Effect of Epidermal Growth Factor on Cu, Zn-Superoxide Dismutase Expression in Cultured Fibroblasts from Rat Skin

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The effects of epidermal growth factor (EGF) on Cu, Zn-superoxide dismutase (SOD) in cultured fibroblasts from rat skin exposed to superoxide anions were studied. Cross-linking of [^{125}I]hEGF using disuccinimidyl suberate and immunoblot analysis using anti-EGF receptor antibody to crude plasma membrane fractions of fibroblasts showed that a 170 kDa EGF receptor protein was present on the membrane, as in A431 cells which over express a specific EGF receptor. The cytosolic SOD enzyme activity in fibroblasts exposed to superoxide anions 24 h after treatment with EGF plus nafamostat (NM), a potent protease inhibitor, was increased 1.6-fold compared to control-treated cells. Treatment with either EGF or NM alone, evoked little increase in SOD enzyme activity. The increase in Cu, Zn-SOD protein levels corresponded to the increase in cytosolic SOD enzyme activity in fibroblasts. The Cu, Zn-SOD mRNA level in fibroblasts treated with EGF plus NM at 3 and 6 h was higher than that of the control. Additionally, levels of [^{125}I]hEGF degradation products released into the medium from fibroblasts exposed to superoxide anions were significantly reduced in the presence of NM. These results suggest that the stabilization of EGF by NM in culture is an important factor in the expression of its effects, and that EGF induces Cu, Zn-SOD expression by accelerating transcription of the Cu, Zn-SOD gene in cells, resulting in their protection from the effects of superoxide anion radicals.

KEY WORDS: EGF; nafamostat; superoxide dismutase; fibroblast.

INTRODUCTION

Epidermal growth factor (EGF), a 53 amino acid polypeptide, has wound healing effects (1–3). In our previous study (4) in which we used a second degree burn model in rats, we found a marked improvement in burn healing following treatment with EGF ointment containing the potent protease inhibitor nafamostat (NM) (5). However, there have been few reports concerning the biochemical mechanism of the EGF-induced healing process following injury.

Oxygen metabolites play an important role in many different biological processes including inflammation, oxygen-induced lung injury, ischemic/reperfusion injuries, aging, and carcinogenesis (6). Recent evidence suggests that the extracellular overproduction of superoxide anions (O_2^-) also occurs in acute inflammatory states such as those resulting from thermal injury and burns (6–8). Superoxide dismutase (SOD), which dismutes superoxide anions into oxygen and hydrogen peroxide, is known to play a pivotal role in protecting cells from free radical damage (9).

Recently, we reported accelerated synthesis of Cu, Zn-SOD protein at burn sites treated with EGF ointment containing NM 1 day after burns (10). However, the effects of EGF on SOD synthesis in burn tissue are still unclear because many endogenous factors participate in wound healing in whole body studies (11). To determine the mechanism of the healing effect of EGF on burn tissue, we investigated the effects of EGF on Cu, Zn-SOD expression in cultured rat skin fibroblasts exposed to enzymatically-generated superoxide anions using the hypoxanthine-xanthine oxidase system as used previously in cell culture experiments (12,13).

MATERIALS AND METHODS

Materials

Human EGF was kindly supplied by Wakunaga Pharmaceutical Co. (Hiroshima, Japan). Nafamostat was purchased from Torii & Co. (Osaka, Japan). Human Cu, Zn-SOD, rabbit anti-human Cu, Zn-SOD antiserum, and human Cu, Zn-SOD cDNA were provided by Asahi Chemical Ind. (Tokyo, Japan). Polyclonal antibody against the EGF receptor (E3138, Lot. 071 H 4808) was purchased from Sigma Chemical Co. (St. Louis, MO). [^{125}I]hEGF (750 Ci/mmol), biotinylated antibody, streptavidin-biotinylated peroxidase complex, Hybond-ECL membrane, and ECL direct nucleic acid labeling and detection system were purchased from Amersham International, plc (Bucks, U.K.). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Flow Laboratories (Rockville, MD). ASF-104 medium was purchased from Ajinomoto Co. (Tokyo, Japan). All other chemicals were of reagent grade.

