The effects of cell adhesion on the growth and protein productivity of animal cells

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Abstract

We investigated the effect of cell adhesion on cell growth and productivity of recombinant protein in Chinese hamster ovary (CHO) cells. Cells cultured in normal tissue culture dishes attached to the dish surfaces and grew as a monolayer, while cells cultured in non-treated dishes proliferated in suspension as single cells without adhering to the dish surfaces. On an agarose-coated dish surface, cell aggregates formed without attaching to the dish. Growth rates in both suspension cultures were slightly lower than those in monolayer culture. Cell cycle analysis indicated that the duration of the G₁ phase in suspension cultures was longer than that in monolayer culture, suggesting that attachment to the substratum mainly affected the transition from the G₁ to the S phase. Consistent with this, CDK inhibitor p27, that inhibits the G₁/S transition, was induced in the cells cultured in suspension. To assess the productivity of recombinant proteins, CHO cells were transfected with a plasmid containing murine interferon γ (mIFN- γ) under the control of the cytomegalovirus promoter. In suspension culture, mIFN- γ productivity was slightly lower than that in the monolayer culture. When protein kinase C was activated by phorbol ester, mIFN- γ production was enhanced in both the monolayer and suspension cultures. However, the productivity in the suspension culture was lower than that in the adherent culture even in the presence of high concentrations of phorbol ester. These results suggested that cell adhesion to the substratum affects various features of CHO cells.

Abbreviations: CDK, cyclin dependent kinase; CHO, Chinese hamster ovary; CMV, cytomegalovirus; mIFN- γ , murine interferon γ ; PKC, protein kinase C; PMA, phorbol myristate acetate; RB, retinoblastoma protein; RSV LTR, Rous sarcoma virus long terminal repeat.

Introduction

Most untransformed cells require anchorage for cell growth. Recently, growth arrested fibroblast cells in suspension culture were analyzed in relation to the various nuclear proteins that control cell growth such as cyclins, cyclin dependent kinases (CDKs), retinoblastoma protein (RB) and CDK inhibitors. Guadagno et al. (1993) reported that the cells in suspension culture expressed only reduced amount of cyclin A, and ectopic expression of cyclin A overcame the arrest. Growth arrest was associated with the reduced amount of cyclin D protein in other cell lines (Böhmer et

al., 1996; Zhu et al., 1996). Fang et al. (1996) reported that reduced kinase activity of cyclin E-CDK2 complex was responsible for growth arrest, which was possibly explained by increased amount of CDK inhibitors p21 and p27. These studies suggest that cell growth is regulated through multiple pathways in suspension culture, therefore, each cell line should be carefully studied to elucidate the mechanism of anchorage dependency.

Chinese hamster ovary (CHO)-K1 cells are widely used as a host for recombinant DNA for basic and applied research fields. CHO cells, which normally grow in an anchorage-dependent manner, are able to

Figure 1. Structure of mIFN- γ expression vector pCMVmIFN γ . mIFN- γ is expressed under the control of the CMV immediate early (IE) promoter.

grow in the absence of anchorage by changing culture conditions. Growth retardation and reduced protein productivity in suspension culture (Chevalot et al., 1994; Cockett et al., 1990) is one of the major problems of CHO cell culture in applied field. However, characteristics of the cells in suspension culture were not examined. In this study, we assessed the effect of cell-adhesion and CDK inhibitor on the growth of CHO cells. We also studied the recombinant protein production by cytomegalovirus (CMV) promoter, which gave higher productivity in CHO cells (Wenger et al., 1994), in terms of cell adhesion and several signal transduction pathways.

