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Role of cartilage-derived anti-angiogenic factor, chondromodulin-I, during endochondral bone formation

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Summary

Objective: Cartilage is a typical avasclar tissue that exhibits powerful resistance to angiogenesis or vascular invasion. We previously identified a cartilage-specific 25 kDa glycosylated protein, chondromodulin-I (ChM-I), as anti-angiogenic factor. Taking advantage of ectopic bone formation and xenograft tumour model by human chondrosarcoma cell line OUMS-27, we examined how ChM-I is involved in switching of angiogenesis in cartilage.

Design: Gene expression pattern of ChM-I was examined in 4-week-old mice and mouse embryos by northern blot analysis and *in situ* hybridization. To evaluate the effect of ChM-I on ectopic bone formation, guanidine extracts of demineralized bone matrix were mixed with the ChM-I-bound heparin-Sepharose beads and were implanted onto the fasciae of back muscle of 6-week old nude mice. To analyse the effect of ChM-I on tumour angiogenesis, the level of ChM-I mRNA in cartilaginous tumours was assessed by competitive PCR, and compared with that of articular cartilage. Then, human chondrosarcoma OUMS-27 cells were inoculated into the back of nude mice to form a tumour about 45 mm^3 in size. Recombinant ChM-I protein was administrated into OUMS-27 xenograft tumours for the initial 5 days to study its effect against tumour-angiogenesis.

Results: ChM-I gene was specifically expressed in cartilage of 4-week-old mice. Eye and thymus were also identified as minor expression sites. However, during endochondral bone development, cartilage changes its character from anti-angiogenic into angiogenic prior to the replacement of calcified cartilage by bone. In embryos, ChM-I mRNA was expressed in proliferative and upper hypertrophic cartilage zones in the developing cartilaginous bone rudiments, but completely abolished in lower hypertrophic and calcified cartilage zones. Purified ChM-I protein apparently inhibited vascular invasion into cartilage induced by the implantation of demineralized bone matrix in nude mice, leading to the inhibition of replacement of cartilage. The level of ChM-I transcripts in the lower-grade chondrosarcomas was substantially reduced to several hundreds or less in the lower-grade chondrosarcomas, compared with that of articular cartilage or other benign cartilage tumours. The local administration of recombinant human ChM-I almost completely blocked tumour angiogenesis and growth in the human chondrosarcoma xenografts in mice.

Conclusions: ChM-I is involved in the anti-angiogenic property of cartilage and its absence creates a permissive microenvironment for vascular invasion into cartilage under physiological and pathological conditions. © 2001 OsteoArthritis Research Society International

Key words: Chondromodulin-I, Angiogenesis inhibitor, Vascular invasion, Chondrosarcoma, Endochondral ossification.

Introduction

Cartilage is generally avascular and exhibits resistance to vascular invasion[1]. However, the phenotypic switching of cartilage from anti-angiogenic to angiogenic phenotype occurs in lower hypertrophic and calcified cartilage zones during endochondral bone formation in the embryo. The endothelial cells are maintained in alternative states of quiescence and angiogenesis by the cumulative level of angiogenic inducer and inhibitor signals supplied by surrounding tissues[2]. This balance switch for angiogenesis may predict that cartilage is penetrated by capillaries by the increase in inducing signals and/or by the decrease in inhibiting signals. Despite the importance of phenotypic switching of cartilage during skeletal development, our understanding of this process is limited.

Despite its apparent avascularity, a number of angiogenic molecules have been found in cartilage such as vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), transferrin and a 120-kDa angiogenic molecule[3–7]. VEGF is produced by hypertrophic chondrocytes undergoing calcification prior to vascular invasion, and then hypertrophic and calcified cartilage zones are invaded by blood vessels[5,7]. Systemic administration of soluble VEGF receptor to 24-day-old mice resulted in the suppression of vascular invasion, which was associated with impaired trabecular bone formation and expansion of hypertrophic zone in epiphyseal growth plate[7]. Overexpression of VEGF in the limb bud resulted in hypervascularization as evidenced by an increase in vascular density in mesenchymal tissues, but not in cartilage[8]. This implies that the withdrawal of anti-angiogenic activity is prerequisite for vascularization of cartilage to occur.

We recently purified a vascular endothelial cell growth inhibitor from fetal bovine cartilage[9], and found it to be identical to chondromodulin-I (ChM-I) which was previously reported as a growth and colony stimulating factor for chondrocytes[10,11]. During embryonic development, ChM-I gene is induced to express in association with chondrogenesis and the active expression is maintained in the

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Bovine   MTENSDKVPIALVGPDDVEFCSPPAYAAVTVKPS-SPARLLKVGAVVLISGAVLLLLGAI  59
Human    MTENSDKVPIALVGPDDVEFCSPPAYATLTVKPS-SPARLLKVGAVVLISGAVLLLFGAI  59
Mouse    MTENSDKVPITMVGPEDVEFCSPPAYTTVTVKPSGSPTRLLKVGAVVLISGAVLLLFGAI  60
Chicken  MAEGSEKVPIARAGPEDVEQGLPPAYTA-AVPPPG-PGRLLKAGATVLIAGALLLLAGAI  58

Bovine   GAFYFWKGSDNHIYNVHYTMSINGKLQDGSMEIDAGNNLETFKMGSGAEEAVEVNDFQNG 119
Human    GAFYFWKGSDSHIYNVHYTMSINGKLQDGSMEIDAGNNLETFKMGSGAEEAIAVNDFQNG 119
Mouse    GAFYFWKGNDNHIYNVHYSMSINGKLQDGSMEIDAVNNLETFKMGSGAEEAIEVNDFKNG 120
Chicken  GAFYFWKATERQVYNVHYTMSINGKVQDGSMEIDAANNLETFKTGSGSEEAVEVHDFQIG 118

Bovine   ITGIRFAGGEKCYIKAQVKARIPEVGTMTKQSISSELEGKIMPVKYEENSLIWVAGDQPV 179
Human    ITGIRFAGGEKCYIKAQVKARIPEVGAVTKQSISSKLEGKIMPVKYEENSLIWVAVDQPV 179
Mouse    ITGIRFAGGEKCYIKAQVKARIPEVGTVTKQSIS-ELEGKIMPANYEENSLIWVAVDQPV 179
Chicken  ITGIRFAGGEKCYIKAQPKARVPEVDAMTKASLSSDLEDEIMPVRFDENSLIWVAADEPI 178

