Vol. 198, No. 1, 1994
January 14, 1994
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 274-280

HISTATIN AS A SYNERGISTIC STIMULATOR WITH EPIDERMAL GROWTH FACTOR OF RABBIT CHONDROCYTE PROLIFERATION

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Received December 8, 1993

SUMMARY: Histatin 5 dose-dependently increased DNA synthesis in rabbit costal chondrocytes in culture. The level of DNA synthesis was stimulated by histatin 5 to about 4 times that of the control. The combination of histatin 5 and epidermal growth factor (EGF) increased the DNA synthesis to about 40 times that of the control while EGF alone stimulated it 15-fold, indicating synergistic stimulation by both factors. Enzyme-linked immunosorbent assay using anti-histatin 5 demonstrated that human serum contained histatin-like substances. These findings suggest that histatins play an important role in chondrocyte proliferation, presumably as a physiological modulator of the action of EGF.

Histatins belong to a family of salivary polypeptides. Some investigators showed that the mRNA for the histatins was expressed in human parotid and submandibular glands but in none of the other tissue studied (1,2). Thus, the function of the histatins is thought to be restricted to saliva. It has not been reported whether histatins exist in other body fluids such as human serum. Histatins have antibacterial and antifungal activities (3-5). We have shown that histatins are potent inhibitors of hemagglutination of *Porphyromonas gingivalis* and coaggregation between *P. gingivalis* and *Streptococcus oralis* (6,7). Moreover, histatins stimulate the release of histamine from isolated rat mast cells (8). However, it remains unclear whether histatins affect other cells.

During endochondral ossification, chondrocytes proliferate, synthesize cartilage matrix components and become hypertrophic; finally, cartilage is replaced by bone. Thus, the proliferation of chondrocytes is the first step for skeletal growth. Using cultured rabbit costal chondrocytes and human chondrocyte-like cell lines, we have

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0006-291X/94 $5.00

shown that various growth factors such as epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insulin-like growth factor-I (IGF-I) and transforming growth factor-β (TGF-β) are mitogens for chondrocytes (9-12). However, there is little report on the modulators of actions of these growth factors on chondrocytes.

In this study, we found that histatin 5 stimulated DNA synthesis in rabbit costal chondrocytes in culture and also synergistically potentiated the stimulatory effect of EGF on DNA synthesis in the cells. We also report on the presence of histatins in human serum.

MATERIALS AND METHODS

Materials: EGF, TGF-β and bFGF were purchased from Collaborative Research Inc. (Bedford, MA). IGF-I was kindly provided by Fujisawa Pharmaceutical Co. (Osaka, Japan). Synthetic histatin 5 (Asp-Ser-His-Ala-Lys-Arg-His-His-Gly-Tyr-Lys-Arg-Lys-Phe-His-Glu-Lys-His-His-Ser-His-Arg-Gly-Tyr) and histatin 8 (Lys-Phe-His-Glu-Lys-His-His-Ser-His-Arg-Gly-Tyr) were purchased from the Peptide Institute (Osaka, Japan). [^{3}H]thymidine was obtained from ICN Pharmaceuticals Inc. (Irvine, CA). Other materials used were commercial products of the highest grade available.

Cell culture: Chondrocytes were isolated from the growth cartilage of the ribs of young male New Zealand rabbits weighing 300-500 g as described previously (13,14). The isolated cells were cultured in Eagle's minimum essential medium (Nissui Pharmaceutical Co., Tokyo, Japan) containing 10% fetal bovine serum (GIBCO, Grand Island, NY). The medium was changed every other day. The cells between passage 0 to 4 were used for the experiments.

Determination of rate of DNA synthesis: DNA synthesis was monitored by determining the Incorporation of [^{3}H]thymidine into acid precipitable materials as described.(15,16)

Collection of human serum and human parotid saliva: Human serum was collected from a 30-year-old healthy human adult. Human parotid saliva was collected as described (17).

Partial purification of histatins from human serum and human parotid saliva: We separated histatins by utilizing their adsorption to heparin affinity gel (18). Human serum (5 ml) or 10-fold concentrated human parotid saliva (5 ml) was applied to a Heparin Sepharose CL-6B column (Pharmacia, Uppsala, Sweden, 1.6 x 3 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.0). The column was washed with 50 ml of the same buffer. Bound materials were eluted with 5 ml of 10 mM Tris-HCl buffer (pH 7.0) containing 1.5 M NaCl. A part of eluate (0.5 ml) was applied on a Sephadex G-25 fine column (Pharmacia, 0.7 x 30 cm) equilibrated with 50 mM ammonium acetate buffer (pH 8.0) and thirty fractions (0.5 ml each) were collected. Absorbance at 220 nm was monitored and materials immunoreactive to anti-histatin 5 antibody were detected by ELISA.

