Journal of Bone and Mineral Metabolism

International Symposium on Cartilage Metabolism

Senri Life Science Center, Toyonaka, Osaka, Japan

November 16–18, 1994

Chair/Fujio Suzuki, Co-Chair/Barbara D. Boyan

Sponsored by Osaka University

Abstracts

Opening Remarks	32
Opening Lecture	32
Plenary Lecture	33
Structure and Function of Cartilage ECM	34
Normal and Pathologic Metabolism of Cartilage	36
Endochondral Bone Formation and Mineralization	44
Regulatory Mechanism of Expression of Cartilage-Phenotype	46

Opening Remarks

Distinguished guests, ladies and gentlemen, Good morning. It is a privilege to welcome you to Osaka for the International Symposium on Cartilage Metabolism 1994. In the last decade or so, there has been a tremendous expansion of knowledge in the various aspects of cartilage research. For example, our knowledge of chondrocyte differentiation, synthesis of extracellular matrices, the structure and function of the epiphyseal growth plate and of articular cartilage has enormously increased. Research in molecular biology on cartilage growth and differentiation has also extensively advanced. These advances will lead to advances in the treatment and prevention of disease processes in which cartilage may be involved. The purpose of this symposium is to bring together cell and molecular biologists, structural chemists, morphologists, and clinical investigators who are concerned about the cartilage metabolism. This symposium will be held in the following topics, 1) Structure and function of cartilage extracellular matrix, 2) Normal and pathologic metabolism of cartilage, 3) Endochondral bone formation and mineralization, 4) Regulatory mechanism of expression of cartilage-phenotype. Therefore, Dr. Barbara Boyan and I hope that our honorable guests coming from abroad and from Japan will update themselves on recent advances in the area of cartilage research.

Furthermore, since this ISCM is the first of its kind, I would like to propose that ISCM will be held consecutively every other year (or every year) and perhaps the next ISCM will be held openly to the public, not semi-closed as this time, somewhere in 1996 or so. I very much hope that this ISCM'94 marks only the beginning of a long and flourishing existence.

This symposium is dedicated to the late Professor Emeritus Shiro Akabori, a great Japanese scientist and the 7th President of Osaka University, for his valuable contributions to life science.

Finally, Dr. Barbara Boyan and I express our gratitude towards Osaka University for the financial help. We also acknowledge with gratitude the invaluable help of the Center for Academic Societies, Osaka and many Japanese pharmaceutical industries. I hope that all of you will enjoy this symposium and happy stay here. Thank you..

Opening Lecture

A HISTORICAL REVIEW OF CARTILAGE RESEARCH IN JAPAN

Fujio Suzuki

Department of Biochemistry, Faculty of Dentistry, Osaka University, Suita, Osaka 565, Japan

The Japanese took over Chinese medicine together with Chinese culture in the 9th century. Tanba, Yasuyori published the first publication in medicine in 982. In the 18th volume of this series, he described bone and articular diseases. Under Dutch influence the Japanese made great progress in surgery in the 17th century. Hanaoka, Seishu (1760-1835) succeeded in preparing an anesthetic substance from herbs. It was made up six mixtures of the extracts of roots, seeds and stalks of plants. He tried it on a woman suffering from breast cancer and removed it by the aid of Dutch style surgical instruments in 1805. His method was tried 37 years before the first use of ether as general anesthesia by Long, C. W. in 1842. We can see in Hanaoka's achievements, fusion of oriental and occidental medicine and also originality and his pioneering spirit.

Ogata, Koan (1810-63) studied Dutch medicine in Osaka, Edo and Nagasaki. After returning home, he opened a private academy "Tekijuku" in Osaka in 1838. Young students inspired by Ogata's high personality and career, gathered to his academy from many areas of the country. From 1838 to 1862, more than a thousand students entered Tekijuku and many graduates made great contribution not only in the field of medicine but also in several other areas in the periods before and after Meiji Restoration. Tekijuku is the forerunner of Osaka University Medical School.

Soon after the Meiji Restoration in 1868, some governmental colleges were established for conducting higher education of western science and technology. As the teaching staff of these colleges a number of scientists were invited from Western countries. In the final stage of the Edo Era, around the middle of the 1860s, the influential power of foreign countries over Japan shifted from the Netherlands to England and France. When the influence of Germany became prominent, many of the leaders of Japanese medicine were trained in Germany.

Among the young scientists who studied in one of those colleges, there was Masamune, Hajime (1896-1959) who was particularly interested in the problem of glycoproteins from cartilage. Masamune graduated from Kyushu Univ. in 1921, but went to Harvard Univ. in 1929 and then to Univ. of Leipzig in 1930 to acquire a fresh knowledge of biochemistry. After coming back to Kyushu in 1931 he started to work on the metabolism of proteoglycans in mammals and soon succeeded in characterizing animal glucuronosidase, glucuronidase, glucosaminase, and acetylglucosaminidase (1934-36), which were really pioneering studies. Masamune promoted to the Professor of Hokkaido Univ. in 1934 and then Tohoku Univ. in 1942. In 1943, a pure form

of chondroitin sulfate was first prepared from nasal cartilage of whale, using Masamune-Ozaki's formalin method. They estimated its structure as (acetylglucosamine-glucuronide sulfate). Levene had asserted that four carbohydrates are conjugated in chondroitin sulfate, but his idea was clearly denied by Masamune. Furthermore, he proposed the general concept of the structure of so-called glycoprotein and predicted the presence of N-glycosidic linkage in some proteoglycans.

Soda, Tokuro and Egami, Fujio (Univ. of Tokyo) reported that squid and horseshoe crab cartilages contain over-sulfated chondroitin sulfates, while chondroitin-6-sulfate isolated from sturgeon cartilage, possesses only 0.87 sulfate residues per repeating unit. Suzuki, Sakaru (Nagoya Univ.) showed that the over-sulfated glycosaminoglycan of shark cartilage contains most of the ester sulfate at position C-6 of acetylglucosamine residues with the remainder substituted at C-2 or C-3 of the glucuronyl units. Murata, Katsumi (Tokyo Univ. of Fine Arts) reported that similar over-sulfated polysaccharide was isolated from human aortic tissue. Suzuki's group also isolated chondroitinase-ABC and chondroitinase-AC from microorganisms, which were very useful tool in the identification of trace amounts of chondroitin 4- and 6-sulfates in mixtures with other glycosaminoglycans. Testicular hyaluronidase was detected by Yoshizawa, Zensaku (Tohoku Univ.). Seno, Nobuko (Ochanomizu Women's Univ.) reported that chondroitin 4-sulfate, chondroitin 6-sulfate, keratan sulfate, and hyaluronic acid were detected in cartilaginous tissue and that sulfate to disaccharide ratios of keratan sulfate from whale cartilage varies from 0.75-1.8. She also found that keratan sulfate contains some minor carbohydrates such as fucose and sialic acid. In whale cartilage sialic acid has been identified as N-acetylneuraminic acid. She found that glycosaminoglycan content tends to be lower in articular cartilage than in most other cartilages.

Concerning biosynthetic pathways of proteoglycans, Suzuki and Strominger found that sulfate is first activated and transferred to the acceptor and that the activated PAPS is formed from inorganic sulfate in a two-step process. Suzuki's group then isolated from squid cartilage a sulfotransferase exhibiting the unique property of catalyzing the introduction of sulfate into position C-6 of a N-acetylglucosamine moiety already bearing a sulfate residue at position C-4. Kimata, Koji (Aichi Medical Univ.) isolated nascent chondroitin sulfate attached to the Golgi membranes of chondrocytes. Kimata's group recently isolated cDNA clones encoding the core protein of proteoglycan-Lb (PG-Lb), which has been shown to be preferentially expressed in the zone of ossifying area of the developing chick embryo cartilage. The homology analysis revealed that chick PG-Lb showed a higher homology to human osteoglycin, formerly called osteoinductive factor, OIF. Kimata's group then identified cDNA clones of proteoglycan-M (PG-M), a large chondroitin sulfate proteoglycan expressed in the pre-chondrogenic condensation area of the developing chick limb buds. The sequence analysis showed that PG-M

showed a high homology to corresponding domains of a human fibroblast large chondroitin sulfate proteoglycan, versican. Therefore, they suggested that versican might be one of the multiple forms of PG-M.

Kimata's group also found the absence of proteoglycan core protein in cartilage from cartilage matrix deficiency mouse (cmd). Sugahara, Kazuyuki (Kobe Pharmaceutical Univ.) detected the defect in PAPS formation in brachymorphic mice. They represent examples of mutations that affect cartilage-specific macromolecules at two levels of the synthesis of proteoglycans, that is, sulfation (brachymorphism) and core protein (cartilage deficiency).

As chondroitin sulfate or hyaluronic acid gradually found a larger market, these commercial preparation became popular materials for the study of glyco-conjugated compounds and various related enzymes in biochemical laboratories. In parallel with these fundamental studies, there has been much work in Seikagaku Kogyo Co. and other industries, using bovine and whale cartilage.

Ogata, Tomosaburo (Univ. of Tokyo) proposed a salivary gland endocrine theory in 1935. His associates observed that the active principle of extracts of the salivary glands of young beef cattle provided a hormone-like substance that they called "Parotin". This substance lowered the serum calcium levels in rabbits by approximately 15 %. Enhancement of the calcification of teeth and bones was another important effect resulting from its administration. Ito, Yosoji then isolated active principles in the form of homogeneous proteins having biological activities similar to those of parotin from bovine, porcine, and equine submaxillary glands, from human saliva, and also from human urine. Parotin generally acts on the mesenchymal tissues, especially the hard and connective tissues, to promote their development and growth.

The primary diseases resulting from parotin deficiency in humans are shown to include chondrodysplasia fetalis, Kashin-Beck's disease (endemic in Northeastern China), the endemic diseases occurring in the volcanic districts in Japan, arthritis deformans, and spondylitis deformans. Parotin was confirmed to be effective in compensatory therapy for the diseases. Endo, Hiroyoshi and Ito, Y., using chick embryo femora cultures, demonstrated the effectiveness of parotin in promoting longitudinal growth and calcium deposition of bone, and suggested that parotin might directly stimulate some hard tissue development sites in vivo. Since then, Endo's group performed extensive studies on cartilage metabolism using tissue and cell cultures. However, we regret extremely that the structure of the so-called parotin has not been characterized yet.

Hiyeda, Kentaro (Kurume Univ.) found that the administration of the extract from refrigerated 16-day chicken embryos into partially resected sites of rabbit or rat long bones stimulates the regeneration of bone and he named this active principle "Osteogemin" (1955). It would have been some premature form of BMP or FGF-like substance, when we think of it now.

The mechanism of endochondral ossification in growth cartilage remains unclear, and whether hypertrophic cartilage cells survive in the bone marrow cavity has been debated repeatedly. Shimomura, Yutaka reported that hypertrophic cartilage transplanted survives to become osteocytes.

Suzuki, Fujio graduated from the Dept. of Bio-Organic Chem., Osaka Univ. but was appointed Assoc. Prof. in the Dept. of Biochem., Faculty of Dentistry in 1966, and so started with Yoneda, Toshiyuki and Shimomura Y. to try some cell biological approach to the mechanism of endochondral bone formation in 1972. First, we found that cultured growth-cartilage cells have a marked osteogenic potential, forming differentiated colonies producing abundant proteoglycans and type II collagen in vitro (1975). We also found that parathyroid hormone (PTH) significantly enhanced proteoglycan synthesis in these cells (1976). This finding may be the first indication of a new role of PTH in differentiation of cartilage.

In 1972, Nevo, Z. and Dorfman, A. reported that exogenous proteoglycans extracted from bovine cartilage stimulate proteoglycan synthesis by cultured chondrocytes. Since then, many investigators have tried to identify such regulatory factors in cartilage. In 1980, we found some somatomedin-like factors in fetal bovine cartilage, named cartilage-derived factor (CDF). In the mean-time, Klagsbrun, M. purified a cartilage-derived growth factor (CDGF) from neonatal cartilage. However, their CDGF was found to be identical to bFGF. Thus, although it has been suspected that factors produced by chondrocytes may regulate cartilage growth and expression, in the last 15 years or more, nobody has succeeded in characterizing an active principle. However, we at last succeeded in the purification and molecular cloning of a cartilage-specific growth modulating matrix component (25 kDa glycoprotein) that functions in growth regulation of the differentiated cartilage cells, and stimulates DNA synthesis of cartilage cells in the presence of bFGF. We named this unique principle "Chondromodulin-I (ChM-I)". In addition, ChM-I inhibited the proliferation of vascular endothelial cells and tube formation. These findings indicate that cartilage-specific ChM-I is bi-functional: modulates cartilage growth and shows angioinhibin function. We also identified another modulator, ChM-II, from the same source. The amino acid sequence of ChM-II (133 amino acid residues) was significantly homologous to the product of chicken promyelocyte-specific *mim-1* gene whose transcription was directly activated by the *v-mylb* oncogene product. ChM-II resulted in increases in both DNA and proteoglycan syntheses in cultured rabbit chondrocytes. However, ChM-II did not inhibit DNA synthesis of endothelial cells but stimulated the differentiation of osteoclasts. Therefore, cartilage-derived matricrine factor, ChM-II would be the first characterized naturally generated, osteopoietic coupling factor in endochondral bone formation.

Plenary Lecture

CARTILAGE MATRIX MOLECULES WITH ROLES IN TISSUE HOMEOSTASIS

Dick Heinegård, Department of Medical and Physiological Chemistry, Lund University, Box 94, S-221 00 Lund, Sweden.

The isolation and characterization of an increasing number of cartilage matrix macromolecules have provided means for identifying distinct compartments and functional units in the tissue. The molecular composition of these compartments is rather different, albeit some of the major macromolecules are common to the different structures.

