—Original Article—

Synthesis of prolyl 4-hydroxylase by cultured chicken corneal cells

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Summary

Prolyl hydroxylase (EC 1.14.11.2) synthesis was examined in the cultured corneas of 17-day-old chick embryos which were cultured in F-12 medium supplemented with fetal calf serum. The amount of prolyl hydroxylase in cells was measured by immunoelectrophoresis using antibodies directed against this enzyme. The results indicated that the amounts of prolyl hydroxylase depended on cell density. The cells were harvested at the early-log phase and plated at high cell density to mimic the culture condition of cells at the stationary phase (crowding). The amount of ^{35}S -prolyl hydroxylase indicated a two-fold increase in the rate of prolyl hydroxylase synthesis after crowding. By use of ^{32}P -labeled cDNA for the β -subunit of prolyl hydroxylase a proportionate increase of the mRNA was noted in accordance with the increase in the rate of prolyl hydroxylase synthesis, suggesting that this enzyme synthesis is regulated at the transcriptional level.

Key words: prolyl 4-hydroxylase, cornea, mRNA, wound healing

Introduction

Prolyl 4-hydroxylase (EC1.14.11.2) is a key enzyme in collagen biosynthesis, in which it catalyses the hydroxylation of proline to hydroxyproline in collagenous polypeptides. The presence of hydroxyproline may allow the collagen molecules to assume a stable triple-helix structure under physiological conditions. The active prolyl hydroxylase is a tetramer consisting of two pairs of non-identical subunits designated α and β ¹⁾. Intracellularly, there are two forms of the enzyme protein. One is active tetramer and the other is inactive free β -subunit. The free β -subunit comprises 40% to 80% of the total enzyme protein in cultured fibroblasts²⁾. The level of free β -subunit in serum seems to be associated with increased collagen synthesis in lung and liver³⁻⁶⁾. The present study was undertaken to clarify whether the prolyl hydroxylase levels are dependent on the cell density in primary corneal cell culture and at which level of cell metabolism they are regulated.

Materials and Methods

(1) Cell culture

Cells were isolated from 17-day chicken embryos by a modification of the Dehm and Prockop procedure⁷⁾. The cells were grown in the F-12 medium supplemented either with 0.2% or 2% fetal calf serum in 150 cm² flasks. Prolyl hydroxylase of the cultured corneal cells at various stages of

growth was measured as follows.

(2) Amount of prolyl hydroxylase

The cells were harvested at various stages of growth and homogenized in lysis buffer at 5×10^7 cells/ml. Aliquots of 40 μ l of the supernatant were used in rocket immuno-electrophoresis to determine the amount of the total enzyme protein. Sheep antisera were prepared by immunizing the sheep with purified prolyl hydroxylase from 17-day-old chick embryos as previously described⁸. Rocket immuno-electrophoresis and crossed immuno-electrophoresis were used to quantitate the total enzyme protein, active tetrameric prolyl hydroxylase and free β -subunit of prolyl hydroxylase. The cell extracts were firstly electrophoresed in 3% polyacrylamide gel and then 0.5% agarose in a solution containing 1 mM Ca lactate and 81 mM tricine buffer, pH 8.6⁹. Electrophoresis was performed at a constant current (100 mM/10 cm in width) for 2 h or until the bromophenol blue migrated 8 cm from the origin. Slices containing the sample were cut off from the gel and transferred to a glass plate (8.5×20 cm). The samples were electrophoresed into a 1% agarose gel in Tris/tricine buffer containing the antisera (2 μ l/ml). The amount of the enzyme protein was estimated by calculating the area under the precipitation arc⁸.

(3) Synthesis of prolyl hydroxylase

Cells at the log-phase were harvested and plated at higher cell density to mimic the stationary phase. After various times of crowding, the cells were labeled with ^{35}S -methionine for an hour. The cells were then collected with a rubber policeman and homogenized in lysis buffer. The amount of ^{35}S -labeled protein was determined by TCA precipitation. The protein contained in the sample was measured by the ninhydrin test using leucine as the standard. The active tetrameric prolyl hydroxylase and free β -subunit were isolated by immunoabsorbent columns. The samples eluted from the affinity columns were then analyzed by SDS-polyacrylamide gel electrophoresis and fluorograms were prepared. The relative amounts of enzyme protein were determined by scanning the fluorograms with a helena and D scanner. The amounts of each ^{35}S -labeled subunits were then calculated.

(4) mRNA of the β -subunit of prolyl hydroxylase

The cells were harvested at various stages after crowding. Total RNA was extracted with 4 M guanidinium isothiocyanate. Ten μ g of total RNA was then blotted to nitrocellulose membrane and hybridized with ^{32}P -labeled cDNA probes for the β -subunit¹⁰. The probes were labeled by nick translation.

Results

The amount of prolyl hydroxylase was measured in the cultured corneal cells at various growth stages. The increase in the amount of prolyl hydroxylase was not demonstrated at the early log or mid phases. However, the amount of prolyl hydroxylase (protein) in the cells was increased at 7 days in the late mid log phase (**Fig. 1**). The amount of prolyl hydroxylase was increased at least 1 h after cell crowding and the protein synthesis was at its maximum 5 h after cell crowding (**Fig. 2** and **Fig. 3**). The amount of β -subunit mRNA of prolyl hydroxylase was at its maximum 2 h after cell crowding, and then it gradually decreased (**Fig. 4**).

