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Hepatocyte growth factor regulates the proliferation and differentiation of cartilage in developing forelimb of mouse embryos in vitro

Ayumu KOBAYASHI[1,2], Osamu AMANO[2,3], Yuji TANI[3], Toshikazu NAKAMURA[4], Shoichi ISEKI[2] and Katsuro TOMITA[1]
[1] Department of Orthopedic Surgery, and [2] Department of Histology and Embryology, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan; [3] Department of Oral Anatomy II, Meikai University School of Dentistry, Sakado, Japan; and [4] Division of Molecular Regenerative Medicine, Course of Advanced Medicine, Osaka University Graduate School of Medicine, Suita, Japan
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ABSTRACT

We examined the role of hepatocyte growth factor (HGF) in the chondrogenesis and endochondral ossification in the forelimbs of mouse embryos by use of immunohistochemistry and organ culture system. In the forelimbs of embryonic day 14 (E14) embryos, intense immunoreactivity for HGF was localized to the chondrocytes located in the proliferative and early hypertrophic zones, and moderate to weak immunoreactivity in the resting and late hypertrophic zones of bone anlagen. Immunoreactivity for the HGF receptor, c-Met, was also localized to the chondrocytes in the resting, proliferative and early hypertrophic zones. In the explants of forelimb buds from E10 embryos cultured for 8 days, exogenous HGF added to the culture media enhanced proliferation of chondrocytes in the forelimb bone anlagen. In contrast, the antisense oligodeoxyribonucleotide (ODN) for HGF as well as the specific HGF antagonist NK4 inhibited proliferation of chondrocytes and caused hypertrophic change and collagen X production, the signs of chondrocyte differentiation, in the arm bone anlagen. Furthermore, the antisense ODN and antagonists for HGF caused a complete lack in the formation of cartilaginous hand and digital bone anlagen. These results suggested that HGF functions in stimulating chondrogenesis and preventing endochondral ossification in the forelimbs of mouse embryos.

Hepatocyte growth factor (HGF) was discovered as a potent mitogen for mature hepatocytes in culture, and considered as a trigger for liver regeneration in vivo (27, 28, 32). HGF is secreted by cells of mesodermal origin as a single-chain precursor which is then processed to yield a heterodimer composed of a large subunit of 69 kDa and a small subunit of 34 kDa. The action of HGF is mediated by its specific receptor c-Met, a protooncogene product (6, 33). The c-Met has a heterodimeric structure consisting of an extracellular subunit of 50 kDa and a membrane-spanning β subunit of 145 kDa, which has a tyrosine kinase domain in its cytoplasmic region (16). Functionally, HGF has been shown to have a mitogenic effect on various types of epithelial cell (28, 32, 40). HGF has also been shown to be identical to "scatter factor", a molecule capable of causing dissociation and migration of epithelial cells by enhancing their motility (20, 47, 54). HGF is also considered to function in mediating epithelial-mesenchymal interactions, because it enhances the branching tubule formation in a three-dimensional collagen matrix by renal tubular epithelial cells (18, 31), hepatic bile duct epithelial cells (20), and mammary gland cells (34). In embryonic tissues, HGF is involved in epithelial-mesenchymal interactions during the development of kidney (9, 41, 55), lung (35), tooth (45, 48), and salivary glands (19, 26). These

Correspondence to: Ayumu Kobayashi, MD
Department of Orthopedic Surgery, Kanazawa University Graduate School of Medical Science, 13-1 Takara-machi, Kanazawa 960-8640, Japan
E-mail: ayu_koba_1122@ybb.ne.jp

observations suggest that HGF plays multiple roles as a mitogen, a motogen, and a morphogen in the organization of multicellular structures (29).

In addition to epithelial-mesenchymal interactions, HGF has been shown to play important roles in mesenchymal-mesenchymal interactions including the hematogenesis (21, 49), angiogenesis (7, 23), myogenesis in limb buds (13) and in tongue (3), chondrogenesis in articular cartilage (50) and in mandibular Meckel's cartilage (2). In articular chondrogenesis, extrinsic HGF stimulates motility, proteoglycan synthesis, and proliferation of cultured articular chondrocytes, and intrinsic HGF mRNA is expressed in the mesenchymal areas of future joint regions in developing limb buds (50). However, the role of HGF in the development of the cartilage of bone anlagen and its endochondral ossification has been unclear. In development of Meckel's cartilage, both HGF and c-Met are expressed in chondrocytes, and exogenous HGF enhances cartilage formation in the mandibular organ culture system (2).

