Arch. Pharm. Res. Vol.20, No.1, pp. 34-38, 1997

Effect of Protease Inhibitors on Degradation of Recombinant Human Epidermal Growth Factor in Skin Tissue

Hae-Won Ryou, Jang-Won Lee, Kyung Ae Yoon, Eun-Seok Park, and Sang-Cheol Chi

College of Pharmacy, Sung Kyun Kwan University, 300 Chunchun-Dong, Jangan-Gu, Suwon, Kyunggi-Do 440-746, Korea

(Received September 10, 1996)

Recombinant human epidermal growth factor (rhEGF), a polypeptide of 53 amino acid residues, is subject to degradation by numerous enzymes, especially proteases, when it is applied on the skin for the treatment of open wound. Amastatin, aprotinin, bestatin, EDTA, EGTA, gabexate, gentamicin, leupeptin, and TPCK were investigated for the possible protease inhibitors, which may use to protect rhEGF from degradation by the enzymes in the skin. Skin homogenates containing protease inhibitors and rhEGF were incubated at 37°C for 30 minutes. After the reaction was stopped with trifluoroacetic acid, the amount of rhEGF remaining in the sample was determined with an HPLC method. The percentages of rhEGF degraded, at the skin/PBS ratio of 0.25, in the mouse, rat, and human skin homogenate were 85%, 70%, and 46%, respectively. The degree of degradation of rhEGF in the cytosolic fraction was higher than that in the membrane fraction and these enzyme reactions were completed in 30 minutes. Bestatin, EGTA, and TPCK showed significant inhibitory effects on the degradation of rhEGF in the two fractions ($p<0.05$), while the other protease inhibitors had no significant inhibitory effects or, even resulted in deleterious effects. Therefore, the formulation containing one or several inhibitors among these effective inhibitors would be a promising topical preparation of rhEGF for the treatment of open wound.

Key words : rhEGF, Skin, Protease inhibitor

INTRODUCTION

Advanced biotechnology has made inherent proteins or peptides commercially available in forms of the recombinant products. Recombinant human epidermal growth factor (rhEGF), a polypeptide hormone of 53 amino acid residues (Araki *et al.*, 1989), is known to stimulate messenger RNA, DNA and protein synthesis in many cell types (Carpenter and Cohen, 1979). In addition, it has various biological functions such as stimulation of keratinocyte division *in vitro* and epidermal regeneration *in vivo* (Brown *et al.*, 1986; Nanney, 1987), activation of a membrane protein kinase (Carpenter, 1985) and inhibition of gastric acid secretion (Gregory, 1977). Physical, chemical and biological properties of rhEGF have been intensively investigated, since EGF may also be useful therapeutically for burn and wound healing, cataract surgery and other ophthalmic applications. (Lynch *et al.*, 1989, Hunt and La Van, 1989).

Correspondence to: Sang-Cheol Chi, College of Pharmacy, Sung Kyun Kwan University, 300 Chunchun-Dong, Jangan-Gu, Suwon, Kyunggi-Do 440-746, Korea

Several investigators reported that the treatment with EGF enhanced epithelial healing in animals (Kiyohara *et al.*, 1994; Schultz *et al.*, 1991). EGF produced at wound cells through autocrine and paracrine mechanisms is known to accelerate the process of wound healing which is a complex process involving inflammation, cell proliferation, extracellular matrix synthesis and remodeling. However, EGF admininistered as an aqueous solution did not produce any significant changes in the healing process (Okumura *et al.*, 1990). It may result from the lack of continuous exposure of residence epidermal cells to EGF and the degradation of EGF by enzymes. Murakami *et al.* (1988) reported the possibility of EGF degradation by enzymes at subcutaneous site. The degradation of EGF was considered to be accelerated at the wound sites due to higher proteolytic activity in cutaneous lesions compared to that in normal tissues (Kiyohara *et al.*, 1994). Therefore, stabilization of EGF at the administration site is an important factor in order to optimize topical formulation of EGF and increase its bioavailability.