A major macromolecular constituent of cartilage extracellular matrix is aggrecan or the large aggregating proteoglycan, distributed throughout the tissue. The other major component is the collagen fibers made up of collagen II molecules with some additional type XI in the same fibers. These fibers have the highest abundance in the interterritorial matrix. They run in different directions, such that they are parallel to the surface in the superficial layer and perpendicular to the surface in deeper layers. The properties of the fibers are modulated by the presence of other molecules on their surface. These molecules include covalently bound collagen IX as well as the proteoglycans decorin and fibromodulin. These diverse molecules have the potential for bivalent action by binding to the collagen II fibers via protein-protein interactions as well as by other interactions between their glycosaminoglycan chain and collagen. This provides an opportunity for linking neighboring fibers. Thereby the effective length of the fibers is extended to provide much improved tensile properties of the tissue. This arrangement appears essential to counteract the swelling properties of the aggrecan molecules and in dissipating load throughout the tissue.

Other molecules in the cartilage matrix include the pentameric cartilage oligomeric matrix protein (COMP or chondrospondin), with particular homology to the thrombospondin molecules, albeit the five subunits (compared to three for thrombospondin) are shorter and only some 85 kDa. There are also distinct sequence differences and the COMP molecule shows a much more narrow tissue distribution. The functions of COMP, that is only detected in cartilage by immunoassay, have not been delineated, although data indicate that the protein can mediate cell binding and may play a role in repair processes of the matrix.

Cartilage matrix contains a number of other molecules detected in cartilage only. Chondroadherin or the 36 kDa protein contains the typical leucine rich repeat domain found in the small proteoglycans. Chondroadherin can mediate chondrocyte attachment, apparently via integrins. A novel 92 kDa protein shows a unique restricted distribution to the middle portion of the articular cartilage. Cartilage also contains small amounts of proteins with a more general distribution between tissues, e.g. thrombospondin and fibronectin. A trimeric slightly basic protein (CMP), although only found in cartilage, is absent from articular cartilage and the intervertebral disc.

By studies of the functions of these matrix molecules, the functional roles of the tissue domains will be delineated.

Structure and Function of Cartilage ECM

16A-1

STRUCTURE AND FUNCTION OF PRECHONDROGENIC MESENCHYME PROTEOGLYCAN, PG-M

Koji Kimata, Tamayuki Shinomura, Kazuo Ito, Masahiro Zako, Minoru Ujita, Naomi Yamakawa, and Yoshihiro Nishida. Institute for Molecular Science of Medicine, Aichi Medical University, Nagakute, Aichi 480-11, Japan

PG-M, a large chondroitin sulfate proteoglycan, was for the first time isolated from chick limb buds (1) and has been shown to be one of major extracellular matrix molecules of the condensation area of undifferentiated mesenchymal cells in limb buds (2). This molecule disappears with cartilage development in an inverse correlation to a dramatic increase of cartilage proteoglycan, aggrecan (2). Such a transient expression of PG-M in limb bud suggests that the formation of a specific extracellular matrix may be necessary for the precartilage condensation process during skeletal cartilage development.

Cell adhesion is a crucial step that regulates a variety of cell behaviors such as cell differentiation and tissue tissue-specific cell function. Michael Sorlush has suggested that modification of cell morphology greatly affect chondrogenic phenotype expression. We previously showed that PG-M had an inhibitory activity for various types of cell-adhesion to extracellular matrix molecules (anti-adhesion activity) and suggested important roles in modulating a variety of such interactions (3). Further, we have recently found that the activity could be due to chondroitin sulfate chains attached onto the core protein (4). These findings suggest that modification of the cell-extracellular matrix molecule interactions by anti-adhesion activity of PG-M may be greatly involved in the condensation process for cartilage development as well as maintenance of cartilage functions.

As one of approaches to the evaluation of this possibility, we have first studied the cDNAs encoding the core proteins of chicken PG-M (5) and mouse PG-M. Protein homology analysis of the deduced amino acid sequences revealed the presence of a link protein-like domain at the N-terminal region and a set of domains at the C-terminal region which are composed of a complement regulatory protein-like domain, a lectin-like domain, and two EGF-like domains from the C-terminus. These domains showed an extremely high homology between chicken PG-M and mouse PG-M and to corresponding domains of human versican (6). In contrast, the chondroitin sulfate-attachment domain at

the middle region of the core protein showed no significant homology among them. In addition, we found that alternative splicing of two different exons (alpha and beta) for the chondroitin sulfate-attachment domain at the middle region generated four different forms of the PG-M core protein (PG-M(V0), PG-M(V1), PG-M(V2), and PG-M(V3)) (see Fig.1) and human versican corresponded to one of short forms (PG-M(V1)). The occurrence in the core protein of regions with such different characteristics, the evolutionary non-conserved and variable domains at the middle region and the conserved and constant domains at the N- and C-terminal regions suggests their distinctive and important functions. An another series of our experiments have shown that the N-terminal and the C-terminal regions function as the hyaluronan-binding domain and the oligosaccharide-binding domain, respectively. Those regions may contribute to the organization of PG-M in the extracellular matrix so as to express its anti-adhesion activity. On the other hand, the variable chondroitin sulfate-attachment domain generated by the alternative splicing may contribute to a regulation of anti-adhesion activity of PG-M which is due to the variation of the amount and/or number of chondroitin sulfate chains per core protein.

We have then investigated what forms of PG-M core protein are involved in the mesenchymal condensation and whether or not

multiple forms of PG-M core protein are regulated in a tissue-and/or time dependent manner. Northern analysis for RNA extracts from various tissue of mouse embryos at various stages using the different domain probes showed that PG-M(V0), the largest and chondroitin sulfate-attachment site-richest form of PG-M, is predominant in limb buds, and also suggested a tissue- and time-dependent variation in the expressions of PG-M multiform. It is likely, therefore, that the anti-adhesion activity of PG-M may vary in spatio-temporal manner, due to the difference in the amount and/or number of chondroitin sulfate chains per core protein and, in limb buds PG-M(V0) possibly with the highest anti-adhesion activity may cause a great change in the mesenchymal cell morphology which is essential for the onset of chondrogenic phenotype expression.

We have recently shown abnormal expression of PG-M in human osteoarthritic cartilage (7) and also in a chicken model of experimental osteoarthritis. Staining with antibodies against the alternatively spliced domains indicated that osteoarthritic chondrocytes synthesized PG-M(V0), suggesting the reappearance of immature cartilage phenotype. Therefore, it is also likely that the formation of an embryonic extracellular matrix comprising PG-M may cause some of abnormal characteristics of osteoarthritic chondrocytes.

References

- (1) Kimata, K., et al. (1986) J. Biol. Chem. 261, 13517-13525
- (2) Shinomura, T., et al. (1990) Anat. Embryol. 181, 227-233
- (3) Yamagata, M., et al. (1989) J. Biol. Chem., 264, 8012-8018
- (4) Shinomura, T., et al. (1993) J. Biol. Chem., 268, 14461-14469
- (5) Sugiura, N., et al. (1993) J. Biol. Chem., 268, 15779-15787
- (6) Zimmermann, D. R., & Ruoslahti, E. (1989) EMBO J., 8, 2975-2981
- (7) Nishida, Y., et al. (1994) Osteoarthritis & cartilage, 2, 43-49

16A-2

STRUCTURAL STUDIES ON THE NOVEL SULFATED TETRASACCHARIDES ISOLATED FROM KING CRAB CARTILAGE CHONDROITIN SULFATE K : DEMONSTRATION OF 3-O-SULFATED GLUCURONIC ACID

Kazuyuki Sugahara¹, Yukako Tanaka¹, Shuhei Yamada¹, Hiroshi Kitagawa¹, and Nobuko Seno².

¹Department of Biochemistry, Kobe Pharmaceutical University, Kobe 658, and ²Department of Chemistry, Ochanomizu University, Tokyo 112, JAPAN.

It has been increasingly evident that chondroitin sulfate (CS) proteoglycans of cartilage are dynamic components which play a multitude of key roles in the normal physiology of cartilage tissues and regulate the biological processes such as cell migration and recognition, extracellular matrix deposition and morphogenesis. Preliminary chemical analyses indicated that unlike CS of many other cartilages, CS K isolated from king crab cartilage seems to contain 3-O-sulfated glucuronic acid residues (1). This novel structure is known to form the unique carbohydrate epitope in nervous tissues, which is recognized by HNK-1 monoclonal antibody (2). During the course of the systematic structural studies of CS from various tissues (3, 4) we isolated two tetrasaccharides from CS K after hyaluronidase digestion. The analysis by degradation enzymes and 500MHz ¹H-NMR unambiguously revealed 3-O-sulfated glucuronic acid residues in these tetrasaccharides.

[Methods] CS K was exhaustively digested with sheep testis hyaluronidase. The digestion was performed by using 3.0 mg of the enzyme and 30 mg of CS K in a total volume of 2.0 ml of 0.15 M NaCl/0.05 M NaH₂PO₄ pH 6.0 at 37°C for 18 hrs. Another 1.5 mg of hyaluronidase was added thereafter and the incubation was continued for 7 hrs, and then the reaction was terminated by heating at 100°C for 1 min. The digest was treated with trichloroacetic acid, neutralized with 1M Na₂CO₃, lyophilized and reconstituted in water. The obtained oligosaccharide fraction was subjected to gel filtration on a column of Bio-Gel P-10 using 1 M NaCl/10% EtOH as eluent. The separated oligosaccharides were pooled, desalted by chromatography on Sephadex G-25, and further fractionated by HPLC on an amine-bound silica column. The NaH₂PO₄ concentration was increased using a linear gradient at a flow rate of 1 ml/min from 16 to 530 mM over 60 min (3). NMR spectra of the oligosaccharides purified by HPLC

were measured with a Varian VXR-500 spectrometer at a probe temperature of 26 or 60°C.

[Results and Discussion] CS K was digested with testicular hyaluronidase and fractionated by gel filtration on a column of Bio-Gel P-10 into three peaks which corresponded to tetra-, hexa- and octasaccharides. In this study, the tetrasaccharide fraction was analysed. Subfractionation of the tetrasaccharide fraction by HPLC yielded two major peaks (peaks B-5 and B-8) accompanied by several

Fig. 1

minor peaks as shown in Fig. 1. These two major peaks were subjected to chondroitinase ACII and chondro-4-sulfatase digestions followed by HPLC analysis, respectively. Peak B-5 was degraded by chondroitinase ACII into ΔDi-4S and a unique saturated disulfated disaccharide, and both resultant disaccharides were sensitive to 4-sulfatase. In contrast, peak B-8 was resistant to chondroitinase ACII although one sulfate group was removed by 4-sulfatase digestion. NMR spectra of the peaks B-5 and -8 oligosaccharides revealed that the compounds in these two peaks contain the following structures with novel 3-O-sulfated glucuronic acid residue(s). In the structural formulas 3S or 4S represents 3- or 4-sulfate group, respectively.

B-5: GlcA(3S)β1-3GalNAc(4S)β1-4GlcAβ1-3GalNAc(4S)

B-8: GlcA(3S)β1-3GalNAc(4S)β1-4GlcA(3S)β1-3GalNAc(4S)

The variation in these two structures may indicate that in the biosynthesis 3-sulfation of GlcA takes place after 4-sulfation of GalNAc residue. It remains to be determined whether these unique structures exist in mammalian tissues and what biological roles they play.

[Ref.] 1) Seno, N., & Murakami, K. (1982) *Carbohydr. Res.*, **103**, 190-194. 2) Ariga, T., Kohriyama, T., Fredro, L., Latov, N., Saito, M., Kon, K., Ando, S., Suzuki, M., Hemling, M.E., Rinehart, K.L., Kusunoki, S., & Yu, R.K. (1987) *J. Biol. Chem.*, **262**, 848-853. 3) Sugahara, K., Ohi, Y., Harada, T., de Waard, P., & Vlieghehart, J.F.G. (1992) *J. Biol. Chem.* **267**, 6027-6035. 4) Sugahara, K., Shigeno, K., Masuda, M., Fujii, N., Kurosaka, A., & Takeda, K. (1994) *Carbohydr. Res.*, **255**, 145-163.

16A-3

FIBROCHONDROCYTES OF HUMAN KNEE MENISCUS SYNTHESIZE AGGREGAN AND TYPE II COLLAGEN

Takaaki Tanaka, Katsuyuki Fujii, Shigaku Sai, Yoshio Kumagai and Kagehisa Murota. Department of Orthopaedic Surgery, Jikei University School of Medicine, Tokyo 105 Japan.

INTRODUCTION

The menisci of the human knee joint are located between the femoral condyles and the tibial plateau. Such a localization results in the menisci being subjected to compressive forces during weight-bearing. It is known that human meniscal cells were shown to synthesize cartilage-like proteoglycan (1). However, metabolism of human meniscal fibrochondrocytes have not been well investigated. The present study was undertaken in order to determine whether human meniscal cells synthesize and express cartilage specific proteoglycan, aggrecan and type II collagen. In addition, the response to meniscal cells to TGF-β was determined in comparison with hyaline cartilage chondrocytes.

MATERIALS AND METHODS

Human knee articular cartilage and medial meniscus were dissected from the amputated legs. Cartilage slices was obtained from femoral condyles, and medial meniscus was cleaned of soft tissues. Middle part of meniscus was divided into two parts (outer 1/3 and inner 2/3) considering the difference in vascular supply. Cartilage and meniscus were treated with DME containing 0.8% pronase for 30 min followed by 0.4% collagenase treatment for 90 min. Chondrocytes and meniscal cells were cultured either in dish or agarose. The cells were labelled with ³⁵S or ¹⁴C-proline, and labelled cell medium was used for proteoglycan or collagen synthesis and non-labelled medium was used for Western blot analysis. Cells were used for extraction of total RNA. Oligonucleotides (20mer) synthesized based on the human aggrecan cDNA (2) and type II collagen cDNA were used for dot blot or Northern blot hybridization. Furthermore,

immunohistochemical analysis was also performed.

RESULTS

After one week in monolayer culture, ³⁵S-proteoglycan synthesis of meniscal cells from the inner 2/3 part was 10 to 20 % higher than that from the outer 1/3. After six weeks in monolayer culture proteoglycan synthesis of both cells was found to remarkably decrease. In contrast, after six weeks in agarose culture, territorial region of cells was strongly stained with alcian blue. Aggrecan expression of the meniscal cells from the inner 2/3 part was approximately 25 % of that of chondrocytes. In the presence of TGF-β aggrecan expression of meniscal cells was up-regulated in a dose-dependent manner as well as chondrocytes, and proteoglycan synthesis was almost parallel to mRNA levels (Fig. 1).