In the present study, we aimed to reveal the role of HGF in the development and endochondral ossification of the cartilage of long bone anlagen. We have examined the distribution of HGF and its receptor c-Met in the cartilage of mouse forelimb buds. Also we have examined the effects of recombinant HGF, HGF antisense oligodeoxyribonucleotide (ODN), and the specific HGF antagonist NK4 on the cartilage in the organ culture system of forelimb buds.

MATERIALS AND METHODS

Mouse embryos. Pregnant time-mated DDY mice were purchased from Japan SLC Ltd. (Hamamatsu, Japan). The day a vaginal plug appeared was designated as embryonic day 0 (E0). At E10 for organ culture and at E14 for immunohistochemistry, the pregnant animals were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/Kg body weight), and their uteri were taken out and placed in cold Hanks' medium (Nissui, Tokyo, Japan). Under a dissection microscope, embryos were carefully collected from the uteri and their membranes were eliminated.

Organ culture of forelimb buds. The forelimb buds from E10 mice were cultured in a serumless, chemically defined medium according to the methods reported by Canoun *et al*. (8). Briefly, E10 DDY mouse embryos at Theiler's stage 18 (52) were isolated and forelimbs at both sides were microdissected and explanted. The explants were supported by Millipore type AABP filters with 0.8 μm pore size and 5 mm diameter on steel rafts and were cultured in BJGb medium (Fitton-Jackson's modified BGJ; Gibco BRL, Gland Island, MY, U.S.A) freshly supplemented with 100 μg/ml ascorbic acid and 100 U/ml penicillin-streptomycin (Gibco BRL) and adjusted to pH 7.4, in an atmosphere of 5% CO_2 and 95% air with 100% humidity at 37°C.

To examine the effect of an excess of HGF on cultured forelimbs, recombinant human HGF at the concentration of 100 ng/ml was added to the medium from the beginning of the culture. Recombinant rat HGF were purified from the culture medium of Chinese hamster ovary (CHO) cells transfected with expression vectors containing human and rat HGF cDNAs (46, 51).

To examine the effect of a deficiency of HGF, antisense ODN for HGF or the specific HGF antagonist NK4 was added to the culture medium at 25 μM and 5 ng/ml, respectively. Synthetic ODNs at 15 nucleotides were designed according to published mouse HGF cDNA sequence (24), and purchased from Japan BioService Co. (Asaka, Japan). To examine the specificity of the antisense ODN, the control ODN with the same size was also prepared. The sequence of the ODNs were as follows: antisense ODN, GGT CCC CCA CAT CAT; and control ODN, GGA CCC CCT CTA CTA. The effectiveness and specificity of the antisense ODN in inhibiting HGF production in mandibular and dental organ culture systems have been confirmed previously (2, 3, 48). NK4 is composed of the N-terminal 447 amino acids of the α-chain of HGF containing the N-terminal hairpin domain and four kringle domains. NK4 binds to the c-Met without activating it, serving as a potent competitive inhibitor of HGF (30).

All experiments were performed with triplicate or more samples for each condition, and repeated 3 times or more. Culture media supplemented with or without HGF, ODNs, or NK4 were changed every other day, and the culture was continued for 8 days.

Histology and immunohistochemistry. The upper limbs of E14 mouse embryos and groups of 3 or more forelimb explants from E10 embryos cultured for 8 days under various conditions were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 4 h, dehydrated in ethanol series, cleared with xylene and embedded in paraffin. The consecutive sections of 5 μm-thickness were cut from each block and mounted on silane-coated glass slides (Dako, Glostrup, Denmark). Some paraffin sections were

stained with hematoxylin-eosin and prepared for histological observation and morphometrical analysis. The rest of the sections were incubated with 3% (v/v) normal porcine serum in PBS for 30 min and then with rabbit anti-HGF antibody (31), rabbit anti-c-Met antibody (Santa Cruz, CA, USA), or rabbit anti-collagen type X antibody (LSL, Tokyo, Japan), each diluted 1 : 200 with PBS, overnight at room temperature. They were then treated for 1 h at room temperature with biotinylated anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) diluted 1 : 200 with PBS. The site of immunoreaction was made visible by incubating the sections with horseradish peroxidase-conjugated streptavidin (Dako) diluted 1 : 300 with PBS for 1 h, then with 0.01% 3', 3'-diaminobenzidine tetrahydrochloride and 0.002% hydrogen peroxide in 0.05 M Tris-HCl (pH 7.6) at room temperature. For immunohistochemical control, the antibodies preabsorbed overnight at 4°C with recombinant human HGF or synthetic peptide for c-Met (Santa Cruz) were used as primary antibodies. Some sections from the cultured forelimbs were prepared for the immunostaining using anti-PCNA antibody (Zymed, Los Angeles, CA, USA) at a dilution of 1 : 50 to detect proliferative cells. Since the anti-PCNA antibody is mouse monoclonal antibody, a mouse section-staining kit (Zymed) was used to devoid the nonspecific immunoreaction.