The purpose of present study was to investigate the enzymatic degradation of rhEGF in the skin homo-

genate and to evaluate the inhibitory effect of various protease inhibitors. The mouse, rat and human skin homogenates were used to study the effect of species variation on the degradation of rhEGF. Various protease inhibitors, known as potent and nontoxic to the body, were selected and evaluated for the possible inhibitors on the protease in the skin tissue, since proteases with specific activity for the degradation of EGF have not been reported. The protease inhibitors employed were serine protease inhibitor, cysteine protease inhibitor, metalloproteinase inhibitor, antibiotics and chelating agent.

MATERIALS AND METHODS

Materials

rhEGF was generously supplied by Daewoong Pharmaceutical Co., Korea. Amastatin (*Streptomyces sp.*), bestatin, ethylene glycol-*bis*-(β-aminoethyl) N,N,N',N'-tetraacetic acid (EGTA), N^a-Tosyl-Phe chloromethyl ketone (TPCK), leupeptin hemisulfate, antipain dihydrochloride, aprotinin lyophilized (bovine lung) were purchased from Calbiochem-Novabiochem Co., USA. Gabexate mesilate was purchased from Wako Pure Chemical Co., Japan, and EDTA and gentamicin sulfate were purchased from Sigma Chemical Co., USA. Trifluoroacetic acid and triethylamine were purchased from Junsei Chemical Co., Japan. The other reagents used were of reagent grade or HPLC grade. All the chemicals were used as received without further purification. Deionized water was prepared with reverse osmosis in house. pH 6.8 phosphate buffered saline (PBS) was used for dilution of reagents and preparation of samples.

Degradation of rhEGF in the skin homogenate

Each skin of rats, mice and humans was pooled into, washed with and homogenized with cool (4°C) PBS using a Teflon-glass homogenizer. The homogenates were centrifuged at 3600 rpm for 10 min and the supernatant was diluted with PBS in the skin/PBS (w/w) ratios of 0.03, 0.06, 0.125, 0.25, 0.5 and 1. Stock solution of rhEGF of 400 μg/ml in water was prepared and stored in the freezer. rhEGF solution was added to each skin homogenate solution to make final concentration of 20 μg/ml and the mixed solutions were incubated at 37°C for 30 min. Twenty μl of 10% trifluoroacetic acid was added to the mixed solution to terminate the degradation reaction. After centrifugation at 4°C, 15000 rpm for 30 min, the amount of rhEGF remaining in the supernatant was assayed using an HPLC method. In order to investigate the effect of incubation time on the degradation, the skin/PBS ratio of 0.3 of rat skin homogenate prepared as the above method was used to determine residual rhEGF after 5, 10, 20, and 30 min incubation time.

Preparation of enzyme solutions

To investigate the effective protease inhibitor on the protection of degradation of rhEGF in the skin, the skin homogenates were separated in the two fractions, membrane and cytosolic fraction. The skins of seven rats were pooled into, washed and homogenized with cool PBS in the skin/PBS ratio of 0.3 using a Teflon-glass homogenizer. The homogenate was centrifuged at 4°C, 3600 rpm for 10 min, and the supernatant was further centrifuged at 4°C, 15000 rpm for 30 min. The resultant supernatant was used as the cytosolic fraction. The precipitate was washed with same PBS as above and the resultant precipitate was used as the membrane fraction.

Screening of protease inhibitors

The substrate solution was prepared with PBS to a final rhEGF concentration of 100 μg/ml. Protease inhibitors were dissolved in water, except TPCK and EGTA in 10% ammonia water and methanol, respectively. The protease inhibitor solutions were diluted with PBS to proper concentrations prior to use. One hundred μl of substrate solution was mixed with 50 μl of inhibitor solution (or water) and 350 μl of enzyme solution. The mixture solution reacted at 37°C for 30 min. The reaction was stopped with 20 μl of 10% trifluoroacetic acid. After centrifugation at 4°C, 15000 rpm for 30 min, the residual rhEGF concentration in the supernatant was determined by HPLC.

