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A STUDY ON THE EXPRESSION OF TYPE I AND TYPE II COLLAGEN GENES AND PROTEINS IN THE DEVELOPING HUMAN MANDIBLE

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Type I and type II collagens are considered the major collagens of bone and cartilage respectively. Monitoring the patterns of those gene and protein expressions during development will provide a basis for the understanding of the normal and abnormal growths. This study was undertaken to investigate the expression of collagen genes and proteins involved in the developing human mandible. Fifty embryos and fetuses were studied with Alcian blue-PAS, Masson's Trichrome, reverse transcription polymerase chain reaction (RT-PCR), Western blot analysis, and Southern blot analysis.

Our results showed that pro-α1(II) collagen gene expression begins in the 5th week. Type II collagen is synthesized in mesenchymal cells in advance of overt chondrogenesis. The gene expression for type II collagen was highest during the appearance of Meckel's cartilage. There was a switch in collagen protein expression from type I to type II during the appearance stage of Meckel's cartilage. The distribution of the mRNA for type II collagen corresponded well with the pattern of type II collagen protein. The endochondral ossification was observed where there was direct replacement of cartilage by bone.

Key words : Human Embryo and Fetus, Mandible, Meckel's Cartilage, Collagen type I and II.

The development and growth of the tissue in the craniofacial skeleton is a complicated process involving a number of changes in the expression of collagen genes as well as transient stages with changes in morphological appearance[22,23,32]. The two major pathways of bone development involve intramembranous or endochondral osteogenesis. During endochondral bone formation, mesenchymal condensation initially leads to formation of a cartilaginous template, which is replaced by bony structures at a later stage[4]. In intramembranous bone formation, however, it has been postulated that osteoblasts of intramembranous bone can potentially express the chondrogenic phenotype. Recent research of the gene expression in extracelluar matrix and developmental morphogenesis has been focused on the more important roles of endochondral bone formation in the mandible[16,27].

The human embryonic mandible is formed from ectomesenchymal cells which originally migrate from the ectodermal neural crest into mesenchymal cells, and these cells differentiate into Meckel's cartilage. It is not entirely clear to what degree Meckel's cartilage contributes to the postnatal mandibular skeleton[34].

Collagens are major structural proteins of the extracellular matrix and the correct temporal and tissue specific expressions of the collagen genes are crucial for the proper development of form during morphogenesis[1,17]. Type II collagen is the major collagenous component of cartilage and has traditionally been considered as very specific for cartilage, synthesized in mesenchymal cells in advance of overt chondrogenesis[9,10,25,29], and may define and determine the sites and timing of chondrogenesis[26].

During limb development, type I collagen disappears from the region where cartilage develops and a synthesis of type II collagen, which is a characteristic of

Table 1. Primer Sequences for Amplification of Type II Collagen cDNA in Human Embryos

Corresponding Position	Sequences
Primer 1 (Exon 1)	5′- CCGCGGTGAGCCATGATTCG- 3′
Primer 2 (Exon 5)	5′- CAGGCCCAGGAGGTCCTTTGGG- 3′

cartilage, begins[5]. The collagen type I and II expressions on the chondroid tissue of mandible in human and cat[15], type I on embryonic rat mandible *in vivo*[35], type II mRNA expression in avian mandibular ectomesenchymal cells *in vitro*[12], type II collagen in cartilage of human fetal long bone[13], and type I, II, III, XI, and XIII collagens in human fetal tissue[19-21] were reported. These studies mainly concentrated on the development of the chick limb bud or were carried out in the developmental stage of the fetal long bone.

Meckel's cartilage is similar to other hyaline cartilage of long bone, however, this cartilage has different origins than their counterparts in the developing long bone. It is important for Meckel's cartilage to determine whether there is any specific significance in the mechanism of chondrogenesis, pattern formation, and regulation of gene in the extracelluar matrix proteins. But little is known about the regulation of collagen gene expression during the development of Meckel' cartilage in the human embryonic mandible.