The statistical analyses were performed to clarify the effects of excess and deficiency of HGF on cultured cartilages. At the 480-fold magnification, five fields in cartilage area were selected randomly from 5 different sections obtained from two different explants. The ratio of the immunopositive cells for PCNA or collagen type X were calculated in each field and expressed as mean ± SD of five fields. The statistical significance was examined with Student's *t*-test, and the values with $p < 0.01$ were considered significant.

RESULTS

Immunohistochemistry for HGF and c-Met in developing forelimb cartilage

To examine the expression of HGF and c-Met, immunohistochemistry using the specific antibodies was carried out in the developing forelimb cartilage in E14 mouse embryos. The specificity of these antibodies in rat and mouse tissues has already been confirmed in our previous studies (1, 2, 3). The immunoreactivity for HGF was observed in the chondrocytes in the resting, proliferating and hypertrophic zones of cartilage in addition to the mesenchymal cells of the interzone and myogenic cells of developing skeletal muscles, whereas cells in the perichondrium were devoid of the immunoreactivity (Fig. 1a). In the resting zone, HGF immunoreactivity was distributed diffusely in chondrocytes, except in those located close to the perichondrium (Fig. 1b). The proliferative chondrocytes forming the longitudinal cartilage columns and the hypertrophic chondrocytes located close to the proliferative zone showed much intenser immunoreactivity than the resting chondrocytes (Fig. 1b). In E14 forelimbs, ossification has not started yet and the hypertrophic zones of proximal and distal sides are located dos-a-dos in the middle portion of developing cartilage (Fig. 1b). The hypertrophic chondrocytes at the bottom of the zones showed weaker HGF immunoreactivity than those at the top of the zones, and some hypertrophic chondrocytes exhibited no immunoreactivity for HGF (arrowheads in Fig. 1c). Immunoreactive materials for HGF were found in the cytoplasm of chondrocytes and not in the cartilage matrix.

The immunoreactivity for c-Met was also localized in the chondryocytes throughout the resting, proliferative and upper hypertrophic zones of the cartilage (Fig. 1d). Unlike the case of HGF immunoreactivity, increase in the intensity of c-Met immunoreactivity in the proliferative and upper hypertrophic zones compared to the resting zone was not apparent (Fig. 1e). In the lower hypertrophic zone, c-Met immunoreactivity was weaker than in other zones and some hypertrophic chondrocytes exhibited no immunoreactivity for c-Met (arrowheads in Fig. 1f). Immunoreactive materials for c-Met were localized both in the cytoplasm and on the cell membrane of chondrocytes and not in the cartilage matrix.

Whole mount Alcian blue staining of cultured E10 forelimb buds of mouse embryo

Fig. 2 demonstrates the forelimb buds of E10 mouse embryos cultured for 8 days. The explants consisted of a large swelling of shoulder girdle, a short bar of arm, and round-shaped hand without digit lobations. The upper surface of the explant was covered with thin ectoderm (Fig. 2a), whereas a large part of the explant was occupied with Alcian blue-stained cartilages representing the forelimb bone anlagen (Fig. 2b). The cartilages consisted of the anlagen for scapula, humerus, radius, ulna, and small bones of hand and digits. Some cartilages were fused each other and hard to identify accurately.

