Cytotechnology **33:** 253–257, 2000.
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Short communication

Effect of 14-3-3 protein induction on cell proliferation of A549 human lung adenocarcinoma

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Received 25 March 1999; accepted 24 July 1999

Key words: lung cancer, proliferation, 14-3-3 protein, tumor development

Abstract

We have previously shown that 14-3-3 protein, a multifunctional adaptor molecule involved in many aspects of signal transduction pathways, is a target antigen for the cancer-associated human monoclonal antibody. Although recent evidences suggest a crucial role of 14-3-3 family members in the control of cell growth and differentiation, their actual contribution toward tumor development is still controversial. In this article, we examined the effect of enforced 14-3-3 overexpression on cell growth of the human lung adenocarcinoma cell line, A549. To address this issue, we obtained 14-3-3 protein-inducible A549 sublines by transfection with 14-3-3 expression vector under the control of dexamethasone-inducible promoter. We found that 14-3-3 protein induction in some of these sublines promoted their cell proliferation. Microscopic observation revealed that morphology of these cells became aggressive multilayer condition, suggesting that malignant phenotypes are also acquired upon ectopic induction of 14-3-3 protein.

Introduction

The 14-3-3 protein is a family of ubiquitously expressed proteins which are highly conserved within eukaryotes (Aitken, 1995). In mammalian cells, at least nine 14-3-3 isoforms have been identified which share quite similar structural properties. These isoforms form homo- or heterodimers *in vivo*, and almost of which exist exclusively in cytosolic fraction.

Several lines of evidence have pointed out the significance of the 14-3-3 protein in the intracellular signal transduction pathways. 14-3-3 interacts with, and activates the Raf-1 oncoprotein, which is an upstream kinase in the mitogen-activated protein kinase (MAP-kinase) pathway (Freed et al., 1994; Irie et al., 1994). In agreement with these evidences, crucial role of 14-3-3 protein in Ras/Raf/MAP kinase cascade has been demonstrated in yeast (Roberts et al., 1997) or *Drosophila* (Kockel et al., 1997). 14-3-3 protein interacts with several other oncoproteins, including c-Bcr, Bcr-Abl (Reuther et al., 1994), and polyoma middle tumor antigen (Pallas et al., 1994). 14-3-3 is also involved in the control of programmed cell death. Zha et al. (1996) have demonstrated that 14-3-3 interacts with the Bcl-2 family member Bad to protect the cells from apoptosis. These data raise the possibility that the 14-3-3 protein plays a part in cell proliferation and oncogenesis.

In previous studies, we have elucidated that 14-3-3 protein is a target molecule for a cancer-related human monoclonal antibody which is reactive with human cancer cell lines, and that the level of 14-3-3 protein is significantly increased in human lung cancer tissues as compared with normal counterpart (Shoji et al., 1994;

Kawamoto et al., 1995). To address the role of 14-3-3 up-regulation in cancer cells, here we examined the effect of ectopic 14-3-3 protein overexpression on the proliferation of A549 human lung adenocarcinoma cells.

Materials and methods

Construction of 14-3-3 protein expression vector

To construct the 14-3-3 protein-inducible expression vecor, *Pst*I/*Eco*RI fragment of the 754 bp 14-3-3 protein-ζ cDNA (Zupan et al., 1992) was cleaved from a previously constructed 14-3-3 expression plasmid (Kawamoto et al., 1995), and subcloned into pBluescript (Stratagene, U.S.A.). Resulting plasmid (pBS-14-3-3) was then digested with *Xba*I/*Sal*I, and the 14-3-3 cDNA fragment was inserted into the *Nhe*I/*Sal*I site of the pMAM-neo (CLONTECH, U.S.A.), which contains the dexamethasone (dex)-inducible mouse mammary tumor virus-derived long terminal repeat (MMTV-LTR) sequence.