In this study we investigated 1) the expression pattern of type II collagen genes 2) the relationships between the gene expression of type II collagen and the development of Meckel's cartilage 3) the expression pattern of type I and II collagen proteins involved during the development of Meckel's cartilage in human embryos, and 4) the appearance of Meckel's cartilage and related morphogenesis in the mandible of human embryo and fetus.

MATERIALS AND METHODS

50 cases of embryo and fetuses were used for the present study. The mandibles were isolated under sterile conditions, rapidly stored in liquid nitrogen at -70℃. Half of the samples were fixed in neutralized buffered paraformaldehyde(3.7%) for 12 hours at room temperature and embedded in paraffin, and 5㎛ sections were cut, stained with Hematoxylin and Eosin, Alcian Blue or Modified Masson's Trichrome, and examined by light microscope.

1. RNA extraction

Total RNA was prepared by the LiCl-urea method from 4-8 week old human embryos from therapeutic abortions (the developmental stage of embryo was determinded according to the cranium-rump length by England's method, 1990[6]). All specimens were obtained with permission of the joint ethical committee of the University Central Hospital and Wonkwang University.

2. Reverse transcription-polymerase chain reaction (RT-PCR)

Reverse transcription of RNA was carried out in a 20㎕ of final volume containing 2㎕ of a 10mM dNTPs mix, 4㎕ of 5X RT buffer (250 mM Tris-Cl, pH 8.3, 375 mM $MgCl_2$, 1㎕ of random primers (25 pmoles/ 25㎕), 2㎕ of RNasin (20 U/㎕), 3㎕ (1㎍/㎕) of denatured total RNA (80℃, 3 min), 7.0㎕ of double distilled water. After adding 1㎕ of reverse transcriptase (200 U/ml), the mixture was spun down, and incubated at 37℃ for 60 min. The reaction was heated at 70℃ for 10 min, then quickly chilled on ice. PCR was performed by using Capillary Thermal Cycler (DAEHAN Medical Co., LTD, Korea). The primers for amplification during the PCR were depicted in Table 1. To the 1㎕ of synthesized cDNA, the following components were added; 1㎕ of dNTP's (2.5 mM), 1.2㎕ of 10X PCR buffer (100 mM Tris-Cl, 30mM $MgCl_2$, 2.5 mg/ml BSA), 1㎕ of primer pair (5 pmoles/ 10㎕), 5.8㎕ double distilled H_2O, 1㎕ of Taq polymerase (0.2U/㎕). After an initial incubation at 94℃ for 20 sec, temperature cycling, at 94℃ for 5 sec, 55℃ for 5 sec, and 72℃ for 10 sec, was carried out. The optimum number of cycles was 45 which was determined experimentally.

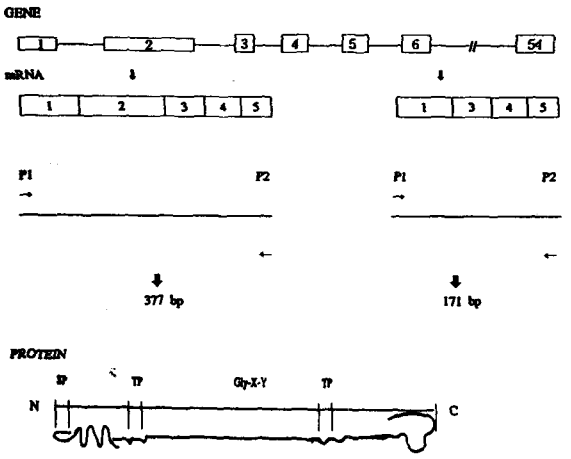

Figure 1. Diagram of Type II Collagen Gene for PCR Strategy. cDNAs were amplified with TaqI polymerase using two separate primers corresponding to sequences within exon 1 to exon 5. The predicted sizes of the PCR products plus and minus exon 2 are 377 and 171 bp, respectively. Exon are indicated as open boxes. SP is the signal peptide, TP is the telopeptide. The regions indicated by the curved lines are globular portions of the protein.

