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Enhancement of the Mitogenic Effect by Artificial Juxtacrine Stimulation Using Immobilized EGF

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Mouse epidermal growth factor (EGF) was covalently conjugated with the water-soluble polymer, poly(acrylic acid) (EGF-PAA), or with the water-insoluble polymer, surface-hydrolyzed poly(methyl methacrylate) (EGF-PMMA). Immobilized EGF (EGF-PMMA) stimulated DNA synthesis in Chinese hamster ovary cells overexpressing EGF receptors in amounts that were 5 to 10% of those of free EGF required for comparable effects. In addition, the maximal mitogenic effect of EGF-PMMA was greater than that of unconjugated EGF or EGF-PAA. EGF, EGF-PAA, and EGF-PMMA induced the autophosphorylation of EGF receptors and the stimulation of mitogen-activated protein kinase. However, whereas the onset of these effects was delayed with EGF-PMMA, they persisted for much longer than those of EGF and EGF-PAA. Unlike EGF and EGF-PAA, EGF-PMMA was not associated with cells after their removal from culture and did not induce receptor internalization. Culturing cells with PMMA-immobilized EGF thus represents a model system for studying "juxtacrine" stimulation of cells by membrane-bound growth factors.

Key words: bioconjugate material, EGF, immobilization, juxtacrine, tissue engineering.

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Cell–cell interactions in which one cell sends a signal to another cell, inducing a change in function of the second cell, are features of various biological phenomena. These interactions are mainly mediated by two categories of proteins, growth factors and cell adhesion molecules. Growth factors are generally secreted as diffusible proteins and transduce proliferation and differentiation signals, while cell adhesion molecules link to extracellular matrices and assemble animal cells into tissues through their adhesive properties. However, these two communication systems substantially overlap (*1-3*). For example, integrins are receptors for cell adhesion molecules, both providing a physical link to the cytoskeleton and transducing signals from the extracellular matrix (*4-6*).

In addition, several growth factors and lymphokines are synthesized as membrane-anchored proteins and the transmembrane forms are biologically active as "juxtacrine stimulators." These include the epidermal growth factor (EGF) family of growth factors (*7*) including transforming growth factor-α (*8, 9*), tumor necrosis factor-α (TNF-α) (*10*), colony-stimulating factor-1 (*11*), and the c-kit ligand (KL) (*12-15*) as reviewed by Massague and Pandiella (*16*). The biological importance of transmembrane KL has been demonstrated *in vivo*. Schmid *et al.* (*17*) have shown that a coculture system of human umbilical cord vein endothelial cells (HUVECs) and Chinese hamster ovary transfectants expressing a noncleavable, exclusively membrane-bound form of TNF-α potently activates HUVECs through the synergistic action of two TNF receptors. The mitogenic effect of heparin-binding EGF-like growth factor has been shown by Higashiyama *et al.* (*18*). Interleukin-1 stimulates cells by means of a juxtacrine mechanism (*19, 20*). These mechanisms were deduced from studies of intercellular regulation by paraformaldehyde-fixed cells that express the growth factors or cytokines.

On the other hand, artificial juxtacrine stimulation has been achieved by several groups. Horwitz *et al.* (*21*) showed that interleukin-2 covalently immobilized on a polystyrene plate maintained the viability of an interleukin-2-dependent cell line. We showed that insulin immobilized on various artificial and biological substrata increases the growth of anchorage-dependent cells, including mouse fibroblast STO cells, bovine endothelial cells, and mouse sarcoma cells, and we also demonstrated that, although the time required for immobilized insulin to stimulate insulin receptors on Chinese hamster ovary (CHO) cells was longer than that required for free insulin, receptor activation persisted longer with the immobilized ligand (*22, 23*).

In this study, mouse EGF was covalently conjugated with two types of synthetic polymers, water-soluble poly(acrylic acid) (PAA) and water-insoluble poly(methyl methacrylate) (PMMA), and the mitogenic effects of the two conjugates were investigated using CHO cells that overexpress EGF receptors (CHO-ER cells). We detected a marked difference in the mitogenic activities of EGF-PAA and EGF-PMMA, indicating that the mode of stimulation is an important determinant of biosignaling effects.