Preparation of rabbit polyclonal antibody to histatin 5: Histatin 5 (2 mg/ml) was mixed with Freund's complete adjuvant and sonicated. The resultant emulsion was injected s.c into male rabbits weighing 3.5 kg. Injection was performed three times 2 weeks apart. The rabbits were bled 5 days after the last injection. The IgG fraction was purified from rabbit antiserum using Econo-Pac Protein A MAPS II kit (Bio-Rad Laboratories, Richmond, CA) and used for immunological assays.

ELISA with anti-histatin 5 antibody: Flat bottom 96-well microtiter plates (Corning Glass Works, Corning, NY) were coated with 100 μl of samples or histatin 5 diluted in 0.05 M carbonate buffer (pH 9.6) at 4°C for 18 h. They were washed three times with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T) and 100 μl of 1% BSA in PBS-T were then added to each well. After incubation at 37°C for 1

h, they were washed three times with PBS-T and incubated with 100 μl of anti-histatin 5 antibody (12μg/ml) at a volume of 100 μl/well at 37°C for 1 h. After washing, the plates were incubated with 100 μl of a 1:1000 dilution of alkaline phosphatase-anti rabbit IgG (H+L) (Zymed Laboratories, Inc., San Francisco, CA) at 37°C for 1 h. Enzyme reaction was carried out in diethanolamine buffer (pH 9.8) using *p*-nitrophenyl phosphate (Sigma, St. Louis, MO) as a substrate. The reaction was stopped with 0.1 N NaOH and the color intensity at 405 nm was measured.

RESULTS

Fig. 1 shows the dose-dependent effects of histatin 5 and/or with EGF on DNA synthesis in rabbit costal chondrocytes. When histatin 5 was added to confluent cultures of the chondocytes, DNA synthesis in the cells increased dose-dependently. The stimulatory effect of histatin 5 was less than that of EGF which is known to be a potent mitogen for chondrocytes (10), but significant at a concentration of 1 mg/ml. The DNA synthesis stimulated by this concentration of histatin 5 was 4.1 times that of the control. When 1 mg/ml of histatin 5 was added to the cultured chondrocytes with 1 ng/ml of EGF, DNA synthesis markedly increased to 41.3 times that of the control. Because one ng/ml of EGF increased DNA synthesis in the cells to 15.3 times that of the control, simultaneous addition of histatin 5 and EGF resulted in a synergistic stimulatory effect on DNA synthesis in the chondrocytes.

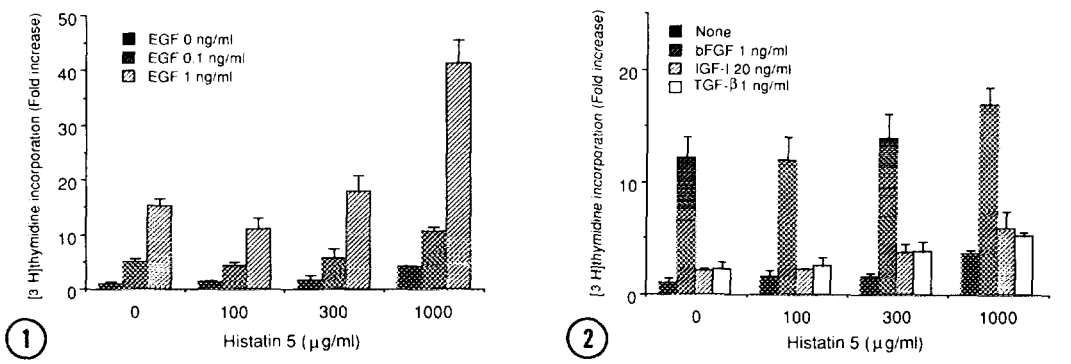

Fig. 1. Effect of histatin 5 alone or with EGF on DNA synthesis in rabbit costal chondrocytes. Rabbit costal chondrocytes were inoculated at a density of 1 x 10^4 cells per 6-mm multiwell plate and cultured in DMEM containing 10% FBS. When they reached confluence, they were washed three times with MEM containing 0.3% FBS and incubated in the same medium for 24 h. The culture medium was then replaced by fresh DMEM containing 0.3% FBS with or without histatin 5 and/or EGF at concentrations indicated. After 21 h, the cultures were labeled with 10 μCi/ml of [^{3}H]thymidine for 3h. Columns and bars represent means and S.D., respectively, for four wells.