Cell Culture

FR (ATCC CRL 1213) fibroblasts from rat skin, and human epidermoid carcinoma cell line A431 (ATCC CRL 1555) were obtained from the American Type Culture Collection and grown in DMEM containing 10% FBS, penicillin (100 units/ml), streptomycin (100 μg/ml), 2 mM glutamine, and a mixture of nonessential amino acids at 37°C in a humidified atmosphere of 5% CO_2. These cells can be propagated for at least 30 passages. When FR cells had grown almost to confluence, the medium containing FBS was replaced with serum-free ASF-104 medium before the experiments, to avoid complication of results by the effects of any growth factors present in FBS. FR cells were exposed to hypoxanthine (final conc, 30 μM)-xanthine oxidase (final conc, 25 mU/ml) as a source of superoxide anion, and then were treated with saline (control), EGF alone (final conc, 10 ng/ml), NM alone (final conc, 40 μg/ml), or EGF plus NM. EGF and NM were dissolved in saline as stock solution.

Detection of EGF Receptor in Cultured Cells

Crude plasma membranes from cultured cells were isolated by a modification of the method of David *et al.* (14).

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EGF receptors (EGFRs) in cultured cells were detected using immunoblotting and cross-linking of labeled EGF. Immunoblotting for EGFRs was performed as described below. Plasma membranes were subjected to 7.5% SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore Co., Bedford, MA) and immunoblotted by the avidin-biotin-peroxidase complex technique using a polyclonal antibody against the EGFRs as the primary antibody, and anti-mouse IgG antiserum as the second antibody. Cross-linking of [^{125}I]hEGF to its receptor was performed by the method previously described (15).

Determination of SOD Protein Expression and Enzyme Activity

Cytosolic proteins were extracted from the cultured cells. Cells were separated with a rubber policeman in phosphate buffered saline (PBS) and centrifuged for 5 min at 1000 rpm. The cell pellets were resuspended in distilled water, sonicated for 15 min and then centrifuged at 14000 rpm for 10 min at 4°C. Supernatants was transferred to clean tubes. The total SOD enzyme activity in supernatants were measured by Oyanagui's method (17). Immunoblotting for Cu, Zn-SOD protein was performed by the method described below. Briefly, the supernatants containing 5 μg of protein were subjected to 15% SDS-PAGE. After electrophoresis, the proteins were transferred to a PVDF membrane. Cu, Zn-SOD protein was then detected using rabbit anti-human Cu, Zn-SOD antiserum as the primary antibody. More than 80% homology has been reported in the amino acid sequences of rat and human Cu, Zn-SOD (18). The Cu, Zn-SOD content of the cell samples was measured using a densitometer (Shimadzu double beam chromatoscanner CS-930; Shimadzu Co., Kyoto, Japan). The supernatants were assayed for protein content using the method of Lowry *et al.* (19) with BSA as the standard.

Northern Blotting for Cu, Zn-SOD mRNA

The total RNA was isolated from cells treated as described above by the method described by Chirgwin *et al.* (20). Total RNA (40 μg) was electrophoresed through 1.0% agarose-formaldehyde gels and transferred onto Hybond-ECL membranes. Hybridization and detection were performed using human Cu, Zn-SOD cDNA as the probe, according to the procedure described by the manufacturers of the ECL direct nucleic acid labeling and detection system. The rat enzyme has been reported to show 86% identity at the nucleotide sequence level with human Cu, Zn-SOD in the coding region (18).

Effects of NM on Cell-Mediated Degradation of [^{125}I] EGF in Culture

The effects of NM on the cell-mediated degradation of [^{125}I]hEGF in FR cells was analyzed by a modification of the method of Kenneth *et al.* (16). Briefly, FR cells exposed to superoxide anion as described above were incubated with or without NM (final conc., 40 μg/ml) and then a mixture of [^{125}I]hEGF (2 ng, 125000 cpm/ng). Unlabeled hEGF (18 ng) was added to the dishes (35 mm diameter), which were then incubated at 37°C for 1 h. The medium (100 μl) was placed in a test tube, and acid-insoluble material precipitated by the addition of an equal volume of ice-cold 20% (w/v) trichloroacetic acid followed by 30 min incubation at 4°C. The precipitate was removed by centrifugation at 14000 rpm for 5 min. Radioactivity in the supernatant and in the precipitate was measured with a gammacounter (1260 Multigamma II, LKB Wallac, Finland), and the amounts of [^{125}I]-labeled degradation products released into the medium were calculated.