Cell culture and transfection

The 14-3-3-inducible expression vector (pMAM-14-3-3) was introduced into A549 human lung adenocarcinoma cells using the calcium phosphate-mediated co-precipitation procedure (Chen and Okayama, 1987). Then drug-resistant cells were selected in culture medium [ERDF medium (Kyokuto Seiyaku, Japan) supplemented with 5% fetal calf serum (FCS; purchased from Whittaker, U.S.A.)] containing 1.4 mg/ml G418 (Sigma, U.S.A.). Limiting dilution was then carried out to obtain G418-resistant individual clones. To isolate 14-3-3 protein-inducible sublines, each clone was cultured in the presence of 100 nM dex (Sigma, U.S.A.) for 48 h to induce MMTV-LTR-driven exogenous 14-3-3 protein expression. Intracellular 14-3-3 protein level was examined by immunoblotting as described below. For cell growth analysis, cell samples (A549 cell-derived 14-3-3 protein-inducible sublines, and mock transfectant) were inoculated in 35 mm dishes (Falcon 3001, Becton Dickinson Labware, U.S.A.) at a cell density of 2×10^4/ml, and cell numbers were counted at two days intervals. Experiments were carried out in duplicate.

Immunoblotting and densitometric analysis

Cellular 14-3-3 protein was detected by immunoblotting using the anti-14-3-3 protein monoclonal antibody (AE6F4; Shoji et al., 1994) as described previously (Kawamoto et al., 1995). Quantification of the level of 14-3-3 protein in each transfectant was carried out using the NIH-image densitometric software for the Macintosh apparatus.

Results

Establishment of 14-3-3 protein-inducible A549 lung carcinoma cells

To obtain ectopic 14-3-3 protein-inducible sublines, A549 lung cancer cells were transfected with the MMTV-LTR-controlled pMAM-14-3-3 expression vector. After selection with G418-supplemented culture, each transfectant was cloned *via* limiting dilution. Western blot analysis indicated that, in thirty G418-resistant clones, three clones (A10, A16, and A20 cells) were shown to increase the level of exogenous 14-3-3 protein (about 1.8-fold: assessed by densitometry) in response to dex addition (Figure 1). We also observed increased endogenous 14-3-3 expression in the control neo pool (transfected with the mock pMAM-neo plasmid), but its induction was slight (about 1.2-fold) as compared to 14-3-3 cDNA-transfected A10, A16, or A20 cells.

14-3-3 protein induction promotes proliferation and malignant morphological change in A549 cells

To examine the effect of 14-3-3 protein induction on the cell growth of A549 carcinoma, 14-3-3 inducible A16 and A20 cells were inoculated at a density of 2×10^4 cells/ml, and their cell numbers were counted at two days intervals. When cultured without dex addition, the growth characters of A16 and A20 cells were similar to that of the control neo pool, confirming that intrinsic growth potentials of these cell lines were equivalent (Figure 2A). When dex was added into the medium to trigger ectopic 14-3-3 induction, initial growth rates of the two clones seemed to become slower than control. However, after day 6, we observed significant growth promotion in the A20 cells, which was not seen in the control neo pool (Figure 2B). This growth promotion was also evident in the A16 cells, although the effect was not so dramatic as A20 cells. Relative growth rates of the A16

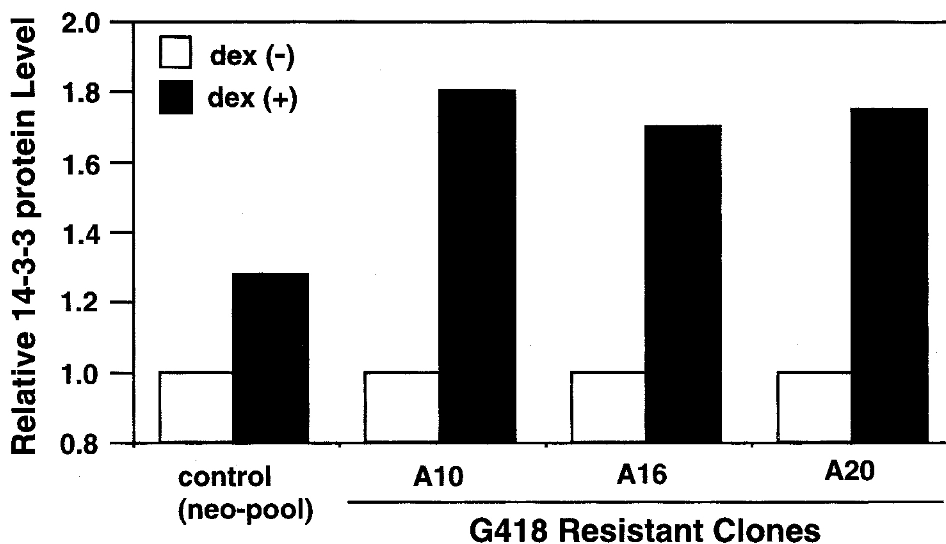

Figure 1. Ectopic 14-3-3 protein induction in A549 human lung carcinoma cells. The dex-inducible 14-3-3 expression vector (pMAM-14-3-3) or the mock pMAM-neo vector was introduced into A549 cells, and individual G418-resistant clones were established by limiting dilution. Intracellular 14-3-3 protein was detected by immunoblotting analysis, and the relative level of 14-3-3 protein expression was determined by densitometry.

