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Surface plasmon resonance-biosensor detects the diversity of responses against epidermal growth factor in various carcinoma cell lines

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ABSTRACT

Surface plasmon resonance (SPR) biosensor detects intracellular signaling events as a change of the angle of resonance (AR). We previously reported that the activation of epidermal growth factor receptor (EGFR) on keratinocytes causes a unique triphasic change of AR, whereas the activation of other receptors, such as IgE receptor and adenosine A3 receptor on mast cells, causes a transient monophasic increase of AR. To study the mechanism of AR changes induced by EGFR activation, we introduced wild and mutated EGFR cDNAs into Chinese hamster ovary (CHO) cells and analyzed changes of AR in response to EGF. CHO cells expressing wild-type EGFR showed a triphasic change of AR, whereas cells expressing kinase-dead EGFR (K721M) showed minimum change of AR. A phosphatidylinositol 3-kinase inhibitor, wortmannin, attenuated the third phase of AR change in CHO cells expressing wild-type EGFR. The pattern of AR change was independent on the concentration of EGF. We also analyzed changes of AR with a nontumorigenic keratinocyte cell line, HaCaT, and several cell lines of carcinoma to explore the feasibility of SPR biosensor as a tool for clinical diagnosis. The activation of HaCaT cells and one out of six carcinoma cell lines showed a full triphasic change of AR. In contrast, five out of the six cell lines showed mono- or bi-phasic change of AR. These results suggest that EGF induces the SPR signals via the phosphorylation of EGFR, and provide a possibility that the SPR biosensor could be applied to the real-time detection and diagnosis of malignant tumors.

1. Introduction

Surface plasmon resonance (SPR) biosensors are capable of characterizing the binding of detectants in the field of resonance on a sensor chip in real-time without any labeling (Homola, 2003). They are useful to study the interactions of biological molecules from proteins, oligonucleotides, and lipids through to small substances, such as phages, viral particles, and cells (Rich and Myszka, 2000). We previously reported that SPR sensors can detect unexpectedly large changes of the angle of resonance (AR), when adherent RBL-2H3 mast cells (Hide et al., 2002) or non-adherent human basophils (Yanase et al., 2007b) were cultured on a biosensor chip and stimulated by antigen, indicating that SPR biosensors could be appropriate for the real time and non-label detection of the activation of living cells. In addition, we recently found that the change of AR reflects intracellular events in living cells rather than changes in the size of the area to which cells adhere (Yanase et al., 2007a), and that the antigen-induced increase of AR in RBL-2H3 cells are largely depend on the activation of PKCβ (Tanaka et al., 2008). RBL-2H3 cells consistently showed a monophasic increase of AR in response to antigen stimulation (Hide et al., 2002). In contrast, PAM212 keratinocytes, which were stimulated with epidermal growth factor (EGF), typically exhibited a triphasic change of AR (Yanase et al., 2007a). However, the exact mechanisms, which cause the differences in patterns of AR change with different types of cells or different types of stimulation, remain undetermined.

Epidermal growth factor receptor (EGFR), one of the ErbB family of receptor tyrosine kinases, is a transmembrane tyrosine kinase receptor promoting proliferation and survival of both normal and cancer cells. EGFR dimerization by its ligand, EGF, causes the phosphorylation of several tyrosine residues, and then recruits and activates the subsequent signaling molecules, such as phospholipase Cγ, c-Cbl, p85 subunit of phosphatidylinositol 3-kinase (PI3K),

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Grb1/2, Shc, and Shp1 (Ono and Kuwano, 2006). Some of the abnormal activation of EGFR in cancer cells is due to an amplification of EGFR, or gain-of-function mutations of EGFR in its tyrosine kinase domain (Normanno et al., 2006). The EGFR-targeted therapies, using small molecule tyrosine kinase inhibitors (erlotinib and gefitinib) or monoclonal antibodies against EGFR (cetuximab and panitumumab), have effectively improved outcomes for patients with colorectal, lung, head and neck, and pancreatic cancers (Laurent-Puig et al., 2009).

In this study, we established exogenous human EGFR-expressing Chinese hamster ovary (CHO) cells and studied an EGF-induced change of AR. We also analyzed the change of AR induced by EGF with several human carcinoma cell lines that express EGFR as well as a nontumorigenic human keratinocyte cell line, HaCaT, and revealed that different types of cells may show different patterns of SPR signals.

2. Materials and methods

2.1. Reagents and antibodies

The recombinant human epidermal growth factor was from R&D systems (Minneapolis, MN, USA). A PI3K inhibitor, wortmannin, was from Sigma–Aldrich (St. Louis, MO, USA). The monoclonal antibody against FLAG was from Stratagene (La Jolla, CA, USA). The polyclonal antibodies against EGFR, phospho-EGFR (pY1148), Akt, and phospho-Akt (pT308) were from Cell Signaling Technology (Beverly, MA, USA).