RESULTS

Few reports (21) exist concerning EGF receptor in rat skin fibroblasts. We investigated whether FR cells have specific receptors for EGF by immunoblot analysis and by cross-linkage of [^{125}I]hEGF. As shown in Figure 1, a band of about 170 kDa was observed in both cell lines by both techniques and this band was abolished when the membranes were preincubated in the presence of excess unlabeled EGF. These results indicate that a specific receptor for EGF is present on the cell membranes of FR cells.

We then studied the effects of EGF and NM on total SOD enzyme activity and Cu, Zn-SOD protein contents in fibroblasts exposed to superoxide anions. Figure 2A shows the total SOD enzyme activity in fibroblasts 24 h after the various treatments. The enzyme activity in cells exposed to superoxide anion (control) was about 1.2-fold that in untreated cells. Following treatment with EGF plus NM, the enzyme activity in cells increased about 2.0-fold that in untreated cells, and about 1.6-fold that in control-treated cells. However, little difference was observed between the enzyme activity in cells treated with EGF alone or NM alone and control-treated cells. Furthermore, the results of the immunoblot analysis for Cu, Zn-SOD protein 24 h after the various treatments showing the changes in Cu, Zn-SOD protein content in the treated cells supported the findings of total enzyme activity (Figure 2B and C).

Northern blot analysis of the total RNA from fibroblasts 3, 6, and 24 h after treatment is shown in Figure 3A. Each lane shows a band of about 0.7 k bp which hybridized with the human Cu, Zn-SOD cDNA probe. This is identical in size to the rodent Cu, Zn-SOD mRNA reported by Delabar *et al.* (18). Figure 3B shows the results of the densitometric measurement of each band. At 3 and 6 h, the Cu, Zn-SOD mRNA levels in control-treated cells were increased in comparison with that in untreated cells. At 3 and 6 h, the Cu, Zn-SOD mRNA levels in cells treated with EGF plus NM were about 3-fold than that in untreated cells. Moreover, at 24 h, the Cu, Zn-SOD mRNA levels in cells treated with EGF plus NM were still about 1.5-fold higher than that in control-treated cells. This level is comparable with the increase in Cu, Zn-SOD protein described above.

We also studied the effects of NM on the degradation of [^{125}I]hEGF in cultures exposed to superoxide anions (Figure 4). The degradation of [^{125}I]hEGF was significantly decreased in the presence of NM.

DISCUSSION

There have been few reports concerning the mechanism of the biochemical EGF-induced healing process following