Materials and methods

Plasmids and reagents

A plasmid containing the CMV promoter, pCMV β , was obtained from Riken Gene Bank (Tsukuba, Japan) and was self-ligated after *NotI* digestion to remove the β -galactosidase gene. Plasmid pBluescript KS vector containing murine interferon- γ (mIFN- γ) cDNA was obtained from Riken Gene Bank (Tsukuba, Japan). mIFN- γ cDNA was excised from this plasmid as an *EcoRI*–*BamHI* fragment and cloned into the plasmid pCMV at the *NotI* site after blunting each end with a Klenow fragment. The structure of the expression

plasmid pCMVmIFN γ , which expresses mIFN- γ under the control of the CMV immediate early promoter, is indicated in Figure 1.

Anti-CDK inhibitor antibodies, anti-p21 (#sc-756) and anti-p27 (#sc-528), were purchased from Santa Cruz Biotechnology (CA, U.S.A.). Antisense S-oligonucleotide for p27 (CACTCTCACGTTTGA-CATCTT), designed to contain the translation initiation site based on the sequence of murine p27, was obtained from Hokkaido System Science Co. (Sapporo, Japan). A random 18-mer of S-oligonucleotide was used as a control.

Cells

CHO-K1 cells were purchased from Riken Gene Bank (Tsukuba, Japan), and were maintained in Ham's F12 medium supplemented with 10% fetal bovine serum. To establish the cell lines producing mIFN- γ , CHO-K1 cells were transfected with plasmid pCMVmIFN γ by the method described previously (You et al., 1999) and selected in the medium containing 500 $\mu\text{g ml}^{-1}$ of G418 (Life Technologies, MD, U.S.A.).

The cells were cultured in Joklik's modified MEM (JRH Biosciences, KS, U.S.A.) supplemented with 5% F12 and 5% fetal bovine serum, final concentration of Ca^{2+} being approximately 0.08 mM. The Ca^{2+} concentration was one fifth of that of normal F12 medium for maintenance. For the adherent culture, cells were cultured in normal tissue culture-treated dishes (IWAKI, Funabashi, Japan) that possess a high density of negative charge. To culture the cells in suspension, dishes were coated with 0.9% agarose gel (TaKaRa, Japan). In some experiments, cells were cultured in a non-treated petri dishes for bacterial culture (IWAKI) that have a low density of negative charge, and therefore a hydrophobic surface.

Assessment of cell growth and protein productivity

The growth rate in each culture condition was assessed by counting viable cells by the trypan blue exclusion method. Cell cycle distribution was analyzed by a standard flow cytometric analysis after staining with propidium iodide. In some experiments, the proliferative response was assessed by ^3H -thymidine uptake: the cells were cultured for 3 days in 200 μl of medium in microtiter plates and then labeled with 9.3 kBq of ^3H -thymidine for 20 h.

The amount of mIFN- γ in the culture supernatant was assessed by two-site enzyme-linked immunosorbent assay using monoclonal antibodies R4-6A2

Figure 2. Effect of culture conditions on the adhesive property of CHO-K1 cells. Cells cultured in Joklik's MEM for 3 days on tissue culture-treated (monolayer, A), non-treated (single-cell suspension, B) and agarose-coated dishes (aggregate, C). The cells in non-treated dishes consisted of completely suspended cells (upper inset of B) and weakly attached cells (lower inset of B) that were indistinguishable in cell shape. Bar: 100 μ m.

(PharMingen, CA, U.S.A.) and biotinylated XMG1.2 (PharMingen) as the coating and detecting antibody, respectively (Abrams et al., 1992).

Figure 3. Reduced growth rate in suspension culture of CHO cells. CHO-K1 cells transfected with pCMVmIFN γ were cultured for the indicated periods in a monolayer ($\square$), a single-cell suspension ($\bullet$), and an aggregated suspension ($\circ$). Viable cell number was assessed by the trypan blue exclusion method.