3. Probe Preparation

Primers were chemically synthesized by using DNA synthesizer (Pharmacia Ltd.). Primer sequences were designed on the basis of GenBank data (Fig 1).

PCR products (377 bp) were prepared by RT-PCR technique, purified by using Geneclean kit, and cloned to Eco RV site within the multicloning site (MCS) of pBluescript II KS(-) vector. Before the cloning to pBluescript II KS(-) vector, the purified PCR products were phosphorylated with polynucleotide kinase and filled with klenow enzyme. The blunted PCR products were extracted with phenol and phenol/chloroform and precipitated with 2 volumes of absolute ethanol. The DNA pellets were dissolved in sterile distilled water. To prepare the vector for cloning, pBluescript II KS(-) vector was purified by ultracentrifugation method (Radloff et al, 1967). The purified vector was linearized with Eco RV and dephosphorylated with bacterial alkaline phosphatase. Bacteriophage T_4 DNA ligase was treated to the mixture of one microgram of the vector and long of the PCR product. An *E.coli* strain, DH5α was transformed with the ligated DNA by calcium chloride method (Hanahan, 1985). After plating the transformed bacterial cells on X-gal/IPTG/ampicillin plate, Colony-PCR was carried out as described in the RT-PCR method. Orientation of inserted DNA was confirmed by appropriate restriction enzyme digestion on the basis of previously reported collagen II cDNA sequence (Baldwin *et al.*, 1989). The fragment isolated from the cloned vector was used as a probe.

4. Southern blot hybridization

The gel from RT-PCR product was then denatured by soaking for 45 min in several volumes of 1.5 M NaCl, 0.5 N NaOH with constant, gentle agitation. The gel was then rinsed briefly in deionized water, and neutralized by soaking for 30 min in several volumes of 1 M Tris-Cl, pH 7.4, 1.5 M NaCl at room temperature with constant agitation. The DNA was then transferred by capillary method to a nylon membrane with transfer buffer (10X SSC) by standard blotting procedures (Maniatis *et al.*, 1982). The DNA was UV cross-linked on to the membrane, and the membrane was prehybridized in prehybridization solution (50% formamide, 6X SSC, 10X Denhardt's solution, 0.5% SDS, 1mg/ml denatured salmon sperm DNA) at 42℃ for 3 hours and then hybridized at 42℃ overnight in the same solution containing [α-^{32}P] dCTP labeled 377 bp cDNA probe. The hybridized membrane was washed three times at 50℃ in 5% SDS/1X SSC and washed once more at 65℃ in 0.1% SDS/0.5X SSC. Kodak XAR film was exposed to the membrane at -70℃ with an intensifying screen.

5. Western blotting

The samples (embryos: 17, 21, 29, 37 mm) were homogenized in 0.2N acetic acid and dialyzed against PBS (phosphate buffer saline, pH 7.4) for 2 hours. Homogenisates and Protein maker (sigma) were prepared with loading buffer and were put down in 8% SDS-polyacrylamide gel. After gel electrophoresis, proteins on the SDS-PAGE were blotted onto a nitrocellulose filter (sigma). 3% skin milk was used for enzyme blocking and the transferred nitrocellulose

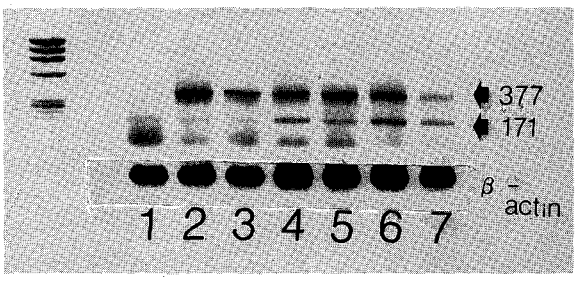

Fig. 2. Exon 1-5 Detection of Type II Collagen Gene According to cranium-rump Length of Human Embryos by RT-PCR.
Lanes : 1; 7 mm (stage 14, 4 wks), 2; 13 mm (stage 17, 5 wks), 3; 17 mm (stage 18, 5 wks), 4; 21 mm (stage 20, 5 wks), 5; 24 mm (stage 21, 6 wks), 6; 29 mm (stage 23, 6 wks), 7; 37 mm (8 wks).