HPLC analysis

The amount of rhEGF in the sample was quantified with an HPLC method as follows. The isocratic HPLC system consisted of a Waters 600 series pump, 481 variable wavelength detector, 746 data module and 7725i Rheodyne injector. A Cosmosil $5C_{18}$-AR column (4.6×150 mm, 5 μm particle size, Nacalai Tesque, Japan) was used. The mobile phase consisted of 1000 ml of 25% (v/v) acetonitrile in water, 9 ml of phosphoric acid (85%) and 22 ml of triethylamine and the final pH was about 6.7. The flow rate was maintained at 0.8 ml/min, the detector wavelength was 214 nm, and the injection volume was 50 μl.

The desired resolution of rhEGF in the sample was obtained using the mobile phase of triethylamine and phosphoric acid solution (Fig. 1). The retention time of rhEGF was from 8 min to 10 min and sensitive for the change of acetonitrile concentration in the mobile phase. The linearity of the method was evaluated by preparation of varing concentrations of rhEGF from 2.5 to 20 μg/ml. The regression line had a corre-

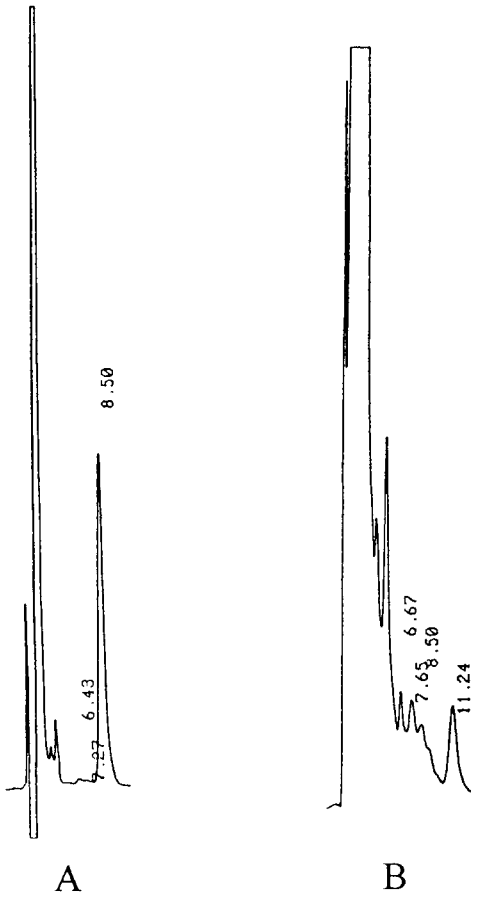

Fig. 1. Representative chromatograms of rhEGF. A: rhEGF in PBS, B: rhEGF in skin homogenate after incubation.

lation coefficient of 0.999. The coefficient of variance and the absolute deviation were less than 5% and 10%, respectively, indicating high reproducibility of HPLC analysis.

Statistics

Dunnet's method of one-way ANOVA was used for the statistical analysis.

RESULTS AND DISCUSSION

Degradation of rhEGF in the skin homogenate

The effects of skin concentration and incubation time on rhEGF degradation in different skin homogenates were investigated, since protease with specific activity for rhEGF degradation in skin has not been reported clearly. Skin concentration was the ratio of skin/PBS and assumed to be proportional to enzyme concentration. The knowledge of activity of protease in skin has been partly informed up to date. Suga (1993) reported that enzyme activity in epidermis was 10 times higher than that in dermis in the study of high molecular weight protease, skin protease. The presence of serine protease in stratum corneum was reported by Suzuki (1993).