Results and discussion

Static suspension culture of CHO cells in dishes

We assessed the effect of the dish surface on the adhesive property of CHO-K1 cells. Cells cultured in the tissue culture-treated dish attached to the dish surface (Figure 2A). On the other hand, when cells were cultured in the non-treated dish, about half of the cells grew in suspension without cell clump formation, referred to hereafter as single-cell suspension (Figure 2B). The other cells in this culture weakly attached to the dish and had a round shape. When cells were cultured in agarose-coated dishes, the cells did not attach to the substratum and cell aggregates were formed (Figure 2C). These results indicate that attachment of CHO-K1 cells to the dishes was mainly controlled by the surface properties of the culture dishes.

Reduced cell growth rate in suspension culture

A plasmid containing mIFN- γ cDNA downstream of the CMV promoter was constructed and transfected into CHO cells and one of the clones was selected for further analysis. The increase in cell number was

examined to estimate the doubling time under various culture conditions (Figure 3). The doubling time in the single-cell suspension was slightly longer than that in the monolayer culture: the doubling time on the tissue culture-treated dish was 27 h, and that on the non-treated dish was about 34 h. The doubling time of the aggregated culture in the agarose coated dish was similar to that of the culture suspended as single cells, suggesting that cell-to-cell attachment did not affect the doubling time in suspension. The reduced growth rate was also confirmed by ^3H -thymidine uptake (data not shown).

Growth retardation in suspension cultures was caused by elongation of the G₁ phase of the cell cycle

To clarify the difference in doubling time under the three culture conditions, the distribution of cells in the cell cycle was examined (Figure 4). When cells were cultured as a monolayer, 43% of the cells were in the G₀/G₁ phase, 33% were in the S phase and 24% were in the G₂/M phase. When cells were cultured in a non-treated dish, the respective percentages were 56, 26 and 18%. When cells were cultured in agarose-coated dish, the respective percentages were 61, 14 and 25%. The cell cycle distribution of suspended cells in non-treated dishes was similar to that of weakly attached cells on non-treated dishes (data not shown). From the distribution, the length of each cell cycle phase was estimated (Figure 4), indicating that the prolonged doubling time in the suspension culture was mainly caused by a prolonged G₁ phase.

The length of the G₁ phase depends on the timing of the G₁/S transition (reviewed in Weinberg, 1995). This transition is promoted primarily by the transcription factor E2F, whose activity is regulated by pRB, cyclins, CDKs and CDK inhibitors. The hypophosphorylated form of RB protein binds to E2F and inactivates it, resulting in G₀ or early G₁ phase arrest. The hypophosphorylated RB, in turn, is phosphorylated by a CDK and cyclin complex and is dissociated from E2F. In this process, cyclins bind to CDKs and activate them, while CDK inhibitors inhibit the kinase activity of the CDK-cyclin complex. Therefore, CDK inhibitors can block cell cycle progression. CDK inhibitors are induced by various stimuli such as DNA damage, serum starvation, contact inhibition and stimulation by TGF- β (El-Deiry et al., 1993; Guan et al., 1994; Hannon and Beach, 1994; Polyak et al., 1994; Toyoshima and Hunter, 1994). The CDK inhibitors p21 and p27 are induced by the lack of substratum

in some fibroblast cell lines (Fang et al., 1996; Zhu et al., 1996). Therefore, we assessed the induction of these inhibitors in CHO-K1 cells. When the cells were grown in a monolayer, p27 was not detected (Figure 5). On the other hand, the p27 level was drastically increased in non-treated or agarose-coated dishes. Another CDK inhibitor p21 was not detected under these culture conditions (Figure 5). These results indicated that the amount of p27 increased when cells grew without adhering to the substratum. To date, the relationship between adherence and the induction of CDK inhibitors has been investigated in several fibroblast cell lines: the expression of CDK inhibitors p21 and p27 were induced by suspension culture in NIH-3T3 cells, untransformed KD cells and HUT-12 cells (a transformed variant of KD cells) but not in rat NRK or PKC-3F4 cells (Fang et al., 1996; Kang and Krauss, 1996; Zhu et al., 1996). At present, the relationship between the expression level of CDK inhibitors and the ability of anchorage-independent growth can be explained only by the characteristics of each cell line. However, the amount of CDK inhibitor appeared to be correlated with their growth potential: cells expressing high levels of CDK inhibitors even in monolayer culture (NIH-3T3, NRK or PKC-3F4) could not grow in suspension, whereas cells with low CDK inhibitors in monolayer culture (HUT-12, CHO) could grow in suspension.