membrane was incubated with primary antibody (type I collagen and type II collagen, dilution: 1:300, Monosan, Holland) for 3 hours. After washing with PBS, the nitrocellulose filter was put in a secondary antibody (alkaline phosphatase conjugated gout IgG anti rabbit, dilution: 1:100, sigma) for 1 hour and was reacted in chromogenic substrate (NBT: 0.3%, BCIP: 0.15%) for 30 min. After the color developing, the protein band was photographed as a record.

RESULTS

1. Expression of type II collagen genes

As shown in Fig. 2. 377 bp (Exon 1-5), type II collagen gene by RT-PCR was not found in a 7 mm embryo, begun to be detected in a 13 mm embryo (stage 17 by Carnegie system, 36 days), and a level of decline was noted in a 37 mm embryo. 171 bp (lacking exon 2) was expressed in all lanes of experimental groups. However, the expression was very weak in the early stages (lane 1-3) but high in the late stage (lane 4-6) of the developing mandible.

In the Southern blotting for RT-PCR products of the exon 1-5 of type II collagen gene, the results of hybridization with 377 bp probe revealed the gene expression in a 13 mm embryo. It was similar to the RT-PCR product, but different expression patterns were detected in developing embryonic stages. For 377 bp, it was decreased during the late stages (lane 5-7) and for 207 bp, highest in the middle stages (lane 3, 4) of experimental groups (Fig. 3, 4).

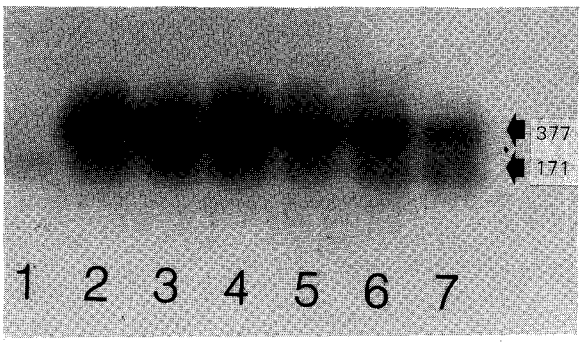

Fig. 3. Southern Blotting for RT-PCR Products of the Exon 1-5 of cDNA Type II Collagen Gene According to cranium-rump Length of Human Embryos.
Lanes : 1; 7 mm (stage 14, 4 wks), 2; 13 mm (stage 17, 5 wks), 3; 17 mm (stage 18, 5 wks), 4; 21 mm (stage 20, 5 wks), 5; 24 mm (stage 21, 6 wks), 6; 29 mm (stage 23, 6 wks), 7; 37 mm (8 wks).

2. Quantitative Relationships between the expression of type II collagen gene and the development of Meckel's cartilage.

In densitogram of southern blotting for the expression of type II collagen gene, the expression amount of 171 bp was 20±2.82 (arbitrary unit) in a 7 mm embryo (4th week) 30±2.89 in a 13 mm, 37±4.16 in a 17 mm, 39±2.48 in a 20.5 mm. This high expression of 171 bp corresponded with the appearance stage of Meckel's cartilage (20.5 mm). After cartilage formation, the result showed a declining tendency for the gene expression during the period of new bone formation (28±2.94 in a 23.5 mm, 23±2.16 in a 37 mm).

In exon 1-5 (377 bp), The highest amount of gene expression was detected during the early stomatodeum period as 65±1.37 mm, 67±3.88 in a 17 mm. Compared to the stomatodeum period, gene expression amounts was reduced during the period of new bone formation (50±2.10 in a 23.5 mm, 53±1.45 in a 37 mm). The amount of the expression was noticeably more increased during the appearance of Meckel's cartilage than in the period of new bone formation (Fig. 4).