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MATERIALS AND METHODS

Materials—Mouse EGF was obtained from Toyobo (Osaka); ^{125}I-labeled EGF from New England Nuclear (Boston, MA); PAA [average molecular mass, 15 kDa; 35% (w/v) in water] and PMMA [average molecular mass, 120 kDa; powder) were from Aldrich (Milwaukee, WI). These materials were used without further purification.

Synthesis of the Soluble EGF Conjugate (EGF-PAA)—Mouse EGF was conjugated with PAA as represented schematically in Fig. 1A.

An aqueous solution of PAA (250 μg/ml, 1 ml) was mixed with 5 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride [water-soluble carbodiimide (WSC)] and the mixture was allowed to stand for 30 min at room temperature. The activated PAA was then rapidly purified by ultrafiltration [Millipore (Bedford, MA) Mole-Cut II; size cutoff, 10 kDa] and mixed with an aqueous solution of EGF (300 μg/ml) in the absence or presence of ^{125}I-EGF. The mixture was incubated at 4°C for 24 h, after which glycine was added to a final concentration of 10 mg/ml to block remaining activated carboxyl groups of PAA. The EGF-PAA conjugate was purified by ultrafiltration and analyzed by high-performance liquid chromatography (HPLC) on a Cosmosil 5Diol-120 column (Nacalai Tesque, Kyoto); material was eluted with 0.02 M sodium phosphate buffer (pH 7.0) containing 0.1 M Na_2SO_4, at a flow rate of 1.0 ml/min and at room temperature, and was detected by measuring the absorbance at 280 nm. EGF-PAA was again subjected to ultrafiltration for sterilization.

Synthesis of the Insoluble EGF Conjugate (EGF-PMMA)—Mouse EGF was immobilized on a surface-hydrolyzed PMMA film as shown in Fig. 1B. The PMMA film was cast by evaporation overnight under vacuum of a 10% (w/v) solution in toluene (200 μl) spread on a cover glass (diameter, 15 or 100 mm). The film was partially hydrolyzed by dipping in 4 M NaOH for 90 min at 50°C, and then washed with water and immersed in 10% (w/v) aqueous citric acid overnight at room temperature. It was washed with double-distilled water until the pH of the washes became neutral (pH 7.0), then stored in double-distilled

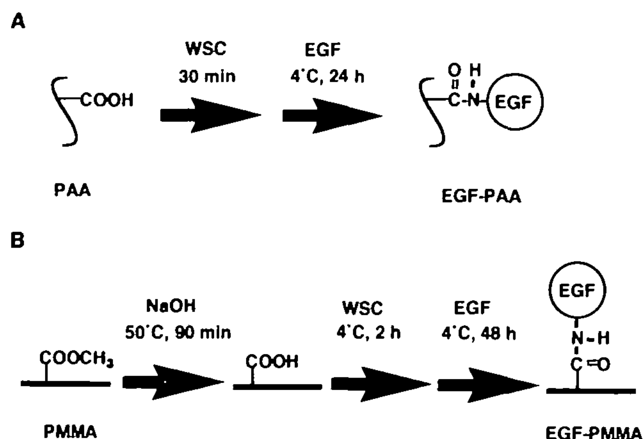

Fig. 1. **Synthetic schemes for water-soluble (EGF-PAA) (A) and water-insoluble (EGF-PMMA) (B) conjugates.** See "EXPERIMENTAL PROCEDURES" for further details. WSC, water-soluble carbodiimide.

water for subsequent experiments. The surface-hydrolyzed PMMA film was incubated at 4°C for 2 h in 0.5 M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 4.5) containing WSC (5 mg/ml) and then transferred to MES buffer containing mouse EGF in the absence or presence of ^{125}I-EGF. After incubation at 4°C for 48 h, the resulting EGF-PMMA film was washed with Ca^{2+}-free and Mg^{2+}-free phosphate-buffered saline (pH 7.4) [PBS(−)] until the release of ^{125}I-EGF became undetectable. Bovine serum albumin (500 μg/ml solution) was also immobilized on PMMA, yielding an albumin-PMMA film, by the same method. The surface-hydrolyzed PMMA film without proteins was also used as a control. All films were sterilized with 70% (v/v) ethanol, and were washed with sterilized PBS(−) before experiments.

