Properties of *ras*-amplified recombinant BHK-21 cells in protein-free culture

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Received 9 November 1998; accepted 8 July 1999

Key words: BHK-21 cells, human monoclonal antibody, *ras* oncogene, synthetic attachment factor

Abstract

We compared serum and protein-free cultures of a *ras*-amplified recombinant BHK-21 cell line (*ras*-rBHK-IgG), which hyperproduces a lung cancer specific recombinant human monoclonal antibody. *Ras*-rBHK-IgG cells were shown to grow well, even in protein-free medium and to be morphologically similar to cells cultured in serum containing medium. However, the growth rate of *ras*-rBHK-IgG cells was considerably slower in protein-free medium, which results in a longer maintenance period compared with cells cultured in serum containing medium. In addition, it was found that antibody production in protein-free culture had a ten times higher maximum than cells cultured in serum containing medium. On the other hand, in high density culture, using the hollow fiber bioreactor system, *ras*-rBHK-IgG cells could be maintained for a month in protein-free culture in contrast with serum culture, which only lasted for half a month. However, the marked increase of antibody production was not observed. A total amount of about 15 mg of the recombinant antibody, obtained in protein-free culture, was about two times of that obtained in serum culture, and was shown to be reactive to lung cancer cells in tissue. From these properties in protein-free medium, it is concluded that protein-free culture of *ras*-rBHK-IgG cells is suitable for middle scale production of recombinant human monoclonal antibody.

Abbreviations: BHK, baby hamster kidney; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; hMAb, human monoclonal antibody

Introduction

With the development of genetic engineering, many biological products have been produced, using cultured mammalian cells (Goldberg et al., 1987; Xiao et al., 1992; Schlapfer et al., 1995; Kong et al., 1998). However, low productivity of mammalian cells remains a major obstacle for the production of useful biologicals, which can be used in the field of clinical diagnostics and therapeutics (Friedman et al.,

1989; Bebbington et al., 1992; Murakami et al., 1997; Miyazaki et al., 1998). We have established a *ras*-amplified recombinant BHK-21 cell line (*ras*-rBHK-IgG) and have shown that cells could hyperproduce the recombinant IgG chimeric human monoclonal antibody (hMAb) AE6F4 using the oncogene activated production system (Yano et al., 1994; Teruya et al., 1995; Shoji et al., 1996).

For the production of such biologicals, using cultured mammalian cells, protein-free medium is not

Figure 1. Photomicrographs of *ras*-rBHK-IgG cells cultured in serum containing or protein-free medium. Cells (1×10^5 cells) were cultured in ERDF medium supplemented with 10% FCS (A) or $80 \mu\text{M}$ iron(III) nitrate (B and C) for 5 days. In protein-free culture, non-coated (B) or FC-106-coated (C) dishes were compared.

only more economical than serum containing medium, but also facilitates the downstream purification of biologicals. *Ras*-rBHK-IgG cells were found to be able to culture in ERDF medium supplemented with $80 \mu\text{M}$ iron(III) nitrate (Inoue et al., 1996a, b). However, protein-free culture of *ras*-rBHK-IgG cells was still not characterized. In this study, we compare serum and protein-free cultures of *ras*-rBHK-IgG cells to clear the properties of the cells in a protein-free culture.

Materials and methods

Materials

A non-protein synthetic attachment factor FC-106, which is a polymethacrylate derivative containing the cell attachment peptide fragment (Arg-Gly-Asp-Ser), was supplied from Fuji Film Co., Ltd.

Cell line and culture condition

A *ras* oncogene-amplified recombinant BHK-21 cell line (*ras*-rBHK-IgG) has been established as reported earlier (Shoji et al., 1996) and is maintained in ERDF medium (Kyokuto Pharmacy Industrial Co.), supplemented with 10% fetal calf serum (FCS) at 37°C in humidified 5% $\text{CO}_2/95\%$ air.

In protein-free culture, cells were plated into 35-mm plastic dishes which were coated with $10 \mu\text{g}/\text{ml}$ of FC-106, and were cultured in 2 ml of ERDF medium supplemented with $80 \mu\text{M}$ iron(III) nitrate at 37°C in humidified 5% $\text{CO}_2/95\%$ air. Cells were also pre-cultured in protein-free medium for 7 days to adapt to protein-free conditions.

High density culture of recombinant BHK-21 cells using a hollow fiber bioreactor system

Ras-rBHK-IgG cells (ca. 1×10^8 cells) were cultured using the hollow fiber bioreactor system, Tecnomouse (INTEGRA Bioscience Co., Germany) as described earlier (Inoue et al., 1996b). The spent medium in the culture cassette was changed every other day, and the antibody concentration in the spent medium was measured by an enzyme-linked immunosorbent assay (ELISA).