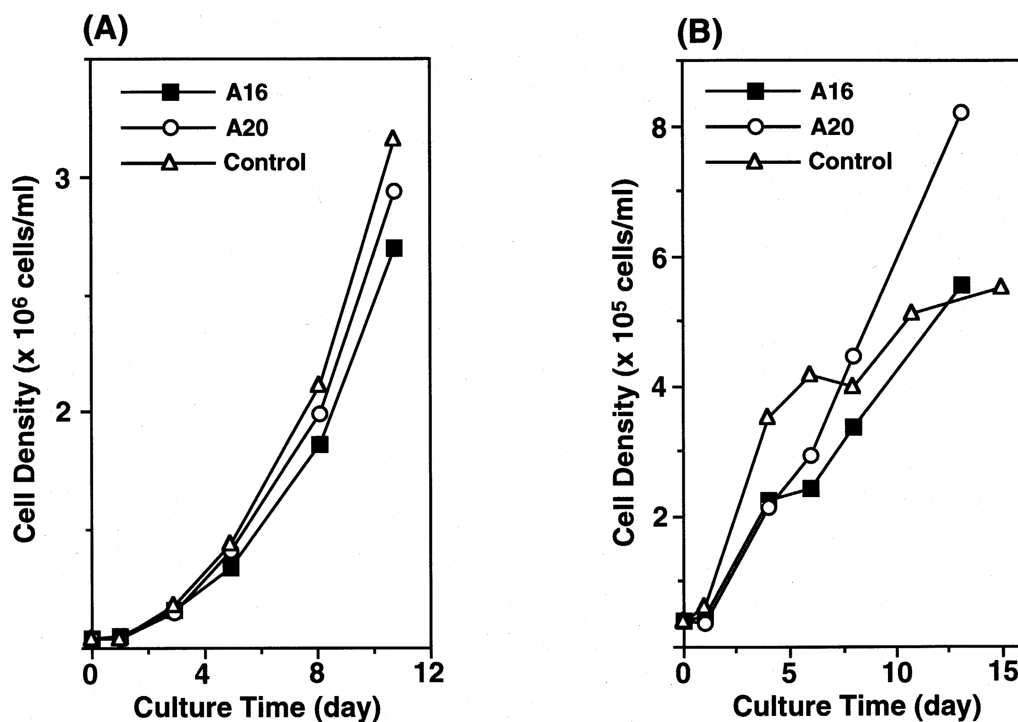

Figure 2. Effect of 14-3-3 protein induction on the cell proliferation of A549 cells. (A) Growth curve of control neo pool (open triangles), or 14-3-3 inducible clones A16 (closed squares), and A20 (open circles) in dex (–) culture. (B) Growth curve of the same cell lines in dex (+) culture. Dex (f.c. 100 nM) was supplemented two days after inoculation.

and A20 cells against the control neo pool after day 6 (calculated by following formula: relative growth rate = inclination of growth curve in A16 or A20 cells after day 6/inclination of growth curve in control after day 6) were 1.6 and 5.0, respectively.