                                             Mature ChM-I
                                      ------>
Bovine   KDNSFLSSKVLELCGDLPIFWLKPTYPKEIQRERRELVRKIVTTTTT----RRLRSG--- 232
Human    KDNSFLSSKVLELCGDLPIFWLKPTYPKEIQRERREVVRKIVPTTTK-----RPHSG--- 231
Mouse    KDSSFLSSKILELCGDLPIFWLKPMYPKEIQRERREVVRNSAPSTTR-----RPHSE--- 231
Chicken  KHNGFLSPKILELCGDLPIFWLRPTYPKDKQRERREMKRNKRQSESNFDAEHRAAAAEEV 238

                                                                **
                      †
Bovine   -PQGTPAP-----GRPNNGTRPSVQEDAEPFNPDNPYHQQEGESMTFDPRLDHEGICCIE 286
Human    -PRSNPGA-----GRLNNETRPSVQEDSQAFNPDNPYHQQEGESMTFDPRLDHEGICCIE 285
Mouse    -PRGNAGP-----GRLSNGTRPNVQDDAEPFNPDNPYHQQEGESMTFDPRLDHEGICCIE 285
Chicken  NTRSTPTQLTQELGPQSNETRPMQQESDQTLNPDNPYNQLEGEGMAFDPMLDHLGVCCIE 298

         *      *   *                   *   *    *
Bovine   CRRSYTHCQKICEPLGGYYPWPYNYQGCRSACRVIMPCSWWVARILGMV 335
Human    CRRSYTHCQKICEPLGGYYPWPYNYQGCRSACRVIMPCSWWVARILGMV 334
Mouse    CRRSYTHCQKICEPLGGYYPWPYNYQGCRSACRVVMPCSWWVARILGMV 333
Chicken  CRRSYTQCQRICEPLLGYYPWPYNYQGCRTACRIIMPCSWWVARILGMV 347
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Fig. 1. Part I.

avascular zone of the developing cartilaginous bone rudiments. However, its expression is completely abolished in the lower hypertrophic and calcified cartilage zones prior to vascular invasion[12,13]. In contrast to normal cartilage, the expression of ChM-I transcripts was only barely detectable in chondrosarcomas which produce a large amount of cartilaginous matrices such as type II collagen and aggrecan[14]. We demonstrate that the local administration of recombinant human ChM-I (rhChM-I) protein dramatically blocked tumour angiogenesis and growth of chondrosarcoma by a xenograft tumour model using human chondrosarcoma cell line OUMS-27[15]. Thus, ChM-I is involved in the anti-angiogenic property of cartilage under both physiological and pathological conditions, and the loss of its expression leads to the creation of an angiogenesis permissive phenotype in cartilage.

Materials and methods

MATERIALS AND CLINICAL SAMPLES

Recombinant human ChM-I was expressed in Chinese hamster ovary cells and purified from the culture supernatant to homogeneity by sequential chromatography as previously described[13]. Clinical samples were obtained from the patients with informed consent. Six cases, each, of grade I chondrosarcoma (CS), of enchondroma (Ench), and of exostosis (Exo) and five cases of articular cartilage (AC) were examined. Each specimen was processed separately for pathological examination and for mRNA extraction. α-[^{32}P]-dCTP (Amersham, Amersham, U.K.) was purchased from Japan Atomic Energy Institute (Tokyo, Japan).

CELL CULTURE

ATDC5 cells were maintained in the medium consisting of a 1:1 mixture of DME and Ham's F-12 medium (JRH Biosciences, Lenexa, KS) containing 5% FBS (JRH Biosciences), 10 μg/ml human insulin (I: Boehringer Mannheim GmbH, Gaithersburg, MD), 10 μg/ml human transferrin (T: Boehringer Mannheim GmbH), 3×10^{-8} M sodium selenite (S: Sigma Chemical, St Louis, MO) and 50 μg/ml kanamycin sulfate (Meiji Chemical Co., Tokyo, Japan), as previously described[16,17]. ATDC5 cells were maintained for 21 days under 5% CO_2 in air. The culture medium was replaced twice a week. Mouse embryonic C3H10T1/2 cells were plated at a density of 6.4×10^3 cells/cm^2 in 6-multiwell plates and grown to confluency in DME containing 10% FBS for 3 days. Mouse osteoblastic MC3T3-E1 cells were plated at a density of 6×10^4 cells/well in 6-multiwell plates and grown to confluency in alpha modified essential medium (αMEM, Dainippon Pharmaceutical, Tokyo) containing 5% FBS for 3 days.

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                                                                        †
Human    EVVRKIVPTT T-----KRP- ---HSGPRSN PGA-----GR LNNETRPSVQ
Bovine   ELVRKIVTTT TT----RRL- ---RSGPQGT PAP-----GR PNNGTRPSVQ
Rabbit   EVVRKTVPT- TT----KRP- ---HSGPRGN PGP-----AR MRNDSRPSVQ
Rat      EVVRSSAPS- TT----RRP- ---HSEPRGN AGP-----GR LSNSTRPSVQ
Mouse    EVVRNSAPS- TT----RRP- ---HSEPRGN AGP-----GR LSNGTRPNVQ
----------------------------------------------------------------
Chicken  EMKRNKRQSE SNFDAEHRAA AAEEVNTRST PTQLTQELGP QSNETRPMQQ

                                           ** *        *   *
Human    EDSQAFNPDN PYHQQEGESM TFDPRLDHEG ICCIECRRSY THCQKICEPL
Bovine   EDAEPFNPDN PYHQQEGESM TFDPRLDHEG ICCIECRRSY THCQKICEPL
Rabbit   EDSEPFNPDN PYHQ-EGESM TFDPRLDHEG ICCIECRRSY THCQKICEPL
Rat      DDEEPFNPDN PYHQQEGESM TFDPRLDHEG ICCIECRRSY THCQKICEPL
Mouse    DDAEPFNPDN PYHQQEGESM TFDPRLDHEG ICCIECRRSY THCQKICEPL
----------------------------------------------------------------
Chicken  ESDQTLNPDN PYNQLEGEGM AFDPMLDHLG VCCIECRRSY TQCQRICEPL

                     *   *     *
Human    GGYYPWPYNY QGCRSACRVI MPCSWWVARI LGMV 120 a.a.
Bovine   GGYHPWPYNY QGCRSACRVI MPCSWWVARI LGMV 121 a.a.
Rabbit   GGYNPWPYNY QGCRSACRVV MPCSWWVARI LGMV 119 a.a.
Rat      GGYYPWPYNY QGCRSACRVV MPCSWWVARI LGMV 120 a.a.
Mouse    GGYYPWPYNY QGCRSACRVV MPCSWWVARI LGMV 120 a.a.
----------------------------------------------------------------
Chicken  LGYYPWPYNY QGCRTACRII MPCSWWVARI MGVV 134 a.a.
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Fig. 1. Part II.