When the recombinant HGF was added to the

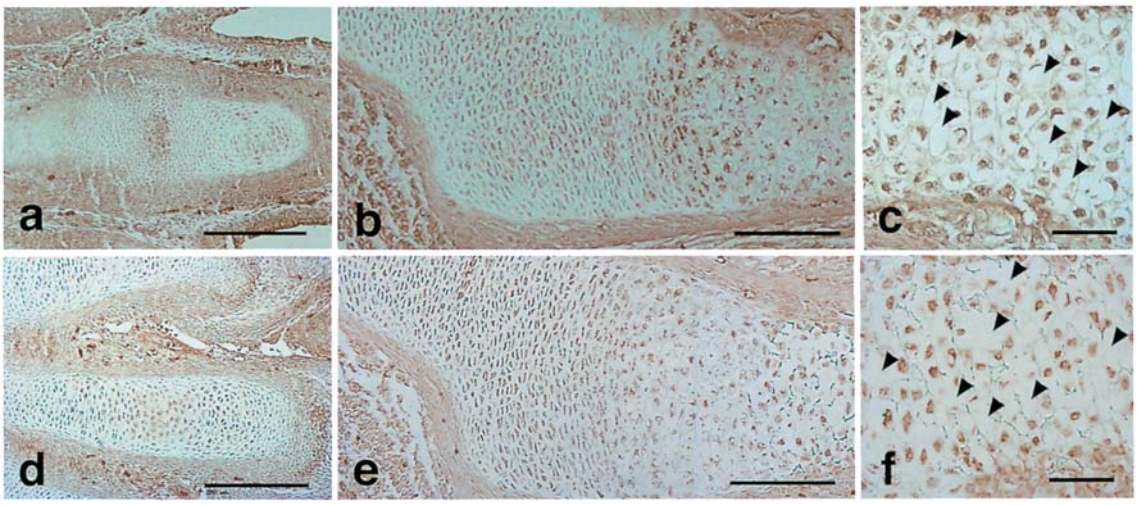

Fig. 1 Micrographs showing immunohistochemistry of the developing cartilage in E14 mouse embryos with anti-HGF (a-c) and anti-c-MET antibodies (d-f). Immunoreactivities for HGF and c-MET are observed in the chondrocytes in the resting, proliferating and hypertrophic zones of cartilage (a, b, d, f), but are absent in the perichondrium and the periphery of cartilage adjacent to the perichondrium (b, e). Some hypertrophic chondrocytes located in the middle of the hypertrophic zone are weak or negative in immunoreactivity for HGF and c-MET (arrowheads in c and f). Scale bar = 200 μm (a, d), 100 μm (b, e) and 50 μm (c, f).

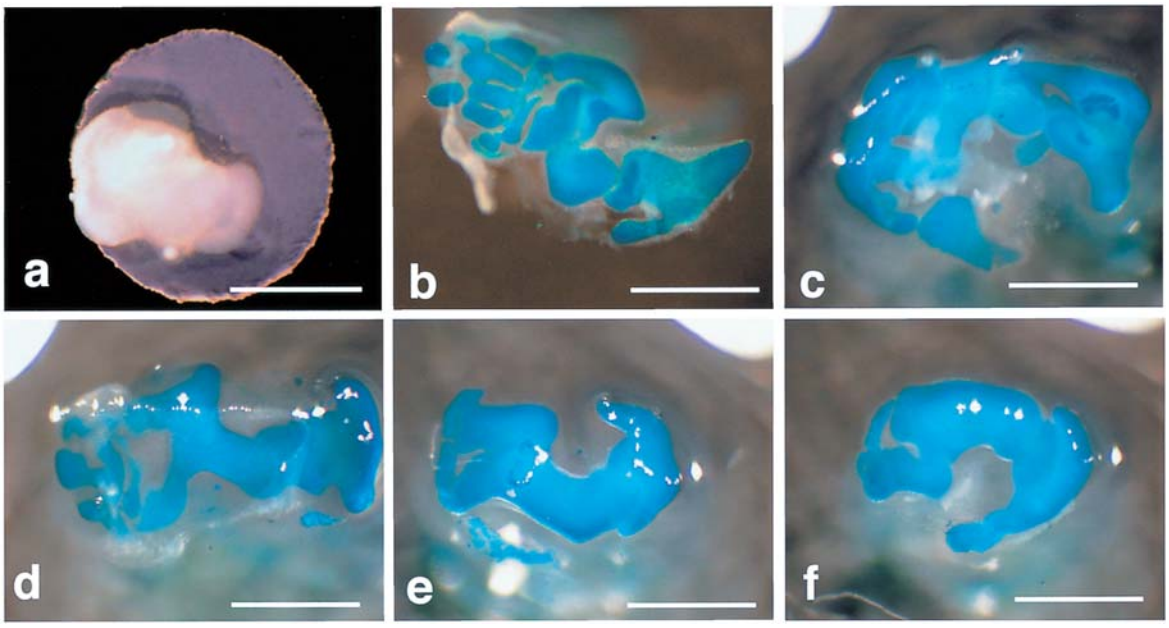

Fig. 2 The whole appearance without staining (a) or with Alcian-blue staining (b-f) of forelimb explants cultured in the absence (a, b), or presence of recombinant HGF (c), HGF control ODN (d), HGF antisense ODN (e) or NK4 (f). The distal end of explants is oriented to the left (a-f). Note that the explant cultured with HGF (c) is larger than the control (b), whereas the explant cultured with HGF antisense ODN (e) or NK4 (f) shows impaired development of the cartilage of digits, and is smaller than the control (b, d). Scale bar = 2 mm (a) and 1 mm (b-f).