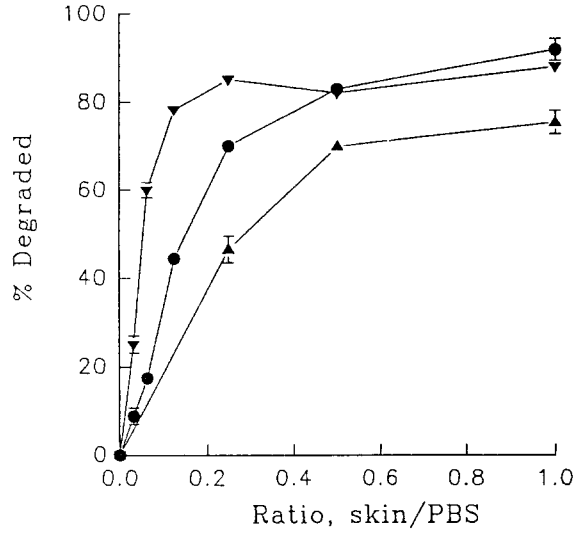

Fig. 2. Degradation of rhEGF in rat, mouse and human skin as a function of skin/PBS ratio in homogenate (mean±S.E., n=3). Key: ●; rat, ▼; mouse, ▲; human

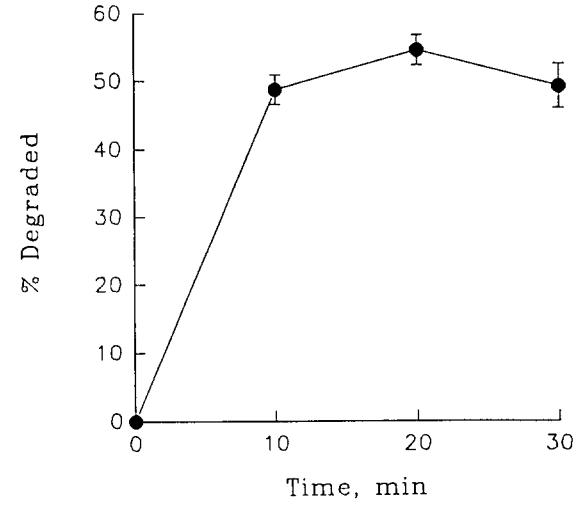

Fig. 3. Profile of degradation of EGF in rat skin homogenate as a function of time incubated (mean±S.E., n=3).

The profile of enzymatically degraded rhEGF percent versus the ratio of skin/PBS (w/w) is present in Fig. 2. The percent of degraded rhEGF in skin homogenates changed with the ratio of skin/PBS due to the difference of enzyme activity in the skins. The degradation of rhEGF in the rat increased with increase in the ratio of skin/PBS up to 0.25 and showed plateau after 0.25 ratio. The rhEGF degradation in the mouse was initially twice higher compared to that in the rat at low ratio of skin/PBS, then it increased slowly after 0.125 ratio and reached plateau at higher of 0.25 ratio. The degradation of rhEGF in human was lower compared to those in rat and mouse. Even

though the percent of the rhEGF degradation in mouse skin was higher compared to that in rat and human skins in the early period, three profiles using rat, mouse, and human skins increased with increase of the skin amount up to the skin/PBS ratio of 0.4 and showed plateau in the range of 0.4 to 1.0 skin/PBS ratios. Therefore, the rat skin was selected for the inhibitor screening study due to the difficulty of the supply of the human skin. The degradation of rhEGF in the skin/PBS ratio of 0.3 was evaluated at given intervals to determine the equilibrium of the incubation time. The percent of rhEGF degradation against the incubation time using 0.3 skin/PBS ratio is plotted in Fig. 3. Incubation of 30 min was considered to be sufficient time for enzymatic degradation, since enzyme reaction was fast and highly efficient. Therefore, the ratio of skin/PBS and incubation time were selected to be 0.3 and 30 min for protease inhibitor screening, respectively.