Fig. 1. Comparison of amino acid sequence of bovine, human, mouse and chicken ChM-I. Conserved amino acid residues are underlined. Transmembrane domains in the precursors are boxed. The potential processing signal (RERR) is indicated by bold type. Positions of Cys residues and the potential N-linked glycosylation in the mature ChM-I domain are denoted by (*) and (†), respectively.

RNA EXTRACTION AND HYBRIDIZATION ANALYSIS

Total RNA was prepared from the cultures of ATDC5 cells or tissues of 4-week DDY[18]. For northern blot analysis, 20 μg of the total RNA was denatured with 6% formaldehyde, fractioned by 1% agrose gel (SeaKem GTG, FMC Bioproducts, Rockland, ME) electrophoresis, and transferred onto Nytran membranes (Schleicher & Schuell, Dassel, Germany) with Turboblotter (Schleicher & Schuell). Hybridization was performed overnight at 42°C with α-[^{32}P]-dCTP labelled cDNA probe (10^6 cpm/ml) in a solution containing 50% formamide, 6×SSPE (0.9 M NaCl, 20 mM NaH_2PO_4, 6 mM EDTA at pH 7.4), 0.1% bovine serum albumin (BSA, Calbiochem-Novabiochem Co., La Jolla, CA), 0.1% Ficoll 400 (Pharmacia, Sweden), 0.1% polyvinylpyrolidone (Wako Pure Chemical), 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA. cDNA probes were labelled with α-[^{32}P]-dCTP (Amercham) by random-primer method using a BcaBEST labelling kit (Takara, Otu, Japan) using the appropriate cDNA fragments; a 1.4 kb EcoRI fragment of pKT1180 as a probe for α1(II)collagen cDNA[19]; a 0.65 kb Hind III fragment of pSAm10h as a probe for α1(X) collagen cDNA[20]; a 2.5 kb EcoRI fragment of rAP54 as a probe for alkaline phosphatase[21]; a 2.2 kb EcoRI fragment of pcDNA1R15B for probing parathyroid hormone (PTH)/PTH related peptide receptor (PTH/PTHrPR) cDNA[22]. After hybridization, filters were washed for 30 min at 55°C in 2×SSPE, 0.1% SDS, then washed for

30 min at 55°C in 0.1×SSPE, 0.1% SDS twice, and exposed to BIOMAX film (Eastman Kodak Co., Rochester, NY) at −80°C.

COMPETITIVE POLYMERASE CHAIN REACTION (PCR)

First strand cDNA was synthesized using SuperScript II RNAse H$^-$ Reverse Transcriptase (Gibco, Grand Island, NY, U.S.A.) with the corrected amount of total RNA isolated from human tissues or culture cells according to the manufactuer's instructions. Portions of total RNA were reverse-transcribed. For the competitive PCR analysis of ChM-I mRNA, the composite primers (5′-CATCGGGGCCTTC TACTTCTCAAGTTTCGTGAGCTGATTG-3′ and 5′-GGCA TGATCTTGCCTTCCAGTTGAGTCCATGGGGAGCTTT-3′) were synthesized using a PCR MIMIC Construction kit (Clontech, CA, U.S.A.). One-fiftieth of the product was re-amplified by using the gene-specific primers (5′-CATCG GGGCCTTCTACTTCT-3′ and 5′-GGCATGATCTTGCCTT CCAG-3′). Following the second PCR amplification, the competitive template was purified by Chroma SPIN+ TE-100 and diluted to 100 attomol/μl for competitive experiments. A fixed amount of cDNA was co-amplified with serial logarithmic dilutions of the competitive template (from 1 to 10^{-6} attomol/μl). The expected sizes of the products were 312 bp and 479 bp for ChM-I and the competitive fragment, respectively. The amplification reaction was carried out with initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec, extension at 72°C for 90 sec and a final extension at 72°C for 4 min. The PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide and photographed. The fluorescence intensity of each PCR product was estimated on NIH image 1.60, as previously described[23].

IN SITU HYBRIDIZATION

Balb/c mouse embryos at days 13.5 and 16 of gestation were collected, and fixed in 4% paraformaldehyde in 0.01 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl (phosphate buffered saline, PBS) overnight at 4°C. Whole embryos were dehydrated in a graded series of ethanol and embedded in paraffin. Sections were cut at 6 μm and were then processed for *in situ* hybridization. For *in situ* hybridization, a 0.5 kb cDNA fragment containing the coding region for mature ChM-I was used as a hybridization probe. A digoxigenin-labelled antisense cRNA probe for mouse ChM-I was prepared with a DIG RNA labelling kit (Boehringer Mannheim, Gaithersburg, MD). Hybridization was performed at 50°C for 16 h. After hybridization, slides were washed under conditions of high stringency. For digoxigenin-labelled probes hybridization was immunohistochemically detected by an alkaline phosphatase-conjugated antibody using a nucleic acid detection kit (Boehringer Mannheim). Appropriate controls to exclude false positive staining due to endogenous alkaline phosphatase activity were performed and were negative. The sections were counterstained by methyl green or haematoxylin.

ECTOPIC BONE FORMATION

Guanidine extract (G-ext) of demineralized bone matrix used in this study was prepared from bovine dehydrated diaphyseal bone powder by the method of Sampath and Reddi[24], and was a generous gift from Dr Y. Kuboki (Hokkaido University Faculty of Dentistry). G-ext (15 mg) was suspended in water (2 ml) in a conical tube and centrifuged at 1500×***g*** for 15 min. The supernatant was discarded, and then mixed vigorously with 200 μl of the hydrated beads of heparin-Sepharose CL-6B (10 mg/ml, Pharmacia) or heparin-Sepharose CL-6B that had previously bound purified ChM-I (2 μg). The suspension was pelleted at 1500×***g*** for 20 min and lyophilized. These lyophilized pellets were implanted onto the fascia of the back muscle at bilateral sites of the male 6-week old nude mice (BALB/c:*nu/nu*, Clea, Osaka, Japan). Nine days or 3 weeks later, anaesthetized mice were photographed by soft X-ray apparatus (Softex, type C-SM) for assessment of bone formation. Then the implants were dissected out, cleaned of adherent tissue, and fixed in neutral formalin. The fixed implants were decalcified, dehydrated and embedded in paraffin. Sections (6 μm thick) were processed for histological examinations.

XENOGRAFT TUMOUR MODEL

Cultured OUMS-27 cells (5×10^6 cells) in 0.1 ml PBS were inoculated subcutaneously in the back of 4-week-old Balb/c nu/nu mice. When tumours developed at the injection sites to become about 45 mm^3 in volume (6 days after inoculation), mice were randomized into two groups. One group received 5 μg rhChM-I in 50 μl PBS, injected subcutaneously around the tumour daily for the initial 5 days. The other group received PBS alone. Tumour volumes were determined as width^2×length×0.52[25]. At the last time point, tumours were excised and fixed for histological examinations.

