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Epidermal Growth Factor Decreases the Level of DNA Topoisomerase IIα in Human Carcinoma A431 Cells

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Human epidermoid carcinoma A431 cells have an extraordinarily large number of epidermal growth factor (EGF) receptors, and their growth is inhibited by EGF, which results in growth arrest at the G1 phase. In order to investigate the EGF-mediated inhibition mechanism, the expression level of DNA topoisomerase (topo) II was analyzed after EGF treatment. As a result, it was shown that EGF treatment lowered the amount of 170 kDa topo II (topo IIα) but not 180 kDa (topo IIβ). However, the A431 cell variant resistant to EGF was not sensitive to EGF treatment. These results suggest that EGF-induced growth arrest of A431 cells may be closely related to the depletion of topo IIα.

Keywords: A431 cells, EGF, Topoisomerase IIα.

Introduction

Studies on signal transduction systems have revealed that the regulatory reactions between receptors and effectors, or the so-called coupling reactions including protein–protein interactions and protein tyrosine phosphorylations, are critical in the responsiveness of the cell. Human epidermoid carcinoma A431 cells, which have an extraordinarily large number of EGF receptors, have provided a unique system to elucidate the relationship between the EGF receptor signal transduction system and cell growth regulation (Gill and Lazar, 1981; Bravo *et al.*, 1985). Although phosphatidyl inositol turnover (Walker and Pike, 1987), phosphorylation of eukaryotic initiation factor 4B (Wolthuis *et al.*, 1993), 12-lipoxygenase transcription (Liu *et al.*, 1997), and hydrogen peroxide generation (Bae *et al.*, 1997) occurred upon EGF stimulation, the EGF inhibited A431 cell proliferation. So far, several papers have reported that EGF induced cell growth arrest at around either G1/S or G2/M during the cell cycle (Macleod *et al.*, 1986; Meyer *et al.*, 1997); however, the mechanism by which EGF works to regulate cell proliferation is still unclear.

Type II DNA topo consists of both 170 kDa (α) and 180 kDa (β) (Drake *et al.*, 1989) and changes the DNA topology which results in the initiation of DNA replication, DNA recombination, and chromosome segregation (Wang, 1996). It has been therefore identified as an intracellular target for a number of important antitumor drugs (Liu, 1989; Lee *et al.*, 1992). Expression of topo IIα has been shown to depend on cell cycles that revealed to peak at the G2/M phase, but topo IIβ expression is parallel through the cell cycle (Heck *et al.*, 1988; Drake *et al.*, 1989). The G2/M phase arrests of A431 cells by EGF indicate that DNA synthesis is not completed or that the maturation step is defective, which is consistent with the finding that EGF regulates topo II activity (Allen *et al.*, 1996). From these backgrounds, it was examined whether EGF affects the molecular changes of the topo II level in A431 cells.

Material and Methods

Materials Human EGF was purchased from Wakunaga pharmaceuticals (Hiroshima, Japan). Aprotinin and leupeptin were from Sigma. Nitrocellulose filters for immunoblotting were purchased from Schleicher & Schuell Inc.

Cell culture and EGF-resistant variant isolation Human epidermoid carcinoma A431 cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium with 4 g/l glucose (DMEM, Gibco, Gaithersburg, USA) supplemented with 10% fetal calf serum (FCS, HyClone) in 10% CO_2. To evaluate EGF-mediated inhibition of cell growth, the cells were plated at 5×10^4 cells/100-mm dish in DMEM with 10% FCS. EGF was added 1 d after plating, and the medium was changed every 2 d. The cells were detached by trypsin-EDTA (Gibco, Gaithersburg, USA) in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution, and mixed with trypsin inhibitor (Sigma, St. Louis, USA). The number of cells was

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counted by a Neubauer Hemocytometer. EGF-resistant A431 variants were obtained by adding EGF at a concentration of 16 nM to the growth medium of wild-type A431 cells. To isolate the EGF-resistant A431 cells, 10^3 parental A431 cells per 100-mm dish were cultured in DMEM–10% FCS containing 16 nM EGF. The surviving colonies were selected and continuously subcultured in DMEM containing 10% FCS and 10 nM EGF for two months. The cells (variant cell number 3) were then stocked in a liquid nitrogen tank.