3. Pattern of Protein Expression for Type I and II Collagens

In protein expression of type I and type II collagens by Western blotting, type I collagen band (84 KD)

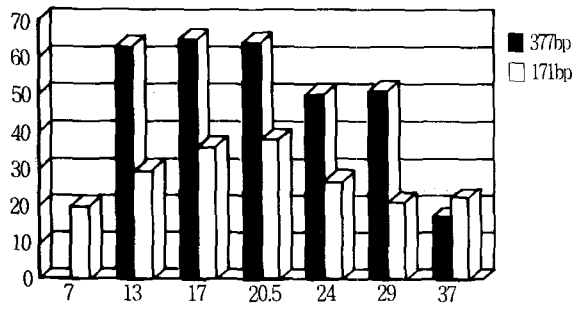

Fig. 4. Densitogram of Southern Blot Analysis for Gene Expression of Type II Collagen According to Developing Mandible of Human Embryos. [(377 bp, exon 1-5 of type II collagen gene. 171 bp, exon 1 to exon 5 lacking in exon 2). Ordinate value is arbitrary unit. Abscissa is cranium-rump length of developmental human embryo(mm)].

was clearly observed in a 17mm embryo, but the expression band detected in a 21mm embryo was very faint. During the appearance stage of Meckel's cartilage, the protein expression of type I collagen was again observed after formation of Meckel's cartilage in 29 mm and 37 mm embryos (Fig. 5B). However, the highest expression of type II (97 KD) was detected in a 21 mm embryo, but noted as a faint band before and after the appearance of Meckel's cartilage (Fig. 5C).

4. Histological Findings on the Meckel's cartilage in the developing mandible.

The stomatodeum with maxillary and mandibular processes was clearly shown in the sagittal section of a 13 mm embryo. Meckel's cartilage was first seen on the mandibular arch with apparent distribution of mandibular nerve in a 20.5 mm embryo (5 weeks later) (Fig. 7). New bone formation was stained deep blue

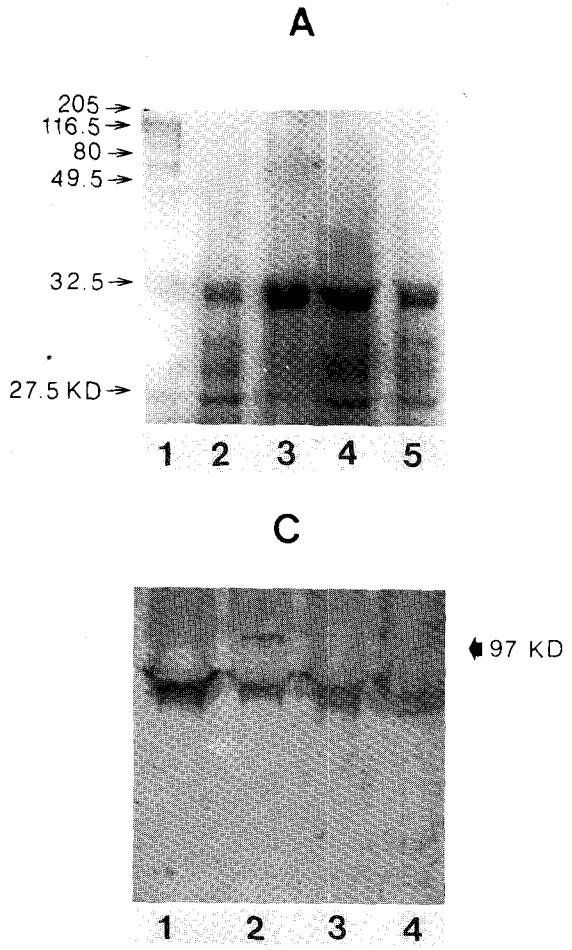

Fig. 5. Protein Expression of Type I and Type II Collagens According to Developing Mandible of Human Embryos by 8 % SDS-PAGE.

A : Coomassi brillant blue staining, B : Western blotting of type I collagen antibody, C : Western blotting of type II collagen antibody.