It is reported that induced expression of p21 and p27 was possibly responsible for growth arrest in suspension culture of KD cells (Fang et al., 1996). To assess whether induced p27 expression caused growth retardation in suspension culture of CHO cells, antisense oligonucleotide, which inhibits protein expression of p27, was added to the culture. A Western blot analysis indicated that 1 μM of antisense p27 reduced the amount of p27 about two thirds (data not shown), although thymidine uptake was not affected in either the suspension culture or the adherent culture. Concentrations of this oligonucleotide up to 20 μM did not affect the growth of CHO cells (data not shown). This result suggests that other factors such as the amount of cyclin and/or CDKs regulate the G₁/S transition in CHO cells.

Reduced protein productivity in suspension culture

A variety of recombinant proteins were expressed under the control of promoters such as the SV40 early, CMV immediate early, β -actin, and Rous sarcoma virus long terminal repeat (RSV LTR) promoters in

Figure 4. Flow cytometric analysis of the cell cycle showed that the reduced growth rate in the suspension culture was caused by elongation of the G₁ phase. CHO-K1 cells were cultured for 3 days. (A) monolayer; (B) single-cell suspension; (C) aggregate. Numbers in parentheses indicate estimated lengths of each phase of the cell cycle in hours.

CHO cells. Among these promoters, it was reported that the CMV promoter was stronger than the other promoters (Wenger et al., 1994) and widely used. Therefore, we selected the CMV promoter in this study.

The mIFN- γ productivity was analyzed by measuring the amount of mIFN- γ in the supernatant of static suspension cultures. As shown in Figure 6A, the production of mIFN- γ in the suspension culture was lower than that in the adherent culture; the mIFN- γ concentration in the suspension cultures was about half of that in the adherent culture. Productivity of mIFN- γ was also slightly but reproducibly lower in the suspension culture (Figure 6B). The amount of mIFN- γ was similar between the culture suspended as single cells and as aggregated cell clumps, suggesting that cell-to-cell attachment did not affect the activity of the CMV promoter under these experimental conditions. Similar results were obtained with other clones transfected with pCMVmIFN γ (data not shown).

PKC activator enhanced mIFN- γ productivity under the control of the CMV promoter both in adherent and suspension cultures

It is possible that the reduced mIFN- γ productivity might be restored by activating some signaling pathway(s) that was deactivated in the suspension culture. Activation of such a pathway may lead to activation of transcription factors. Therefore, we examined the effects of several signal-transduction pathway effect-

ors on the productivity. There are several binding sites for transcription factors in the CMV promoter: these include four sites for CREB and NF- κ B, two sites for AP-1 and single site for SP1, SRF and CBP (reviewed by Stinski et al., 1993). As shown in Figure 7A, dibutyryl cAMP, which activates protein kinase A and its downstream transcription factor CREB, did not affect the mIFN- γ productivity in either the adherent culture or the suspension culture. Similarly, the calcium ionophore A23187, which activates AP-1, CREB and NF- κ B with an elevation of intracellular calcium ion concentration, did not alter the amount of mIFN- γ . On the other hand, phorbol myristate acetate (PMA), one of the phorbol esters that activate protein kinase C (PKC) resulting in activation of AP-1 and NF- κ B, significantly enhanced mIFN- γ production in the adherent culture. Our results are consistent with the report by Paterson et al. (1994). In addition, PMA enhanced mIFN- γ production in suspension culture. It is reported that PKC is activated by extracellular matrix fibronectin (Vuori and Ruoslahti, 1993). This suggests that a modest activation of PKC by adhering fibronectin increased the protein productivity of the adherent cultures above the basal level of the suspension cultures. Therefore, we then examined the dose effects of PMA on mIFN- γ productivity. As shown in Figure 7B, PMA enhanced the production of mIFN- γ in a dose-dependent fashion in both the adherent and suspension cultures. Since mIFN- γ was lower in the suspension culture than in the adherent culture even in the presence of high concentrations

