Peptide immobilized on gold particles enhances cell growth

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Abstract A multivalent ligand of thrombopoietin (TPO) was prepared by immobilization of mimetic peptides on gold particles. An effective peptide ligand containing cysteine was designed to enhance the growth of TPO-sensitive cells. The peptide was then immobilized on gold particles by self assembly. The multivalent ligand enhanced the growth of TPO-dependent cells and its activity was more than that of the monovalent ligand.

Keywords Thrombopoietin · Multivalency · Gold particle · Bioconjugate · Cell proliferation

Introduction

Thrombopoietin (TPO) is a glycoprotein consisting of 332 amino acids, active at many stages in the development of megakaryocytic progenitors, leading to the production of platelets (Kaushansky 2008).

Therefore, recombinant human (rh) TPO has been tested as a treatment for patients with thrombocytopenia, including those receiving nonmyeloablative chemotherapy (Nash et al. 2000). However, with a protein-based drug, some difficulties must be taken into consideration for future drug development, such as the limited administration methods and the development of an antibody against TPO (Sakai et al. 2005). Thus, small peptide or nonpeptide molecules that mimic the activity of TPO have been investigated (Duffy et al. 2001).

Several small peptides of 14–16 amino acid residues and their dimers have been reported as potent agonists of the TPO receptor c-Mpl (Cwirla et al. 1997), with in vivo efficacy in mice. Recently, nonpeptide synthetic compounds, such as a benzodiazepine derivative, hydrazinonaphthalenes (Kimura et al. 1998), azonaphthalenes (Duffy et al. 2002), and a substituted thiazole (Inagaki et al. 2004), have been reported to have TPO-like activity. Sakai et al. (2005) reported the isolation of xanthocillins from a marine fungus, *Basipetospora* sp., as TPO mimics and discussed xanthocillins as a novel class of c-Mpl agonist.

In addition to these drug discovery efforts, it is possible to enhance drug activity by modification of the drug itself. Natural and synthetic multivalent ligands can function as potent inhibitors or effectors of biological processes (Huskens 2006; Kiessling et al. 2006; Wall et al. 2008). Because they present multiple copies of a receptor-binding element, multivalent

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ligands can bind to receptors with high avidity and specificity, thereby acting as powerful inhibitors. At the same time, multivalent ligands can be potent effectors that promote a specific biological response via signal transduction.

In this study, a peptide ligand that acts as a TPO mimetic was immobilized on gold particles to form a multivalent ligand. Considering that colloidal gold has been safely used to treat rheumatoid arthritis for half a century (Root et al. 1954; Merchant 1998), multivalent ligands based on gold particles are expected to be candidates for medical applications.

Materials and methods

Materials

rhTPO was purchased from CosmoBio Co. (Tokyo, Japan). TPO-mimicking peptides (Table 1) were synthesized at the Brain Research Institute of RIKEN (Wako, Japan). Spherical gold particles (diameter, 1.5–3.0 μm) were purchased from Sigma-Aldrich (Lot #03212BC, St Louis, MO, USA).

Immobilization

The gold particles were washed with Piranha solution (30% H_2O_2 : H_2SO_4 = 1:3) and rinsed several times with water. Various concentrations of peptide solution were added to the suspensions. After the suspension had been incubated at 20 $^\circ\text{C}$ for 1 h, it was washed with phosphate-buffered saline repeatedly by centrifugation at 15,000g for 5 min until no further peptide was released from the immobilized particles. The lack of release was confirmed by staining the supernatant using Coomassie Protein Assay Kits (Bradford method; Pierce, Rockford, IL, USA). The amount of immobilized peptide was also determined using these assay kits. After immobilization, the mixture was centrifuged at 15,000g for

5 min at room temperature and part of the supernatant was taken for assay. The amount of immobilized peptide was determined by the difference in peptide solution concentration before and after the addition of gold particles.

Cell culture

The FDCP-hM15 cell line, which proliferates in the presence of TPO, was kindly supplied by the Kirin Brewery Co. (Tokyo, Japan) (Morita et al. 1996). The cells were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum (Gibco, Invitrogen, Carlsbad, CA, USA) and 1% penicillin G-streptomycin (Gibco). The cell suspension (1 mL of 1×10^5 cells/mL) was added to the wells and incubated in a humidified atmosphere of 5% CO_2 at 37 $^\circ\text{C}$ for the required time. After incubation, the number of cells was counted using a cell counting kit (Dojindo, Kumamoto, Japan).