Screening of protease inhibitors

As a result of protease inhibitor screening, the effect of each protease inhibitor on the enzymatic degradation of rhEGF in the membrane fraction was determined (Table I). The inhibitory effect of the concentration of rhEGF solution measured without addition of skin and protease inhibitors was assigned to one hundred precent. The inhibitory effect of the concentration of skin/rhEGF solution measured without addition of protease inhibitors was assigned to zero percent. Among the serine protease inhibitors, only TPCK had a significant inhibitory effect of 66%, and aprotinin and gabexate did not. Among cysteine protease inhibitors, antipain had a high significant inhibitory effect of 57% and leupeptin did not show any significant change compared to control. Among metalloproteinase inhibitors, bestatin had the inhibitory effect of 35% but amastatin did not. Among chelating agents, EGTA had the inhibitory effect of 17% due to the formation of complex with metal ions which are necessary for the activation of enzymes, but EDTA did not (Table I). Increase in gentamicin which is antibiotics gave the deleterious effect on the degradation of rhEGF (data not shown).

Table I. Inhibitory effects of serine, cycteine and metalloproteinase inhibitors, antibiotics and chelating agents on rhEGF degradation in membrane and cytosolic fraction (n=4)

Group	Inhibitor	% Inhibited	
		Membrane Fraction	Cytosolic Fraction
Serine protease inhibitor	aprotinin	9.20±4.27*	-4.84±0.23
	gabexate	-4.64±3.38	27.77±1.59
	TPCK	65.86±6.09	73.69±2.48
Cysteine protease inhibitor	antipain	57.34±2.01	-8.39±0.81
	leupetin	-4.66±13.35	5.70±0.76
Metalloproteinase inhibitor	amastain	-4.97±5.95	0.95±1.98
	bestatin	35.39±5.31	28.31±0.41
Antibiotics	gentamicin	-15.01±1.50	-94.29±0.07
Chelating agent	EDTA	5.81±2.27	-87.16±2.56
	EGTA	16.57±1.24	76.72±0.70

*mean±S.E.

The effect of protease inhihitors on rhEGF degradation in the cytosolic fraction was determined (Table I). Among serine protease inhibitors, gabexate and TPCK had the significant inhibitory effects of 28 and 74%, respectively. Cysteine protease inhibitors such as antipain and leupeptin did not have any significant effects. Among metalloproteinase inhibitors, bestatin had the inhibitory effect of 28%, but amastatin did not. Gentamicin in cytosolic fraction also resulted in the acceleration of rhEGF degradation as like its effect in membrane fraction. Among chelating agents, EDTA accelerated the rhEGF degradation but EGTA had the significant inhibitory effect of 77% (Table I).

The degradation of rhEGF in cytosolic fraction was higher compared to that in membrane fraction, since the enzymes in cytosolic fraction are considered to be more active compared to those in membrane fraction. The cytosolic enzyme activities were also different from the membrane activities due to differences in the existence, number and quantity of enzymes in the two fractions. Consequently, the high effective protease inhibitors against degrading rhEGF enzymes in all the two fractions were bestatin, EGTA and TPCK. Therefore, the formulation containing one or several inhibitors among these effective inhibitors would be a promising topical preparation of rhEGF for the treatment of open wound.

CONCLUSIONS

The enzymatic degradation of rhEGF in different skin homogenates was investigated and various protease inhibitors were screened for the protection from the degradation of rhEGF by enzymes.

The degradation of rhEGF was dependent on the activities of enzymes in skin and was higher in cytosolic fraction compared to that in membrane fraction due to higher enzyme activities in cytosolic fraction. In the protease inhibitors screening study, cytosolic enzyme activities are considered to be different from the membrane activities in the existence, number and quantity of enzymes in the two fractions Serine protease is considered to most active for rhEGF degradation. Bestatin, EGTA and TPCK may use in topical formulation to protect rhEGF from the enzymatic degradation in cytosolic and membrane fractions, since they had higher inhibitory effect in the both fractions and low toxicity.

ACKNOWLEDGMENTS

This work was supported by the research grant from Daewoong Pharmaceutical Co. Ltd.