Immunoblotting Cells were plated at 5×10^4 cells per 100-mm dish, cultured for 24 h, and then treated with or without 10 nM EGF for a further 24 h. The cells were then washed with 10 ml of ice-cold 20 mM HEPES (pH 7.4) containing 120 mM NaCl and all the subsequent steps were performed at 4°C. The cells were harvested with 200 μl of lysis buffer [10 mM HEPES-NaOH (pH 7.4), 100 mM NaCl, 2% SDS, 16 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)] and then boiled for 3 min. The lysates were sonicated for 60 s and centrifuged at $10{,}000 \times g$ for 10 min. The protein concentrations were measured using a protein assay kit (Pierce, Rockford, USA). Constant amounts of cleared lysates (40–80 μg per lane) were subjected to 7.5% SDS-polyacrylamide gel elecrophoresis (PAGE), and then transferred to the filter. Immunoblotting was carried out using the ECL immunoblot detection system (Amersham Life Science Co., Buckinghamshire, UK) with anti-human topo IIα, β and α/β specific antibodies (a kind gift of N. Nozaki, Nagoya University) and horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad, California, USA) for detection.

Results

Proliferation-dependent differential expression of topo IIα To know the expression level of topo II during the proliferation stage, the whole cell lysates of A431 cells were extracted and immunoblotted at various time intervals using anti-topo IIα/β specific antibody. As shown in Fig. 1, the level of the 180 kDa topo IIβ showed parallel through the growth phase, while the amounts of 170 kDa topo IIα were extremely lowered at both the initial and plateau phases than the exponentially growing phase. That is, the ratio of expression levels between α and β were quite different along the cell growth. This result is consistent with the previous reports that the proliferation specific expression of two enzymes is suggestive of a unique function in cell proliferation. Consequently, topo IIα might have a key role in cell growth control (Heck *et al.*, 1988; Hsiang *et al.*, 1988; Drake *et al.*, 1989; Woessner *et al.*, 1991).

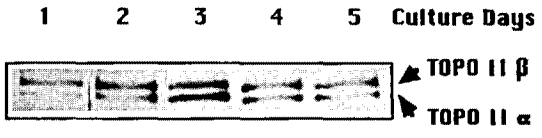

Fig. 1. Proliferation-dependent expression of topo IIα. A431 cells were seeded at a density of 5×10^4 cells /100-mm dish and then whole proteins were extracted everyday. The separated proteins were subjected to immunobloting with anti-topo IIα/β specific antibody, which recognizes both topo IIα and β. Each lane number represents the day of culture.

Decrease in the level of topo IIα by EGF Since topo IIα is expressed in a proliferation-dependent manner and EGF results in growth arrests in A431 cells, the expression level of topo IIα was surveyed at the exponentially growing stage upon EGF treatment. The expression level of topo IIα in EGF-treated A431 cells was drastically reduced. As shown in Fig. 2, immunoblotting of the cell extracts with anti-topo IIα specific antibody showed reduced amounts of topo IIα, i.e., less than 10% of that in normal A431 cells. However, it was never seen in topo IIβ (Fig. 2).

No changes in the level of topo IIα in the EGF-resistant variant To further elucidate the relationship between the EGF dependent growth-inhibitory mechanism and the level of topo IIα, several lines of EGF-resistant variants were isolated by chronic exposure to 10 nM EGF (see Materials and Methods). Among them, variant cell number 3 (V#3) was selected for further analysis. V#3 showed proliferative growth rates in the presence of 4 and 16 nM EGF in a dose-dependent manner, whereas parental A431 cells were severely inhibited. Subsequently, the amount of topo IIα of V#3 was examined after addition of 10 nM EGF. As shown in Fig. 3, topo IIα levels were not affected by EGF in contrast to a marked reduction of parental A431 cells. Changes in the expression levels of topo IIβ were not shown in both cell lines (data not shown). Clearly, EGF was involved in the changes of topo IIα levels. These results indicated that the EGF-mediated A431 cell growth arrest is possibly caused by the depletion of topo IIα, which might result in an incomplete DNA synthesis or a defect in the DNA maturation step.