Cell Culture—CHO-ER cells (2.5×10^5 wild-type human EGF receptors per cell), established as previously described (*24, 25*), were cultured under 5% CO_2 at 37°C in Ham's F-12 medium supplemented with 10% (v/v) fetal bovine serum (Life Technologies, Grand Island, NY).

Assay of DNA Synthesis—CHO-ER cells were harvested from culture flasks by treatment with PBS(−) containing 0.15% (w/v) trypsin (2,000 unit/g) and 0.02% (w/v) ethylenediaminetetraacetic acid (EDTA), suspended in Ham's F-12 medium, and incubated at 37°C for 48 h under 5% CO_2 in 24-well plates (10^5 cells per well) in the presence of various test agents. The cells were lysed by heating in 1 M NaOH (300 μl) at 70°C for 30 min or until complete lysis was confirmed with a light microscope. Lysates were then neutralized with 1 M HCl (300 μl) and homogenized. The homogenate (100 μl) was added to 3 ml of a solution containing 100 mM NaCl, 10 mM EDTA, 10 mM Tris-HCl (pH 7.0), and 4′,6-diamidino-2-phenylindole dye (100 ng/ml). The amount of DNA in cells was determined from the amount of DNA-dye complex (*26*); the fluorescence intensity at 450 nm was measured at an excitation wavelength of 360 nm.

Assay of EGF Receptor Autophosphorylation—CHO-ER cells that had been deprived of serum overnight were suspended in Ham's F-12 medium (5×10^5 cells/ml), and the cell suspension (20 ml) was incubated at 37°C in dishes containing the standard PMMA film or test samples. After various times, the cells were rapidly frozen in liquid nitrogen and stored at −80°C. Cells were subsequently lysed in a solution containing 50 mM 2-[4-(2-hydroxyethyl)-1-piperazyl]ethanesulfonic acid (HEPES)-NaOH (pH 7.5), 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM leupeptin, and aprotinin (10 μg/ml). The lysates were centrifuged at $12,500 \times g$ for 15 min at 4°C, and the resulting supernatants, which were clear, were incubated for 90 min at 4°C with 1 μg of antibodies to the EGF receptor (Ab-1, Oncogene Science, Uniondale, NY) coupled to protein G-Sepharose (Pharmacia Biotech, Uppsala, Sweden). Immunoprecipitates were washed three times with a solution containing 20 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, and 1 mM sodium orthovanadate, and then boiled for 3 min in SDS electrophoresis sample buffer. Samples were then subjected to SDS-polyacrylamide gel electro-

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phoresis, and the separated proteins were transferred to a nitrocellulose filter. The filter was probed with polyclonal antibodies to phosphotyrosine and detected as described previously (*24, 25*).

MAP Kinase Activity Assay—CHO-ER cells that had been deprived of serum overnight were incubated at 37°C for various periods in Ham's F-12 medium in 24-well plates in the presence of test agents. Cells were 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 EGTA, and 1 mM PMSF, and the lysate was centrifuged at 12,500$\times g$ for 15 min at 4°C. The MAP kinase activity of $p44^{mapk}$ was measured in immunoprecipitates prepared from cell lysate supernatants with polyclonal antibodies to MAP kinase (αC92) (*27*). The immunoprecipitates were incubated for 10 min at room temperature with 1 μCi of [γ-^{32}P]ATP in a solution containing 25 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM dithiothreitol, 40 μM ATP, 0.5 mM EGTA, and myelin basic protein (MBP) (1 mg/ml) as a substrate (*28*). Under these conditions, the MBP phosphorylation showed a linear time course for >20 min. The reaction was terminated by spotting 15 μl of reaction mixture onto P-81 phosphocellulose paper (Whatman International, Maidstone, UK), which was then washed three times with 0.75% phosphoric acid and once with acetone. The papers were dried, and ^{32}P was quantitated by Cerenkov counting.

