time course of (a) ERK/MAPK and (b) p38 MAPK activation by diffusible EGF (■), diffusible NGF (●), and immobilized EGF (▲).** $n = 3$.

cell-matrix interface, *etc.* The present study demonstrates the importance of the inhibition of down-regulation in that immobilized EGF regulated the cell differentiation.

It is known that some membrane-anchored growth factors, such as the members of the EGF family, are biologically active in their transmembrane forms, which is termed "juxtacrine stimulation" (*22*, *23*). The mouse phenotype carrying the *steel dickie* (Sl^d) mutant allele provides a dramatic example of the importance of membrane-anchored growth factors in mammalian development (*24–26*). The EPH-related receptor tyrosine kinase requires transmembrane forms to act (*27*). The transmembrane form of the tumor necrosis factor (TNF) elicits qualitatively different TNF responses, such as rendering resistant tumor cells sensitive to TNF-mediated cytotoxicity (*28*). Recently, it was reported that membrane-anchored EGF induces apoptosis (*29*). However, these juxtacrine stimulation mechanisms have been deduced from experiments using paraformaldehyde-fixed cells, which contain membrane-anchored growth factors (*23*). That method alone is not sufficient to prove the effects of non-diffusible growth factors because it is impossible to neglect the release of fixed materials. The present investigation using micropattern-immobilized EGF clearly demonstrates the effect of non-diffusible biosignal molecules by comparing EGF-immobilized regions with non-immobilized regions.

Traverse *et al.* (*30*) and Dikic *et al.* (*31*) reported the neural differentiation of PC12 cells triggered by the overexpression of EGF and insulin receptors, respectively. They concluded that the overexpression induced long-lasting activation of ERK/MAPK, similar to that induced by NGF. Therefore, it was concluded that PC12 cells can either differentiate or proliferate in response to growth factor stimulation, depending on the strength or duration (or both) of the stimulus. Although the growth factor receptor-mediated differentiation of PC12 cells requires the integration of other signals with the Ras/Raf/MAPK pathway, as reported by Vaillancourt *et al.* (*32*), the present study indicates not only that the neural differentiation of PC12 is caused by the long-lasting activation of MAPK but also that changing the stimulation mode switches the transcriptional regulation of PC12 cells.

The present investigation demonstrates that cell differentiation can be regulated by biomaterials with immobilized growth factors on the surface. Recently, various types of micro-engineered biomaterials have been developed (*10*, *33*, *34*). The micropattern immobilization of biosignal molecules provides a new tool with which to regulate fine tissue formation.

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