culture media, the entire forelimb cartilage became longer than control and curved in shape (Fig. 2c). In contrast, the antisense ODN for HGF induced impaired development of the cartilage of digits, whereas development of the cartilage of scapula and arm appeared normal (Fig. 2e). The control ODN for HGF did not affect the development of forelimb cartilage (Fig. 2d). NK4, the pharmacological antag-

onist of HGF, induced the same result as that induced by HGF antisense ODN (Fig. 2f). These results suggest that the development of cartilaginous anlagen of forelimbs is positively regulated by HGF, and HGF is essential for the formation of digital bone anlagen.

Histology of cartilages in cultured forelimbs

The cultured forelimbs of E10 mouse embryos were prepared for the conventional histology with hematoxylin-eosin staining. In the control and HGF control ODN-administered explants, chondrocytes were distributed uniformly in the cartilaginous anlagen (Fig. 3a, d). Chondrocytes were located in small lacunae, and a few lacunae contained two chondrocytes. No signs for endochondral ossification as seen in E14 forelimbs were recognized in the cultured forelimbs. In the explants administered with recombinant HGF, the chondrocytes and cartilaginous lacunae were smaller in size and more densely packed than those of control, and many lacunae contained two or more chondrocytes (Fig. 3b, c). This suggested that HGF had induced mitosis of chondrocytes in cultured forelimbs. When the endogenous HGF was inhibited by HGF antisense ODN or NK4, the number of chondrocytes was smaller, and the size of cartilaginous lacunae was larger than those of the control (Fig. 3e, g). Furthermore, hypertrophic chondrocytes with enlarged lacunae were observed in the middle portion of cartilaginous anlage (Fig. 3f).

Chondrocyte proliferation in cultured forelimbs

To detect the proliferative activity of chondrocytes, the sections of cultured explants were immunostained with anti-PCNA antibody. In the untreated control and HGF control ODN-treated groups, only a small number of chondrocytes were stained for PCNA (Fig. 4a, c). Recombinant HGF added to the culture media induced marked increase in the number of PCNA-positive chondrocytes (Fig. 4b). When HGF antisense ODN or NK4 was added to the culture media, no or very few chondrocytes were immunopositive for PCNA (Fig. 4d, e). The statistical analyses showed that the labeling index of HGF-administered group was twice that of the control group (Fig. 4f). The labeling indexes of HGF antisense ODN- and NK4-treated groups were about 40% and 20% of the control group, respectively ($p < 0.05$,