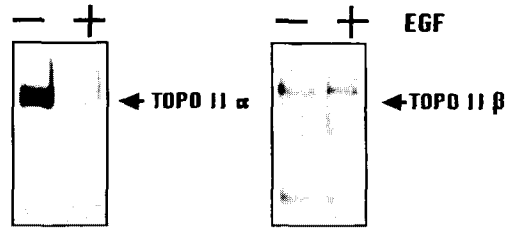

Fig. 2. EGF reduced the expression level of topo IIα. A431 cells were cultured for 24 h at a density of 5×10^4 cells /100-mm dish in DMEM containing 10% FCS. The cells were then treated with or without 10 nM EGF for a further 24 h. Whole cell extracts were separated on a 7.5% SDS-PAGE and electroblotted onto the nitrocellulose filter. Immunoblotting was carried out using anti-topo IIα and β specific antibodies, respectively.

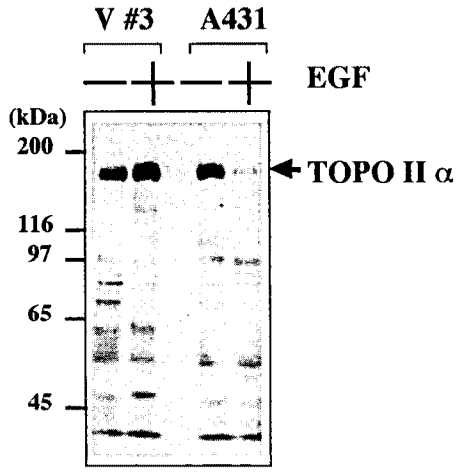

Fig. 3. The amount of topo IIα was not reduced in variant #3. Both cell lines (wild-type A431 and EGF-resistant variant) were treated with 10 nM EGF for 24 h, and the level of topo IIα was examined by immunoblot analysis using topo IIα specific antibody.

Discussion

The present study revealed a preferential decrease of topo IIα upon EGF-treatment. This EGF-mediated inhibition is not limited to A431 cells and has also been shown in other squamous cell carcinomas which have an increased number of EGF receptors (Kamata *et al*, 1986; Allen *et al.* 1996). However, the increased number of receptors is not the primary cause of EGF-mediated inhibition of cell growth (Rizzino *et al.*, 1988). Topo IIα is expressed in proliferating cells or tissues and its amount peaks at the G2/M phase in the cell cycle, while the level of topo IIβ is contant (Woessner *et al.*, 1991). In order to clarify the growth-inhibitory mechanism, the molecular behavior of topo IIα, which is known to be expressed in a cell-cycle-dependent manner, was examined by EGF treatment. As a result, it was shown that EGF selectively decreased the amounts of topo IIα but not IIβ. It has also been established that the molecular behaviors of the α and β-isoforms of topo II are quite different from each other. Also, the mRNA transcripts of topo IIα decreased gradually to 47% of untreated cells after incubation of cells with EGF for up to 24 h (data not shown). The decrease might be caused by an inhibition of translational levels or accelerated degradation of protein. Still, it remains to be elucidated whether EGF acts as a negative regulator for topo IIα expression or as a positive regulator for some proteases. Since topo II has been identified as an intracellular target molecule of antitumor drugs (Liu, 1989), the present findings may contribute to this field.

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References

Allen, G. C., Lubas, S., Wax, M. K., and Devore, R. F. (1996) Epidermal growth factor regulates topoisomerase II activity and drug sensitivity in human KB cells. *Otolaryngol Head Neck Sur.* **114**, 785–792.

Bae, Y. S., Kang, S. W., Seo, M. S., Baines, I. C., Teckle, E., Chock, P. B., and Rhee, S. G. (1997) Epidermal growth factor-induced generation of hydrogen peroxide. Roles in EGF receptor-mediated tyrosine phosphorylation. *J. Biol. Chem.* **272**, 217–221.

Bravo, R., Burckhardt, J., Curran, T., and Muller, R. (1985) Stimulation and inhibition of growth by EGF in different A431 cell clones is accompanied by the rapid induction of c-*fos* and c-*myc* protooncogenes. *EMBO J.* **4**, 1193–1197.