Fig. 1 Amounts of prolyl 4-hydroxylase in cultured corneal cells at various stages of growth

Fig. 3 Rate of the α and β -subunit synthesis of prolyl 4-hydroxylase after cell crowding

Fig. 2 Detecting of the tetramer and free β -subunit of prolyl 4-hydroxylase by immuno-electrophoresis

Fig. 4 mRNA level of the β -subunit of prolyl 4-hydroxylase after cell crowding

Discussion

The regulation in the synthesis of prolyl hydroxylase was examined in cultured corneal cells. According to the present results, the enzyme production was turned on as the cell reached the stationary phase. The amount of prolyl hydroxylase protein in cultured corneal cells was increased after crowding and the mRNA synthesis was completed within 3 h after the induction.

It has been reported that an increase in collagen synthesis is usually accompanied by an increase in the level of prolyl hydroxylase¹¹⁾. For example, the amount of prolyl hydroxylase in the cartilage and tendons of chick embryos reaches a maximum when the cells are most active in procollagen biosynthesis. The relationship between prolyl hydroxylase and collagen synthesis has

also been found to be true in such pathological conditions as liver fibrosis¹¹⁾.

Schwarz et al.³⁾ reported that a cell-density dependency of prolyl hydroxylase was revealed in the collagen synthesis by cultured avian tendon fibroblast. The levels of prolyl hydroxylase in L-929 fibroblasts were cell density-dependent and the amount of enzymes was increased when the cells approached a confluent monolayer¹²⁾. Cell density could be playing an important role in the regulation of collagen synthesis⁷⁾. Procollagen synthesis by tendon fibroblasts was strikingly enhanced at the stationary phase by the increase of procollagen mRNA in the cells^{1,11)}. In the present study, an increase of prolyl hydroxylase level in cultured corneal cells was correlated with cell crowding and with the increasing of the β -subunit mRNA. These results suggest that this enzyme synthesis is regulated at the transcriptional level.

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References

- 1) Kivirikko, K.I. and Myllylä, R.: Posttranslational enzymes in the biosynthesis of collagen; Intracellular enzymes. *Methods Enzymol.*, **82**, 245~304, 1982.
- 2) Kao, W.W.-Y., Berg, R.A. and Prockop, D.J.: Ascorbate increases the synthesis of procollagen hydroxyproline by cultured fibroblasts from chick embryo tendons without activation of prolyl hydroxylase. *Biochim. Biophys. Acta*, **411**, 202~215, 1975.
- 3) Schwarz, R.I. and Bissell, M.J.: Dependence of the differentiated state on the cellular environment; Modulation of collagen synthesis in tendon cells. *Proc. Natl. Acad. Sci. USA*, **74**, 4453~4457, 1977.
- 4) Tuderman, L. and Kivirikko, K.I.: Immunoreactive prolyl hydroxylase in human skin, serum and synovial fluid; Changes in the content and components with age. *Eur. J. Clin. Invest.*, **7**, 295~299, 1977.
- 5) Giri, S.N., Misra, H.P., Chandler, D.B. and Chen, Z.: Increases in lung prolyl hydroxylase and superoxide dismutase activities during bleomycin-induced lung fibrosis in hamsters. *Exp. Mol. Pathol.*, **39**, 317~326, 1983.
- 6) Colquhoun, J.E. and Dresden, M.H.: Prolyl hydroxylase activity during newt forelimb regeneration. *Dev. Biol.*, **95**, 193~199, 1983.
- 7) Dehm, P. and Prockop, D.J.: Synthesis and extrusion of collagen by freshly isolated cells from chick embryo tendon. *Biochim. Biophys. Acta*, **240**, 358~369, 1971.
- 8) Hebda, P.A., Ebert, J., Chou, L.K.L., Shieids, M. and Kao, W.W.-Y.: The association between prolyl hydroxylase metabolism and cell growth in cultured L-929 fibroblasts. *Biochim. Biophys. Acta*, **758**, 128~134, 1983.
- 9) Monthony, J.F., Wallace, E.G. and Allen, D.M.: A non-barbital buffer for immunoelectrophoresis and zone electrophoresis in agarose gels. *Clin. Chem.*, **24**, 1825~1827, 1978.
- 10) Kao, W.W.-Y., Nakazawa, M. and Aida, T.: Isolation of cDNA and genomic DNAs of β -subunit of chicken prolyl hydroxylase. *Connect. Tissue Res.*, **18**, 157~174, 1988.
- 11) Pierce, R.A., Glaug, M.R. and Deak, S.B.: Increased procollagen mRNA levels in carbon tetrachloride-induced liver fibrosis in rats. *J. Biol. Chem.*, **262**, 1652~1658, 1987.
- 12) Kao, W.W.-Y. and Berg, R.A.: Cell density-dependent increase in prolyl hydroxylase activity in cultured L-929 cells requires de novo protein synthesis. *Biochim. Biophys. Acta*, **586**, 528~536, 1979.

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