Growth and secretion of erythropoietin of Chinese hamster ovary cells coexpressing epidermal growth factor receptor and erythropoietin genes: Design of cells for cell culture matrix

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Abstract

Chinese hamster ovary (CHO) cells were transfected with both genes encoding erythropoietin (Epo) and epidermal growth factor receptor (EGFR). The transfection of the Epo gene was confirmed by an enzyme-linked immunoassay. Overexpression of EGFR was confirmed by Western blotting of EGFR. The transfected CHO cells were cultured in serum-free medium in the presence of soluble epidermal growth factor (EGF) or immobilized EGF. The CHO cells overexpressing EGFR grew in the presence of less EGF than the cells not overexpressing EGFR. In addition, the growth of EGFR-overexpressing CHO cells was enhanced in the presence of immobilized EGF more efficiently than in the presence of soluble EGF. The amount of Epo secreted from the cells increased linearly with the increase of growth rate. Consequently, culture of CHO cells coexpressing Epo and EGFR on EGF-immobilized matrix was the most efficient for Epo production.

Introduction

Polypeptide growth factors were believed to transmit their signals by diffusional stimulation such as paracrine, endocrine, or autocrine. However, an important variation became known in the stimulation mode of signaling: certain growth factors, including epidermal growth factor (EGF) family, exist as membrane-anchored forms that bind and activate membrane receptors on adjacent cells. This process, termed juxtacrine stimulation, can deliver spatially localized intercellular stimuli while supporting adhesive interactions (Bosenberg and Massague, 1993; Massague and Pandiella, 1993). An increasing number of growth factors that derive from membrane-anchored precursors and growth factors which function both as soluble and as membrane growth factors have been described in the

past several years (Tajima et al., 1998; Tanaka et al., 1998).

On the other hand, we found that covalently immobilized growth factors can stimulate cell growth (Ito et al., 1991). Micropattern immobilization of growth factors demonstrated the signal transduction without enhancement of cell adhesion (Ito et al., 1998). By using the matrices immobilized with growth factors, no protein supplements were needed in the medium and the immobilized growth factor was repeatedly utilized like enzymes immobilized in bioreactors (Ito et al., 1996).

Recently, in addition to the matrix design, cell lines for cell culture engineering have been designed by several researchers. Ito et al. (1995) reported that overexpression of bcl-2, an apoptosis-suppressing gene, prolonged the viable culture period of hybridoma and enhanced antibody production. It was reported that

Figure 1. Relative growth rates of CHO (a), CHO-Epo (b), and CHO-Epo-EGFR cells (c) in the presence of soluble (○), or photo-immobilized EGF (●). The relative cell growth rate calculated from the photometric absorbance of cells cultured in the tissue culture plates in the absence of EGF was taken as 1.0. Bars represent the standard deviations, $n = 6$.

overexpression of epidermal growth factor receptor (EGFR) amplified normal epidermal growth factor (EGF) signal transduction (Fiore et al., 1987; Reddy et al., 1994), and the metabolic and mitogenic effects of both insulin and insulin-like growth factor were enhanced by transfection of insulin receptors into NIH3T3 fibroblasts (Hofmann et al., 1989).

In the present study, both culture matrices and cells were designed to achieve an efficient production of protein by cell culture engineering. As a target protein, erythropoietin (Epo) was chosen. Epo is one of the principal cytokines involved in the regulation and maintenance of a physiological level of circulating erythrocytes. Epo supports the survival of erythroid precursor cells and stimulates their proliferation and differentiation through interaction with the Epo receptor integrated into the plasma membrane (Krantz, 1991). Therefore, the recombinant Epo has been produced by cell culture engineering for use in therapy and research (Glover, 1984). For the production of

proteins, serum-free cell culture engineering is preferred, taking into consideration the high cost of serum and the interference with purification of the secreted proteins.

The genes of Epo (for Epo production) and EGFR (for enhancement of sensitivity to EGF-immobilized matrix) were coexpressed in Chinese hamster ovary (CHO) cells and the cells were cultured on the immobilized EGF.