REFERENCES CITED

Araki, F., Nakamura, H., Nojima, N., Tsukumo, K. and Sakamoto, S., Stability of recombinant human epidermal growth factor in various solutions. *Chem. Pharm. Bull.*, 37, 404-406 (1989).

Brown, G.I., Curtsinger, L.J. and Brightwell, JR., Enhancement of epidermal regeneration by biosynthetic epidermal growth factor. *J. Exp. Med.*, 163, 1319-1324 (1986).

Carpenter, G., Epidermal growth factor: biology and receptor metabolism. *J. Cell. Sci.*, Suppl 3, 1-9 (1985).

Carpenter, G. and Cohen, S., Epidermal growth factor. *Ann. Rev. Biochem.*, 48, 193-216 (1979).

Greaves, M.W., Lack of effect of topically applied epidermal growth factor (EGF) on epidermal growth in man *in vivo.*, *J. Invest. Dermatol.*, 5, 101-103(1980).

Gregory, H., Holmes, J.E. and Willshire, I.R., Urogastrone levels in the urine of normal adult humans. *J. Clin. Endocrinol. Metab.*, 45, 668-672 (1977).

Hunt, T.K. and La Van, F.B., Enhancement of wound healing by growth factors. *N. Engl. J. Med.*, 321, 111-112 (1989).

Kiyohara, Y., Komada, F., Iwakawa, S. and Katsuhiko, O., Improvement in wound healing by epidermal growth factor (EGF) ointment: efficacy of EGF ointment containing protease inhibitor, nafamostat, for ischemic ulcer (decubitus) in rats. *Yakuzaigaku*, 54, 117-121 (1994).

Kobayashi, S., Kondo, S. and Juni, K., Study on pulmonary delivery of salmon calcitonin in rats; Effects of protese inhibitors and absorption enhancers, *Pharm. Res.*, 11, 1239-1243 (1994).

Lynch, S.E., Colvin, R.B. and Antoniades, H.N., Growth factors in wound healing. *J. Clin. Invest.*, 84, 640-646 (1989).

Murakami, T., Kawakita, H., Kishimoto, M., Higashi, Y., Amagase, H., Hayashi, T., Nojima, N., Fuwa, T. and Yata, N., Intravenous and subcutaneous pharmacokinetics and rectal bioavailability of human epidermal growth factor in the presence of absorption promoter in rats. *Int. J. Pharm.*, 46, 9-17 (1988).

Nanney, L.B., Epidermal growth factor-induced effects on wound healing. *Clin. Res.*, 35, 706a (1987).

Okumura, K., Kiyohara, Y., Komada, F., Iwakawa, S., Hirai, M. and Fuwa, T., Improvement in wound healing by epidermal growth factor (EGF) ointment. I. Effect of nafamostat, gabexate, or gelatin on stabilization and efficacy of EGF. *Pharm. Res.*, 7, 1289-1293 (1990).

Schultz, G., Rotatori, D.S. and Clark, W., EGF and TGF-α in wound healing and repair. *J. Cell. Biochem.*, 45, 346-352 (1991).

Suga, Y., Takamori, K. and Ogawa, H., Skin proteasomes (high molecular weight protease): Purification, enzymologic properties, gross structure and their tissue distribution., *J. Invest. Dermatol.*, 101, 346-351 (1993).

Suzuki, Y., Nomura, J., Hori, J., Koyama, J., Takahashi, M. and Hori, I., Detection and characterization of endogenous protease associated with desquamation of stratum corneum., *Arch. Dermatol. Res.*, 285, 372-377 (1993).

Takeyama, M., Ishida, T., Kokubu, N., Komada, F., Iwalawa, S., Okumura, K. and Hori, R., Enhanced bioavailability of subcutaneously injected insulin by pretreatment with ointment containing protease inhibitors., *Pharm. Res.*, 8, 60-64 (1991).