Results

COMPARISON OF AMINO ACID SEQUENCES OF MATURE CHM-I AMONG SPECIES

Mature ChM-I was coded as the C-terminal portion of a larger precursor (334–335 amino acid residues in mammals and 347 amino acid residues in chicken), which is assumed to be a transmembrane protein[10,12,13,26,27]. The membrane spanning domain was well conserved between species. The mature ChM-I sequence was preceded by a processing signal (RERR), which was completely conserved between species (Fig. 1). Most amino acid substitutions were found in the *N*-terminal hydrophylic domain of the mature ChM-I, but an *N*-glycosylation site (NXT) is completely conserved (Fig. 1). The C-terminal two-thirds of mature ChM-I, which contained all eight cysteine residues, were also well conserved, suggesting a functional importance of this hydrophobic domain.

TISSUE DISTRIBUTION OF CHM-I GENE EXPRESSION

We examined the ChM-I gene expression in various tissues of 4-week-old mice by northern blot analysis (Fig. 2). As previously reported[10,12], ChM-I gene was abundantly expressed in epiphyseal cartilage and primary growth plate chondrocytes (Fig. 2). Although the type II collagen gene was expressed in various tissues during embryonic development[28], its expression was restricted to cartilage in 4-week-old mice. In contrast, expression of

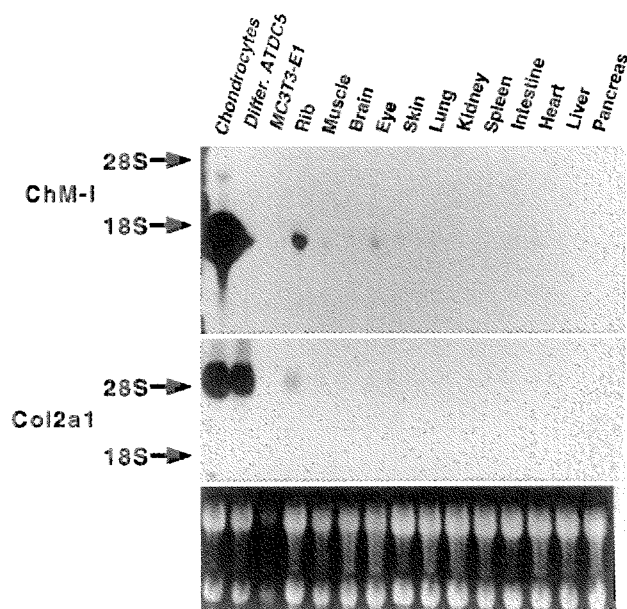

Fig. 2. Northern blot analysis of ChM-I mRNA in various tissues of 4-week-old mice. Total RNA (20 μg) isolated from the indicated tissues of DDY mice, and hybridized with mouse ChM-I cDNA or rat type II collagen cDNA. The equivalent loading of each RNA was verified with ethidium bromide staining (bottom panel). The positions of 28S and 18S ribosomal RNAs are indicated.

ChM-I gene was also detected in eye, an organ avascular tissues (Fig. 2).

EXPRESSION OF CHM-I GENE DURING ENDOCHONDRAL BONE FORMATION

In situ hybridization clearly indicated that the expression of ChM-I mRNA was associated with cartilage and that the increase in the ChM-I transcripts was associated with expansion of cartilaginous areas in the body (Fig. 3A). Positive signals were also found in the cartilaginous rudiment of the occipital bone, but more likely to be minimal or absent in sclerotome prior to overt chondrogenesis at day 11 of gestation in mouse embryo (data not shown). During overt chondrogenesis expression of the ChM-I gene was found in all cartilaginous structures such as the vertebral column, the chondrocranium, tracheal rings and ribs. In the developing cartilaginous bone rudiments, positive signals were detected in proliferating and mature cartilage zones, but absent in the notochord at day 13.5 of gestation (Fig. 3B). A low level of hybridization signals was detected in immature osteoblasts in the transitional zone between cartilage and membranous bone at this stage (Fig. 4C). At day 16 of gestation, chondrocytes in the centre of vertebral body begin to be hypertrophic and then vascular invasion and ossification begins to develop within a day. At this stage of development, a high level of ChM-I gene expression was detected from the proliferative to the early hypertrophic zone of cartilage (Figs 4A, B). However, the ChM-I mRNA expression was completely abolished in the late hypertrophic and calcified zone, in which vascular invasion occurs (Fig. 4A,B).

As reported previously[16,17,29], the mouse embryonal carcinoma-derived cell line ATDC5 provides an excellent *in vitro* model for analysis of the expression pattern of genes related to skeletogenesis. We examined the expression pattern of the ChM-I gene during chondrogenesis of ATDC5 cells, compared with the expression of type II collagen, PTH/PTHrP receptor, type X collagen and ALP genes. Transcripts for the ChM-I gene were undetectable in undifferentiated ATDC5 cells, C3H10T1/2 cells and MC3T3-E1 cells (Fig. 5). However, following the induction of type II collagen and PTH/PTHrP receptor genes, the expression of ChM-I gene was induced during chondrogenic differentiation of cells (Fig. 5). The ChM-I mRNA level reached a maximum in ATDC5 cells on day 15 (Fig. 5), in parallel with formation of cartilage nodules in culture. As differentiated ATDC5 cells became hypertrophic as evidenced by the increase in type X collagen and ALP mRNA levels, the ChM-I mRNA expression declined (Fig. 5). *In situ* hybridization analysis revealed that ChM-I transcripts were present in differentiated cells of cartilage nodules, but absent in undifferentiated cells surrounding nodules[12]. Thus, the expression of ChM-I gene was clearly associated with chondrogenic differentiation and maturation of ATDC5 cells.

BLOCKAGE OF AN ENDOCHONDRAL PATHWAY OF BONE FORMATION BY CHM-I

When bone morphogenetic protein (BMP) is implanted subcutaneously, ectopic bone is formed through an endochondral pathway of bone formation. BMP first induces chondrogenesis at a local site to form ectopic cartilage[30]. Upon vascular invasion, cartilage is then resorbed by chondroclasts supplied by haematopoietic precursor cells. Therefore, by using ectopic bone formation as a model, we studied the effect of ChM-I on enodochondral bone formation using a crude BMP preparation, i.e. a guanidine extract (G-ext) of demineralized bone matrix.