Fig. 2. Effect of combinations of histatin 5 and other growth factors on DNA synthesis in rabbit costal chondrocytes. Experimental conditions are the same as those in the legend of Fig. 1 except that bFGF (1ng/ml) , IGF-I (20ng/ml) or TGFβ (1ng/ml)were used instead of EGF.

To determine whether this synergism is specific for EGF, rabbit chondrocytes were treated with histatin 5 and other mitogens for chondrocytes. As shown in Fig. 2, IGF-I and TGF-β weakly increased DNA synthesis in rabbit chondrocytes while bFGF dramatically increased their DNA synthesis (12.2-fold stimulation at 1 ng/ml) as did EGF. Even when histatin 5 was added with these growth factors, only additive effects on DNA synthesis were observed (Fig. 2).

Because histatins are believed to be a family of salivary polypeptides, the finding that histatin 5 stimulated DNA synthesis in chondrocytes suggests that histatins act on the cartilage through the circulation. Therefore, we next examined whether or not serum contains histatins. When the heparin-bound fraction of human serum was applied on a Sephadex G-25 gel filtration column, a small peak with absorbance at 220 nm was observed between fraction numbers 15 and19. ELISA revealed that these fractions contained a material(s) reacting to anti-histatin 5 antibody (Fig. 3 A). Another small peak detected by the ELISA was present at fraction 10. Human parotid saliva also showed a similar gel filtration pattern. There were also two peaks detected by ELISA using anti-histatin 5 antibody (Fig. 3 B). One was observed between fractions 9 and 11 and the other was fractions 15 to 19. As shown in Fig. 3 C, the antibody used for ELISA reacted to authentic histatin 5. A peak of histatin 5 (MW: 3,036) detected with O.D. 220 appeared between fraction numbers 11-16 and a peak detected by ELISA was also observed at the same position. On the other hand, a broad peak of histatin 8 (MW: 1,562) observed between fractions 16 and 25 was not detected by ELISA. These findings indicate that rabbit antibody to histatin 5 used in this study had high immunoreactivity to histatin 5, but not to histatin 8.

DISCUSSION

In this study, we showed that histatin 5 alone increased DNA synthesis in rabbit costal chondrocytes and synergistically potentiated EGF-stimulated DNA synthesis in the cells (Fig. 1). When we examined the effects of EGF and its combination with histatin 5 on the number of cultured chondrocytes, similar results were observed (data not shown), suggesting that histatin 5 has a mitogenic effect on chondrocytes. Because the increase in DNA synthesis by histatin 5 alone was significant but slight when compared with those of EGF and bFGF, the synergistic effect with EGF would be more important under physiological conditions. Among various mitogens for chondrocytes investigated, the synergism on DNA synthesis was specific for EGF (Fig. 2). In this regard, it is noteworthy that EGF is also abundant in salivary glands.

EGF has been found in all fluids in a body. Its level in mouse and human blood is 1ng/ml and 0.1-0.2 ng/ml, respectively. Therefore, EGF has been suggested to act on chondrocytes *in vivo* as a physiological mitogen. In the present study, we found that histatin-like substances were also present in human serum, suggesting that histatins also play an important role for chondrocyte proliferation as a physiological modulator of action of EGF.

