Enhanced antibody production of human-human hybridomas by retinoic acid

Yuichi Inoue^{1*}, Mihoko Fujisawa², Masahiro Shoji³, Shuichi Hashizume³, Yoshinori Katakura² & Sanetaka Shirahata²

¹ Department of Biochemical Science and Technology, Faculty of Agriculture, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan; ² Graduate School of Genetic Resources Technology, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan; ³ Morinaga Institute of Biological Science, 2-2-1 Shimosueyoshi, Tsurumi-ku, Yokohama 230-0012, Japan

(* Author for correspondence; E-mail: inoue@chem.agri.kagoshima-u.ac.jp)

Received 28 July 1999; accepted 14 January 2000

Key words: antibody production, human monoclonal antibody, hybridoma, retinoic acid

Abstract

The enhancement of human monoclonal antibody production by retinoic acid (RA) was evaluated using the human-human hybridoma cell line BD9 under serum-free culture condition. The amount of human IgG secreted by BD9 hybridomas was enhanced about eight-fold by treatment with 10^{-7} M of RA for 4 days. Northern blot analysis showed that both mRNA levels of the IgG light and heavy chains were markedly increased by RA when compared with control without RA treatment. On the other hand, it was found that continuous treatment of cells with RA was not always required to exhibit the enhancing effect, suggesting that RA may act as a trigger for IgG gene expression. The comparison between extra- and intracellular IgG amounts by immunoblot analysis suggests that the secretion rate of IgG may be accelerated by RA treatment. These results suggest that RA may be an effective culture additive for efficient production of human monoclonal antibody using human-human hybridomas.

Abbreviations: EBV, Epstein-Barr virus; ELISA, enzyme-linked immunosorbent assay; hMAb, human monoclonal antibody; Ig, immunoglobulin; RA, retinoic acid; SDS, sodium dodecyl sulfate

Introduction

Human monoclonal antibodies (hMAbs) have been much more preferably used for therapy in human than those of murine origin in order to avoid adverse immune effects. Human-human hybridoma provides an ideal system for producing hMAb. The hMAb produced by the human-human hybridoma cell line BD9 has been shown to be useful for immunocytological detection of some lung cancer cells in tissues (Shoji et al., 1994). However, antibody productivities of human-human hybridomas are too low to obtain a sufficient amount for clinical trials. Some investigators have ever searched substances which enhance antibody production of hybridomas to produce hMAb efficiently. Aotsuka et al. (1991) found that retin-

oids such as retinol and retinoic acid (RA) could enhance the hMAb production of some human-human hybridoma cell lines. We have recently reported that retinyl acetate may be effective for middle scale production of hMAbs by the human-human hybridoma cell line AE6 (Inoue et al., 1999). However, how retinoids enhance antibody production by human-human hybridomas was still not understood. An understanding of the regulation of antibody production by RA leads to optimization of antibody productivity in hybridoma culture system. In this study, we examined the enhancing effect of RA in the process of antibody synthesis using BD9 hybridomas.

Materials and methods

Cell line and cell culture

The human-human hybridoma cell line BD9, which produces the lung cancer associated antibody, was generated by fusing peripheral blood lymphocytes from a healthy adult with a T lymphoblastoid cell line A₄H₁₂ derived from Molt4 cells using an *in vitro* immunization method (Kawahara et al., 1992). Cells were maintained in ERDF medium (Kyokuto Pharmaceutical Industrial. Co., Tokyo, Japan) supplemented with 5 µg/ml insulin (I), 20 µg/ml human transferrin (T), 20 µM ethanolamine (E) and 25 nM sodium selenite (S) (ITES-ERDF medium), at 37 °C in humidified 5% CO₂/95% air.

Retinoic acid

All-trans-Retinoic acid (Wako Pure Chemical Industries Ltd, Osaka, Japan) was dissolved in ethanol at a concentration of 1 mM, and stored at -20 °C in small aliquots under light protection. For each experiment, stock aliquots were serially diluted with ethanol, and immediately added to the culture medium at final concentrations of 10⁻⁹ to 10⁻⁶ M.