Since ChM-I rapidly diffuses out of the pellet, we used heparin-Sepharose beads as a carrier. Three nude mice were employed in the following implantation experiment. Heparin-Sepharose beads alone did not interfere with ectopic bone formation when they were premixed with G-ext (15 mg) and pelleted. By 3 weeks after implantation of G-ext onto the fasciae of back muscle of 6-week old nude mice, formation of osteoid and trabecular bone was histologically confirmed. Bone marrow was well developed in the centre of implants. Alcian blue-positive cartilaginous tissue occupied less than 5% of total area of the section. On the other side of the animals, we implanted the G-ext (15 mg) that had been mixed with the ChM-I (2 μg)-bound heparin-Sepharose beads. After 3 weeks of implantation, the bony parts of the implants covered only about 30% of total area of section (Fig. 6B). Cartilage persisted around the ChM-I containing beads (Fig. 6B). These results indicate that ChM-I, when exogenously added to demineralized bone matrix, inhibited an transition from cartilage to bone along the endochondral pathway of bone formation.

LOSS OF CHM-I EXPRESSION IN CHONDROSARCOMA

We evaluated the ChM-I mRNA level in chondrosarcomas by competitive PCR, and compared it with that of benign cartilaginous tumours and normal articular cartilage[14]. Each clinical specimen was diagnosed pathologically and processed separately for mRNA extraction. Tumour grades were assigned on the basis of Evans's report[31]. In all cartilaginous tumour samples and articular cartilage used here, expression of α1(II) collagen and

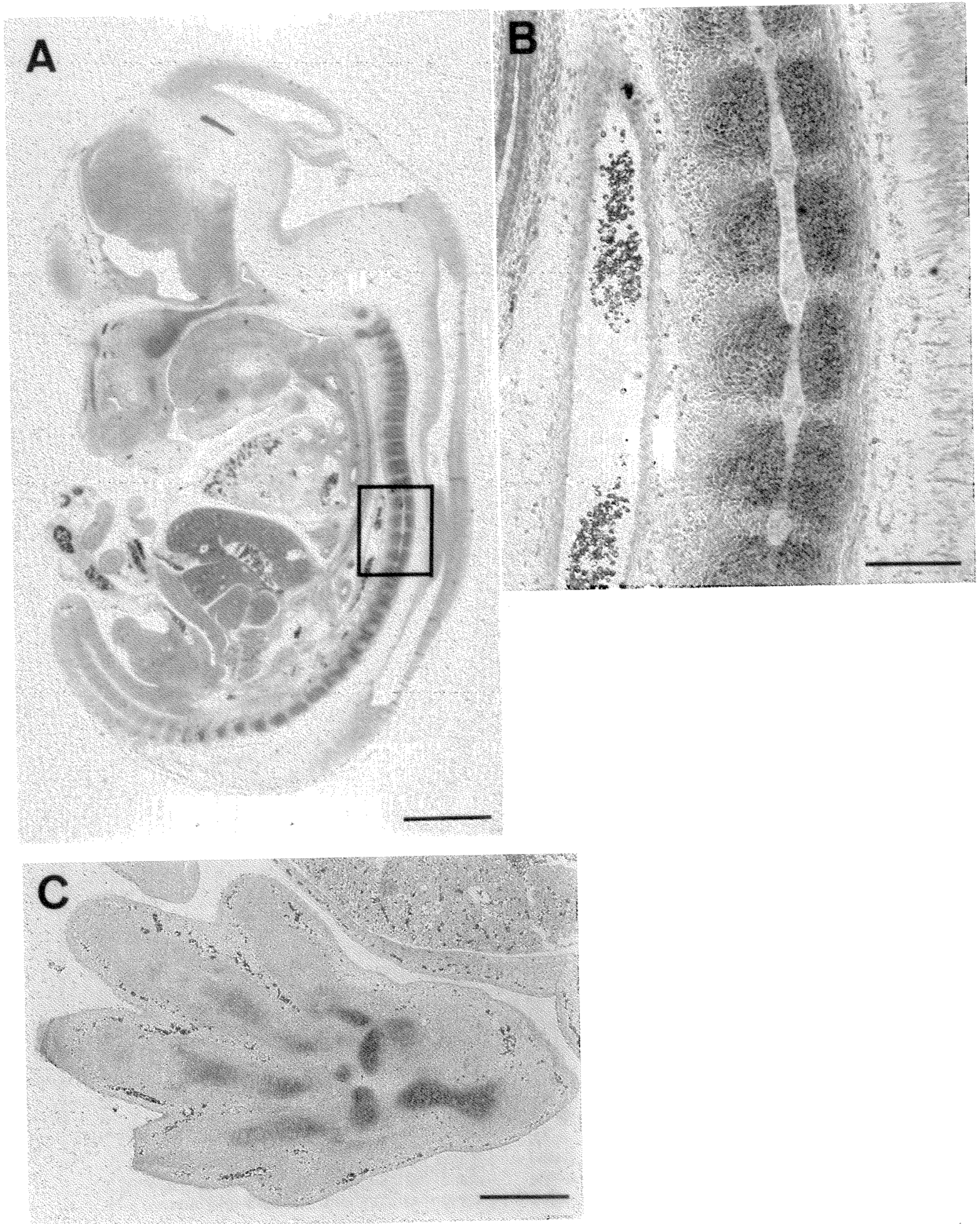

Fig. 3. *In situ* hybridization for ChM-I transcripts in mouse embryos at day 13.5 of gestation. In (A), the sagital section of the mouse embryo at day 13.5 was hybridized with the antisense ChM-I cRNA probe. There were obvious hybridization signals in the cartilaginous bone rudiments. The framed area is shown at a higher magnification in (B). In (C), the section of hindlimb also shows the positive signals in cartilage. The sections were counter-stained with methyl green or haematoxylin. Bars, 1000 μm in (A), 150 μm in (B), and 400 μm in (C).