Cell Association Assay—CHO-ER cells that had been deprived of serum overnight were incubated for 48 h at 37°C in 24-well plates (10^6 cells per well) containing Ham's F-12 medium and various ^{125}I-labeled samples. Cells were harvested by incubation at 37°C for 1 h with PBS(−) containing 0.02% (w/v) EDTA. The absence of cells remaining on the film was confirmed with a light microscope.

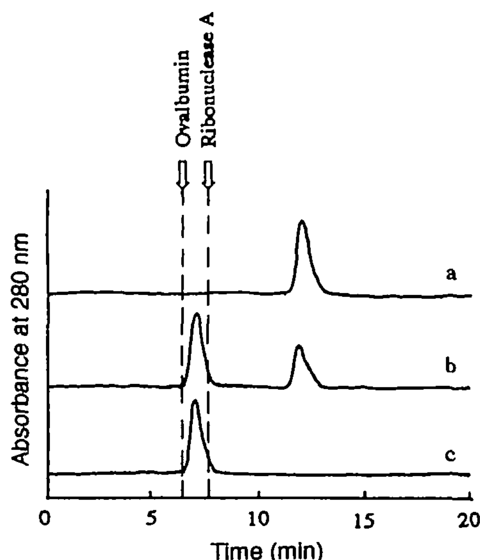

Fig. 2. **HPLC elution profiles of EGF and EGF-PAA.** Mouse EGF (curve a), the reaction mixture after incubation of EGF with PAA (curve b), and EGF-PAA after purification by ultrafiltration (curve c) were analyzed by HPLC as described in "EXPERIMENTAL PROCEDURES." Dotted lines indicate the retention times of the reference proteins ovalbumin (43 kDa) and ribonuclease A (13.7 kDa).

The harvested cells were washed three times with PBS(−) and then lysed with 1 M NaOH. The radioactivity associated with lysates was measured with a γ-counter. The radioactivity associated with the EGF-PMMA film was also measured.

Binding Assay—CHO-ER cells that had been deprived of serum at 37°C for 24 h were incubated in 24-well plates containing Ham's F-12 medium in the presence of test agents. Cells were harvested by incubation at 37°C for 1 h with PBS(−) containing 0.02% (w/v) EDTA, washed three times with ice-cold Ham's F-12 medium containing 20 mM HEPES-NaOH (pH 7.4) and 0.1% (w/v) bovine serum albumin, and then incubated at 4°C overnight in the same solution (100 μl) containing 0.1 nM ^{125}I-EGF. Nonspecific binding was assessed in the presence of 1 μM unlabeled EGF. Cells were washed three times with ice-cold PBS(−), then lysed with 1 M NaOH and the radioactivity associated with the lysates was measured with a γ-counter. The amount of lysate protein was determined with the Bio-Rad (Hercules, CA) protein assay.

RESULTS

Preparation of Soluble and Insoluble EGF Conjugates—Mouse EGF was conjugated with PAA as shown in Fig. 1A. HPLC analysis of the reaction mixture revealed a peak, in addition to that of EGF, between 43 and 13.7 kDa corresponding to the product of the reaction between the only amino group of mouse EGF (at the NH_2-terminus) and a carboxyl group of PAA (Fig. 2). The EGF-PAA conjugate could be separated from unreacted EGF by ultrafiltration. The content of EGF in PAA-EGF was calculated to be 40.8 μg per 100 μg of PAA, indicating that 1.02 molecules of EGF were coupled to 1.00 molecule of PAA.

EGF was also immobilized on a surface-hydrolyzed PMMA film as shown in Fig. 1B. As for EGF-PAA, WSC was used to couple a carboxyl group of hydrolyzed PMMA to the amino group of EGF. Newly prepared EGF-PMMA films were routinely washed until release of the growth factor was not detectable.