Measurement of IgG concentration and cell number

The concentrations of IgG in culture medium were measured by an enzyme-linked immunosorbent assay (ELISA) as described previously (Shoji et al., 1994), using anti-human immunoglobulin (Ig) antibody (#4100; TAGO, Burlingame, CA, USA) as the first antibody, and anti-human Ig peroxidase conjugate antibody (#2390; TAGO, Burlingame, CA, USA) as the second antibody. Cell number was counted by using a cell counter, and viability was determined by the trypan blue dye exclusion method. All experiments were done in triplicate and the average value was used. Standard deviations were within 10%.

Dose effect of RA on IgG production

Cells were plated in 35 mm plastic dishes at initial density of 1 × 10⁵ cells/ml. Cells were cultured in ITES-ERDF medium supplemented with RA at various concentrations of 10⁻⁹ to 10⁻⁶ M for 4 days. Control received an equal amount of ethanol without RA. To compare the enhancing effect, treatment of cells with retinyl acetate (10⁻⁷ to 10⁻⁴ M) was similarly performed. On day 4, viable cell density and IgG concentration were examined.

Northern blot analysis

Total RNA was recovered from 6 × 10⁵ cells using TRIZOL reagent (GIBCO BRL, Tokyo, Japan). Two micrograms of RNA were loaded on 1.5% agarose-formaldehyde gel and the separated RNA was transferred onto Hybond N+ membrane (Amersham, Buckinghamshire, UK). The blot was hybridized with the specific probe for BD9 γ heavy or κ light chain variable region (Larrick et al., 1989), of which the 3'-end was labelled with fluorescein-11-dUTP using the 3'-oligolabelling system (Amersham). Signal detection was performed using the Gene Images CDP-Star detection module (Amersham) according to the manufacturer's protocol. As a control, β-actin was similarly detected using the commercial β-actin specific DNA probe (Wako). Signal intensity was measured with the Kodak EDAS system.

Effects of RA treatment time on IgG production

Cells were pretreated with 10⁻⁷ M of RA for 1, 2 or 4 days, washed thoroughly, and replated in 35 mm plastic dishes at 1 × 10⁵ cells/ml in ITES-ERDF medium without RA. On day 4, viable cell density and IgG concentration were examined. A non-treated culture was also done as control. Enhanced rate of IgG production by RA was estimated by comparison between test and control without pretreatment.

Immunoblot analysis

Cells (6 × 10⁵ cells) were lysed by sample buffer for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Electrophoresis of cell lysate and the spent medium was performed under reduced condition by the Leammli method (1970) with a 10% gel, then proteins were electrotransferred onto a nitrocellulose membrane (Towbin et al., 1979). The IgG heavy and light chains were detected using horseradish peroxidase conjugated anti-human heavy and light chain antibodies, respectively, with chemiluminescence reagent as a substrate (DuPont, Wilmington, DE, USA). Signal intensity was measured with the Kodak EDAS system.

Figure 1. Dose effect of RA on IgG production of BD9 hybridomas. Cells (1×10^5 cells/ml) were cultured in ITES-ERDF medium supplemented with RA (■) at indicated concentrations for 4 days. To compare the enhancing effect, retinyl acetate (○) was also tested.

Figure 2. Northern blot analysis of IgG production of BD9 hybridomas. (a) Northern blotting, (b) enhanced rate of mRNA level by RA. Cells (1×10^5 cells/ml) were cultured in ITES-ERDF medium supplemented with or without 10^{-7} M RA for 4 days. Total RNA was recovered from 6×10^5 cells, and used for Northern blotting (See Materials and methods). Signal intensity was measured with the Kodak EDAS system. Enhanced rate of mRNA level by RA was calculated by comparison of the signal intensity between control and test.

Figure 3. Effect of treatment time with RA on IgG production of BD9 hybridomas. Cells were pretreated with 10^{-7} M of RA for indicated days, washed thoroughly, and replated at 1×10^5 cells/ml in ITES-ERDF medium without RA. On day 4 after replating, IgG concentration was determined. Enhanced rate of IgG production by RA was estimated by comparison between test and control.