Materials and methods

Materials

Mouse EGF was purchased from Toyobo (Osaka, Japan). ¹²⁵I-EGF, of which tyrosine residues were labeled with radioactive iodine by chloramine T method, was purchased from Daiichi Chem Ltd. (Tokyo, Japan). MicroscintTM-20 was a product of

Figure 2. The secretion of Epo from CHO cells cultured in the presence of soluble (○), or immobilized EGF (●); CHO-Epo cells cultured in the presence of soluble (△), or immobilized EGF (▲); and CHO-Epo-EGFR cells cultured in the presence of soluble (□), or immobilized EGF (■). The concentration of soluble EGF is calculated by dividing the amount with 1 ml and the surface concentration of immobilized EGF by dividing with 1.77 cm². Bars represent the standard deviations, n = 6.

Figure 3. The relationship between Epo secretion and growth rate of CHO-Epo cells cultured in the presence of soluble (○), or immobilized EGF (●); and CHO-Epo-EGFR cells cultured in the presence of soluble (△), or immobilized EGF (▲). Bars represent the standard deviations, n = 6.

Canberra Company (The Netherlands). A cell counting kit was purchased from Dojin Chem (Kumamoto, Japan). Ham's F12 medium was purchased from Nissui Pharm. Co. Ltd. (Tokyo, Japan).

Transfection of cells with the Epo gene

Epo expression plasmid was constructed under control of the CMV promotor as reported previously (Moon et al., 1997). Lipofectamine reagent forming liposome (Felgner et al., 1989) was used to transfect the Epo gene (pCMV-Epo) into CHO-K1 (CHO) cells. CHO cells were cultured in Ham's F-12 serum-free medium containing pCMV-Epo/lipofectamine mixture at 37 °C, in a 5% CO₂ atmosphere for 6 h. Then the medium was changed to Ham's F-12 medium containing 10% fetal bovine serum (FBS), and the cells were selected by adding Geneticin (G-418, 400 µg ml⁻¹). The selected cells were screened for secretion of Epo by enzyme-linked immunoassay (Goto et al., 1989). The CHO cells transfected with pCMV-Epo are referred to as CHO-Epo.

Epo measurement with enzyme-linked immunoassay

The amount of Epo was measured by an improved method of a sandwich-type enzyme-linked immunoassay using two monoclonal antibodies, R2 and R6, that recognize different epitopes of Epo (Goto et al., 1989). This improved method measures Epo as low as 100 microunits ml⁻¹ (1 pg ml⁻¹). The amount of Epo was calculated from the standard curve drawn using recombinant human Epo (Goto et al., 1988) as the standard, which was standardized with the Second International Standard Reference Preparation for human Epo.

Transfection of CHO-Epo cells with the EGFR gene

Plasmids carrying EGFR cDNA were kindly provided by Drs Masato Kasuga and Yoshinori Okabayashi. DH5 α was transformed with PRC/CMV/EGFR and the resulting plasmid DNA was purified and used for transfection into CHO-Epo cells. The method of transfecting PRV/CMV/EGFR into CHO-Epo cells was the same as the transfection of pCMV-Epo into CHO cells. Stable colonies were selected and screened for expression of EGFR by Western blotting. The CHO-Epo cells overexpressing EGFR are referred to as CHO-Epo-EGFR cells. The EGFR was assayed by Western blotting.

Preparation of photo-immobilized EGF and Cell culture

The photo-immobilized EGF was prepared as previously reported (Chen et al., 1997). CHO cells, CHO-Epo cells and CHO-Epo-EGFR cells were subcultured in Ham's F12 medium containing 10% (v/v) FBS. The cells were harvested from culture flasks with PBS containing 0.15% (w/v) trypsin (2000 unit/g) and 0.02% (w/v) ethylenediaminetetraacetic acid (EDTA), without either calcium or magnesium ions. The cells were washed once with Ham's F12 containing FBS and once with Ham's F12, respectively. They were suspended in Ham's F12 and adjusted to a concentration of 2×10^5 cells ml⁻¹, which was counted with a hemocytometer. The cell suspension (100 µl) was mixed with 900 µl of Ham's F12 and the mixture was added to each well of 24-well polystyrene plates containing either soluble or immobilized EGF. The cells were incubated under 5% CO₂ in air at 37 °C for 48 h. The cells were also incubated in a 24-well polystyrene plate in the absence of EGF as a control. The cell growth was photometrically estimated using the cell counting kit (Ishiyama et al., 1993, 1996).