Measurement of antibody production

Antibody concentrations were determined by ELISA as reported earlier (Shoji et al., 1994), using an anti-human IgG (γ) antibody (4100, TAGO) as the first antibody, and anti-human IgG (γ) peroxidase conjugate antibody (2390, TAGO) as the second antibody.

Figure 2. Growth curve of *ras*-rBHK-IgG cells cultured in serum-containing or protein-free medium. Cells were plated at initial cell number of 1×10^4 (■), 1×10^5 (△), or 1×10^6 (●) cells, and then cultured in ERDF medium supplemented with 10% FCS (left figure) or 80 μ M iron(III) nitrate (right figure). In protein-free culture, FC-106 coated dishes were used.

Figure 3. Antibody production of *ras*-rBHK-IgG cells cultured in serum-containing or protein-free medium. Cells were plated at initial cell number of 1×10^4 (■), 1×10^5 (△), or 1×10^6 (●) cells, and then cultured in ERDF medium supplemented with 10% FCS (left figure) or 80 μ M iron(III) nitrate (right figure). In protein-free culture, FC-106 coated dishes were used. Antibody concentration in the spent medium was measured by ELISA.

Reactivity of recombinant hMAb with lung cancer tissues

The binding activity of the recombinant hMAb to formalin-fixed paraffin-embedded adenocarcinoma tissue sections was examined by the indirect immunohistochemical analysis described by Murakami et al. (1985), where the peroxidase-labeled anti-human γ antibody was used as the second antibody. The bound recombinant hMAb was visualized by the enzymatic reaction using 3, 3'-diaminobenzidine as the substrate

for the antibody detection.

Results and discussion

Protein-free culture of *ras*-rBHK-IgG cells

Ras-rBHK-IgG cells could be easily cultured under protein-free conditions, and their morphology in protein-free culture was similar to that of cells cultured in serum containing medium (Figure 1). *Ras*-transformed cells are known to have low growth factor

Figure 4. Antibody production of *ras*-rBHK-IgG cells cultured at high density using the hollow fiber bioreactor system. Cells (ca. 1×10^8 cells) were cultured using the hollow fiber bioreactor system. The spent medium was changed every other day, of which antibody concentration was measured by ELISA.

Figure 5. Reactivity of recombinant human antibody with lung cancer cells in tissue. The binding activity of recombinant hMAb to formalin-fixed paraffin-embedded adenocarcinoma tissue sections was examined by the indirect immunohistochemical analysis. A: human IgG control, B: recombinant hMAb from serum culture, C: recombinant hMAb from protein-free culture.

requirements because they stimulate their own growth via the autocrine mechanism (Sporn et al., 1985; McKay et al., 1986; Shirahata et al., 1990). In our protein-free medium, cells could grow even from a low initial cell number as 1×10^4 cells.

Cell maintenance in protein-free medium was superior to in serum containing medium because cell

growth rate in protein-free medium was considerably slower in serum containing medium (Figure 2). Takahashi et al. (1994) reported that growth rate suppression of cultured mammalian cells enhanced protein productivities. We could also observe that antibody production in protein-free culture had a ten times higher maximum than in serum culture (Figure 3). The

increased rate of antibody production tend to be larger at the higher cell densities.

High density culture of ras-rBHK-IgG cells using a hollow fiber bioreactor system

Ras-rBHK-IgG cells were cultured at high density using the hollow fiber bioreactor system. In protein-free culture, however, the marked increase of antibody production, observed in a plastic dish, was not observed (Figure 4). Yamaguchi et al. (1997) reported that the direct contact of *ras*-rBHK-IgG cells, or at a very close distance between the cells is important for the enhancement of the hMAb productivity in protein-free culture, using collagen gel particles. In our case, however, cell density during cultivation was not known because the inner site of the culture cassette of the bioreactor system could not be observed.

On the other hand, cells could also be cultured in protein-free medium for a month using the bioreactor system, while cells cultured in serum containing medium was viable for only half a month (Figure 4). This difference is probably originated from cell growth rate in the limited surface area for cell attachment. The total amount of hMAb, produced during protein-free culture, was about 15 mg and corresponded to two times the serum culture. The specificity activity of antibody, produced in protein-free culture, was the same as that of antibody produced in serum culture by ELISA against the putative antigen, the 14-3-3 protein (data not shown). The histochemical analysis indicated that the recombinant hMAb obtained was specifically reactive to lung cancer cells in tissue, contrasted with control human IgG (Figure 5). From these results, it is concluded that protein-free culture of *ras*-rBHK-IgG cells is suitable for middle scale production of recombinant hMAb.

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