Figure 4. Immunoblot analysis of IgG production of BD9 hybridomas. (a) Immunoblotting, (b) secretory efficiency. Cell culture was performed according to the experiment of Figure 3. Immunoblotting was done as indicated in Materials and Methods section. Signal intensity was measured with the Kodak EDAS system. Secretory efficiency was estimated as extracellular(E)/intracellular(I) value.

Results and discussion

Effects of RA on IgG production and mRNA levels

The enhancing effect of RA on IgG production by BD9 hybridomas was observed among all RA concentrations ranging from 10^{-9} to 10^{-6} M. IgG production of BD9 hybridomas was enhanced up to eight-fold by treatment with 10^{-7} M of RA for 4 days. The enhancing effect of RA was stronger than that of retinyl acetate, which enhanced IgG production up to five-fold at 10^{-5} M (Figure 1). In Epstein-Barr virus (EBV)-transformed B cells, the action and metabolism between RA and retinol were shown to be different (Buck et al., 1991). RA was considered to be suitable for efficient hMAb production rather than retinyl acetate. The enhancement by RA was not due to the increase of cell growth because the growth of BD9 hybridomas was suppressed by RA (data not shown). Takahashi et al. (1994) have reported that growth rate suppression of hybridomas enhances antibody productivity.

Northern blot analysis showed that both mRNA levels of the IgG heavy and light chains were markedly increased by RA when compared with control without RA treatment (Figure 2a, b). In addition, similar results were obtained when cells were treated with retinyl acetate (data not shown). Retinoids have been shown to transcriptionally regulate many genes (Desai et al., 1996). Our findings further expanded these facts.

Effects of RA treatment time on IgG production and secretory rate

Treatment of cells with RA for 4 days was not always required to exhibit the enhancing effect. RA treatment was enough for only one day (Figure 3). Retinoids have been reported to act as a trigger for many cellular processes, including cellular differentiation (Rius et al., 1992) and embryogenesis (Eichele et al., 1989). This observation suggests that RA may also act as a trigger for IgG gene expression.

Immunoblot analysis showed that the secretion efficiency of the IgG heavy and light chains was raised by RA treatment (Figure 4a, b). The difference of signal intensity between heavy and light chains in Figure 4a was considered to be caused by that of the sensitivity of the antibodies used for detection. In the heavy chain, in particular, the intracellular accumulation was little observed in spite of increased transcription levels by RA (Figure 4a). Assembly of IgG in endoplasmic reticulum (ER) has been shown to

proceed through the formation of a heavy chain dimer to which each one of the light chains is then sequentially disulfide-bonded (Baumal et al., 1973). Bibila et al. (1991) have described a model that the processing of the heavy and light chains in the ER to give the assembled antibody was the rate-determining step in the secretion step. Therefore, our experimental data suggest that the secretion rate of the assembled IgG may be accelerated by RA.

Mechanisms underlying the enhancing effect of RA

There are two possible mechanisms for the enhancing effect of RA on antibody production. First, RA exerts its effects by binding to RA receptor (RAR), which interacts with DNA at specific promoter sequences, called RA response elements, to regulate gene expression. Most of the cells of the hematopoietic system have been shown to express RARs (Dolcetti et al., 1998). Therefore, enhancement of Ig gene expression in human-human hybridomas may be associated directly with nuclear receptors such as RAR. Second, RA is also known as an important regulator of cytokine production in normal B cells (Blomhoff et al., 1992). Thus, RA may induce cytokine(s) which activate Ig gene expression of human-human hybridomas. Ballou et al. (1996) reported that the enhancing effects of RA on antibody synthesis by EBV-transformed B cells were mediated, at least in part, by interleukin-6 (IL-6). Makishima et al. (1992) reported that IL-6 is antiproliferative to a mouse hybridoma cell line and promotive for its antibody productivity. However, the mediation of IL-6 was still not confirmed in human-human hybridomas. To prove these possibilities, further study on the relationship of RAR or cytokine to Ig gene expression should be required.