PMMA films on which various amounts of EGF were immobilized were obtained by changing the feed concentra-

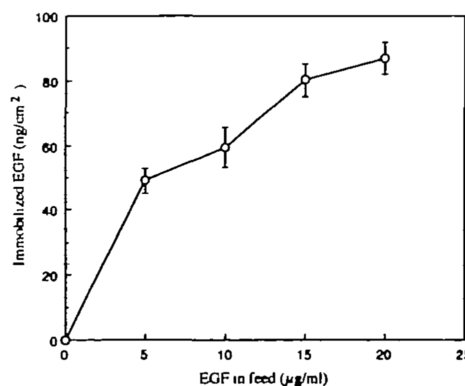

Fig. 3. **Effect of the feed concentration on the amount of EGF immobilized on PMMA films.** Surface-hydrolyzed PMMA films were incubated with various concentrations of ^{125}I-EGF, and the amount of immobilized ligand was calculated from the amount of radioactivity associated with the film after washing. Data are means ± SD (n=5).

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tion of EGF (Fig. 3). The amount of immobilized EGF increased with increasing concentration of EGF in the feed. The maximal amount of immobilized EGF (~90 ng/cm^2) was about two-thirds of the value (130 ng/cm^2) calculated by assuming the formation of an EGF monolayer on the surface of the matrix.

It is important to confirm that the immobilized ligand does not subsequently dissociate from the PMMA film (*21*). We therefore incubated immobilized ^{125}I-EGF with culture medium containing 10% (v/v) serum or with serum-free medium at 37°C for 48 h. In neither instance was the presence of radioactivity in the medium or a decrease in the amount of immobilized ^{125}I-EGF on the film observed. In addition, incubation with 4 M urea and 8 M guanidine hydrochloride for 48 h did not induce leakage of immobilized ^{125}I-EGF.

Mitogenic Activity of EGF Conjugates—The amount of total DNA in CHO-ER cells cultured for 48 h in the presence of EGF or EGF conjugates was determined by DNA-dye complex formation (Fig. 4). EGF-PMMA stimulated DNA synthesis at concentrations one-tenth to one-twentieth of those of free EGF required for comparable effects; the maximal effect of EGF-PMMA was greater than that of free EGF. In contrast, the mitogenic effect of EGF-PAA was not very different from that of free EGF. The effects of free EGF and EGF-PAA were determined in the presence of control surface-hydrolyzed PMMA films. A PMMA film containing immobilized albumin had no effect on DNA synthesis.

Effect of EGF Conjugates on EGF Receptor Autophosphorylation and MAP Kinase Activation—Transduction of the mitogenic signals of EGF-PAA and EGF-PMMA was first examined at the level of autophosphorylation of EGF receptors. Quiescent CHO-ER cells were stimulated for various periods with unconjugated EGF, EGF-PAA, or EGF-PMMA, lysed, and subjected to immunoprecipitation with antibodies to the EGF receptor. Tyrosine-phosphorylated receptors were then detected by immunoblot analysis with antibodies to phosphotyrosine (Fig. 5). Both unconjugated EGF and EGF-PAA rapidly (within 6 min) induced receptor autophosphorylation, an effect that was no longer apparent after 24 h. In contrast, the effect of EGF-PMMA on receptor autophosphorylation was apparent at 1 h, but not at 6 min, and was still evident at 24 h.