Microscopic inspection further confirmed this 14-

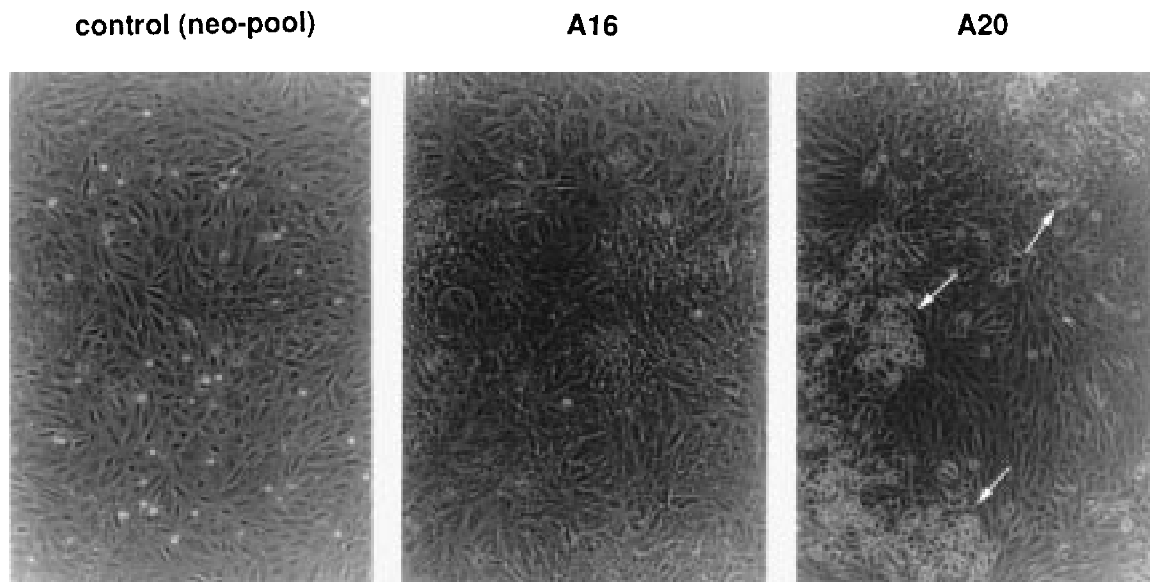

Figure 3. Induction of 14-3-3 protein in A549 cells results in a malignant-like morphology. The photographs shown here were taken twenty days after dex addition. Left panel; control cells (neo-pool) transfected with the mock pMAM-neo plasmid, middle and right panel; 14-3-3-inducible A16 and A20 cell lines, respectively. Aggressive foci formation seen in A20 cells is shown arrowed (right panel). Magnification of each view is ×100.

3-3 induction-associated growth promoting effect. Morphology of the 14-3-3-induced A16 and A20 cells became a multilayer condition, whereas no such sign was observed in control neo pool (Figure 3). Moreover, in A20 cells, we found aggressive foci formation (shown arrowed, right panel in Figure 3), implicating that their malignancy also increased upon ectopic 14-3-3 protein induction.

Discussion

Present data suggest that enforced expression of 14-3-3 protein promotes cell proliferation and induces a malignant morphological character in A549 lung cancer cells. This result together with previous observations that 14-3-3 protein level is increased in human lung cancer tissues (Shoji et al., 1994; Nakahashi et al., 1997) raise a possibility that 14-3-3 induction in cancer cells plays a role in aggressive tumor development. Suen et al. (1995) have reported that 14-3-3 cDNA transfection is insufficient to transform NIH-3T3 fibroblast, suggesting that 14-3-3 protein by itself cannot act as an oncoprotein. Rather, our data suggest that 14-3-3 protein might be involved in the maintenance of malignant phenotypes in immortalized or transformed cells.

Molecular basis for the observed mitogenic effect of 14-3-3 protein overexpression is presently unknown. One plausible explanation is that ectopic 14-3-3 induction modulates the biological activity of its binding partners to trigger cell proliferation. Identified 14-3-3 partners include several oncoproteins (Freed et al., 1994; Irie et al., 1994; Reuther et al., 1994; Pallas et al., 1994), signal transducers (Bonnefoy-Berand et al., 1995; Liu et al., 1996), and cell cycle- or apoptosis regulators (Zha et al., 1996; Conclin et al., 1995), all of which possess the potential to regulate cell proliferation. Moreover, it has been demonstrated that 14-3-3 specifically recognizes target molecules *via* the consensus motif including a phosphoserine residue (Muslin et al., 1996). Therefore, it is also possible that unknown Ser-phosphorylated 14-3-3-binding partners, which are involved in serine/threonine kinase-mediated signaling pathway, play a part in this mitogenic effect. To test these possibilities, as well as to further elucidate detailed molecular mechanism underlying the growth-regulatory effect of 14-3-3 protein, we are now trying to construct a more critical inducible system, such as utilizing the tetracycline repressor/operator-regulated expression system (Gossen and Bujard, 1992).

Acknowledgements

We wish to thank Dr H. Tachibana for his helpful discussion and Mr. Perry A. Seto for reviewing the manuscript. S. K and N. I. equally contributed to this work. S. K. was supported from the Japan Society for the Promotion of Science.

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