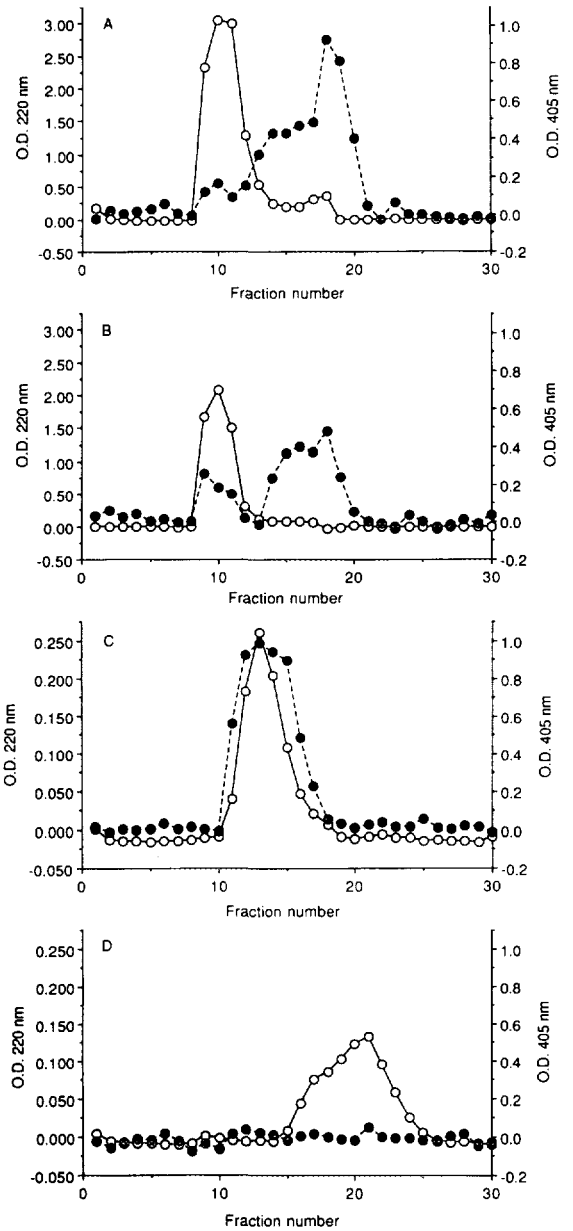

Fig. 3. Sephadex G-25 column chromatography of human serum (A), human saliva (B), histatin 5 (C) and histatin 8 (D). Heparin-bound materials of human serum (5 ml) and 10-fold concentrated human parotid saliva (5 ml) were eluated with 5 ml of 10 mM Tris-HCl buffer (pH 7.0) containing 1.5 M NaCl and a part of eluate (0.5 ml) was subjected to Sephadex G-25 gel filtration as described in the Materials and Methods. Half ml of 10^{-4}M histatin 5 and histatin 8 were used for gel filtration. To determine the elution profiles of peptides, absorbance at 220 nm was monitored (○). ELISA reactivity of anti-histatin 5 antibody (●) was measured at 405 nm.

As shown in Fig. 3, gel filtration chromatography of the heparin-bound fraction of human serum and human parotid saliva revealed one major broad peak and one minor peak detected by ELISA using anti-histatin 5 the molecular weights of which

were estimated to be 1,500 to 3,000 and >5,000. Troxler et al. have established that there are twelve histatins in human parotid secretions and have clarified their structural interrelationships (19). What types of histatins these immunoreactive peptides are remains unknown.

Previous studies have shown that 6 to 10 mg of the histatin can be isolated from 100 ml of stimulated saliva (18,20). We used 5 ml of human serum and 5 ml of 10-fold concentrated human saliva as starting materials, subjected one tenth of each bound fraction to a Sephadex G-25 fine column chromatography and obtained slightly more immunoreactive material from human serum than from 10-fold concentrated saliva. These findings suggest that human serum contains at least 0.6 to 1 mg/ml of histatin-like substances, although it is difficult to estimate the exact to 1 mg/ml of histatin-like substances, although it is difficult to estimate the exact amount of histatins by this method. Significant stimulation of DNA synthesis by histatin 5 was observed at a concentration of 1 mg/ml (Fig. 1 and 2), suggesting the physiological importance of the mitogenic effect of the histatins.

It is unknown how histatins act on the chondrocytes. The presence of EGF receptors on cultured rabbit costal chondrocytes was demonstrated in the previous study (10). Therefore, it would be interesting to investigate whether histatin 5 has any effect on the expression of the EGF receptor or EGF binding to its receptors. Receptors for histatin 5 might be present on cultured chondrocytes.

In conclusion, this is the first report to demonstrate the presence of histatins in human serum, that physiological concentrations of histatin 5 stimulated DNA synthesis in cultured chondrocytes and that its effect was marked in the presence of a physiological concentration of EGF. Since EGF stimulates the proliferation of epithelial cells and mesenchymal cells (21) and since the oral cavity is exposed to histatins provided by both saliva and serum, it would be very interesting to examine the effect of histatins on epithelial as well as mesenchymal cells in the oral cavity.

ACKNOWLEDGMENTS

We thank Dr. M.-I. Enomoto for technical support and Prof. Fujio Suzuki for valuable advice and support of this work.

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