Total collagen synthesis of meniscal cells from the inner 2/3 part was not different from that from the outer 1/3. Western blot analysis showed that type II collagen was synthesized from meniscal cells and the amount was decreased after a long term culture (Fig. 2). Expression of type II collagen was also recognized by immunohistochemical analysis. Anti-type II collagen antibody staining showed that pericellular region and a part of extracellular matrix were positive. This staining pattern was almost consistent with alcian blue staining.

DISCUSSION

Previously it is reported that the amount of glycosaminoglycans in meniscus was approximately 10 % of that in hyaline cartilage (3). The present study showed that the amount of ³⁵S-proteoglycan synthesized by cells from the inner 2/3 part was greater than that from the outer 1/3; this value was approximately 30 % of chondrocytes. The expression of aggrecan mRNA was consistent with ³⁵S-incorporation. These data indicate that meniscal cells from the inner 2/3 part is more chondrocytic. In addition, in the presence of TGF-β proteoglycan synthesis was stimulated in a dose-dependent manner. Thus, human meniscal cells from the inner 2/3 part are like chondrocytes

in terms of their expression of aggrecan and the response to TGF-β. The present study also showed that type II collagen was expressed from human meniscal cells and was decreased after a long term culture. This is a similar phenomenon found in chondrocytes during dedifferentiation. Type II collagen synthesis of meniscal cells relative to chondrocytes was less than aggrecan synthesis. However, pericellular region and extracellular matrix stained with type II collagen antibody was also stained with alcian blue. It is shown that human meniscal cells express both aggrecan and type II collagen but expression type II collagen was less.

Fig. 1

Fig. 1. The effect of TGF-β on proteoglycan synthesis of human articular chondrocytes and meniscal cells.

Fig. 2

Fig. 2. Western immunoblot of type II collagen present in the media of monolayer culture. (A) chondrocytes. (B) meniscal cells, 4 weeks. (C) meniscal cells, 1 week

REFERENCES

- (1) Roughley J.P. et al., (1981) *Biochem. J.* **197**, 77-83
- (2) Dooge K.J. et al., (1991) *J Biol Chem.* **266**, 894-902
- (3) Arnosky S. et al., (1988) *Meniscus*, 487-537

Normal and Pathologic Metabolism of Cartilage 16P1-1

CURRENT CONCEPTS OF AGGREGAN CATABOLISM

John D. Sandy, Kelli A. Garcia, John T. Gordy and Anna H.K. Plaas.

Shriners Hospital for Crippled Children, Tampa Unit, and College of Medicine, University of South Florida, Tampa Florida, 33612, U.S.A.

Introduction

Aggrecan is the major space-filling proteoglycan of articular and growth cartilage matrix. The bulk of the mass of the aggrecan molecule is provided by its substituent chondroitin sulfate (CS) which, due to electrostatic repulsive forces between chains, provides the tissue with compressive resistance. Maintenance of the aggrecan concentration in articular cartilage is therefore critical to tissue function. Modulation of the concentration and structure of aggrecan in the growth plate matrix would also appear to be a critical feature of growth plate maturation and matrix calcification. The metabolism of aggrecan in cartilage is a function of the resident chondrocytes which have the capacity to synthesize and assemble aggregates (each typically composed of 50-100 aggrecan monomers bound non-covalently to link protein on a hyaluronan backbone) and also to degrade these multimeric structures. The mechanism by which chondrocytes catabolize aggregates is presently unclear and represents the central target of our investigations.

The aggrecan core protein has two globular domains at the NH₂ terminus which are referred to as G1 and G2 and these are connected by a proteolytically-sensitive segment known as the interglobular domain (IGD). Analysis of aggrecan fragments from a range of catabolic systems has indicated that a novel proteinase (aggrecanase), which cleaves the Glu 373 - Ala 374 bond of the interglobular domain (IGD) of aggrecan, plays a central role. Cleavage at this site separates the CS-substituted region of aggrecan from the G1 domain and so effectively destroys the function of the molecule. Such chondroitin sulfate-substituted catabolic products which result from aggrecanase action, and which initiate at Ala³⁷⁴, are released from articular cartilage explants, growth plate explants, and are present in human synovial fluids from both osteoarthritis and inflammatory joint diseases (1,2).

In order to further study this activity we have developed a culture system in which different cells can be assessed for their capacity to degrade aggrecan by the aggrecanase-dependent pathway.

Methods:

Chondrosarcoma cell lines (LTC and Rx) were provided by Dr. James Kimura, Henry Ford Hospital, Detroit. The protocol for preparation of alginate beads was essentially as described (3). Briefly, cells were entrapped at a concentration of 4×10^6 cells/ml in 1.2% alginate beads

with exogenous 35S-labelled rat aggrecan aggregates. Beads (about 20 per culture) were maintained in 0.5 ml DMEM/10% FBS for 48h, switched to serum-free medium (+/- 1μM retinoic acid (RA)) and medium collections were made for 14 days and frozen after addition of protease inhibitors. Aggrecan products were chondroitinase-digested and portions were fractionated on SDS-PAGE (4-12% gradient gels). Fragments were visualized on Western blots by chemiluminescent detection with a peptide-specific antiserum to rat aggrecan G1 domain (anti-CATEGQVRVNSIYQDKVSL) and with an antiserum (anti-NITEGE, supplied by Dr. Jeff Weidner of Merck, Rahway, NJ) which specifically detects aggrecanase-generated G1 domain.

Results :

We have previously shown (4, 5) that LTC cells in monolayer respond to RA addition by release of a 68kD product which represents the aggrecan G1 domain generated by aggrecanase action. When Rx cells were maintained in alginate beads containing exogenous 35S-labelled aggregates, and treated with retinoic acid, the cells responded catabolically by increased release of aggrecan fragments into the medium. Calculations based on the release curves indicated that the half-life of radiolabelled aggrecan in control beads was about 42 days and this was reduced to about 28 days in the presence of RA.

Western blot analysis of aggrecan fragments in medium from day 2 thru day 14 from parallel experiments (see Figure) showed that the pattern of immunoreactive fragments released was markedly altered by RA treatment. Control release products (left panel) were detected first on day 6 and were characterized by a high molecular weight aggrecan core species and a mixture of fragments of intermediate size. However, in the presence of RA (right panel), two major

products were generated which were essentially undetectable in controls. Firstly, a 220 kD species, which is also a major catabolic product of monolayer culture (4). This product apparently represents G1-bearing aggrecan which has been truncated from the C-terminal end of the core protein. Secondly, a 68kD product, which also reacted with the anti-NITEGE antiserum (not shown) and which therefore represents the G1 domain resulting from aggrecanase-mediated cleavage of aggrecan at the Glu 373 - Ala 374 bond.

Conclusion :

The results show that Rx chondrocytes maintained in alginate beads under these conditions will respond catabolically to RA treatment by degradation of aggrecan via an aggrecanase-mediated pathway. Moreover, the cells use this pathway to degrade aggrecan supplied to the beads exogenously. This culture system should therefore facilitate the search for aggrecanase activity in different cell populations and also allow for an evaluation of the effects of aggrecan substrate structure on the efficiency of the aggrecanase-mediated process.

References :

- (1) Sandy J.D. et al. (1991) J. Biol. Chem. 266, 8683-8685.
- (2) Sandy J.D. et al. (1992) J. Clin. Invest. 89, 1512-1516.
- (3) Guo J et al. (1989) Conn. Tiss. Res. 19, 277-297.
- (4) Sandy J.D. et al. (1993) Trans. Orth. Res. Soc. 19, 49.
- (5) Lark MW, Sandy JD et al. (unpublished).

16P1-2

PERICELLULAR MATRIX REMODELLING AND THE ROLE OF THE CHONDROCYTE IN THE GENESIS OF OSTEOARTHRITIS

C. Anthony Poole.

Department of Anatomy, School of Medicine, University of Auckland, PO Box 90214, Auckland, New Zealand.

INTRODUCTION

Articular cartilage plays a major role in joint mechanics, and is primarily responsible for the smooth transmission of compressive forces to the rigid underlying bone. Matrix integrity is critical for load bearing, and long term mechanical perturbation of the matrix is one of the key factors in the induction of osteoarthritic degeneration. Several studies now indicate the chondrocyte plays a key role in the initiation of matrix remodelling, a process involving the complex interaction of cytokines, growth factors, matrix metalloproteinases and tissue enzyme inhibitors. In particular, the degradation of pericellular type II collagen has been identified as one of the earliest recognisable structural changes to occur during matrix remodelling (1).

Previous studies have established the chondrocyte and its pericellular microenvironment represents the chondron, a natural microanatomical unit of adult articular cartilage which can be extracted by slow speed serial homogenisation (2). Immunohistochemical studies have characterised the heterogeneous composition of the pericellular microenvironment, and in particular the distribution of the minor collagens types VI and IX. In isolated chondrons extracted from normal articular cartilage, types VI and IX collagens are restricted to the pericellular capsule of the chondron (3). By comparison, histochemical analysis of chondrons isolated from osteoarthritic cartilages has identified structural and temporal changes which characterise the transition from a normal chondron to a swollen chondrocyte cluster typical of osteoarthritic pathology (4).

METHODS

To better define the response of cartilage matrix components to degenerative remodelling, we examined changes in the distribution of collagen types VI and IX in five dogs euthanised for spontaneous osteoarthritis of the stifle joint. Cartilage erosion varied from unaffected normal cartilage at the periphery, to complete eburnation of bone in the centre of the plateau. Intact samples of cartilage and underlying bone were removed from the centre and periphery of each plateau, fixed in 4% paraformaldehyde for 1 hour, washed and the samples divided in two. One portion was equilibrated in 20% DMSO, and stored frozen at -20°C, prior to cryosectioning at 10-25μm. The other portion was mounted wet in a vibratome, and thick slabs of cartilage (35-100μm) prepared for confocal microscopy. Cartilage remaining on the plateau was resected at the tidemark, the chondrons extracted, and plated in agarose gel as previously described (5). Antibodies directed against types VI and IX collagens were probed with a range of fluorescent or electron dense secondary antibodies, and the samples examined by light, confocal and transmission electron microscopy as previously described (3).

RESULTS

Type VI Collagen: Testicular hyaluronidase digestion was an essential prerequisite for exposure of type VI epitopes, revealing a discrete pericellular distribution in both unaffected (figure 1a) and involved cartilage. By contrast, omission of the hyaluronidase digestion produced variable results. In unaffected cartilage from the periphery, no type VI staining was observed. Conversely, in regions of surface fibrillation, or fissure formation, strong staining for type VI collagen was evident (figure 1b), while unaffected regions of the matrix remained unstained. The remodelling of pericellular type VI

collagen was restricted to a 'reactive layer' characterised by the presence of chondrocyte clusters at the eroding surface. Type VI collagen content in this 'reactive' layer increased as the microenvironment expanded to accommodate the continued cell proliferation, but remained almost exclusively associated with the surface of the chondrocytes (figure 1a, b). Osteoarthritic chondrons isolated from these samples provided superior images of the close inter-relationship between the chondrocyte and type VI during pericellular remodelling (figure 1c).

Figure 1a

Figure 1b

Figure 1c

Type IX Collagen: Hyaluronidase digestion was also required to expose type IX epitopes, and patterns of staining similar to those described above were seen when enzyme extraction was omitted. However, type IX collagen remodelling in the 'reactive' layer appeared distinctly different. First, there was an abrupt transition from discrete pericellular, to strong extracellular, staining at the junction between normal cartilage and the advancing fibrillation front (figure 2a). Second, type IX remodelling coincided with expansion of the pericellular microenvironment and the initiation of chondrocyte division (figure 2b). Finally, as the proliferating cell clusters enlarge toward the eroding surface, pericellular staining for type IX collagen was significantly reduced, while extracellular staining was substantially increased. Near the eroding articular surface, a matrix space completely devoid of type IX staining was typically observed between the weakly stained clusters and the intensely stained matrix (figure 2b). Isolated osteoarthritic chondrons reflected this spectrum of type IX collagen remodelling (figure 2c), and the progressive restriction of type IX collagen staining to a residual calyx typical of chondrocytes at the eroding surface (figure 2b).

Figure 2a

Figure 2b

Figure 2c

DISCUSSION

This study has shown that the 'minor' pericellular collagens, type VI and type IX, are differentially affected during the matrix remodelling which accompanies spontaneous canine osteoarthritis. It also demonstrates that minor collagen remodelling was limited to a 'reactive' matrix layer between the eroding articular surface and the advancing margins of fibrillation or fissure involvement. Invariably, remodelling of both collagen species was associated with the initial cell division and the continued proliferation of chondrocytes within this 'reactive' layer. Outside this involved surface region, the distribution of types VI and IX collagens appeared unaffected, and they were typically concentrated in the chondron with little staining in the bulk extracellular matrix.

Within this 'reactive' layer, the pericellular remodelling of types VI and IX collagens appeared quite different. At its advancing edge, recruitment of new chondrons into the degenerative processes was accompanied by expansion of the pericellular microenvironment and increased staining for both collagen species, even in the absence of hyaluronidase digestion. Dividing cells initially retained a portion of the expanded microenvironment, but as chondrocyte proliferation proceeds to cluster formation, pericellular type IX collagen became restricted to a narrow calyx around each cell, while extracellular type IX collagen increased significantly. By contrast, the bulk of type VI collagen remained closely associated with the cell surface during cluster formation, but it too was ultimately restricted to a narrow calyx around each chondrocyte at the eroding surface.