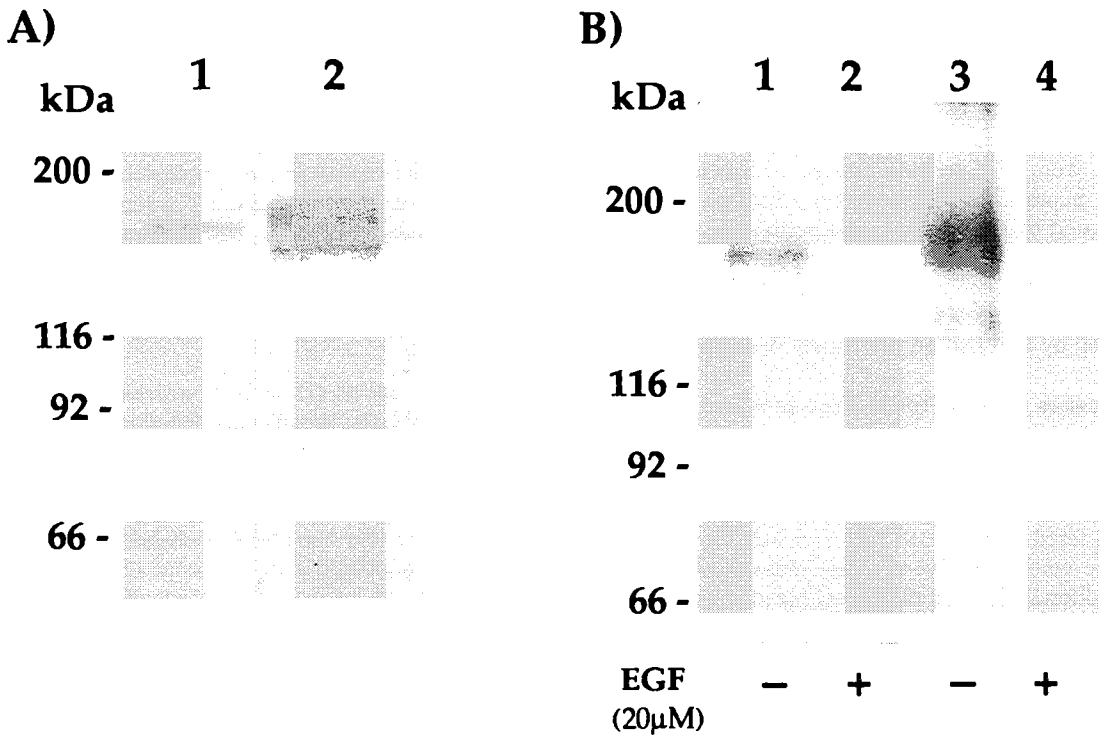

Fig. 1. Detection of EGF receptor in cultured cells. A) Immunoblotting analysis: lane 1; FR cells, and lane 2; A431 cells. B) Disuccinimidyl suberate cross-linking of [^{125}I]-hEGF: lanes 1 and 2; FR cells and lanes 3 and 4; A431 cells, the presence (+) and absence (−) of excessive amounts of unlabeled hEGF.

injury. We previously reported that systemic levels of plasma epinephrine, cortisol, and glutamic-oxalacetic transaminase were improved by the topical application of EGF ointment containing a protease inhibitor, nafamostat (NM), in burn and open wound models in rat (22). Recently, we also reported accelerated synthesis of Cu, Zn-SOD protein at burn sites treated with EGF ointment containing NM 1 day after lesion (10). However, the healing effect of EGF in burn

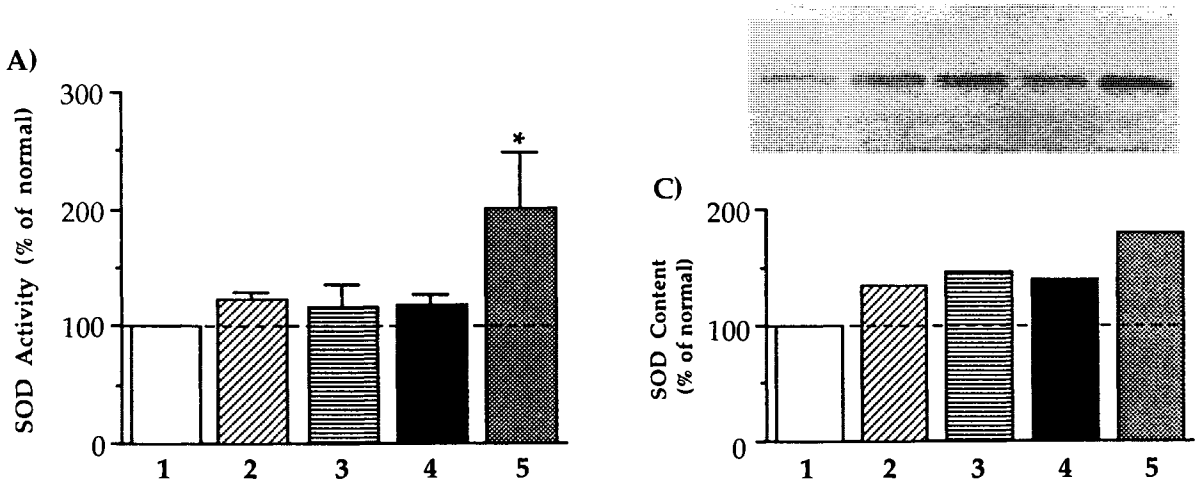

Fig. 2. Effects of EGF and NM on total SOD enzyme activity (A) and Cu, Zn-SOD protein content (B and C) 24 h after treatments in FR cells exposed to superoxide anion. A) Total SOD enzyme activity: column 1; normal (untreated cells), column 2; control (cells exposed to superoxide anions), column 3; EGF (cells treated with EGF after exposure to superoxide anions), column 4; NM (cells treated with NM after exposure to superoxide anions), column 5; EGF plus NM (cells treated with EGF and NM after exposure to superoxide anions). Results are expressed as the mean ± SD (n = 3). Statistical analysis of the data was performed by analysis of variance (ANOVA). *$p < 0.05$ compared with control. B) Immunoblot analysis for Cu, Zn-SOD. Each sample (5 μg protein) was applied onto SDS-PAGE for immunoblot analysis: lane 1; normal, lane 2; control, lane 3; EGF, lane 4; NM, lane 5; EGF plus NM. C) Cu, Zn-SOD protein content: column 1; normal, column 2; control, column 3; EGF, column 4; NM, column 5; EGF plus NM.