Results and discussion

Cell transfection and resultant cell lines

CHO cells were transfected with the Epo gene by using lipofectamine reagent forming liposome, and the cells expressing the gene (CHO-Epo) were selected by addition of Geneticin. The secretion of Epo from the selected CHO-Epo cells was confirmed by an enzyme-linked immunoassay. Since the transfectants were selected during culture in the presence of Geneticin, 100% of the colonies secreted erythropoietin. CHO-Epo cells were further transfected with EGFR by the liposome method. The overexpression of EGFR was confirmed by Western blotting of EGFR. CHO-Epo-EGFR cells expressed more EGFR than either CHO or CHO-Epo cells. However, in this case, since the transfectants were not selected during culture process, 23% of the colonies expressed the EGFR.

Cell growth

The relative cell growth rates of the three cell lines in the presence of soluble EGF or photo-immobilized EGF are shown in Figure 1. Photo-immobilized EGF stimulated the growth of the three cell lines more

efficiently than soluble EGF. This enhancement was considered to be due to inhibition of down-regulation of EGFR and the highly local concentration of EGF in contact with the cells, as previously described (Ito et al., 1991, 1996; Chen et al., 1997).

The EGF dependence of growth of CHO and CHO-Epo cells was similar. This result indicates that the transfection of Epo gene did not significantly affect the cell growth. In contrast, the EGF dependence of growth of CHO-Epo-EGFR cells was different from that of CHO or CHO-Epo cells. Less soluble EGF or photo-immobilized EGF was required to stimulate the growth of CHO-Epo-EGFR cells than CHO or CHO-Epo cells. In addition, the maximum cell growth rate of CHO-Epo-EGFR was higher than that of CHO or CHO-Epo. This result indicates that overexpression of EGFR not only enhanced the concentration effect of EGF, but also enhanced the stimulation strength. Consequently, the growth rate of CHO-Epo-EGFR on the photo-immobilized EGF was the highest among the growth rates of cells in the tested systems.

Secretion of Epo

The amount of Epo secreted into the culture medium was determined and is shown in Figure 2. No significant amount of Epo was detected in the medium in which CHO cells were cultured. In contrast, CHO-Epo and CHO-Epo-EGFR cells secreted significant amounts of Epo into the medium. The amount of Epo increased with increasing EGF concentration, and reached a maximum. In addition, CHO-Epo-EGFR cells secreted more Epo than CHO-Epo cells. The overexpression of EGFR enhanced the sensitivity of cells to EGF.

Immobilized EGF enhanced the Epo secretion from the cell lines. Consequently, the largest amount of Epo was secreted from CHO-Epo-EGFR cells cultured on photo-immobilized EGF. This result was considered to be due to growth enhancement. Therefore, the relationship between the growth and secretion was plotted as shown in Figure 3. The cell specific productivity of Epo increased linearly with the increase of cell growth. CHO-Epo and CHO-Epo-EGFR cells produced 0.185 and 0.189 pg/cell·3 days, respectively. The transfection with EGFR did not significantly affect the secretion ability of the cell.

The present study demonstrated that a cell culture system using overexpression of EGFR and immobilized EGF achieved high production of Epo from the system. In the past, matrix design for cell culture

engineering, design of bio-compatible materials, has been carried out by many researchers. The present study indicates that the design of matrix, together with the design of matrix-compatible cells, was useful for construction of a cell culture system. The combination of immobilization of cell growth factors with the transfection technique is, therefore, a good means to establish a new cell culture system.

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