Type IX is a FACIT collagen involved in limiting the diameter of heterotypic collagen fibrils composed of types II and XI collagens (6). These fine heterotypic fibrils provide the architectural integrity of the chondron, but are vulnerable to attack by a range of matrix metalloproteinases, notably stromelysin (7). The synthesis of metalloproteinases is up-regulated by IL-1 (8), and it is probable that the reduction in pericellular type IX collagen results from metalloproteinase induced catabolism. Similar type IX remodelling has recently been induced in isolated chondrons cultured in the presence of IL-1 (work in progress). The high extracellular concentration of type IX is more difficult to explain, and further work is required to establish if it is the result of increased type IX synthesis, or increased concentrations of catabolic type IX fragments trapped in the bulk extracellular matrix.

In contrast, type VI collagen is not degraded by IL-1 induced metalloproteinases, but rather, by the serine proteinases, typically associated with the inflammatory response which accompanies osteoarthritis (9). While type VI collagen is obviously remodelled in the 'reactive' layer, it remains closely associated with the swollen microenvironment and the chondrocyte calyx, with little staining in the bulk matrix. These results suggest that serine proteinase could regulate type VI collagen remodelling to ensure the integrity of the expanding cell cluster, and the continued survival of individual chondrocytes confined within.

On the basis of these and other studies (4), a three phase hypothesis for chondron degeneration has been developed. First, cytokine-induced disruption of pericellular collagens allows expansion of pericellular proteoglycans causing the chondron to swell. Second, reduction in the steric hindrance exerted by degraded fibrillar collagens stimulates the progenitor chondrocyte to divide. Finally, physicochemical changes in the pericellular microenvironment promote the cartilage catabolism and chondrocyte proliferation typical of osteoarthritic cartilage pathology. The mechanisms responsible for the initiation and progression of this pericellular remodelling have yet to be fully elucidated.

Supported and funded by the Health Research Council of New Zealand. Antibodies supplied by Drs S. Ayad and D. Hartman are gratefully acknowledged.

(1). J. Clin. Invest. 83: 440, 1989. (2). J. Orthop. Res. 6: 408, 1988. (3). J. Cell Sci. 113: 1101, 1992. (4). Arth. Rheum. 43: 22, 1991. (5). J. Histochem. Cytochem. 39: 1175, 1991. (6). Curr. Opin. Struct. Biol. 3: 430, 1993. (7). J. Biol. Chem. 266: 5625, 1991. (8). Matrix 13: 95, 1993. (9). Biochem. Biophys. Res. Comm. 191: 1230, 1993.

16P1-3

A PERSPECTIVE ON METALLOPROTEASE REGULATION IN GROWTH PLATE PHYSIOLOGY

David S. Howell, Ofelia E. Muniz, Zvi Schwartz*, Barbara D. Boyan*, and David D. Dean*. GRECC, V.A. Medical Center and Univ. of Miami, Miami, USA and *Department of Orthopaedics, University of Texas Health Science Center, San Antonio, USA.

The extracellular matrix of calcifying growth plate cartilage (longitudinal septa) are an intricate mesh of collagen networks, proteoglycans and multiple other proteins, which we believe must undergo metabolic changes before calcification can occur. Most of these changes occur opposite the hypertrophic cells, which through enlargement and elongation can accelerate longitudinal bone growth (1). During relentless growth plate advancement in the rat, elimination of over 40% of the extracellular proliferative cell zone matrix occurs within a few hours to accommodate the 5-10 fold enlargement of chondrocytes (2). Proteolytic enzymes accomplish these processes, as part of degradative cascades (2). Breakdown of the septal extracellular matrix involves many proteolytic enzymes and a variety of substrates. Most of these enzymes belong to 1 of 2 families, the cartilage serine proteases, e.g., plasminogen activator (uPA) or other serine proteases and the matrix metalloproteases (MMPs), (3) in cartilage. At least 9 different MMPs have been reported. Prominent among these are collagenase stromelysin, gelatinase A or 72K gelatinase, and these are the principal enzymes studied in our laboratory. We have focused on accelerated collagenolysis by interstitial collagenase, as well as activity of stromelysin and gelatinase A - the components detected by acid and neutral metalloprotease assays respectively (4). The catalytic activity of these proteases is tightly regulated, and they are secreted as inactive proenzymes. The procollagenase appears from our studies to be regulated predominantly by interleukin 1 β rather than interleukin 1 α or TNF α (5). The process is independent of the presence of vitamin D metabolites in regard to adequate cell enlargement and matrix removal capability (6). However, increased matrix degradation can occur in the presence of phosphate deficiency by increased active collagenase and suppression of TIMP in the presence of 24,25(OH) $_2$ D $_3$ (7). This phenomenon is not observed in the presence of calcium deficiency and adequate phosphate level. The activation of the collagenase must occur at the plasma membrane surfaces, because micropuncture fluid assays of fluid opposite hypertrophic cells shows the presence of latent, but no active collagenase (2,8). We found that cultured growth cartilage cells chondrocyte plasma membranes lack the presence of plasminogen activator at sites where activation of collagenase would be essential in hypertrophic cells, but was present in matrix vesicle membranes at sites where capillary invasion could provide the substrate plasminogen. The presence of plasminogen activator (PA) neutral metalloprotease in matrix vesicles in a totally active form suggest that plasmin is an MMP activator there. Hypothetically, a new metalloprotease

discovered in cancer cells, MTMMP (Sato, H. et al. (1994) Nature 370:61-65), a membrane bound metalloprotease could account for activation of collagenase at hypertrophic cell sites where the plasminogen fails to penetrate the cartilage. Upregulation of procollagenase and stromelysin in RNA and downregulation of TIMP 1 and 2 mRNA have been recently demonstrated in rat growth plate chondrocytes (7). The ultimate fate of proteoglycan and their role in mineralization remain in dispute. Our view is that proteoglycan may function both for calcium storage (9), and for inhibition of mineralization (9). The presence of proteoglycan including some stored calcium in the form of "tightly knit" aggregate molecules in a gel of 50% concentration of dry weight estimated from the authors' previous studies (9) would make diffusion of ions impossibly slow, unless channels to the available capillary lymph are opened for fast ion exchange. The finding of a complete proteolytic system in cultured matrix vesicles seems to support the hypothesis that following the breakdown of the phospholipid membranes by increased phospholipase A2, and release of active proteases from the vesicles of crystal proliferation and expansion of mineral phase ensues.

References

1. Hunziker, E.B. Schenk, R.K., & Cruzorne, L.M. (1987) J. Bone Joint Surg. 69A:162-173.
2. Dean, D., Muniz, O.E., Berman, I., Pita, J.C., Carreno, M.R., Woessner, J.F., Jr., & Howell, D.S. (1985) J. Clin. Invest. 76:716-722.
3. Woessner, J.F., Jr. (1991) FASEB J. 5:2145-2154.
4. Wilhelm, S.M., Shao, Z.H., Housley, T.J., Separak, P.K., Baumann, A.P., Gunja-Smith, Z. & Woessner, J.F., Jr. (1993) J. Biol. Chem. 268:21906-21913.
5. Arsenis, C., Dean, D.D., Muniz, O.E. & Howell, D.S. (1990) Trans. Orthop. Res. Soc. 15:159.
6. Dean, D.D., Muniz, O.E., & Woessner, J.F., Jr. (1990) Matrix 10:320-330.
7. Grumbles, R.M., Dean, D.D., Muniz, O.E., Masse, P.G., Roos, B.A. & Howell, D.S. (1994) Trans. Orthop. Res. Soc. 19:418.
8. Dean, D.D., Muniz, O.E., & Howell, D.S. (1989) Matrix 9:366-375.
9. Howell, D.S. & Dean, D.D. (1992) In Disorders of Bone and Mineral Metabolism (F.L. Coe, M.J. Favus, Ed.), pp. 313-353. Raven Press, New York.
10. Dean, D.D., Schwartz, Z.V.I., Muniz, O.E., Gomez, R., Swain, L.D., Howell, D.S. & Boyan, B.D. (1992) Bone Min. 17:2-176.

16P2-1

STIMULATION OF TIMP-1 PRODUCTION BY ONCOSTATIN M IN HUMAN ARTICULAR CARTILAGE

Masayuki Shimmei and Osamu Nemoto

Department of Orthopaedic Surgery, National Defense Medical College, Saitama 359, JAPAN.

[INTRODUCTION]

Oncostatin M (OSM), 28-kDa glycoprotein, is a newly characterized cytokine which shares structural and functional homology to IL-6¹⁾. It is well known that tissue inhibitor of metalloproteinase -1 (TIMP-1) is a potent inhibitor of activated matrix metalloproteinases (MMPs) and involved in the control of extracellular matrix degradation in chondrocytes²⁾. Furthermore, the production of MMPs and TIMP-1 have been thought to be regulated by various cytokines³⁾. Since TIMP-1 expression has been shown to be up-regulated by IL-6⁴⁾, it should be meaningful to study the effects of OSM on TIMP-1 production by chondrocytes.

[MATERIALS AND METHODS]

Patella cartilage from 6 cadavers were used as tissue and cell culture. Cartilage slices were incubated in the DMEM containing 2% FBS with various cytokines for 10 days. For chondrocyte culture, finely diced tissues were treated with 0.4% pronase E for 1.5 hours, followed by digestion with 0.025% collagenase P for over night. Dissociated cells were seeded at the density of 5×10^5 cells/ml and cultured with DMEM + 10% FBS. After confluency, cultured medium was changed to DMEM + 2% FBS and stimulated with cytokines for 48 hours. Both tissue and cell culture supernatant were tested for MMP-3 and TIMP-1 production by EIA kits. As for the production of MMP-1,2 and 9, western blot analysis was performed by using anti-human MMP-1,2 and 9 monoclonal Ab, respectively. To study the effects of OSM on TIMP-1 mRNA expression, northern blot analysis was prepared by standard techniques and hybridized with human TIMP-1 cDNA probe.

[RESULTS]

As shown in Fig. 1, OSM stimulated TIMP-1 production in a dose dependent manner. Enhancement of TIMP-1 production was evident at 1 ng/ml and further elevated at 10 ng/ml and 50 ng/ml of OSM. Stimulatory effects in TIMP-1 mRNA expression were also confirmed by northern blot analysis. In contrast, OSM did

not induce MMP-1,2,3 and 9 production in both tissue and cell culture. When comparing the effects of OSM with other cytokines such as IL-1, IL-6, LIF and TGF-beta on TIMP-1 production, TIMP-1 mRNA expression was substantially stimulated by OSM, TGF-beta and somewhat less by IL-6.

[DISCUSSION]

We demonstrated here that OSM stimulated TIMP-1 production, showing the same degree as that of TGF-beta, whereas the production of MMPs were not changed by OSM. These findings lead us to speculate that OSM might have possible roles in the modulation of cartilage matrix degradation, through induction of TIMP-1 production in chondrocytes.

[ACKNOWLEDGEMENTS]

We thank Prof. Seiki (Kanazawa Univ.) for kindly providing human MMPs, TIMP-1 cDNA probes and Fujii. Chem. Ind. Ltd. for supplying MMP-3 and TIMP-1 EIA kits.

[REFERENCES]

- Rose, T.M., and Bruce, A.G. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**: 8641-8645.
- Pelletier, J.-P., F. Mineau, M.-P. Faure, and J. Martel-Pelletier. (1990) *Arthritis Rheum.* **33**: 1466-1472.
- Shimmei, M., Masuda, K., Kikuchi, T., and Shimomura, Y. (1990) *Semin. Arthritis Rheum.* **4**: 16-20.
- Lotz, M. and Guerne, A. (1990) *J. Biol. Chem.* **266**: 2017-2020

Fig. 1 Effects of OSM on TIMP-1 Production (cell culture)

16P2-2

MATRIX METALLOPROTEINASES AND CARTILAGE DEGRADATION DURING INFLAMMATION

Karen A. Hasty, Tomohiko Hirose, Ken Kikuchi and Carlo L. Mainardi, Departments of Anatomy and Neurobiology and Medicine, University of Tennessee, Memphis, Tennessee, USA 38104 and the Department of Orthopaedic Surgery, Juntendo University, Tokyo, JAPAN.

Using the animal model of collagen-induced arthritis, we have described two phases of cartilage matrix destruction; initial loss of proteoglycan followed by erosion of the underlying collagen matrix (1). Loss of collagen is felt to be the irreversible step in degradation of cartilage matrix. We have undertaken study of the primary structure of collagenase, the matrix metalloproteinase responsible for degradation of type II collagen, to define the structural determinants important in recognition and cleavage of its collagen substrate. To address this question, we generated truncated mutants of neutrophil collagenase (TrNC) with varying deletions in the carboxy-terminal (C-terminal) domain and chimeric molecules between neutrophil collagenase (NC) and stromelysin (Strom), and assayed the expressed enzymes against type I collagen and the general substrate, casein (2).

[Methods] The cDNAs of the mutants were cloned into a pcDNA1 expression vector and transfected into COS-7 cells. We tested enzymatic activity in the culture supernatants against type I collagen and the general substrate casein. The amount of the enzyme was normalized by Western blot analysis with antiserum raised against a synthetic peptide of NC and by autoradiography of culture supernatants of COS-7 cells labeled with [³⁵S]-methionine. To further define sequences critical for substrate recognition, chimeric enzymes were constructed by fusing cDNA representing the carboxy-terminal portions of stromelysin to the collagenase fragments representing the amino-terminal sequence. This allowed conservation of the cysteine loop at the carboxy terminus.

The development of chimeric metalloproteinases with and without collagenase activity allowed us to test the hypothesis that substrate specificity, i.e. cleavage of native type I collagen, is directly related to substrate binding. To test this hypothesis, recombinant enzymes were incubated with native reconstituted collagen fibrils at 22°C for 45 minutes

and the bound enzyme quantitated using an ELISA technique. Substrate binding was assessed before and after incubation with aminophenyl mercuric acetate to detect differences in active and latent enzymes.

[Results] Significant reduction in enzymatic activity against type I collagen was seen with subsequent deletion of carboxy terminal sequences. The deletion of the four terminal amino acids including cysteine⁴⁶⁴ resulted in significant reduction in collagenase activity. Cysteine⁴⁶⁴ may stabilize a critical loop at the carboxyl end of the molecule by interacting with the cysteine at position 279. Further deletion resulted in loss of substrate specificity. TrNC 262 did not show any activity against type I collagen but degraded casein. This suggests that the catalytic region of the molecule remained intact and distinct from the site of substrate recognition. Chimera NC326 / Strom exhibited significant collagenase activity while NC258 / Strom did not show any activity against type I collagen.