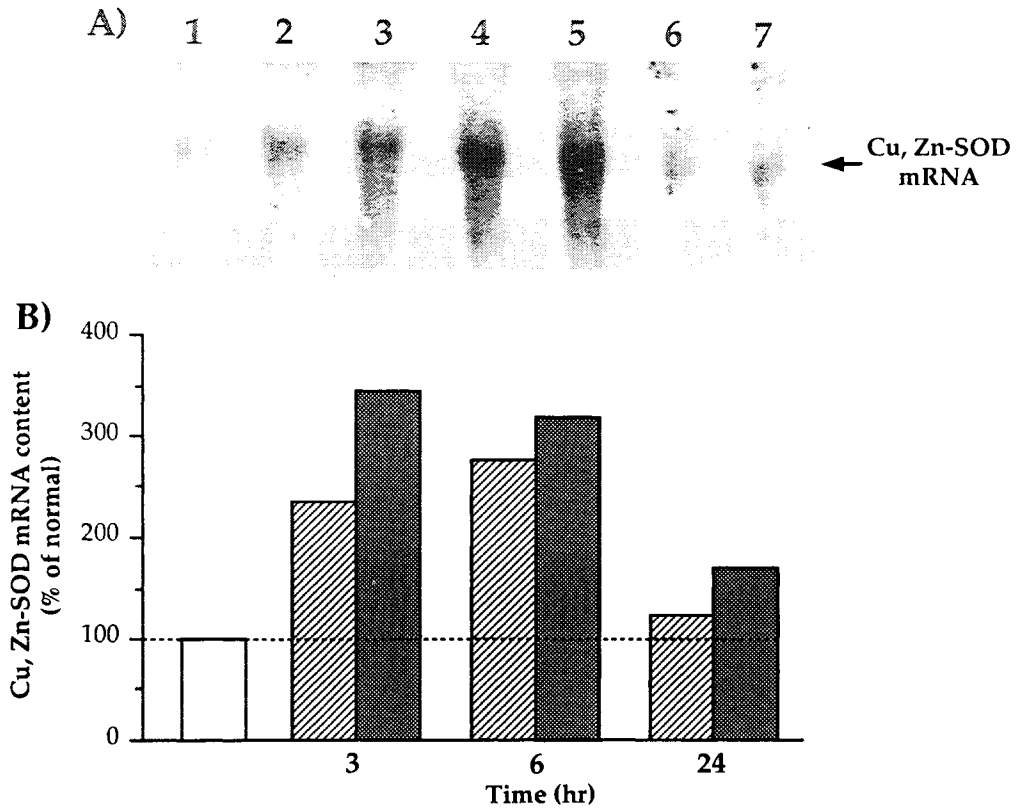

Fig. 3. Effects of EGF and NM on Cu, Zn-SOD mRNA levels in FR cells exposed to superoxide anions. A) Northern blot analysis. Each sample (40 μg RNA) was electrophoresed through a 1.0% agarose-formaldehyde gel for Northern blot analysis: lane 1; untreated, lanes 2, 4 and 6; control-treated cells 3, 6 and 24 h after treatment, respectively, lanes 3, 5 and 7; cells treated with EGF plus NM 3, 6 and 24 h after treatment. B) Cu, Zn-SOD mRNA contents: open column; untreated cells, diagonally-barred column; control-treated cells, hatched column; EGF plus NM-treated cells.

tissue is still unclear because many endogenous factors participate in wound healing in whole body studies (11). The purpose of the present study was to determine the mechanism of the healing effect of EGF. Thus, we investigated the effects of EGF on Cu, Zn-SOD expression in cultured rat skin fibroblasts 24 h after various treatments.