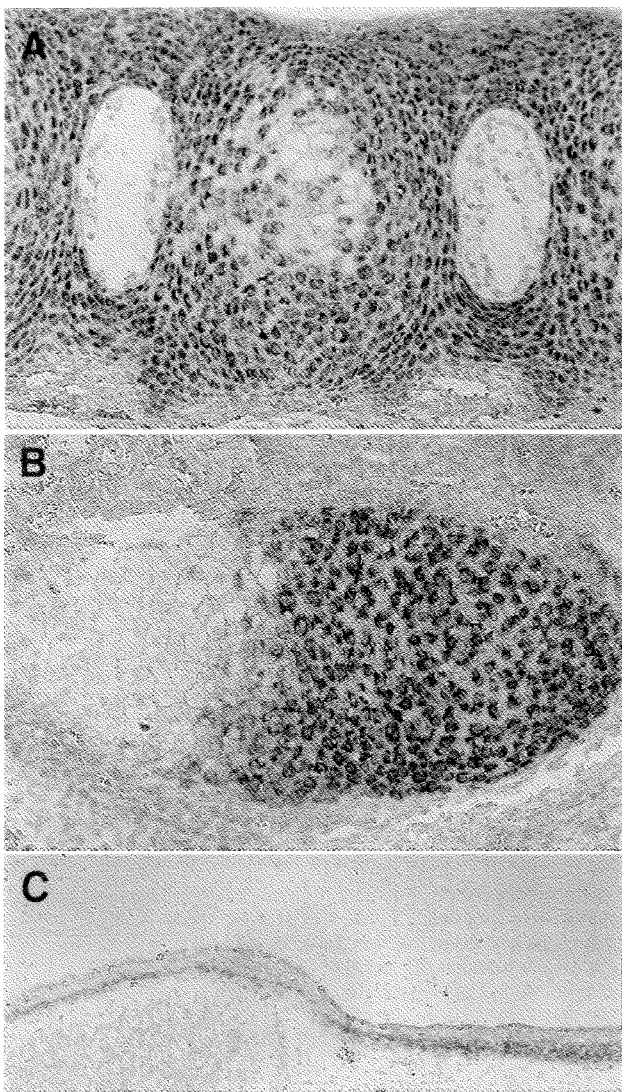

Fig. 4. *In situ* hybridization for ChM-I transcripts in mouse embryonic tissues. In (A), vertebral columns at day 16 of gestation were hybridized with the antisense probe. The expression of the ChM-I transcripts declined in the late hypertrophic chondrocytes at the centre of vertebral columns. In (B), Meckel's cartilage at day 16 of gestation is shown. Positive hybridization signals were detected in the prehypertrophic and early hypertrophic zones, but were completely abolished in the late hypertrophic zone. In (C), in addition to the chondrocranium, immature osteoblasts in the dermocranium were also hybridized with the probe at day 13.5 of gestation. The sections were counter-stained with methyl green or haematoxylin. Bars, 100 μm in (A) and (B); 200 μm in (E).

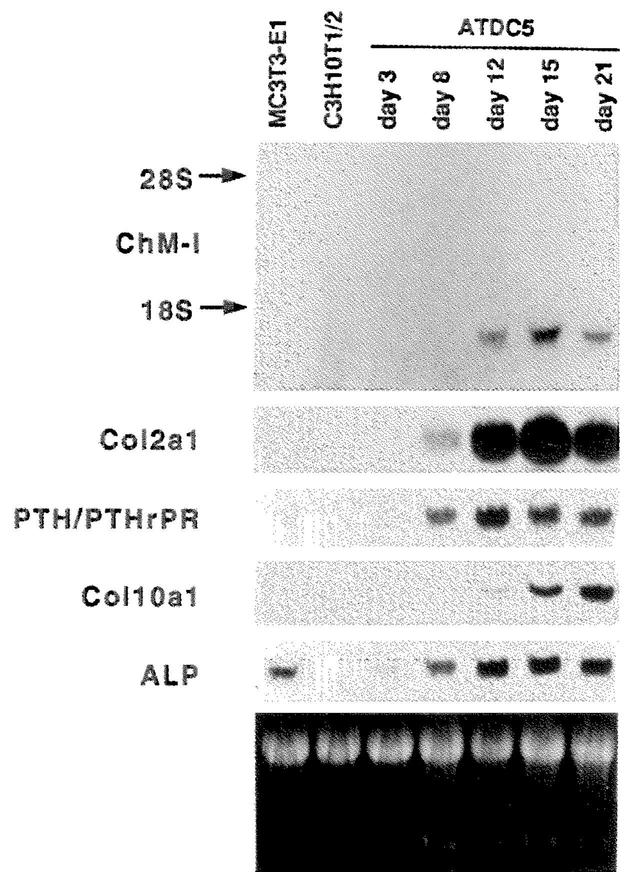

Fig. 5. Expression pattern of ChM-I mRNA along the course of differentiation of mouse chondrogenic cell line ATDC5. Total RNA (20 μg) was isolated from MC3T3-E1 osteoblastic cells, C3H10T1/2 cells, or ATDC5 cells on the indicated day of culture, and hybridized with cDNA probes for mouse ChM-I (ChM-I), rat type II collagen (Col2a1), rat parathyroid hormone (PTH)/PTH related peptide receptor (PTH/PTHrPR), mouse type X collagen (Col10a1), and rat alkaline phosphatase (ALP).

aggrecan mRNAs was readily detected (data not shown). Moreover, ChM-I mRNA was also detected in enchondroma, exostosis, and articular cartilage. However, the mRNA level of ChM-I gene was extremely low in grade I and II chondrosarcomas, and undetectable in grade III chondrosarcoma. Competitive PCR indicated that the ChM-I mRNA level was from 1/100 to 1/3500 less than that of enchondroma, exostosis, or articular cartilage (Fig. 7). Thus, the ChM-I gene expression was downregulated in chondrosarcomas although cartilage markers were highly expressed.

SUPPRESSION OF TUMOUR ANGIOGENESIS BY RECOMBINANT CHM-I PROTEIN

Taking advantage of human chondrosarcoma cell line OUMS-27 (15), we tested the effect of rhChM-I on tumour angiogenesis *in vivo*. OUMS-27 cells were derived from grade III chondrosarcoma tissue from a patient and develops grade II chondrosarcoma, when subcutaneously inoculated onto nude mice[15]. Here we inoculated 5×10^6 OUMS-27 cells in the back of nude mouse. When cells formed a tumour about 45 mm^3 in size, mice received 5 μg rhChM-I dissolved in PBS at the site of the tumour for the initial 5 days. The tumour in the control mice was considerably invaded by capillaries (Fig. 8A), whereas in the treated mice vascular invasion was visibly blocked (Fig. 8B). The increase of tumour size was significantly suppressed by ChM-I treatment (Fig. 8C). The wet weight of the ChM-I treated tumour was about 30% of that in the control mice, when tumour tissues were recovered on day 29[14].