2.2. Human carcinoma cell lines and a nontumorigenic human keratinocyte cell line, HaCaT

The gastric cancer cell lines, MKN-1, MKN-7, TMK-1, and MKN-28 (Yoshida et al., 1990) were generous gifts from Dr. Yasuhiko Kitadai, Department of Medicine and Molecular Science, Hiroshima University Graduate School of Biomedical Sciences. The prostate cancer cell lines, DU145 and LNCap, were kindly provided from Dr. Hiroaki Yasumoto, Department of Urology, Graduate School of Medical Sciences, Hiroshima University. DU145 cells were maintained in MEM supplemented with 10% fetal calf serum (FCS) and antibiotics. All other cancer cell lines were maintained in RPMI1640 supplemented with 10% FCS and antibiotics. HaCaT cells (Boukamp et al., 1988) were maintained in DMEM supplemented with 10% FCS and antibiotics.

2.3. EGFR expression vectors

The human EGFR cDNA (pco12 EGFR) was provided by DNA Bank, BioResource Center, RIKEN (Tsukuba, Ibaraki, Japan), and the cDNA was amplified by PCR with the following primer pair, 5′-TGCAGGATATCGCTCTTCGGGGAGCAGCGATG-3′ and 5′-CCGCTCGAGTGCTCCAATAAATTCACTGCTTTG-3′. The PCR product was digested with *EcoR V* and *Xho I* and ligated into the same sites of pCMV-Tag4 vector (Stratagene) for the addition of C-terminal FLAG epitope. The EGFR with the mutation on the ATP binding site (K721M) (Chen et al., 1987) was generated by the QuikChange® Site Directed Mutagenesis Kit (Stratagene) and the following primer pair; the sense primer: 5′-GTTAAAATTCCCGTCGCTATCATGGAATTAAGAGAAGCAACATCTC-3′, the antisense primer: 5′-GAGATGTTGCTTCTCTTAATTCCATGATAGCGACGGGAATTTTAAC-3′.

2.4. Transfection

CHO-K1 cells (RCB0285) were provided by RIKEN Cell Bank (Tsukuba, Ibaraki, Japan) and maintained with Ham's F-12 (Invitrogen) containing 10% FCS and antibiotics. Cells were transfected with FLAG-tagged EGFRs and were selected by 1 mg/ml G418 for two weeks. The clones that stably expressed the FLAG-tagged EGFRs were selected by immunoblotting using anti-FLAG antibody.

2.5. Western blot analysis

Cells were washed twice with ice-cold phosphate buffered saline and lysed in the lysis buffer (25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% nonidet P-40, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 25 μg/ml aprotinin, and 2 μg/ml pepstatin) (Hiragun et al., 2006) and incubated for 30 min on ice. The cell lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membrane (Immobilon-P; Millipore, Billerica, MA, USA). The membrane blots were probed with the indicated primary antibodies, and the immunoreactive proteins were visualized by the use of horseradish peroxidase-conjugated secondary antibodies and chemiluminescence.

2.6. Flow cytometric analysis

The CHO cells expressing wild-type EGFR or EGFR-K721M were cultured on RepCell™ plates (CellSeed, Tokyo, Japan), detached at low temperature, and then stained with PE-conjugated anti-EGFR monoclonal antibody (BD Bioscience, Franklin Lakes, NJ, USA) or its isotype control. The surface expression of EGFR was determined with FACSCalibar™ (BD Bioscience).

2.7. SPR analysis

The method measuring the change of AR of living cells in response to antigen stimulation was described previously (Hide et al., 2002; Yanase et al., 2007a). Briefly, on the day before experiments, cells were seeded on biosensor chips (1.2×10^4 cells/60 μl/site) and cultured overnight. The sensor chip was equipped in a flow-cell unit of the SPR apparatus, SPR-CELLIA (Moritex, Nagoya, Japan). The cells placed in the chambers were preincubated for 20 min with glucose saline/pipes buffer (Hiragun et al., 2006) and stimulated with 10 ng/ml of EGF for 10 min. The change of AR during cellular activation was monitored by SPR-CELLIA for up to 30 min.

3. Results

3.1. CHO cells expressing the wild type EGFR and HaCaT cells show triphasic changes of AR in response to EGF

CHO cells, which do not possess endogenous EGFR (Chen et al., 1987; Krug et al., 2002), were transfected and the CHO cell clone stably expressing the exogenous wild type human EGFR was established. The mock or EGFR-transfected cells were stimulated with EGF and the change of AR was monitored by the SPR biosensor. As shown in Fig. 1A, EGFR-transfected cells showed a typical triphasic change in response to EGF. The first phase was short, tiny spike like increase in response to EGF. The second phase was the following quick decrease, and the third phase was the relatively slow increase of AR close to the base line. HaCaT is a spontaneously transformed human epithelial cell line from adult skin, and it is immortal but nontumorigenic (Boukamp et al., 1988). HaCaT cells also showed the triphasic change of AR in response to EGF (Fig. 1B).