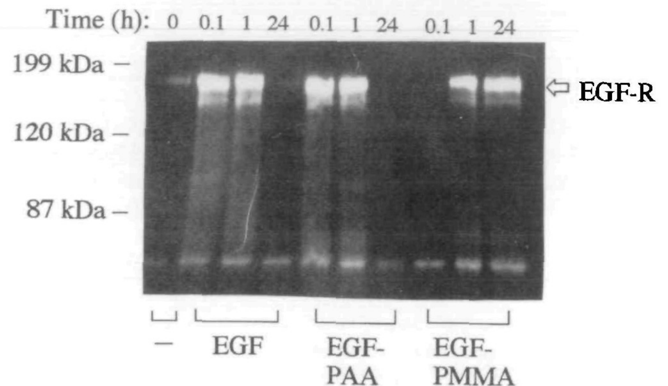

Fig. 5. **Time course of EGF receptor autophosphorylation in CHO-ER cells stimulated with EGF or its conjugates.** Quiescent CHO-ER cells (10^7 cells/dish) were cultured at 37°C for the indicated periods with the standard PMMA film in the absence (−) or presence of EGF (6 μg per dish), the standard PMMA film and EGF-PAA (6 μg of EGF per dish), or EGF-PMMA film (6 μg of EGF per dish). Cell lysates were subjected to immunoprecipitation with antibodies to the EGF receptor, and the tyrosine-phosphorylated receptors were detected by immunoblot analysis with antibodies to phosphotyrosine. EGF-R, EGF receptor.

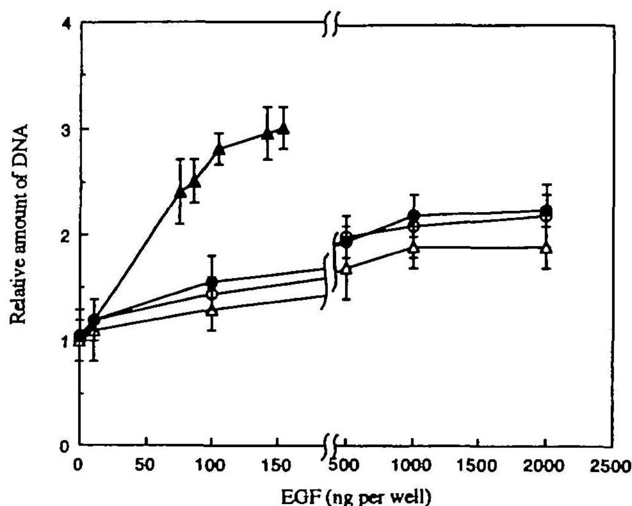

Fig. 4. **Effect of EGF conjugates on DNA synthesis in CHO-ER cells.** CHO-ER cells (10^5 cells per well) were cultured in Ham's F-12 medium at 37°C for 48 h in the presence of control PMMA film and free EGF (○), albumin-PMMA film and free EGF (●), control PMMA film and EGF-PAA (△), or EGF-PMMA film (▲). Cells were lysed and homogenized, and the amount of total DNA was determined by complex formation with 4′,6-diamidino-2-phenylindole. The amount of total DNA in cells grown in the presence of the standard PMMA film without EGF was taken as unity. Data are means±SD (n=8).

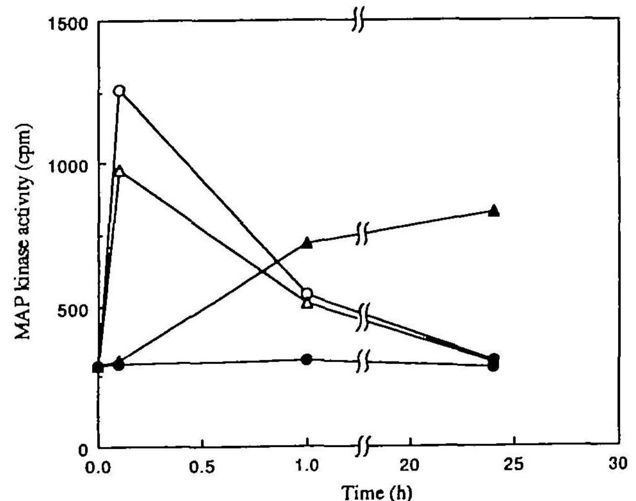

Fig. 6. **Time course of MAP kinase activation in CHO-ER cells stimulated with EGF or its conjugates.** CHO-ER cells were incubated for the indicated periods at 37°C with the standard PMMA film and free EGF (100 ng per well) (○), the standard PMMA film and EGF-PAA (100 ng of EGF per well) (△), the EGF-PMMA film (100 ng of EGF per well) (▲), or the albumin-PMMA film in the absence of EGF (●). Cell lysates were subjected to immunoprecipitation with antibodies to p44^{mapk}, and kinase activity was measured in terms of incorporation of ^{32}P from [γ-^{32}P]ATP into MBP. Similar results were obtained in two additional experiments.