Figure 5. Western blot analysis of CDK inhibitors. Cells were cultured for 3 days in a monolayer (M), a single-cell suspension (S), and an aggregated suspension (A). Nuclear extracts were subjected to Western blotting to detect cdk inhibitors p27 and p21. A nuclear extract of CV-1tsTλ that expressed p21 (Mizuarai et al., 1999) cultured at 33 °C was loaded to indicate the expected size of p21 (C).

Figure 6. Reduced protein productivity in suspension culture of CHO cells. CHO-K1 cells transfected with pCMVmIFN γ were cultured for the indicated periods in a monolayer (□), a single-cell suspension (●), and an aggregated suspension (○). (A) Quantity of produced mIFN- γ in each supernatant. (B) Productivity of mIFN- γ estimated from the results shown in Figures 3 and 6A.

(A) **(B)**

Figure 7. Enhancement of mIFN- γ production by PKC activator PMA. (A) Effect of the reagents modulating intracellular signaling pathway on mIFN- γ production in monolayer and aggregate cultures. Cells were cultured for 1 day either with $2 \mu\text{g ml}^{-1}$ of PMA, 1 mM of dibutyl cAMP (dbcAMP) or 100 nM of A23187, and supernatants were harvested to quantify the amount of mIFN- γ . (B) Dose response curve of PMA. Cells died in the presence of more than $10 \mu\text{g ml}^{-1}$ of PMA (not shown).

of PMA, the difference in productivity could not be explained by the activation of PKC alone. Other pathways might be responsible for this difference. One such pathway may be the MAP kinase cascade, which is known to be activated by integrins which is surface receptor of extracellular matrix. Further studies will be needed to answer this question. However, these results suggest that enhancement of the PKC pathway, such as by transfecting cDNA of some subtypes of PKC, might improve the productivity controlled by the CMV promoter, even for cells growing in suspension. We studied the activity of CMV promoter because it is one of the strongest promoters and widely used. However, our findings might lead to improve protein productivity driven by another promoters. Up to now, several promoters such as SV40 early, β -actin and RSV LTR promoters, in addition to CMV promoter were used for the expression of recombinant proteins in CHO cells. Among them, SV40 early promoter contains AP-1 sites and are activated by PMA (Angel et al., 1987). Therefore activating PKC pathway might lead enhanced protein production in another promoter system. We are now examining to clarify this point.

Conclusion

In this study, we have estimated the effect of cell adhesion on CHO cell growth and on recombinant protein production cultures. The growth rate and protein productivity in suspension culture of CHO cells under the control of the CMV promoter were lower than those in the adherent culture. The decreased growth rate in the suspension culture was accompanied by enhanced expression of CDK inhibitor p27 and was not recovered by the addition of various reagents including activators of signal transduction pathways such as PMA, ionophore and dibutyryl cAMP and the antisense oligonucleotide of the CDK inhibitor p27. On the other hand, the decreased protein productivity was partly recovered by the addition of the PKC activator PMA. High concentrations of PMA did not abolish the decrease of productivity, but they were effective in both the suspension and adherent cultures. This suggests that modification of the PKC pathway may improve the protein productivity by CMV promoter. We will analyze the role of cytoskeletal proteins such as actin and its associated proteins in maintaining cell shape and anchorage dependency. These studies may provide useful information for overcoming the problems with suspension culture of CHO-K1.

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