We demonstrated here that RA could enhance transcription and secretion steps at least in the process of antibody synthesis by the human-human hybridoma cell line BD9. These results provide direct evidence for enhancement of hMAb production by RA. In addition, the enhancement by RA has been found in some other human-human hybridoma cell lines (Aotuska et al., 1991). Therefore, RA may be effective as a culture additive for efficient production of monoclonal antibodies using human-human hybridomas.

References

- Aotuska Y and Naito M (1991) Enhancing effects of retinoic acid on

- monoclonal antibody production of human-human hybridomas. *Cell Immunol* 133: 498–505.
- Ballow M, Xiang S, Wang W and Brodsky L (1996) The effects of retinoic acid on immunoglobulin synthesis: role of interleukin 6. *J Clin Immunol* 13: 171–179.
- Baumal R and Scharff MD (1973) Synthesis, assembly and secretion of mouse immunoglobulin. *Transplant Rev* 14: 163–183.
- Bibila TA and Flickinger MC (1991) A model of interorganella monoclonal antibody transport and secretion in mouse hybridoma cells. *Biotechnol Bioeng* 38: 767–780.
- Blomhoff HK, Smeland EB, Erikstein B, Rasmussen AM, Skrede B, Skjonsberg C and Blomhoff R (1992) Vitamin A is a key regulator for cell growth, cytokine production, and differentiation in normal B cells. *J Biol Chem* 267: 23988–23992.
- Dolcetti R, Zancai P, Cariati R and Boiocchi M (1998) In vitro effects of retinoids on the proliferation and differentiation features of Epstein-Barr virus-immortalized B lymphocytes. *Leukemia Lymphoma* 29: 269–281.
- Desai D, Michalak M, Singh NK and Niles RM (1996) Inhibition of retinoic acid receptor function and retinoic acid-regulated gene expression in mouse melanoma cells by calreticulin. *J Biol Chem* 271: 15153–15159.
- Eichele G (1989) Retinoids and vertebrate limb pattern formation. *Trends Genet* 5: 246–251.
- Inoue Y, Fujisawa M, Kawamoto S, Shoji M, Hashizume S, Fujii M, Katakura Y and Shirahata S (1999) Effectiveness of vitamin A acetate for enhancing the production of lung cancer specific monoclonal antibodies. *Cytotechnology* 31: 77–83.
- Kawahara H, Shirahata S, Tachibana H and Murakami H (1992) *In vitro* immunization of human lymphocytes with human lung cancer cell line A549. *Hum Antibod Hybridomas* 3: 8–13.
- Larrick JW, Danielsson L, Brenner CA, Wallace EF, Abrahamson M, Fry KE and Borrebaeck AK (1989) Polymerase chain reaction using mixed primers: cloning of human monoclonal antibody variable region genes from single hybridoma cells. *BioTechnology* 7: 934–938.
- Laemmli UK (1970) Cleavage of structural proteins during assembly of head of bacteriophage-T4. *Nature* 227: 680–685.
- Makishima F, Terada S, Mikami T and Suzuki E (1992) Interleukin-6 is antiproliferative to a mouse hybridoma cell line and promotive for its antibody productivity. *Cytotechnology* 10: 15–23.
- Rius C and Aller P (1992) Vimentin expression as a late event in the in vitro differentiation of human promonocytic cells. *J Cell Sci* 101: 395–401.
- Shoji M, Kawamoto S, Sato S, Kamei M, Kato M, Hashizume S, Seki K, Yasumoto K, Nagashima A, Nakahashi H, Suzuki T, Imai T, Nomoto K and Murakami H (1994) Specific reactivity of human antibody AE6F4 against cancer cells in tissues and sputa from lung cancer patients. *Hum Antibod Hybridomas* 5: 116–122.
- Takahashi K, Terada S, Ueda H, Makishima F and Suzuki E (1994) Growth rate suppression of cultured mammalian cells enhances protein productivities. *Cytotechnology* 15: 57–64.
- Towbin H, Staehelin T and Gordin J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 76: 4350–4353.