Interestingly, all activated recombinant enzymes exhibited binding to collagen, although only two, wild type collagenase and NC326/Strom, had been shown to appreciably degrade the substrate. The latent wild type recombinant NC did not bind to collagen. By contrast, chimerae with or without collagenase activity bound to collagen in both latent and activated forms. The binding to collagen therefore did not seem to correlate with the ability to degrade collagen.

The binding of both latent and active enzymes led us to hypothesize that chimerae bearing the carboxyl domain of stromelysin were binding to collagen in a manner which differed from wild type collagenase which bound only when active. To investigate this hypothesis, we quantitated the binding of the recombinant enzymes to collagen which had been completely digested with collagenase under conditions which preserved the native confirmation of the three quarter and one quarter fragments. Wild type NC bound insignificantly to collagenase derived collagen fragments while both chimerae with the stromelysin derived carboxyl terminal domain bound to collagen fragments.

[Discussion] Our data suggest that substrate-specificity for interstitial collagen is determined by a 62 amino acid sequence in the C-terminal domain of NC and is dependent upon the integrity of a disulfide-defined loop at the C-terminus for maximal activity. This suggests that collagen binding of the chimerae is related to the carboxyl terminus of stromelysin, does not correlate with collagenase activity and is not dependent on the

presence of the cleavage site on collagen. By contrast, wild type collagenase does not bind significantly in its latent form, recognizing the collagen monomer only when activated. The binding to active enzyme is dependent on the presence of an intact cleavage site in the collagen, no doubt relating to the release of the processed substrate.

[Acknowledgments] This work was supported by grants from the National Institutes of Health (AI22603 and AR39166) and the Arthritis Foundation.

[References]

1. Hasty, K. A., Reife, R. A., Kang, A. H. and Stuart, J. M. (1990). Arth. Rheum. 33, 388-397.
2. Hirose, T., Patterson, C., Pourmotabbed, T., Mainardi, C. L. and Hasty, K. A. (1993). Proc. Natl. Acad. Sci. USA 90, 2569-2573.

16P2-3

CARTILAGE-DERIVED INHIBITORS OF ANGIOGENESIS

Marsha A. Moses, Department of Surgery, Harvard Medical School and The Children's Hospital Medical Center, Boston, MA, 02115, USA

Angiogenesis, the process of new capillary growth from pre-existing vessels, is important in a number of important physiological events, both normal and pathological. When it occurs under normal conditions, angiogenesis is a stringently controlled process. Under pathological conditions, these controls fail. There exist a number of serious diseases, characterized by persistent and deregulated neovascularization, which have recently been termed "angiogenic diseases", including a number of connective tissue pathologies (1).

Motivated by our interest in understanding the mechanisms underlying the control of angiogenesis, we have studied cartilage, an *avascular* tissue. We have demonstrated that cartilage contains a potent inhibitor of neovascularization in vivo using the chick chorioallantoic membrane assay and the rabbit corneal pocket assay (2). To understand the mechanism by which CDI (Cartilage-Derived Inhibitor) may exert its anti-angiogenic effect, we have tested it in a number of in vitro angiogenesis assays including those which measure capillary endothelial cell proliferation, migration, and capillary tube and sprout formation. The inhibitory activity of CDI in these systems will be presented in detail.

Interestingly, we have found that CDI is also an inhibitor of matrix metalloproteinase activities. These studies represent the first demonstration that a metalloproteinase inhibitor, with no other known biological activity, can inhibit angiogenesis. Further studies from our laboratory have demonstrated that cultures of scapular chondrocytes which were established and maintained serum-free, produce an anti-angiogenic factor which shares the same biological activities as CDI (3).

We have recently become interested in understanding the role of CDI

and its target metalloproteinases in the switch to the angiogenic phenotype. One of the model systems that we are utilizing is the development of chondrosarcoma, the *vascularized* tumor of cartilage. We have developed a system to study the transition from normal cartilage to chondrosarcoma in vivo and have also established cell lines representative of these various transition states for in vitro study as well (4). Data will be presented which suggest that one means of controlling the deregulated vascular growth characteristic of many angiogenic diseases may be at the level of the control of matrix metalloproteinase activity.

References

1. Folkman, J. and Klagsbrun, M. (1987) Science 235, 442-447.
2. Moses, M.A., Sudhalter, J. and Langer, R. (1990) Science 248, 1408-1410.
3. Moses, M.A., Sudhalter, J. and Langer, R. (1992) J. Cell Biol. 119, 475-482.
4. Moses, M.A. and Shing, Y. (1994) Biochem. Biophys. Res. Comm. 199, 418-424.

18P1-1

FURTHER CHARACTERIZATION OF A HUMAN CHONDRO-SARCOMA-DERIVED CHONDROCYTIC CELL LINE: HCS-2/8

Masaharu Takigawa¹, Kojiro Takahashi¹, Tohru Nakanishi¹, Takako Hattori¹, Yusuke Kimura¹, Hai-Ou Pan², Akihiro Kinoshita³, Kaoru Nakajima⁴, Fujio Suzuki⁴, Shintaro Nomura⁵, Tokutaro Okawa⁶ and Jing-de Zhu⁷ ¹Dept. of Biochemistry, Okayama University Dental School, Okayama 700, JAPAN, ²Drug Discovery Research Laboratories, Hoechst Japan, Kawagoe 350-11, JAPAN Depts. of ³Pediatric Dentistry and ⁴Biochemistry, Faculty of Dentistry and ⁵Faculty of Medicine, Osaka University, Suita, Osaka 565, JAPAN, ⁶Osaka City University Medical School, Osaka 545, JAPAN and ⁷Chester Beatty Laboratories, London SW3 6JB, UK

[Introduction]

HCS-2/8 cell line is the first immortal cell line carrying high abilities to synthesize cartilage-type, large proteoglycans (PGs) and collagen types II and XI (1,2). These characteristics suggest that this cell line is useful for studies on human chondrocyte metabolism. However, the cell line also forms chondrosarcomas in nude mice (1-3). Therefore, to use the HCS-2/8 cell line as a experimental model(s) for studies on human chondrocytes and/or human chondrosarcomas, further characterization of this cell line is indispensable. Here in this report, we describes characteristics of the cell line in more detail.

[Results and Discussion]

Characteristic of proteoglycans (PGs) produced by HCS-2/8 cells HCS-2/8 cells produced proteoglycan (PG) aggregates composed of PG monomers, hyaluronic acid and a link protein. Using glycerol gradient centrifugation and size exclusion chromatography, the PG monomers synthesized by the cells were identified as cartilage specific, large PG monomers. Northern blot analysis also revealed gene expression of aggrecan. The major glycosaminoglycans (GAGs) in the PG monomers was about 38 kDa when determined by Superose 12 FPLC. The GAGs were mainly composed of chondroitin-6-sulfate. The PG monomers contained a little keratan sulfate. The cells synthesized a single link protein with a molecular weight of 42kDa. These findings indicate that HCS-2/8 cells synthesize PGs of which composition and structure are similar to those in normal cartilage.

Autocrine regulation of expression of the differentiated phenotype of chondrocytes by insulin-like growth factors (IGFs) Both IGF-I and II increased PG synthesis in HCS-2/8 cells. In contrast to stimulatory effects of IGFs on rabbit costal chondrocytes, the stimulatory effect of IGF-II on PG synthesis in HCS-2/8 cells was more potent than that of IGF-I. Binding and crosslinking studies revealed that HCS-2/8 cells had both IGF-I and IGF-II receptors. The cells expressed mRNAs of IGF-I and IGF-II and produced IGF-I and IGF-II proteins. Mannose-6-phosphate, which is known to bind IGF-II receptors, stimulated PG synthesis and potentiated IGF-II-stimulated PG synthesis. Anti-IGF-I receptor antibody inhibited PG synthesis. These findings suggest that IGFs act on HCS-2/8 cells as autocrine differentiation factors and that IGF-II which had been believed to act through IGF-I receptors stimulates PG synthesis via IGF-II receptors.

Regulation of Proliferation and Differentiation by Other Factors Like normal chondrocytes, PG synthesis in HCS-2/8 cells was stimulated by insulin and transforming-growth factor (TGF)- β and inhibited by basic FGF (bFGF) and retinoic acid. In addition, TGF- β , bFGF and retinoic acid stimulated the DNA synthesis or proliferation of HCS-2/8 cells. However, there were some subtle differences between responsiveness of HCS-2/8 cells to these factors and that of normal rabbit chondrocytes. For instance, the stimulatory effect on DNA synthesis in HCS-2/8 cells was observed only in sparse cultures. TGF- β stimulated DNA synthesis in the cells cultured even in serum-free medium.

In contrast to normal chondrocytes, HCS-2/8 did not respond to parathyroid hormone, EGF or interleukin 1. We will discuss these differences in detail.

Species specific cytokines interferons, especially, interferons α and β , dramatically inhibited PG and DNA syntheses in HCS-2/8 cells.

Cell Density-Dependent Expression of Differentiation-Related Genes The HCS-2/8 cells expressed many genes related to chondrocyte differentiation such as collagen types II, X and XI, vitamin D₃ receptor, matrix Gla protein (MGP), IGF-I and II and their receptors and alkaline phosphatase (ALPase). Expression of these genes was depend on cell density in culture. During the rapidly growing phase and subconfluent phase of cultures, HCS-2/8 cells were in slightly elongated polygonal shape and had very little extracellular matrix but already expressed aggrecan and type II collagen genes. As they approached to confluence, they became polygonal and surrounded by extracellular matrix and their PG synthesis reached a maximum. In this phase, MGP was strongly expressed. The cells then became multilayered and formed nodules which showed strong metachromasia when stained with toluidine blue. In this phase, the cells expressed vitamin D₃ receptor (VDR) and type X collagen which have shown to be expressed in hypertrophic chondrocytes. The cells also expressed mRNA of IGF-I and II and IGF-type I and II receptors. However, in the absence of ascorbic acid (Vit. C), ALPase activity which is a marker of calcification was low. When the cells were treated with Vit. C for more than one week, mRNA level and enzyme activity of ALPase markedly increased. These findings suggest that HCS-2/8 cells in growing, subconfluent, confluent and overconfluent cultures correspond to proliferating, differentiating, maturing and hypertrophic chondrocytes, respectively, and that ascorbic acid is essential for further differentiation along the process of endochondral ossification.

Proto-oncogene Expression As described above, HCS-2/8 is a stable human chondrosarcoma cell line with many chondrocytic characteristics. However, the cells also has the capacity to form chondrosarcomas in nude mice. Therefore, we also examined transcript levels of 21 proto-oncogenes by Northern analysis. Using 10 μ g total RNA, we found no detectable transcripts of 9 proto-oncogenes (*c-sis*, *c-met*, *c-src*, *c-lyn*, *c-lgr*, *c-ros*, *c-pim*, *Blym* and *N-myc*) but detected transcripts of 12 other proto-oncogenes (*int-2*, *erbB*, *c-abl*, *c-raf-1*, *c-fyn*, *K-ras*, *H-ras*, *c-mos*, *c-myc*, *c-myb*, *c-fos*, and *c-jun*). In the over-confluent phase, the levels of *c-fos* and *c-raf-1* which were reported to be expressed in hypertrophic chondrocytes were increased some dozens and about 5 times, respectively, while the level of *c-abl* was about 1/5th of that in the sparse, subconfluent and confluent phases of culture. The level of *int-2*, a FGF-related proto-oncogene, increased about 10-fold in the confluent and overconfluent

phases of *in vitro* culture. We will discuss physiological and pathological significances of the changes in expression of these proto-oncogenes.

In conclusion, the HCS-2/8 line had many characteristics very similar to those of normal chondrocytes, indicating that the cell line should be useful for studies on human chondrocytes. On the other hand, the cell line had also some characters different from those of normal chondrocytes. Further studies on these differences should be helpful in understanding the tumorigenicity of human chondrosarcomas.

[References]

1. Takigawa, M., Tajima, K., Pan, H.-O., Enomoto, M., Kinoshita, A., F. Suzuki, Takano, Y. & Mori, Y. (1989) Cancer Res. 49, 3996-4002.
2. Enomoto, M. & Takigawa, M. (1992) In Biological Regulation of The Chondrocyte (M. Adolphe, Ed.) pp. 321-338. CRC Press., Boca Raton, FL.
3. Takigawa, M., Pan, H.-O., Kinoshita, A., Tajima, K., & Takano, Y. (1991) Int. J. Cancer, 48, 717-725.
4. Takigawa, M., Pan, H.-O., Enomoto, M., Kinoshita, K., Nishida, Y., Suzuki, F., & Tajima, K. (1990) Anticancer Res., 10, 311-316.
5. Zhu, J.-D., Pan, H.-O., Suzuki, F. & Takigawa, M. (1994) Jpn. J. Cancer Res., 85, 364-371.

Table 1. Cell density-dependent expression of differentiation-related genes and proto-oncogenes by HCS-2/8 cells

Phase in culture	growing	subconf.	confluent	overconfluent
Aggrecan mRNA	+	+++	++	+
α 1(I) mRNA	++	++	+	+
α 2(XI) mRNA	ND	+	++	++
α 1(X) mRNA	ND	ND	ND	+
MGP mRNA	+	++	+++	ND
VDR mRNA	+	+	+	+++
ALPase mRNA	ND	ND	+	+
IGF-I & II mRNA	+	+	+	+++
IGF-I & II R mRNA	ND	ND	ND	+
<i>int-2</i> mRNA	+	+	++++	++++
<i>c-fos</i> mRNA	+	+	+	+++
<i>c-abl</i> mRNA	+++	+++	++	+
<i>c-raf-1</i> mRNA	+	++	++	+++
Stage in differentiation	proliferation	differentiation	maturation	hypertrophy

ND: not determined.