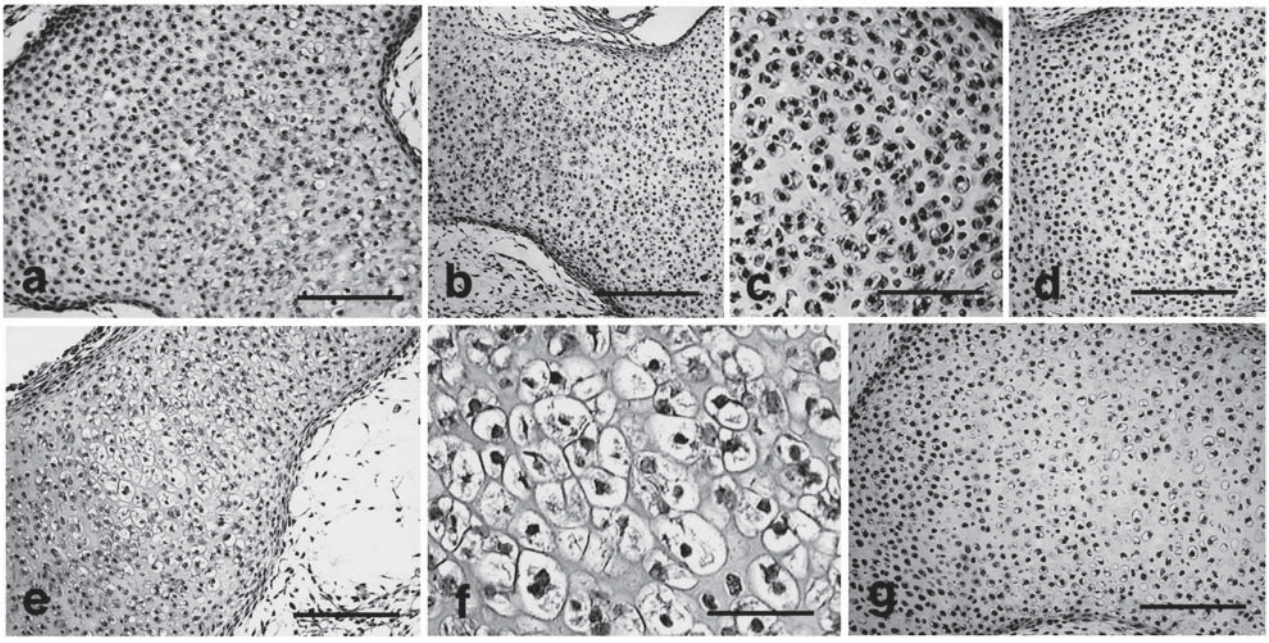

Fig. 3 Micrographs showing H-E staining of the developing cartilages of forelimbs from E10 mouse embryos cultured in the absence (a), or presence of recombinant HGF (b, c), HGF control ODN (d), HGF antisense ODN (e, f) or NK4 (g). c and f are the higher magnifications of b and e, respectively. No sign of endochondral ossification is recognized in the control cartilage without treatment. The cartilage cultured with recombinant HGF (b, c) has numerous cartilaginous lacunae that are smaller in size and higher in the proportion of ones containing two or more chondrocytes to ones containing single chondrocyte, as compared with the control (d). In contrast, the cartilage cultured with HGF antisense ODN (e, f) or NK4 (g) has the lacunae smaller in number and larger in size, containing single hypertrophic chondrocyte (f). Scale bar = 50 μm (a, b, d, e, g), 25 μm (c) and 20 μm (f).

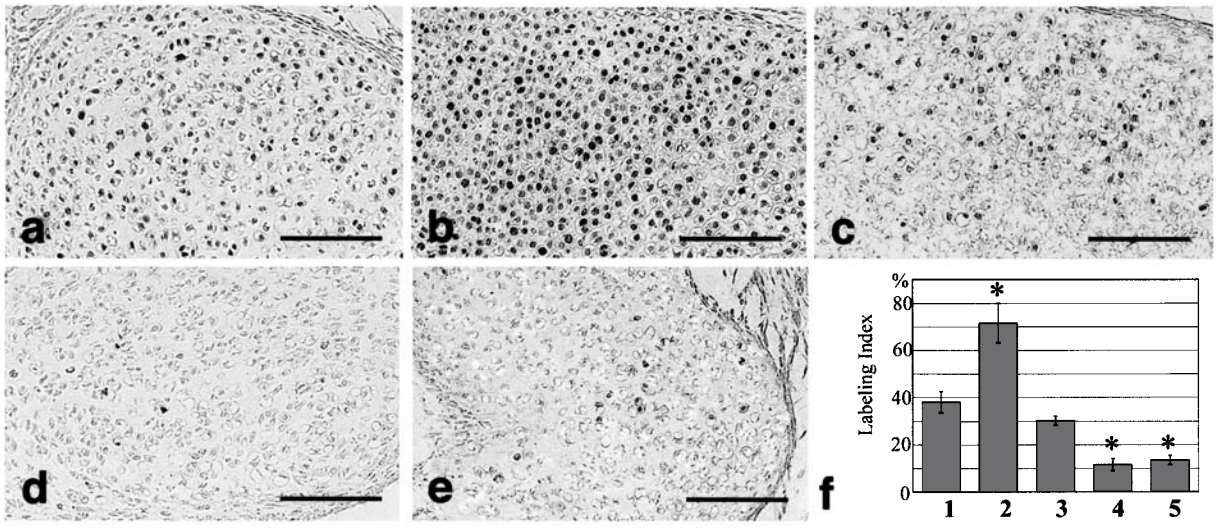

Fig. 4 Micrographs showing PCNA immunohistochemistry of the developing cartilages of forelimbs from E10 mouse embryos cultured in the absence (a), or presence of recombinant HGF (b), HGF control ODN (c), HGF antisense ODN (d) or NK4 (e), and a graph showing PCNA labeling index of cells in the developing cartilage in the absence (1) or presence of recombinant HGF (2), HGF control ODN (3), HGF antisense ODN (4) or NK4 (5). $^{*}p < 0.05$ compared to the control; (n = 3). Scale bar = 50 μm (a-e).