A : Lane 1 ; protein standard marker, Lane 2 ; 17 mm (stage 18, 5 wks), Lane 3 ; 21mm (stage 21, 6 wks), Lane 4 ; 29 mm (stage 23, 6 wks), Lane 5 ; 37 mm (8 wks). B, C : Lane 1 ; 17 mm, Lane 2 ; 21 mm, Lane 3 ; 29 mm, Lane 4 ; 37 mm.

Abbreviations
MC Meckel's cartilage, DL Dental lamina, EO Endochondral ossification, NB New bone formation
Mx Maxilla, Mn Mandible, T Tongue

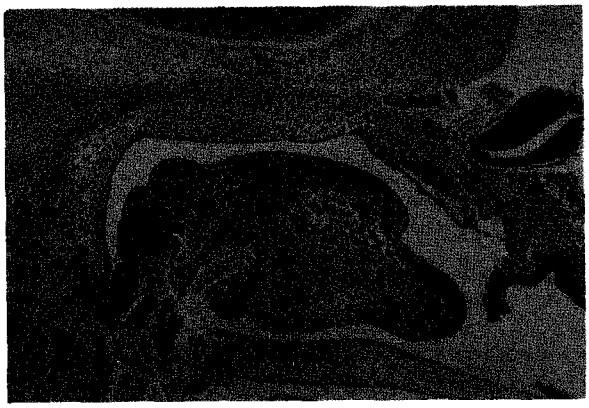

Fig. 6. Microphotography of sagittal section through a 16 mm embryo showing the stomatodeum (H & E, X 100)

Fig. 7. Microphotography of a 21 mm embryo showing bud stage of tooth germ & Meckel's cartilage in mandible (Alician Blue, X 200)

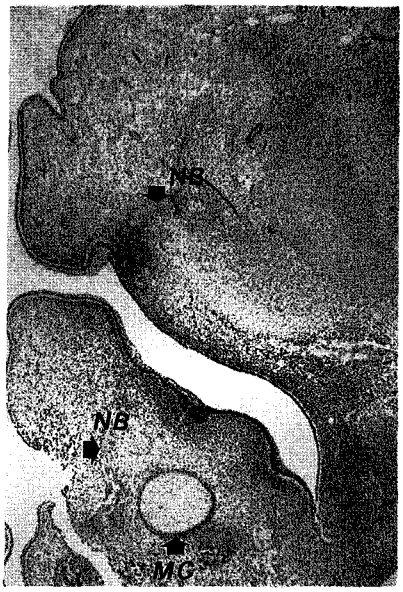

Fig. 8. Microphotography of a 23 mm embryo showing developing mandible. Meckel's cartilage and developing new bone(stained purple) were observed.(Alician Blue, X 100)

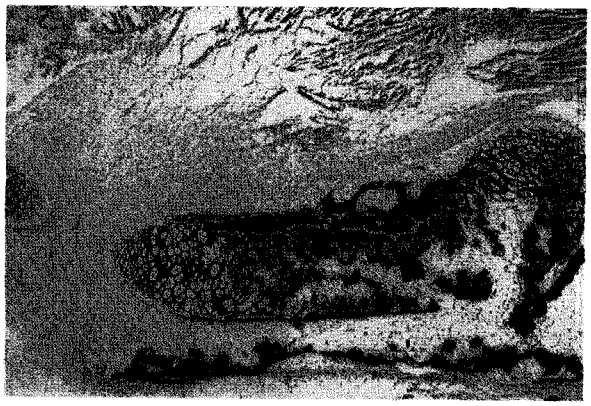

Fig 9. Microphotography of 13 week fetus showing degeneration of Meckel's cartilage. As the cartilage is degenerated, the space previously occupied by cartilage becomes filled with new bone. (H & E, X 40).

and purple by Goldman's M.T and Alcian blue stains (Fig. 8). It was initiated at the outer side of the middle portion of Meckel's cartilage in 21-37 mm embryos (6-8 weeks). The endochondral ossification was observed where there was direct replacement of cartilage by bone (Fig. 9).