18P1-2

MOLECULAR CLONING AND FUNCTIONAL EXPRESSION OF HUMAN CHONDROMODULIN-I(hChM-I) AND SEQUENCE ANALYSIS OF AN UPSTREAM REGION OF hChM-I GENE

Kazuhiro Takahashi, Junko Suzuki, Takaharu Kohno, Akiko Mori, Jun Kondo, Yuji Hiraki* and Fujio Suzuki*. Research Center, Mitsubishi Kasei Corporation, Kanagawa 227, JAPAN. *Department of Biochemistry, Faculty of Dentistry, Osaka University, Osaka 565, JAPAN

Growth and maturation of epiphyseal growth-plate cartilage play an important role in endochondral bone formation. Previously we reported purification of growth promoting activity from an extract of fetal bovine epiphyseal cartilage, which we named Chondromodulin-I (ChM-I) (1). Subsequent cloning of bovine ChM-I (bChM-I) cDNA revealed bChM-I consist of 121 amino acids with three possible glycosylation sites and is coded as the C-terminal part of a larger precursor. On northern blot analysis, ChM-I mRNA was specifically expressed in cartilage. Recently we succeeded in the molecular cloning of human ChM-I (hChM-I) cDNA. From its nucleotide sequence, primary structure of hChM-I was deduced, and compared with its bovine counterpart.

[Methods] *Screening of human genomic Library* : A lambda EMBL3 genomic library of human placenta was obtained from Clontech (Palo Alto). The library was screened by hybridization with a probe labeled by a random labeling method (2). The EcoRI-EcoRI fragment derived from the bovine ChM-I cDNA clone (1) was used as probe. Hybridization-positive phage clone was isolated by repeated plaque purification.

Nucleotide Sequencing : Appropriate DNA fragments were subcloned into pBlueScript SK(-) and sequenced by the chain termination method (3) modified for Dupont Automatic nucleotide sequencer.

RNA purification and RT-PCR : Total RNA was extracted from

human chondrosarcoma tissues by the guanidine-thiocyanate method (4). The chondrosarcoma cDNA was synthesized by Reverse transcriptase with the annealed anchor primers consisted of primer 1 (5'-CTCGAGGCCATGGCGGCCGCTTTTTTTTTTTTTTTTTTTTTTTT-3') and primer 2 (5'-GCGGCCGCCATGGCCCTCGAG-3'), using total RNA extracted from chondrosarcoma as a template. PCR reaction was carried out by the following procedure. The primer 3 (5'-AGTCTCCAAGTGCCTCACT-3'), which was synthesized as an upstream region primer on the basis of human genomic sequence, and the primer 4 (5'-CGAGGCCATGGCGGCC-3') corresponding to a part of the primer 2 were annealed with chondrosarcoma cDNA. Amplification by PCR was performed 35 cycles as described previously (5), using a Geneamp Kit (Perkin-Elmer-Cetus). The nested PCR followed by using the primer 5 (5'-CATGACAGA GAACTCCGA-3') near the initiation-codon ATG and the primer 6 (5'-ACACCATGCCAGGATGC-3') which was synthesized on the basis of the nucleotide sequence surrounding the stop-codon in bChM-I cDNA.

3'-RACE (Rapid Amplification of cDNA End method) : After nucleotide sequencing of the entire coding region of hChM-I PCR clones described above, a pair of primer was designed for 3'-RACE (6): primer 7 (5'-CCCTAGACTGGATCAC-3') and primer 8 (5'-CGAGGCCATGGCGGCC-3'). Thirty-five cycles of PCR yielded a DNA fragment of 3' untranslated sequence.

[Result and Discussion] Our previous study indicates that bChM-I is coded as a C-terminal region (121 amino acids) of a larger precursor. The precursor protein consists of 335 amino acids, and has a hydrophobic transmembrane domain at near N-terminal part which is rich in valine, leucine and isoleucine. Thus ChM-I precursor is assumed to be expressed as a transmembrane protein. Mature ChM-I protein occurs by proteolytic digestion at the potential processing signal (KEIQRRER underlined in Fig. 1) which precedes the mature bChM-I sequence. This signal sequence is homologous to a processing signal of human complement C5

precursor and can be recognized by a certain endoprotease (7). Three possible glycosylation sites were identified at T⁹, T¹² and N⁹ in bChM-I. N-terminal two third of bChM-I precursor is assumed to reside on cell membrane. We termed this portion of the precursor as chondrosurfactant protein (Ch-SP) (1), whose biological function remains unknown.

Fig. 1 Comparison of Amino Acid Sequences

The upper and lower sequences represent primary amino acid sequences of bChM-I and hChM-I precursors, respectively. Computer analysis was done by GENETYX-MAC (Software Development Co., Ltd. Tokyo Japan). Processing signal sequences are underlined.

```

1' MTNSDKVPIALVGPDDVEFCSPPAYAAVTVPKSSPARLLKVGAVVLISGAVLLLLGATG
*****
1'' MTNSDKVPIALVGPDDVEFCSPPAYATLTVKPSPARLLKVGAVVLISGAVLLLLGATG

61' AFYFWKGSNDHIYNVHYTMSINGKLQDGSMEIDAGNNLETFKMGSGAEAEVDFQNGI
*****
61'' AFYFWKGSNDHIYNVHYTMSINGKLQDGSMEIDAGNNLETFKMGSGAEAEAVDFQNGI

121' TGIRFAGGEKCYIKAQVKARIEVGTMTKQSISSLEGKIMPVKYEENSLIWWAGDQPVK
*****
121'' TGIRFAGGEKCYIKAQVKARIEVGTMTKQSISSLEGKIMPVKYEENSLIWWAGDQPVK

181' DNSFLSSKVLLECGDLPFWLKYPTYPEIKRERRELVKIVTTTTRRLRSGPQGTAPG
*****
181'' DNSFLSSKVLLECGDLPFWLKYPTYPEIKRERRELVKIV-PTTTRKPHSGPRSNPGAG

241' RPNNGTRPSVQEDAEPFNPDPNYPHQEGESMTFDPRLDHEGICCECRRSYTHCQKICEP
** *****
240'' RLNNETRPSVQEDSAFNPDPNYPHQEGESMTFDPRLDHEGICCECRRSYTHCQKICEP

301' LGGYHPWPYNYQGCRSACRVIMPCSWWVARILGMV
**** *****
300'' LGGYHPWPYNYQGCRSACRVIMPCSWWVARILGMV

```

PCR amplification yielded three independent clones corresponding to a coding region of hChM-I cDNA. Figure 1 shows the amino acid sequence of major type cDNA clone, hChM-I 3-6. The amino acid sequence of hChM-I precursor was 92% identical to that of bChM-I precursor.

Unexpectedly, however, sequence homology between mature hChM-I and its bovine counterpart was only 86% which was lower than that of the entire precursor (Fig. 1). Most amino acid substitutions (16 amino acid residues) in mature ChM-I were found in the N-terminal domain of the molecule which contains all three glycosylation sites. One amino acid deletion was also identified in this domain of the molecule. Bovine ChM-I has a cluster of Thr residue (T⁸ to T¹²) in the N-terminal domain. One of the residue (T⁹) was glycosylated. In human ChM-I, this cluster was replaced by PTTT due to a substitution and deletion of one T residue. Moreover, a sequence (QGTPAP) surrounding the other O-glycosylation site (T¹²) in bChM-I was replaced by RSNPGA. N-Glycosylation motif (NGT) in bChM-I changed into NET which is still valid as an N-glycosylation motif. In contrast, C-terminal half of the mature ChM-I molecule, which contains all eight cysteine residues, was completely conserved except for one amino acid residue. Previous chemical analysis of bChM-I indicated that these eight cysteine residues are linked by disulfide-bonding. Taken together, this domain of the molecule probably fulfills structural requirements for expression of its biological activities.

On the other hand, the present sequence comparison revealed a remarkable conservation (96% identity) of a sequence corresponding to Ch-SP. This high degree of sequence conservation implies functional importance of this putative transmembrane protein, Ch-SP, which remains to be clarified.

[Acknowledgements] We thank Dr. Y. Kotoura (Kyoto Univ. Medical School) for providing us with human chondrosarcoma tissues. This work was supported in parts by the Foundation for Growth Science and The Mitsubishi Foundation.

[References]

- Hiraki, Y., et al. (1991) *Biochem. Biophys. Res. Commun.* **175**, 971-977.
- Feinberg, A. P., and Vogelstein, B. (1983) *Anal. Biochem.* **132**, 6-13.
- Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5463-5467.
- Chirgwin, J. M., et al. (1979) *Biochemistry* **18**, 5294-5299.
- Okada, H., et al. (1989) *Biochem. Biophys. Res. Commun.* **162**, 1553-1559.
- Frohman, M. A., et al. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 8998-9002.
- Hosaka, M., et al. (1991) *J. Biol. Chem.* **266**, 12127-12130.
- Howgood, S. and Clements, J. A. (1990) *J. Clin. Inve.* **8**, 1-6.

18P1-3

RESISTANCE OF HUMAN OSTEOARTHRITIC CHONDROCYTES TO GROWTH HORMONE AND INSULIN-LIKE GROWTH FACTOR-1.

Johanne Martel-Pelletier, Sylvain Doré, Jean-Pierre Pelletier. Louis-Charles Simard Research Center, Notre-Dame Hospital, University of Montreal, Montreal H2L 4K8, CANADA.

In degenerative joint diseases, such as osteoarthritis (OA), the balance between the synthesis and degradation of matrix macromolecules is disturbed and in the evolved stage of the disease the degradative process exceeds the reparative one, leading to a total loss of cartilage and eburnation of bone. It is, therefore, of interest to understand the influence of growth factors on this equilibrium. Several growth factors are known to modulate cartilage homeostasis. The growth hormone (GH) and the insulin-like growth factor (IGF-1) appear to be the most important growth factors affecting the anabolism of the major molecules found in cartilage.

We investigated the anabolic role of GH and IGF-1 in normal and OA human adult cartilage. Since the action of these growth factors is mediated through membrane receptors, we first looked at their expression and presence, and investigated their biological response in human normal and OA chondrocytes.

[Methods] Articular cartilage specimens were obtained either from patients undergoing knee replacement or from normal individuals. Chondrocytes were released from the cartilage by sequential enzymatic digestion, and seeded at high density. All the binding experiments were performed on primary cell culture in which, unless otherwise mentioned, fetal calf serum was removed 24 hours prior to the experiment. For the binding assay, cells were incubated with increasing concentrations of labeled rhGH or rhIGF-1 in PBS, pH 8.0 containing 1% BSA for 4 hours at 22°C with or without 100-fold excess of radioinert GH or IGF-1. Competition experiments for IGF-1 were carried out by incubating chondrocytes with ¹²⁵I-IGF-1 in the presence or absence of increasing concentrations of rhIGF-1, rhIGF-II and rhInsulin. Affinity cross-linking experiments were performed with the cross-linker disuccinimidyl suberate after an incubation of chondrocytes with ¹²⁵I-IGF-1 in the presence or absence of IGF-1. Furthermore, the chondrocytes were subjected to SDS-PAGE electrophoresis and autoradiography. The IGF receptor type was investigated by incubating chondrocytes with a specific monoclonal antibody against human type I IGF receptor (α IR3) 1h prior to the receptor binding assays.

Total RNA was extracted from freshly released chondrocytes and mRNA used for Northern blot or PCR analysis.

GH biological response was monitored by the IGF-1 determination. Total IGF-1 was extracted and levels determined by a radioimmunoassay. IGF-1 biological activity was determined using incorporation of radioactive proline after IGF-1 stimulation.

[Results] In order to investigate the putative action of GH on human adult cartilage, we first examined the expression and presence of GH receptor in normal and OA chondrocytes. Results showed that human adult chondrocytes possess the GH receptor mRNA. However, binding assays with ¹²⁵I-GH failed to demonstrate specific binding in either normal or OA chondrocytes.

Incubation of chondrocytes with rhGH (0-100 ng/ml) over a period of 8 days failed to show an increased IGF-1 secretion of both chondrocyte types. However, these articular chondrocytes showed their ability to secrete *de novo* IGF-1. Importantly, the IGF-1 basal secretion was significantly ($p < 0.005$) enhanced in OA cells than in normal ones on any given day.

IGF-1 binding experiments in normal and OA chondrocytes demonstrate that non specific binding is less than 5%. Specific binding is dose-responsive and saturable for both cell types. Scatchard curve yielded linear plots indicating a single binding site. Binding experiments revealed that the receptor affinity (Kd) for normal chondrocytes is 1.4 ± 0.4 nM with $26.8 \pm 5.5 \times 10^3$ binding sites/cell. OA chondrocytes showed a lower affinity with a Kd of 15.4 ± 4.7 nM and a higher receptor density, $1,178.3 \pm 299.5 \times 10^3$ sites/cell. Biological responses with radiolabeled proline after IGF-1 stimulation revealed a non responsiveness by OA chondrocytes, which contrasts with the 2.5 fold enhancement activity in normal chondrocytes.

The type I IGF receptor characterization of normal human chondrocytes revealed that both IGF-1 and IGF-II displaced radiolabeled IGF-1 in a comparable manner; however, insulin at high concentrations weakly displaced IGF-1. Moreover, the anti-type I IGF receptor antibody effectively blocked (77%) specific binding in normal chondrocytes. Northern blot analysis with an antisense mRNA probe specific for type I IGF receptor, detected the signal at 11 Kb. Covalent cross-linking analysis on a 6% polyacrylamide gel revealed two bands, one of 130 kDa and another of approximately 300 kDa, characteristic of the type I receptor. Using a gel of 13%, bands were revealed and reflected the presence of the binding protein (IGFBP). Comparison between normal and OA chondrocytes showed an increased intensity in bands corresponding to the IGFBP-3, on the cells as well as in the culture medium.