Fig. 4f). These results suggest that the proliferative activity of chondrocytes in cultured forelimbs are positively regulated by HGF.

Expression of collagen type X in cultured forelimbs

To examine the differentiation/hypertrophy of chondrocytes, the expression of collagen type X was examined by immunohistochemistry. Collagen type X is expressed predominantly by hypertrophic chondrocytes (11). Compared with the small number of collagen type X-immunoreactive chondrocytes observed in the control and HGF control ODN-treated groups (Fig. 5a, c), much smaller number of cells in HGF-administered group (Fig. 5b) and much larger number of cells in HGF antisense ODN and NK4-treated groups (Fig. 5d, e) were immunopositive for collagen type X. Statistically, about 10% in the control and HGF control ODN-treated groups, 3% in HGF-administered group ($p < 0.05$ vs. control), and 50–70% in HGF antisense ODN- and NK4-treated groups ($p < 0.05$ vs. control) of chondrocytes were immunopositive. These results suggest that HGF negatively regulates the expression of collagen type X as a marker of chondrocyte differentiation.

DISCUSSION

Endochondral ossification consists of three processes: the chondrocyte proliferation, differentiation (hypertrophy) and subsequent osteogenesis accompanied by angiogenesis (36, 38). Past studies have implicated cartilaginous HGF in pathogenesis of osteoarthritis (22, 37, 39, 53), but the role of HGF in normal development of cartilage and endochondral ossification has not been clarified yet. The present study has shown that the combination of immunohistochemistry and functional organ culture is useful for addressing this issue. However, the present organ culture system reproduces only the first half of the process of endochondral ossification, i.e., the proliferation and differentiation of chondrocytes. Investigation of the latter half, i.e., osteogenesis, may require another approach.

The study using in vivo embryonic limbs has demonstrated intense immunoreactivity for both HGF and its receptor c-Met in proliferative and early hypertrophic chondrocytes and moderate to weak immunoreactivity in resting and late hypertrophic chondrocytes. These results imply roles of the HGF/c-Met system in the proliferation and differentiation of chondrocytes in the cartilaginous anlagen for long bones. Consistent with this, the organ culture study has demonstrated that exogenous HGF causes enhanced proliferation of chondrocytes, whereas antisense ODN and the specific antagonist of HGF cause impaired proliferation as well as enhanced hypertrophy and collagen X production of chondrocytes, evidence for cartilaginous differentiation. In articular and Meckel's cartilages, HGF is also known to stimulate proliferative activity as well