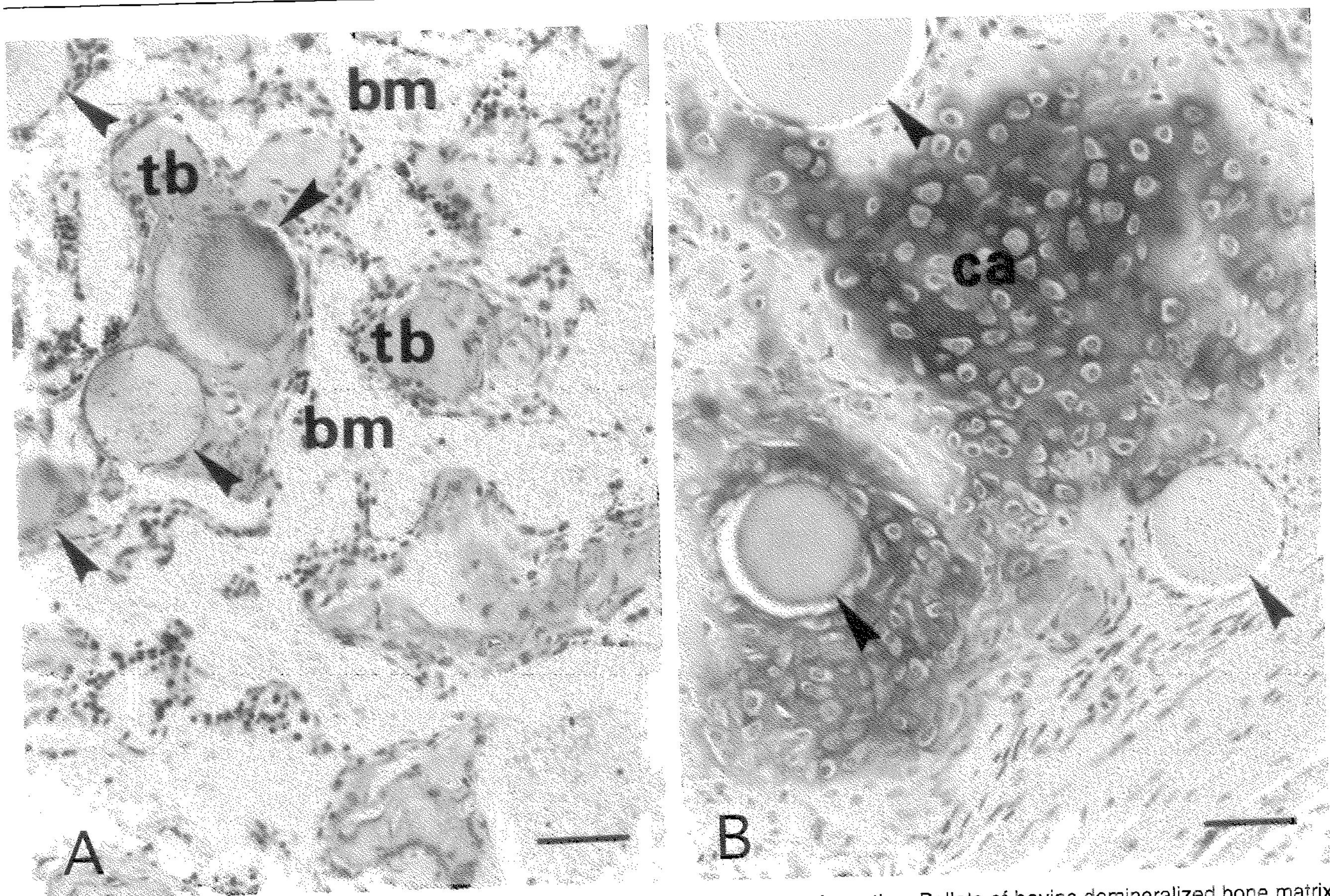

Fig. 6. Effect of ChM-I on the replacement of cartilage by bone during ectopic bone formation. Pellets of bovine demineralized bone matrix (DBM) were implanted into the fascia of the back muscle of nude mice. Implants were recovered 3 weeks after implantation. Sections of the implants were stained with Alcian blue and Kernechtrot. In (A), trabecular bone (tb) and bone marrow (bm) developed in the pellet of DBM (15 mg) mixed with heparing-Sepharose beads alone. In (B), typical cartilage (ca) induced in the pellet of DBM (15 mg) were still remained 3 weeks after implantation, when the pellets had been mixed with bovine purified ChM-I (2 μg)-bound heparin-Sepharose beads. Arrowheads indicate heparin-Sepharose beads in the implanted DBM beads. Bars, 50 μm.

Discussion

As one can see in tumour angiogenesis[2], the total balance of angiogenic inducers and inhibitors controls whether or not a certain tissue is vascularized. Hyaline cartilage is a typical avascular tissue and remarkably resistant to vascular invasion[1]. However, during endochondral bone formation in the embryo, cartilage undergoes phenotypic switching from anti-angiogenic to angiogenic as cells undergo late-phase differentiation of cartilage to become hypertrophic and calcifying cells (Fig. 9). In cartilaginous rudiments, vascular invasion is a pivotal event which triggers the replacement of cartilage by bone.

Chondrocytes generally express FGF-2 as a powerful angiogenic factor[6]. In the resting and proliferating zones of cartilage, FGF-2 was confined to cell surface or pericellular space with scarcely any in inter-territorial space. As chondrocytes became hypertrophic and calcified, FGF-2 diffuses into the interterritorial space and became amenable to interactions with capillaries[6,9]. Moreover, another angiogenic factor VEGF is also expressed in association with cellular hypertrophy of chondrocytes[5,7]. These results suggest that the expression and localization of angiogenic inducers in cartilage underlies switching of cartilage phenotype from anti-angiogenic to angiogenic. In fact, when VEGF-A gene was conditionally inactivated in mice at the type II collagen-expressing sites, the hypertrophic cartilage zone evidently expanded in the growth plates[32]. Systemic administration of a soluble VEGF receptor-1 (Flt-(1-3)-IgG) chimaeric protein exhibited similar phenotype in mice due to a delay of vascular invasion[7].