[Conclusion] This study showed the expression of GH and IGF-1 receptor mRNA in human adult normal and OA chondrocytes. However, no relationship between GH stimulation and IGF-1 production could be observed, suggesting that GH receptor is not present or is inactive in these adult cells. The higher IGF-1 production of OA chondrocytes than normal, could indicate that factors other than GH modulate IGF-1 production in this diseased tissue. Interestingly, human OA chondrocytes in contrast to normal ones are unresponsive to the IGF-1 treatment despite an increase in IGF-1 binding on these diseased cells. Our results suggest that the increase of IGF-1 binding may involve an enhancement of IGFBP in the OA articular cartilage. By binding IGF-1, the IGFBP will diminish its bioavailability and consequently prevent its anabolic action.

Thus, in spite of a higher secretion of IGF-1 in OA chondrocytes compared to normal ones, the unresponsiveness of OA cells to IGF-1 might explain, at least in part, the progression of this degenerative disease.

18P2-1

HUMAN SYNOVIAL FIBROBLAST COEXPRESS INTERLEUKIN-1 RECEPTOR TYPE I AND TYPE II: THE ENHANCED BINDING TO INTERLEUKIN-1 IN OSTEOARTHRITIC CELLS IS RELATED TO AN INCREASED LEVEL OF TYPE I RECEPTOR.

Jean-Pierre-Pelletier, M'Bark Sadouk, François Mineau, Kayghobad Kiansa, Ginette Tardif, Johanne Martel-Pelletier. Louis-Charles Simard Research Center, Notre-Dame Hospital, University of Montreal, Montreal H2L 4K8, CANADA.

Osteoarthritis (OA) is a common rheumatic disorder characterized mainly by a progressive erosion of articular cartilage which is believed to be related to the proteolytic degradation of extracellular matrix. Previous studies have shown the presence of an increased level of latent and active metalloproteases (MMP) in OA cartilage (1). Proinflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF- α) are believed to mediate the catabolism in OA tissue (2). IL-1 has been detected in OA synovial tissue (1) and has been shown to induce, *in vitro*, the synthesis of MMP by synovial cells and chondrocytes.

The action of IL-1 is mediated by the binding of the cytokine to specific membrane receptors. Two distinct IL-1 receptors have been identified and cloned. IL-1-RtI (80 kDa) is preferentially expressed on T lymphocytes while IL-1-RtII (68 kDa) is expressed on B lymphocytes. The role of IL-1 in OA and rheumatoid arthritis is widely documented (3), but little data is available for IL-1-R in these arthropathies. A recent study from our laboratory has demonstrated that an increased level of IL-1-RtI in OA chondrocytes could play a role in the pathophysiology of this disease (4).

In the present study, we have investigated the number and the level of expression of IL-1-R in normal human and OA synovial fibroblasts, as well as examined the type of receptor which mediates the biological response of these cells.

[Methods] Synovial tissue specimens were obtained from OA patients undergoing total knee joint replacement and normal controls at autopsy. Specimens were dissected and cells were released by sequential enzymatic digestion (5) with 1 mg/ml pronase for one hour followed by six hours with 2 mg/ml collagenase at 37°C in DMEM supplemented with 10% heat inactivated FCS and antibiotics. Synoviocytes were then seeded at high density and allowed to grow for five days in DMEM containing 10% FCS and antibiotics before the binding assay. Twenty four hours prior to the assay, the medium was removed and the cells incubated in serum-free medium.

Direct binding experiments were performed under saturating conditions (4). Monolayer cultures of synoviocytes were washed in PBS and incubated in the binding buffer (PBS,

10 mM HEPES, 0.5% gelatin, pH 7.4) with 125 I-IL-1B (up to 250 pM) with or without 100-fold molar excess of unlabeled rhIL-1B. The data were analyzed by the LIGAND computer program.

Binding experiments were also done in the absence or presence of anti-IL-1-RtI or anti-IL-1-RtII antibodies: (0 - 40 μ g/ml). Non immune rat IgG was used as a control.

For polymerase chain reaction (PCR), 2 μ g of total RNA from confluent cultures of primary OA and normal synoviocytes was converted to single-stranded cDNA with 45 U of M-MLV reverse transcriptase. Amplification of cDNA was carried out using specific oligonucleotide primers: IL-1-RtI or IL-1-RtII.

For slot blot analysis, the total RNA was isolated from confluent cultures of primary OA and normal synoviocytes, and the experiment performed on a nylon membrane. The membranes were hybridized 32 P-labeled IL-1-RtI riboprobe. GAPDH probe was also used as a control of the RNA quantity loaded onto the slots.

Biological activity was measured on synoviocytes after being incubated with increasing concentrations of anti-IL-1-RtI, or anti-IL-1-RtII, or non immune isotype antibody 1 hr prior addition of rhIL-1B (200 pg/ml). Cells were incubated at 37°C for 24 h. Supernatants were harvested and tested for PGE₂ by a radioimmunoassay.

The statistical analysis was done using Student's 2-tailed t-test. P values less than 0.05 were considered significant.

[Results] The binding data revealed the presence of a single class of high affinity IL-1-R with no difference in the receptor affinity between OA and normal cells. The number of receptors per cell was, however, significantly higher ($p < 0.004$) in OA synoviocytes than in normal controls; 2534 ± 187 versus 1310 ± 96 . This increase in the level of IL-1-R was transient since the binding level was similar to normal controls after the OA cells were transferred into a second passage. PCR data revealed that the IL-1-RtI and IL-1-RtII are coexpressed in both OA and normal synoviocytes, although the level of IL-1-RtI mRNA was much more predominantly expressed in all samples. However, no difference in the level of IL-1-RtII mRNA was observed between OA and normal synoviocytes, as determined by slot blot analysis. A blocking antibody against the IL-1-RtI completely inhibits, in OA and normal synoviocytes, the receptor binding and the PGE₂ production stimulated with IL-1B. Treatment with the anti-IL-RtII was ineffective.

[Conclusion] These findings indicate that the IL-1-RtI is upregulated in OA synoviocytes and is responsible for mediating the biological activation of these cells by IL-1. This phenomena is probably secondary to an abnormality in the post-transcriptional regulation of IL-1-RtI gene.

Although the IL-RtII gene is (weakly) expressed in these cells, this receptor type does not seem to be responsible for any significant binding of the natural ligand or to be responsible for IL-1 biological effect.

[References]

1. Dean, D.D. (1991) *Semin. Arthritis Rheum.* **20** (Suppl 2), 2-11.
2. Pelletier, J.P., DiBattista, J.A., Roughley, P., McCollum, R. & Martel-Pelletier, J. (1993) *Osteoarthritis, Edition of Rheumatic Disease Clinics of North America* (R.W. Moskowitz, Ed.) W.B. Saunders Company Publishers, Philadelphia, pp 545-568.
3. Arend, W.P. & Dayer, J.M. (1990) *Arthritis Rheum.* **33**, 305-315.
4. Martel-Pelletier, J., McCollum, R., DiBattista, J.A., Faure, M.P., Chin, J.A., Fournier, S., et al. (1992) *Arthritis Rheum.* **35**, 530-40.
5. Martel-Pelletier, J., McCollum, R., Pelletier, J.P. (1993) *Biochim. Biophys. Acta.* **1175**, 302-305.

18P2-2

Nitric Oxide and Chondrocytes

Martin Lotz, University of California, San Diego

Osteoarthritis (OA) is the most common musculoskeletal disorder and most cases of OA develop as a function of increasing age. Age-related changes in articular cartilage include altered composition of extracellular matrix and, as a result, impaired mechanical properties. Cartilage of older individuals also shows a profound reduction in thickness and cellularity.

Our hypothesis suggests that, as a function of aging, cartilage cellularity decreases, cartilage repair mechanisms decompensate and this leads to the development of OA. Based on this concept we examine stimulators and inhibitors of chondrocyte proliferation and changes which develop during aging of human articular cartilage.

A growth factor response profile was established for primary human articular chondrocytes. The three isoforms of TGF β were the most potent stimuli. IGF, PDGF and bFGF also function as chondrocyte growth factors but are much weaker than TGF β . IL-1 inhibits chondrocyte proliferation induced by serum or TGF β and this represents the most important difference to fibroblasts where IL-1 stimulates proliferation.

Human articular chondrocytes are the major intraarticular cell type capable of inducible nitric oxide (NO) synthesis. NO synthesis and expression of mRNA for the inducible nitric oxide synthase (iNOS) are stimulated by IL-1, TNF or LPS but not by chondrocyte growth factors such as TGF β or IGF-1. NO inhibits chondrocyte proliferation when administered from exogenous sources such as sodium nitroprusside or when induced by IL-1. The antiproliferative effects of IL-1 on chondrocytes are blocked by NOS inhibitors. NO activates prostaglandin production in chondrocytes and this is a downstream event in the antiproliferative effects of IL-1 and NO. In constant to chondrocytes, fibroblasts do not produce detectable levels of NO in response to IL-1 and this difference may be one explanation for the differential growth regulatory effects of IL-1 in these two mesenchymal cell types. In addition to the inhibition of chondrocyte proliferation NO also induces DNA fragmentation and ultrastructural changes characteristic of apoptosis.

Based on these observation we suggest that the production of NO is one factor which contributes to the rapid age-related decline in chondrocyte proliferative potential and cartilage cellularity. This impairs the ability of cartilage to repair tissue injury which ultimately leads to the development of osteoarthritis.

Endochondral Bone Formation and Mineralization 17A-1

BIFUNCTIONAL ROLE OF CHONDROMODULIN-I (ChM-I) IN
ENDOCHONDRAL BONE FORMATION

Yuji Hiraki, Tatsuya Koike, Hiroyuki Inoue, Jun Kondo*, Chisa Shukunami and Fujio Suzuki
Department of Biochemistry, Osaka University Faculty of Dentistry,
Osaka 565, JAPAN. *Research Center, Mitsubishi Kasei Corporation,
Kanagawa 227, JAPAN.

Endochondral bone formation is initiated by formation of cartilaginous rudiment in which chondrocytes rapidly grow to become matrix-producing mature cells. It has been noted that a certain component(s) cartilage-matrix acts as an autocrine growth-regulator which mediates cell-matrix interactions in cartilage. Cartilage is one of the main sources of basic fibroblast growth factor (bFGF). It is known as a unique but potent autocrine factor which stimulates anchorage-independent growth, i.e. colony formation in soft agar (1). Tissue extract of cartilage, however, contains chondrocyte-colony stimulating activity which is distinct from bFGF. On the other hand, bFGF is also known as a potent angiogenesis inducer. In spite of abundant presence of bFGF, cartilage is maintained avascular until it calcifies. This phenomenon has been explained by a putative angiogenesis inhibitor in cartilage. In fact, cartilage-extract inhibited DNA synthesis and growth of vascular endothelial cells (2).

Previously we reported a cartilage-specific glycoprotein, i.e. chondromodulin-I (ChM-I), which synergistically stimulated DNA synthesis of chondrocytes in the presence of bFGF (3). We report here that ChM-I was identified as a potent colony stimulating factor for chondrocytes. Moreover we identified ChM-I as a growth inhibitor for vascular endothelial cells present in cartilage-extract.

[Methods] *Colony-formation Assay*: Growth-plate chondrocytes were isolated from the ribs of young New Zealand rabbits, as described (4). A 0.5-ml of base layer of 0.72% agarose (Sigma, Type VII) in Ham's F-12 medium supplemented with 5 % FBS, 2×10^{-7} M hydrocortisone, 60 μ g/ml transferrin (Medium A) was added to a 12-well plate. Chondrocytes (5×10^3) were suspended in 0.5-ml of 0.41% agarose in Medium A, and used as an overlay. Growth factors were dissolved in 1-5 μ l of Medium A with 0.5% bovine serum albumin and added only once on the second day after cell seeding. Cultures were maintained for 8-10 days without addition of any growth factors.

DNA Synthesis Assay of Cultured Endothelial Cells: Vascular endothelial cells were isolated from bovine carotid arteries by the

scrapping method (5). The cells (4×10^3 cells/well) were inoculated in a 96-multiwell plate, and cultured in 100 μ l MEM containing 20% FBS. When culture became subconfluent, the medium was replaced. Sample solution (1-3 μ l) was then added to the culture. After incubation for 12 hr, the cells were labeled with [3 H]thymidine (0.5 μ Ci/well) for another 4 hr. Radioactivity incorporated into DNA was counted in a scintillation spectrometer.

[Results] Rabbit growth-plate chondrocytes were suspended in purified agarose and plated in a 12-well plate. Without bFGF, none of cells grew to form a colony even after incubation in the presence of 5% FBS for 8 days, although the cells were viable. Basic FGF (0.4 ng/ml) markedly stimulated formation of chondrocyte-colonies (Fig. 1). On the other hand, ChM-I(600 ng/ml) clearly stimulated colony formation of the cells. Its activity was weaker than that of bFGF. However, in the presence of bFGF, ChM-I synergistically stimulated colony formation. On Day 4, synergistic action was already evident. After incubation for 8 days, ≈ 900 cells out 5000 formed colonies which were apparently larger than those formed by bFGF alone.

Fig. 1. Effect of ChM-I on colony formation of rabbit chondrocytes in agar.

Guanidine-extract from fetal bovine cartilage contains growth-inhibitor(s) for cultured vascular endothelial cells (2). We purified the growth-inhibitory activity from the guanidine-extract by monitoring inhibitory activity on DNA synthesis of cultured vascular endothelial cells, and identified ChM-I as an active component in the extract by amino acid sequence analysis. As shown in Fig. 2, purified ChM-I inhibited DNA synthesis of endothelial cells cultured in the presence of 20% FBS in a dose-dependent manner. The inhibitory effect was detectable at a concentration of ≈ 100 ng/ml (≈ 4 nM), and became

maximal at ≈ 1 μ g/ml (≈ 40 nM). The data indicated that ChM-I is one of the potent inhibitor for growth of endothelial cells.

Fig. 2. Effect of ChM-I on DNA synthesis of vascular endothelial cells.

[Discussion] Cartilage is known as an avascular tissue which is exceptional among tissues of mesodermal origin. Chondrocytes exhibit autonomous growth character *in vitro* which exemplified by feasibility of suspension culture and colony formation in soft agar. Autocrine growth regulation is assumed behind the autonomous growth of chondrocytes. We demonstrated that ChM-I was a potent colony stimulating factor for chondrocytes in the presence of bFGF, while it inhibited growth of vascular endothelial cells. Thus a cartilage-specific growth modulator, ChM-I, acted bifunctional depending on cell-type. Chondromodulin-I plays an important role in morphogenesis of cartilage.