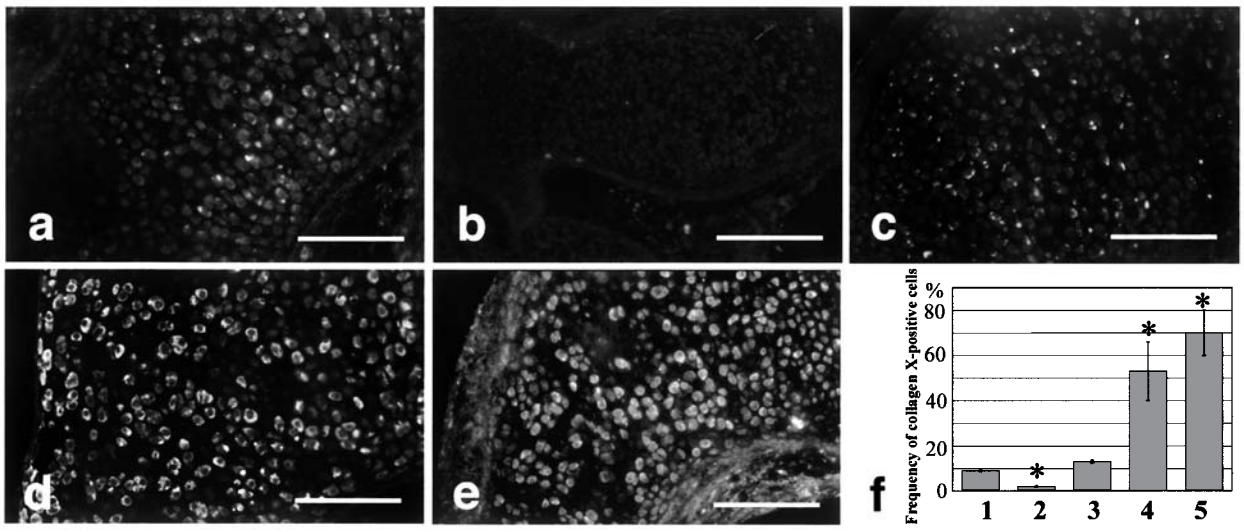

Fig. 5 Fluorescent micrographs showing collagen X immunohistochemistry of the developing cartilage in forelimb from E10 mouse embryos cultured in the absence (a), or presence of recombinant HGF (b), HGF control ODN (c), HGF antisense ODN (d) or NK4 (e), and a graph showing the frequency of collagen X- immunopositive chondrocytes (f) in the developing cartilage in the absence (1) or presence of recombinant HGF (2), HGF control ODN (3), HGF antisense ODN (4) or NK4 (5). $^{*}p < 0.05$ compared to the control; (n = 3). Scale bar = 50 μm (a-e).

as cell motility and matrix production of chondrocytes (50). The present in vivo and vitro results suggest that the intrinsic HGF produced by chondrocytes exerts an autocrine/paracrine function on chondrocytes in stimulating their proliferation while inhibiting their differentiation, resulting in prevention of endochondral ossification in the anlagen of forelimb long bones.

Rather paradoxically, the hypertrophic chondrocytes, especially those in the upper portions of the hypertrophic zone, show considerable expression of HGF and c-Met. Since HGF is known as a potent mitogen and motogen of endothelial cell (7, 23), HGF secreted by hypertrophic chondrocytes may function in stimulating vascular invasion into the hypertrophic zone that is known to occur prior to the onset of osteogenesis at the final step of endochondral ossification (5). However, this function of HGF could not be confirmed in the present organ culture system, which failed to reproduce the process of vascular invasion and osteogenesis even after 8 days of incubation.

Lack of digital and hand bone anlagen in the forelimb explants cultured with HGF-antisense ODN or HGF antagonist has been revealed in the present study. This indicates that in the distal end portions of forelimbs HGF is indispensable to initiation of chondrogenesis rather than development of preexisting cartilage. In the developing limb buds, the mesoderm-derived mesenchymal cells aggregate each other to form mesenchymal condensations that give rise to cartilaginous bone anlagen. Takebayashi *et al*. (50) reported a strong expression of HGF mRNA in the mesenchyme condensations planned to form digital and hand bones. Production of intrinsic HGF in these areas might be responsible for hand formation. In this conjunction, the role of apical ectodermal ridge (AER) that covers the end portions of forelimbs is notable. In developing chick embryo, removal of AER causes lack of limb/wings formation (42, 43). This is probably interpreted by production and secretion of fibroblast growth factor (FGF) 2 in AER, which then stimulates HGF production in the mesoderm adjacent to AER (44).

In addition to HGF, other cell growth factors and transcriptional factors, including bone morphogenetic proteins (BMP), epidermal growth factor (EGF), FGF, Indian hedgehog (Ihh), parathyroid hormone-related peptide (PTHrP), TGF-α, TGF-β, vascular endothelial growth factor (VEGF) and Sox family, are postulated to be involved in chondrogenesis and endochondral ossification (12, 14, 17, 25). PTHrP and its receptor are considered to play important roles in both proliferation and differentiation of chondrocytes (4, 5). PTHrP-deficient mice exhibit abnormalities in endochondral ossification, such as occurrence of non-hypertrophic but proliferative chondrocytes in the hypertrophic zone (4). VEGF is produced in hypertrophic chondrocytes and functions as paracrine factor in stimulating capillary in-

vasion and osteogenesis in the growth plate (15). Furthermore, VEGF may function as autocrine factor in stimulating chondrocyte differentiation (10). Such dual function of VEGF in endochondral ossification in paracrine and autocrine fashions is similar to that of HGF as revealed in the present study, although the autocrine function of HGF resides in preventing chondrocyte differentiation rather than stimulating it.

The normal process of endochondral ossification is achieved by successive steps of chondrocyte proliferation, differentiation (hypertrophy), and subsequent osteogenesis accompanied by vascularization (38). The present study has suggested that HGF is a factor regulating the balance among such steps of endochondral ossification.

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