As shown in Figs 2 and 5, ChM-I mRNA was specifically expressed in chondrocytes, but not in osteoblasts. Induction of the gene expression apparently associated with formation of type II collagen-expressing chondrocytes and declined as the cells became type X collagen-expressing hypertrophic chondrocytes[12]. In developing cartilaginous rudiments, expression of ChM-I gene is restricted to the avascular zone of cartilage including resting proliferating and prehypertrophic cells (Figs 3 and 4). Its expression was abolished in the late hypertrophic and calcified zone of cartilage just prior to vascularization (Fig. 4)[12,13]. Mature ChM-I protein was deposited in the inter-territorial space of cartilage matrix[9]. Thus, the negative side of angiogenic balance switch also dramatically changed during vascular invasion into cartilaginous bone rudiments (Fig. 9). Accordingly, when exogenous ChM-I protein was added, vascular invasion was apparently inhibited or delayed (Fig. 6)[12]. In contrast, malignant cartilaginous tumour readily accept vascular invasion, with which chondrosarcomas rapidly grow and metastasize. Loss of ChM-I expression may account for the angiogenic phenotype of

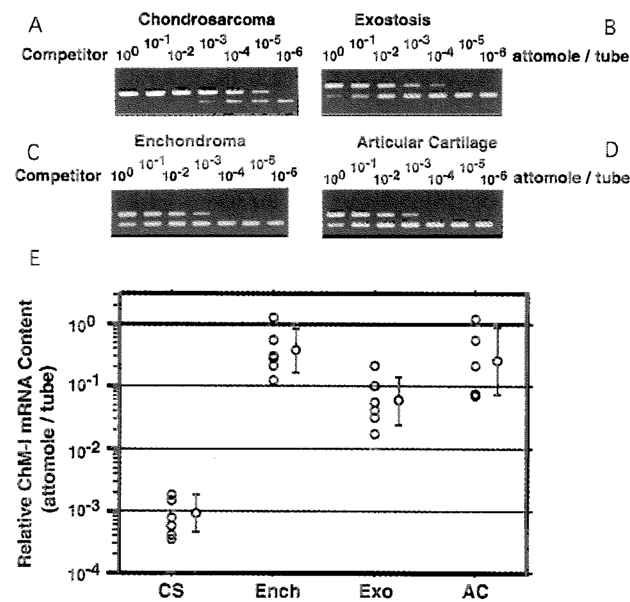

Fig. 7. ChM-I gene expression lost in human chondrosarcoma. Total RNA was isolated from human grade I chondrosarcoma (CS), enchondroma (Ench), exostosis (Exo), and articular cartilage (AC). The relative level of ChM-I mRNA was estimated by competitive PCR using cDNA from grade I CS and other cartilaginous tissues. Each reverse-transcribed cDNA was co-amplified by gene specific primers in the presence of a 10-fold dilution series of competitive template (upper panels). Estimated ChM-I mRNA levels were plotted in a logarithmic scale with means±S.D. (n=6 for grade I CS, n=6 each for Ench and Exo and n=5 for AC) in the lower panel.

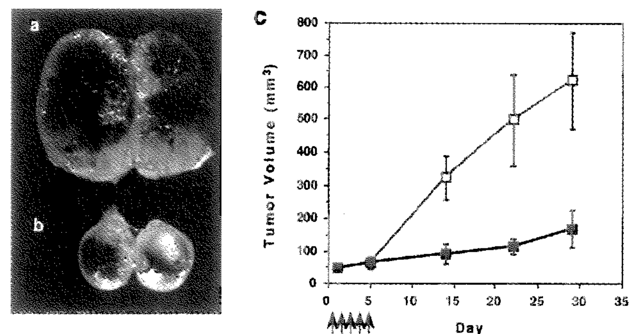

Fig. 8. Effect of rhChM-I on OUMS-27 human chondrosarcoma xenografts in nude mice. OUMS-27 cells (5×10^6 cells) were inoculated subcutaneously in the back of 4-week-old nude mice. Gross appearance of tumours excised on day 29 and cross-sections of the representative tumour treated with PBS alone (a) or with rhChM-I (b) are shown. In (c), when the tumour volume reached about 45 mm^3, each mouse was injected with 50 μl PBS alone (□) or 50 μl PBS containing 5 μg rhChM-I (■) around the tumour for the initial 5 days, as indicated by arrows. Values represents means±S.D. (n=5).

chondrosarcoma (Fig. 7)[14]. Treatment with ChM-I protein reverted the angiogenic phenotype of chondrosarcoma to be anti-angiogenic, which interfered with the growth of tumour (Fig. 8).

In addition to the gene expression of angiogenesis inducers and inhibitors, remodelling of cartilage matrix is undoubtedly important for the angiogenic switch to shift from anti-angiogenic to angiogenic. For example, VEGF synthesized in hypertrophic cartilage has to be activated[5]. Moreover, it is believed that ChM-I synthesized in chondro-

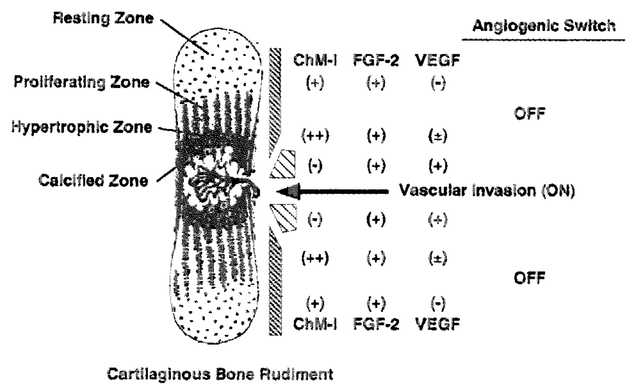

Fig. 9. Angiogenic switching of cartilage phenotype prior to vascular invasion into calcified cartilage during endochondral bone formation. Angiogenic FGF-2 are widely express in cartilage, periosteum, bony collar and surrounding soft tissue. VEGF expression is augmented as chondrocytes matures to become hypertrophic. Tissue-specific expression of ChM-I confers resistance of hyaline cartilage to vascular invasion. Accumulation of ChM-I protein in the inter-territorial matrix of cartilage serves as a barrier to endothelial cell proliferation. Upon the loss of ChM-I gene expression and degradation of mature ChM-I protein, calcified cartilage accepts the ingrowth of capillaries.

cytes is integrated in cartilage matrix by interaction with some anchoring matrix components[9]. ChM-I protein was clearly degraded in matrix at the late-hypertrophic and calcified cartilage zone[9]. Therefore, matrix remodelling has to be critically involved in the release of anti-angiogenic and angiogenic molecules from the extracellular matrix. Matrix metalloproteinases may play a role in this process. Vu and coworkers reported that inactivation of MMP-9 gene in mice resulted in delay of vascular invasion into growth plates[33]. In this context, it is important to study how MMPs interact with and metabolize ChM-I in cartilage matrix for elucidation of molecular machinery of angiogenic switching.

In developing limb buds, *de novo* formation and regression of blood vessels occur in close vicinity and simultaneously during formation of the cartilaginous bone rudiment[34]. The vascular system is developed by sprouting angiogenesis from the aorta in association with limb bud outgrowth. We reported that the expression of ChM-I mRNA was first recognized in the avascular peripheral zone of mesoderm just beneath the apical ectodermal ridge in chicken limb buds[27]. Later the ChM-I gene is expressed in the area of mesenchymal condensation where vascular regression occurs prior to overt chondrogenesis[27,35]. These results suggest that ChM-I may participate in the control of vascularity also in the extra-cartilaginous tissues.

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