The results are given as means $\pm$ standard deviations (SD). Statistical differences were determined with Student's two-tailed *t*-test. Scheffe's method was used for multiple comparisons at a significance level of 95%.

Results and discussion

Biological activity of peptides

Figure 1 shows the activity of rhTPO and its peptide derivatives. rhTPO had the highest activity. The peptide sequence of TPO-P is the same as that of AF12505, reported by Cwirla et al. (1997). They reported that the activity of AF12505 was about 10^{-4} times as high as that of rhTPO, calculated from the proliferative response of Ba/F3 hTPOR cells. In the present study, the activity of TPO-P was also about 10^{-4} times as high as that of rhTPO, although the cells used for the estimation in the present study (FDCP-hM15) (Morita et al. 1996) were different. This result indicates that the activity estimation using FDCP-hM15 cells is similar to that using Ba/F3 hTPOR cells.

The peptide derivative TPO-P-Cys had about 10^2 times higher activity than TPO-P, whereas the activity of TPO-P-Lys was similar to that of TPO-P. We considered that TPO-P-Cys formed a dimer

Table 1 Prepared peptide derivatives

Abbreviation	Peptide sequence
TPO-P (AF12505)	IEGPTLRQWLAARA
TPO-P-Lys	IEGPTLRQWLAARAK
TPO-P-Cys	IEGPTLRQWLAARAC

Fig. 1 Proliferative response of FDCP-hM15 cells to peptides TPO-P, TPO-P-Lys, TPO-P-Cys, and rhTPO. $n = 3$

with enhanced activity. Cwirla et al. (1997) reported that a type of dimer of AF12505, AF13948, had high activity, which was close to that of rhTPO.

Preparation of peptide immobilized on particles

Because TPO-P-Cys has a cysteine residue at the carboxy terminus, it was used for the immobilization of the peptide onto the gold surface. It is known that the S–H bond of the cysteine cleaves and the molecule chemisorbs to the surface through a covalent Au–S bond. The amount of immobilized peptide ligands was measured. When the peptide concentration was 1 mg/mL, a gold particle concentration of 10 mg/mL was sufficient to achieve almost quantitative immobilization of the whole amount of peptide on the particle surfaces.

Biological activity of immobilized peptide

First, we confirmed that no immobilized peptide was released during the cell culture. The biological activity of the immobilized TPO-P-Cys was measured using nonlabeled peptide and the results are shown in Fig. 2. The immobilized TPO-P-Cys had higher activity than soluble TPO-P-Cys, although the activity of immobilized TPO-P-Cys was lower than that of rhTPO.

The effects of multivalent ligands have been discussed by many researchers (Huskens 2006; Kiessling et al. 2006; Ito 2008; Wall et al. 2008). They govern many interactions between proteins and small molecules, between proteins or antibodies and

Fig. 2 Proliferative response of FDCP-hM15 cells to soluble TPO-P-Cys peptide, TPO-P-Cys immobilized on gold particles, and soluble rhTPO. $n = 3$

cell membranes, between viruses and cells, etc. There are multiple possible mechanisms underlying the enhanced activity produced by multivalency resulting from tethering, including higher overall ligand affinity attributable to avidity effects, forced receptor multimerization, stabilization of the receptor–ligand complex, and the inhibition of receptor–ligand internalization (Ito 2008).

Recently, gold particles have been investigated for medical applications. In fact, colloidal gold has been safely used to treat rheumatoid arthritis for half a century (Root et al. 1954; Merchant 1998). Recent work has indicated that pegylated gold nanoparticles (colloidal gold particles coated with a protective layer of polyethylene glycol [PEG]) exhibit excellent in vivo biodistribution and pharmacokinetic properties after systemic injection (Paciotti et al. 2006; James et al. 2007; Qian et al. 2008).

This study demonstrated the preparation of a peptide immobilized on gold particles, and showed that this conjugate had enhanced biological activity caused by multivalency.

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