Drake, F. H., Hofmann, G. A., Bartus, H. F., Mattern, M. R., Crooke, S. T., and Mirabelli, C. K. (1989) Biochemical and pharmacological properties of p170 and p180 forms of topoisomerase II. *Biochemistry* **28**, 8154–8160.

Gill, G. N. and Lazar, C. (1981) Increased phosphotyrosine content and inhibition of proliferation in EGF-treated A431 cells. *Nature* **293**, 305–307.

Heck, M. M. S., Hittelman, W. N., and Earnshaw, W. C. (1988) Differential expression of DNA topoisomerase I and II during the eukaryotic cell cycle. *Proc. Natl. Acad. USA.* **85**, 1086–1090.

Hsiang, Y.-H., Wu, H.-Y., and Liu, L. F. (1988) Proliferation-dependent regulation of DNA topoisomerase II in cultured human cells. *Cancer Res.* **48**, 3230–3235.

Kamata, N., Chida, K., Rikimaru, K., Horikoshi, M., Enomoto, S., and Kuroki, T. (1986) Growth-inhibitory effects of epidermal growth factor and overexpression of its receptors on squamous cell carcinomas in culture. *Cancer Res.* **46**, 1648–1653.

Kozma, S. C., Ferrari, S., and Thomas, G. (1989) Unmasking a growth factor/oncogene-activated S6 phosphorylation cascade. *Cell Signal.* **1**, 219–225.

Lee, F. Y., Flannery, D. J., and Siemann, D. W. (1992) Modulation of the cell cycle-dependent cytotoxicity of adriamycin and 4-hydroperoxy cyclophosphamide by novobiocin, an inhibitor of mammalian topoisomerase II. *Cancer Res.* **52**, 3515–3520.

Liu, L. F. (1989) DNA topoisomerase poisons as antitumor drugs. *Annu. Rev. Biochem.* **58**, 351–375.

Liu, Y. W., Chen, B. K., Chen, C. J., Arakawa, T., Yoshimoto, T., Yamamoto, S., and Chang, W. C. (1997) Epidermal growth factor enhances transcription of human arachidonate 12-lipoxygenase in A431 cells. *Biochim. Biophys. Acta.* **1344**, 38–46.

Macleod, C. L., Luk, A., Castagnola, J., Cronin, M., and Mendelsohn, J. (1986) EGF induces cell cycle arrest of A431 human epidermoid carcinoma cells. *J. Cell. Physiol.* **127**, 175–182.

Meyer, K. N., Kieldsen, E., Straub, T., Knudsen, B. R., Hickson, I. D., Kikuchi, A., Kreipen, H., and Boege, F. (1997) Cell cycle-coupled relocation of type I and II topoisomerase and modulation of catalytic enzyme activities. *J. Cell Biol.* **136**, 775–788.

Rizzino, A., Ruff, E., and Kazakoff, P. (1988) Isolation and chracterization of A431 cells that retain high epidermal growth factor binding capacity and respond to epidermal growth

factor by growth stimulation. *Cancer Res.* **48**, 2377–2381.

Walker, D. H. and Pike, L. (1987) Phosphatidylinositol kinase is activated in membranes derived from cells treated with epidermal growth factor. *Proc. Natl. Acad. Sci. USA* **84**, 7513–7517.

Wang, J. C. (1996) DNA topoisomerase. *Annu. Rev. Biochem.* **65**, 635–692.

Woessner, R. D., Mattern, M. R., Mirabelli, C. K., Johnson, R. K., and Drake, F. H. (1991) Proliferation-dependent and cell cycle-dependent differences in expression of the 170-kilodalton and 180-kilodalton forms of topoisomerase-II in NIH-3T3 cells. *Cell Growth and Differ.* **2**, 209–214.

Wolthuis, R. M. F., Cremers, A. F. M., Kasperaitis, M. A. M., Mast, C. V. D., Voorma, H. O., and Boonstra, J. (1993) Epidermal growth factor stimulates phosphorylation of eukaryotic initiation factor 4B, independently of protein kinase C. *Biochim. Biophys. Acta.* **1177**, 160–166.