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TABLE I. **Fate of EGF conjugates during interaction with CHO-ER cells.** Quiescent CHO-ER cells (10^6 cells per well) were incubated for 48 h at 37°C in the presence of the standard PMMA film and free EGF (10 ng per well), the standard PMMA film and EGF-PAA (10 ng of EGF per well), or the EGF-PMMA film (10 ng of immobilized EGF per well); all EGF preparations were labeled with ^{125}I. The amounts of radioactivity associated with harvested cells and with the EGF-PMMA film were measured with a γ-counter. Data are means±SD ($n=5$).

Sample	^{125}I-EGF associated with cells (% of total)	^{125}I-EGF remaining on film (% of initial value)
EGF	8.4±0.8	
EGF-PAA	5.5±1.1	
EGF-PMMA	0.0±0.5	99.9±1.2

TABLE II. **Binding of ^{125}I-EGF to CHO-ER cells after interaction with EGF conjugates.** Quiescent CHO-ER cells were incubated for 24 h at 37°C on the standard PMMA film with free EGF (150 ng per well) or with EGF-PAA (150 ng of EGF per well), on the EGF-PMMA film (150 ng of immobilized EGF per well), or on the albumin-PMMA film without EGF. Cells were harvested, washed, and then incubated with 0.1 nM ^{125}I-EGF at 4°C overnight. Binding was calculated by dividing the amount of radioactivity in cell lysates by the amount of lysate protein, and is expressed as a percentage of that apparent with quiescent CHO-ER cells not previously exposed to EGF. Data are means±SD ($n=3$).

Sample	^{125}I-EGF specific binding (% of control)
EGF	25±7
EGF-PAA	16±4
EGF-PMMA	93±5
Albumin-PMMA	97±2

Signal transduction downstream of the EGF receptor was assessed in terms of activation of MAP kinase. Thus, after incubation of quiescent CHO-ER cells with unconjugated EGF, EGF-PAA, or EGF-PMMA, cell lysates were subjected to immunoprecipitation with antibodies to MAP kinase. The activity of MAP kinase in immunoprecipitates was determined by phosphorylation of MBP (Fig. 6). The MAP kinase activity in immunoprecipitates from CHO-ER cells treated with free EGF was maximal at 6 min and had decreased to control values by 24 h. Stimulation of MAP kinase by EGF-PAA showed a similar time course, but the maximal effect was less than that of unconjugated EGF. In contrast, EGF-PMMA induced a gradual activation of MAP kinase, with the maximal effect apparent at ~24 h. Albumin-PMMA films had no effect on MAP kinase activity.

Effect of EGF Conjugates on the Fate of EGF Receptors—Quiescent CHO-ER cells were cultured for 48 h with free ^{125}I-EGF, or with EGF-PAA or EGF-PMMA prepared from ^{125}I-EGF. Radioactivity was associated with cells incubated with either free EGF or EGF-PAA, but not with cells incubated with EGF-PMMA (Table I). In addition, the amount of radioactivity associated with EGF-PMMA was not decreased after incubation with cells.

The fate of EGF receptors after interaction with EGF derivatives was examined with a ^{125}I-EGF binding assay. Quiescent CHO-ER cells were incubated with unconjugated EGF, EGF-PAA, EGF-PMMA, or albumin-PMMA at 37°C for 24 h. The treated cells were harvested, washed, and then incubated overnight at 4°C with 0.1 nM ^{125}I-EGF. Cells treated with either unconjugated EGF or EGF-PAA showed markedly decreased binding of ^{125}I-EGF relative to control, untreated cells, whereas prior exposure to EGF-PMMA or albumin-PMMA had no effect on binding (Table II). It was considered that, although EGF-PAA was taken into the cells by endocytosis after internalization, EGF-PMMA was neither taken up nor internalized.

DISCUSSION

EGF binds to the cell surface receptors, resulting in the formation of complexes that are internalized and sorted, with some receptors being recycled to the cell surface. However, it is not known which steps in this process are required for signal transduction. The necessity for internalizing the EGF-receptor complex can be investigated by immobilization on a substrate, which inhibits EGF internalization.