Our knowledge of the physical, biochemical and cellular events in burned skin is limited. It is probable that the direct effects of heat and secondary effects of various inflammatory mediators such as cytokines generated as a result of burning are relevant. Marklund (23) reported that extracellular SOD expression in human dermal fibroblasts was regulated by many cytokines, but that Cu, Zn-SOD activity was not affected. Visner *et al.* (24) demonstrated a dramatic induction of Mn-SOD mRNA expression in response to interleukin-1 and tumor necrosis factor in epithelial cells, but these stimuli had no effect on the corresponding mRNA levels of Cu, Zn-SOD. In a preliminary study, we also observed that Cu, Zn-SOD protein levels were not increased in cells treated with interleukin-1 alone or in combination with EGF (data not shown). These findings indicate that cytokines such as interleukin-1 have little direct effects on the synthesis of Cu, Zn-SOD protein in cultured cells. Therefore, we speculate that the cytokines generated by inflammation stimulate the production of reactive oxygen species by migrating lymphocytes and macrophages at the burn site, and that the fibroblastic cells are then affected by these reactive oxygen species. In the present study, rat skin fibroblasts were exposed to the hypoxanthine-xanthine oxidase system which generates superoxide anions *in vitro*.

Little difference was observed in total SOD enzyme activity and in Cu, Zn-SOD protein levels between cells treated with EGF (10 ng/ml) alone, NM alone and control-treated

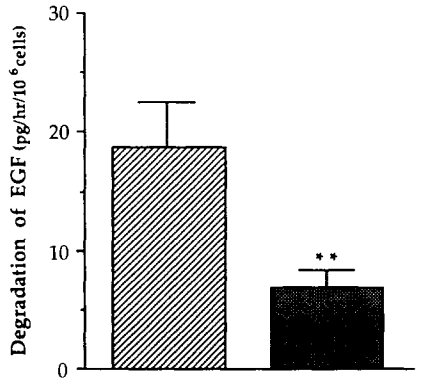

Fig. 4. Effects of NM on cell-mediated degradation of [^{125}I]-hEGF in culture medium exposed to superoxide anions. Diagonally-barred column; control, hatched column; NM. Results are expressed as the mean ± SD (n = 4). Statistical analysis of the data was performed by Student's *t* test. **p < 0.01.

cells. However, we observed a slight increase in total SOD activity at a high concentration of EGF (100 ng/ml) (data not shown). The degradation of EGF in culture exposed to superoxide anion was also significantly decreased in the presence of NM. High proteolytic activity in inflamed and/or wound tissue has been reported (25,26). The present findings indicate that stabilization of EGF in culture is also required for expressing the effects of EGF itself, as observed in previous *in vivo* studies (4,10,27), and that the increase in total SOD enzyme activity is due to an increase in Cu, Zn-SOD protein in fibroblasts.

Since several plasma protease inhibitors interact with cytokines and growth factors, their actions being additive or synergistic (28), an increase in SOD activity and protein levels may be due to synergistic effects of EGF and NM. However, the mechanism of this synergistic effect remains to be determined. It was not known whether Cu, Zn-SOD protein levels are regulated via the transcription rate of the Cu, Zn-SOD gene or via the inhibition of degradation of the protein. We therefore studied the effects of EGF and NM on Cu, Zn-SOD mRNA levels in fibroblasts exposed to superoxide anions. At 3 and 6 h, a marked increase in Cu, Zn-SOD mRNA levels in fibroblasts treated with EGF plus NM was observed compared with that of controls. These results indicate that EGF induces Cu, Zn-SOD mRNA expression at an early period following stimulation by superoxide anion, and that the observed increase in Cu, Zn-SOD expression is not due to inhibition of Cu, Zn-SOD degradation by NM but to accelerated transcription of the Cu, Zn-SOD gene. However, Amstad *et al.* (29) reported that H_2O_2 is the major intracellular oxidant, and that the balance of Cu, Zn-SOD and catalase was more important than the level of Cu, Zn-SOD alone in their study using mouse epidermal cells transfected with human Cu, Zn-SOD cDNA. In the present study, we did not examine the effects of EGF on catalase expression. Further study of the other cellular antioxidant enzymes is necessary.

In conclusion, the present results suggest that the stabilization of EGF by NM in culture is an important factor in the expression of its effects, and that EGF induces the expression of Cu, Zn-SOD protein in fibroblasts, thus protecting them from the effects of superoxide anion radicals. This induction of Cu, Zn-SOD expression was found to be due to accelerated transcription of the Cu, Zn-SOD gene. The present findings in cell culture support those of our previous *in vivo* study (10).

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