DISCUSSION

Analysis of the regulation of gene expression and pattern of bone formation during development of the craniofacial skeleton will provide a basis for understanding normal growth and osteochondrogenesis. The development of the human mandible involves a number of transient stages with changes in morphological appearance and in the composition of extra-

cellular matrix[8,22].

Type II collagen may define and determine the sites and timing of chondrogenesis[26]. Analysis of the early stages around the 7th week of gestation revealed that for type II collagen a strong hybridization signal limited the chondrogenic tissue during the development of the human long bone[13]. We investigated gene expression of type II collagen in the mandibular portion of the craniofacial skeleton according to developing embryonic stages. Our result showed that the gene expression for type II collagen begins early in the 5th week. Type II collagen is synthesized in mesenchymal cells in advance of overt chondrogenesis [9,10,25,28], appears transiently in advance of overt chondrogenesis[32], and increases during the course of chondrogenesis[9]. A low level of type II collagen mRNA is present prior to the onset of chondrogenesis and increases just before onset[12]. In our results, Exon 1-5 (377 bp) expression of type II collagen gene was first detected in a 13 mm embryo and showed the highest expression amount during the appearance of Meckel's cartilage.

Mandibular mesenchymal cells differentiate into the original cartilaginous skeleton of the mandible - Meckel's cartilage. Meckel's cartilage was entirely formed in a 8 to 11 mm embryo at stage 16, 37 postconceptional day[16], in the 7th week of intrauterine life with a 16 mm embryo[3], but had not yet formed in a 19 mm[7] embryo. In our study, we observed the appearance of Meckel's cartilage in a 20.5 mm embryo.

The differences in results according to various researchers may be explained by differences in methods for determining embryo length as well as the freshy and intact condition of the embryo at the time of measurement.

Type II collagen revealed exon 2 encoding a 69 amino acid cysteine-rich domain in the NH_2-terminal propeptide and is subject to tissue-specific alternative splicing (Ryan and Sandell, 1990). Fetal and juvenile human chondrocytes contain both transcripts, although the alternatively spliced form is more abundant in juvenile mRNA. mRNA lacking the second exon is a relatively late event during chondrogenesis in chick limb[14], also the expression of exon 2 may be a marker for a distinct population of chondrocytes[18]. We found that mRNA lacking exon 2 (171 bp) was expressed at an earlier stage (early 4th week) than 377 bp (including exon 2) expression on the contrary to the results in the chick (Nah & Upholt, 1991). However, this expression was very weak in the early stages and was highest in the middle stages of embryonic development. The highest expression of 171 bp gene corresponded with the appearance stage of Meckel's cartilage in a 20.5 mm of embryo. After appearance of cartilage, our results showed a declining tendency in the amouts of gene expression during the period of new bone formation in a 37 mm (8 weeks). Results are similar to those investigations of Sandbeg and Vuorio, 1987. Type II collagen was negative in fingers of 15 week fetus[21], and had almost disappeared in the 15 week fetus in human limb[13]. Because our experiment was only for embryonic periods, it is necessory to confirm this result through further study.

In the chicken limb, type I collagen disappears from the region where cartilage develops and synthesis of type II collagen, which is characteristic of cartilage, begins, and the distribution of the mRNA for type II collagen corresponds well with the pattern of type II collagen synthesis by Devlin et al., 1988. In our study, protein expression of type I and type II collagens by Western blotting revealed that type I collagen band (84 KD) was observed in a 17 mm embryo, but the expression band was detected as very faint in a 21 mm, the appearance stage of Meckel's cartilage. Type II (97 KD) was highly detected in a 21 mm of embryo, but appeared as a faint band before and after the appearance of Meckel's cartilage. There was a switch for the expression of collagen proteins from type I to type II during the appearance stage of Meckel's cartilage.

The mRNA and protein for type II collagen expression was highest during the appearance of Meckel's cartilage. The distribution of the mRNA for type II collagen corresponded well with the pattern of type II collagen protein by Western blot analysis, suggesting control at the level of transcription and mRNA accumulation.

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