EGF-conjugates with fluorescein (*29, 30*), urogastrone (*31*), ricin A (*32–34*), diphtheria toxin (*35*), *Pseudomonas* exotoxin (*36*), *Pseudomonas* endotoxin (*34*), β-amanitin-poly-L-ornithine (*37*), dextran (*38–40*), and fusion peptides (*41*) bind to EGF receptors and exhibit varying degrees of mitogenic activity. Since the conjugates thus constructed by modifying the N-terminal of EGF retained biological activities, we modified the N-terminus in this study. Two types of conjugates were synthesized. One was diffusible in solution and the other was immobilized on a substrate. Both the EGF-synthetic polymer conjugates described were mitogenic and activated the EGF signaling pathway in CHO-ER cells with distinct time courses.

The mitogenic activity of EGF-PAA was lower than that of free EGF, possibly because of steric hindrance attributable to PAA interfering with binding to EGF receptors. In contrast, the maximal mitogenic effect of immobilized EGF (EGF-PMMA) was greater than that of free EGF and required less growth factor. Thus, the local concentration of EGF on the PMMA film surface is sufficient to promote effective interaction with EGF receptors of adsorbed cells.

The greater mitogenic effect of EGF-PMMA is probably attributable to the more protracted activation of the EGF signaling pathway apparent with this conjugate than with either free EGF or EGF-PAA. This persistent activation in turn, probably results from the fact that the complex of immobilized EGF with the EGF receptor cannot be internalized and the receptor is therefore not down-regulated. It is also possible that by preventing the lateral diffusion of activated receptors in the cell membrane, immobilized EGF reduces the chance that the receptors will encounter inhibitory regulators such as tyrosine phosphatases or serine/threonine kinases.

Wakshull and Wharton (*42*) have shown that concanavalin A stabilizes the complex of EGF with its receptor, thus inhibiting receptor endocytosis and that the presence of the stabilized ligand-receptor complex at the cell surface resulted in an acute biological response to EGF (RNA, but not DNA synthesis). However, cells expressing recombinant internalization-deficient mutant EGF receptors are fully activated by EGF (*43–46*). Various native extracellular matrices and adhesion factors activate intracellular signaling pathways by interacting with integrins, without inducing integrin internalization (*6, 47, 48*). In addition, some growth factors and cytokines stimulate cells in a juxtacrine manner. These results demonstrated that signal transduction does not necessarily require internalization of the signal ligands.

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This study demonstrated an artificial juxtacrine stimulation with immobilized EGF. Considering the persistent activation by immobilized EGF, juxtacrine stimulation is thought to differ from stimulation with diffusible ligands (paracrine, endocrine, and autocrine) in both intensity and duration. PC12 cells either differentiate or proliferate in response to growth factor stimulation, depending on the intensity or duration (or both) of the stimulus, as indicated by the time course of MAP kinase activation (*49-52*). It is possible that the stimulation modes are related to gene expression.

The interaction of immobilized biosignals with cells was originally investigated by fixing insulin to microporous polysaccharide gels (*53*). Nerve growth factor immobilized on a Sepharose gel was also biologically active (*54*). However, the usefulness of this approach was limited by problems such as detachment of the biosignal molecules from the polysaccharide gel and difficulties of cells gaining access to the hormone because of gel porosity (*55-58*). Because most of the immobilized biosignals were located in the pores of the gels and could not interact with cells, it was impossible to quantify their biological effects. We evaluated the relationship between the amount of EGF and its biological effect by immobilizing it on a smooth (nonporous) surface.

Although it is difficult to be certain that no immobilized EGF is released from the substratum (this situation is the same as in the case of juxtacrine stimulation using formalin-fixed transfectant cells), this study shed light on the mechanism of action of immobilized EGF by demonstrating its considerable mitogenic activity and the different time course of key signal transduction events. The immobilization of growth factors on an insoluble supporting matrix is an important technique for elucidating the sites and mechanisms of signal transduction and for developing clinical therapeutics such as drug delivery systems, tissue regeneration systems, and bioartificial organs.

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