[Acknowledgments] This work was supported in parts by the Mochida Memorial Foundation for Medical and Pharmaceutical Research and the Senri Life Science Foundation.

[References]

1. Kato, Y., Iwamoto, M. & Koike, T. (1987) *J. Cell. Physiol.* **133**, 491-498.
2. Takigawa, M., et al. (1988) *J. Bone Min. Metab.* **6**, 83-92.
3. Hiraki, Y., et al. (1991) *Biochem. Biophys. Res. Commun.* **175**, 971-977.
4. Shimomura, Y., Yoneda, T. & Suzuki, F. (1975) *Calcif. Tissue Res.* **19**, 179-187.
5. Hagiwara, H., et al. (1984) *Thrombosis Res.* **33**, 363-370.

17A-2

ROLE OF RETINOIC ACID AND ATP IN TERMINAL DIFFERENTIATION AND MINERALIZATION OF CARTILAGE

Irving M. Shapiro, Masashi Hatori, Cristina Teixeira, Masahiro Iwamoto* and Maurizio Pacifici

School of Dental Medicine, University of Pennsylvania, Philadelphia, PA 19104 USA, * Department of Anatomy and Cell Biology, Osaka University Faculty of Dentistry, Osaka 565 JAPAN.

In the epiphyseal growth plate, chondrocyte maturation and the subsequent mineralization of the extracellular matrix is accompanied by dramatic alterations in energy metabolism. To explore the relationship between these two events, we used retinoic acid (RA) to promote chondrocyte maturation in culture. Using this system we asked the following questions: (i) does RA treatment of cultured chondrocytes *in vitro* induce a change in energy status similar to that seen in the hypertrophic region of the maturing growth cartilage *in vivo*; (ii) do chondrocytes secrete nucleotides; (iii) does the presence of extracellular nucleotides modulate chondrocyte function? [Methods] Maturing chondrocytes were isolated from the cephalic region of Day 18 chick embryo sterna and grown for 7-14 days in monolayer until confluent; the cells were then treated with 10-300 nM RA. Immature chondrocytes from the caudal region of sternum were grown in parallel and served as control cells for the study. At the end of the culture period, cells were harvested using a rapid freezing technique. The energy status of the cells was determined using HPLC; pyruvate kinase and glycolytic enzyme activities were evaluated using biochemical techniques. In addition, we measured the lactate and adenine nucleotide content of the medium. [Results and Discussion] We found that in maturing cephalic cell cultures, RA had a rapid and profound effect on oxidative metabolism. The retinoid caused a reduction

in the energy charge ratio (ECR) and the ATP/ADP ratio, and a sharp decrease in cell ATP levels. Maximum inhibition was observed when the RA concentration was 10-35 nM. Compared with the adenine nucleotides, creatine phosphate levels were decreased to a lesser extent by RA, although there was substantial inhibition of creatine kinase activity. We expected to find a compensatory elevation in glycolytic activities; however, the lactate levels in the medium of the treated cells indicated that anaerobic glycolysis was depressed. In contrast to the cephalic chondrocytes, when caudal cell cultures were treated with RA, lactate formation was stimulated and there was minimal effects on oxidative metabolism. The unique energy state of the RA-treated chondrocytes was termed *minimal energy* state. To determine the mechanism of inhibition of glycolysis, we measured the activity of pyruvate kinase in RA-treated cephalic cells. We found that the activity of this key glycolytic enzyme was profoundly and rapidly inhibited by the retinoid (Figure 1). Since there was a profound decrease in cell adenine nucleotide levels we considered the possibility that adenine nucleotides may be required for events that lead to terminal differentiation. Analysis of nucleotide levels in the culture medium indicated that in parallel with the change in energy status there was an increase in the medium ATP, ADP and AMP values. ATP was rapidly, but incompletely, degraded into ADP and AMP. To test the hypothesis that extracellular nucleotides modulated chondrocyte function, we examined the effect of ATP on three major determinants of chondrocyte function: alkaline phosphatase activity, the cell proliferation rate and mineral deposition. For these studies, since ATP is rapidly hydrolyzed in culture, we used concentrations of ATP that were supra-physiological. We found that the nucleotide caused a slight change in cell

Figure 1

number. This effect may not be due to ATP itself, since it was mimicked by Pi. We noted that ATP regulated alkaline phosphatase activity as well as serving as a substrate for the enzyme. In addition, we observed that in the presence of ATP, the mineral formed in the culture was a very poorly crystallized apatite. Results of the investigation suggest that RA exerted a profound effect on chondrocyte metabolism and that in concert with the metabolic changes, extracellular ATP modulated selected aspects of chondrocyte function and mineral formation and maturation.

17A-3

ROLES OF PARATHYROID HORMONE AND THYROID HORMONE IN THE CONTROL OF ENDOCHONDRAL BONE FORMATION

YUKIO KATO

Department of Biochemistry, School of Dentistry, Hiroshima University, Hiroshima, 734, JAPAN

Parathyroid hormone (PTH), PTH-related peptide (PTH-rp) and thyroid hormone have various actions on cultured chondrocytes. PTH1-34 and PTH1-84 stimulated DNA synthesis in fetal chondrocytes, stimulated selectively cartilage-matrix proteoglycan synthesis in fetal and postnatal chondrocytes. Furthermore, in growth plate chondrocytes, they suppressed the increases in alkaline phosphatase activity and in type X collagen and 1 α -25-dihydroxyvitamin D3 receptor levels and abolished mineralization, all of which occurred in parallel untreated cultures in the hypertrophic stage. In contrast, the middle and carboxyl-terminal fragments of PTH tested had little effect on proteoglycan synthesis or alkaline phosphatase activity. Furthermore, PTH-receptor levels were up-regulated in the matrix-forming zone of growth plates. The stage-dependent increases in PTH/PTH-rp receptor levels may localize the hormone stimulations of DNA and proteoglycan syntheses and inhibition of precocious hypertrophy in the matrix-forming zone of growth plates. PTH also stimulated the syntheses of matrix metalloproteinases in chondrocyte cultures. The significance of the PTH stimulation of matrix proteinases remains unknown.

In cultures from resting cartilage of ribs, thyroid hormone, concanavalin A and BMP-2 acted on the permanent chondrocytes to initiate the terminal differentiation program and to synthesize alkaline phosphatase, whereas PTH1-34 suppressed the enzyme induction even in the presence of concanavalin A, the most potent stimulator of chondrocyte hypertrophy. These findings suggest that PTH/PTH-rp is involved in the inhibition of hypertrophy in the permanent cartilaginous region of the rib cartilage.

In this study, the effect of thyroid hormone on proteoglycan degradation was also examined. In growth plate chondrocyte cultures with 10% serum, thyroid hormone had a marginal effect on proteoglycan synthesis, alkaline phosphatase activity or syntheses of type II and X collagen in growth plate chondrocyte cultures. Nevertheless, thyroid hormone, at low concentrations, markedly stimulated degradation of cartilage-matrix proteoglycan. The levels of stromelysin and gelatinases in chondrocyte cultures treated with thyroid hormone were, however, similar to those in the hormone-free cultures. Furthermore, injections of thyroid hormone into rats with hypothyroidism stimulated removal of proteoglycans from the presumptive ossifying region of cartilage, resulting in the onset of endochondral bone formation. These findings suggest that thyroid hormone plays a crucial role in the control of proteoglycan metabolism in growth plates.

Acknowledgment---This study was carried out by many members of Department of Biochemistry, School of Dentistry, Hiroshima and Osaka Universities.

REFERENCES

- Suzuki, F., et al., FEBS Lett., 70, 155-158, 1976
- Kato, Y., et al., Endocrinology, 122:1991-1997, 1988
- Koike, T., et al., J. Clin. Invest. 85:626-631, 1990
- Kato, Y., et al., Endocrinology, 127:114-118, 1990
- Iwamoto, M., et al., J. Biol. Chem. 269:17245-17251, 1994

17A-4

PARATHYROID HORMONE-RELATED PEPTIDE AND ENDOCHONDRAL BONE DEVELOPMENT

Gino V. Segre, James D. Deeds, and Kaechong Lee. Endocrine Unit, Massachusetts General Hospital, Department of Medicine, Harvard Medical School, Boston, MA02114, USA.

Parathyroid hormone-related peptide (PTHrP) was originally identified as a causative agent of humoral hypercalcemia of malignancy, a common paraneoplastic syndrome. The N-terminal 1-13 portion of PTHrP has striking sequence homology with parathyroid hormone (PTH), and PTH and PTHrP have been shown to bind to and to activate a common, cloned receptor (1). Whereas PTH functions as a systemic regulator of calcium homeostasis through its action on receptors expressed in bone and kidney, PTHrP has been postulated to function mainly as a paracrine/autocrine regulator of cellular growth and differentiation, through actions mediated at least partly by activation of the common receptor it shares with PTH. This hypothesis is based first on the fact that both PTHrP and its receptor genes are expressed in a wide variety of normal fetal and postnatal tissues. Second, this hypothesis is supported by the observation that either overexpression or null mutation of the PTHrP gene in mice results in abnormal fetal development; targeted overexpression of PTHrP in keratinocytes in transgenic mice results in abnormal hair-follicle development (2), and targeted disruption of the PTHrP gene is lethal and associated with skeletal dysplasia in homozygous mutants (3).

If PTHrP acts as a paracrine/autocrine factor through the activation of common PTH/PTHrP receptors during fetal development, the expression of these two genes must be spatially and temporally correlated. In this study we first examined the localization of transcripts for both PTHrP and PTH/PTHrP receptors in endochondral bones during fetal development of rats. Then we analyzed the skeletal abnormalities of "PTHrP-less" mice morphologically and at the molecular level by comparing developing long bones of normal and mutant fetuses.

[Methods] The proliferative status of chondrocytes was analyzed by H4 histone mRNA expression (a marker for cells in the S-phase of the cell cycle), and their differentiation was analyzed by the expression of transcripts for Types II, and X collagen, PTH/PTHrP receptor, and osteopontin. Apoptosis of chondrocytes also was examined by 3'-end-labeling of DNA fragments using Biotin-dUTP (TUNEL assay).

[Results] 1. PTHrP and its receptor mRNA expression during normal endochondral bone development. Transcripts for both PTHrP and its

receptor were highly expressed in cell-type and stage-specific manners during endochondral bone formation. Since the patterns as well as the sites of expression changed over developmental stages, we first characterized the different stages of endochondral bone formation by examining the expression of chondrocyte/osteoblast differentiation/proliferation "marker" genes in order to place cells that express PTHrP and its receptor transcripts in the context of endochondral bone development. Chondrocytes in developing cartilage were orderly stratified into three layers; (i) early chondrocytes are proliferating, as evidenced by H4 histone mRNA expression, (ii) hypertrophic chondrocytes, which express Type X collagen mRNA, but do not express H4 histone mRNA, and (iii) terminal hypertrophic chondrocytes, which express osteopontin mRNA. Maturing chondrocytes reside at the border between layers (ii) and (iii), and express alkaline phosphatase mRNA. At early stages before chondrocyte maturation, PTHrP mRNA was expressed in early chondrocytes, and in focal areas of the perichondrium, while its receptor mRNA was expressed in early chondrocytes and surrounding mesenchymal cells. At later stages (after chondrocyte maturation), PTHrP was expressed in focal areas of the perichondrium and in hypertrophic chondrocytes, while its receptor mRNA was most intensely expressed in non-proliferating, maturing chondrocytes. PTH/PTHrP receptor mRNA expression dramatically decreased in hypertrophic chondrocytes as they enlarged and was not detectable in the mineralized layer, where osteopontin and PTHrP mRNAs were highly expressed. 2. Molecular analysis of abnormal endochondral formation in "PTHrP-less" mice. The orderly stratification of chondrocytes was dramatically altered in the mutant long bones. First, layer (i) was markedly shortened in the mutant throughout the time course studied (from day 15 to day 18). Second, while there was little overlap among cells in layers (i), (ii), and (iii) in normal mice, chondrocytes in mutant long bones were disordered with marked overlap among layers. The loss of orderly stratification in the mutant was more evident in the earlier fetuses. Moreover, while few hypertrophic chondrocytes in layers (ii) and (iii) were apoptotic in the normal long bones, most hypertrophic cells in the mutant long bones were apoptotic from day 15 to day 17.

[Discussion] The close temporal and spatial relationships between gene expression of PTHrP and its receptor during fetal skeletal development are consistent with the hypotheses that PTHrP acts as a paracrine/autocrine peptide factor in endochondral bone development and that a common receptor mediates both the paracrine/autocrine PTHrP signal and the endocrine PTH signal. The changing patterns of the localization of transcripts for these two genes suggest that PTHrP is likely to have roles in

the early stages that are distinct and different from its roles in later stages. This is consistent with the most obvious abnormalities in "PTHrP-less" bones, where the proliferating zone of epiphyseal cartilage is markedly shortened, and both mineralization of cartilage and replacement of cartilage by bone appeared to be accelerated (3). Since PTH/PTHrP receptor mRNA expression dramatically decreases in the mineralized layer in normal cartilage, it is likely that the activated PTH/PTHrP receptor is involved in retarding cartilage matrix mineralization. If cartilage mineralization occurs prematurely, the bone is shortened and malformed, as occurs in PTHrP-less mutants. These data, when taken together, suggest that PTHrP regulates not only the proliferation and differentiation of chondrocytes, but also cartilage mineralization and its replacement by bone.

[References]

1. Jüppner, H., et al. (1991) *Science* **254**, 1024-1026.
2. Wysolmerski, J. J., et al. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 1133-1137.
3. Karaplis, A., et al. (1994) *Genes & Development* **8**, 277-289.

Regulatory Mechanism of Expression of Cartilage-Phenotype

17P1-1

ENVIRONMENTAL CONTROL OF CHONDROCYTE DIFFERENTIATION

Peter Bruckner, Jean Farjanel*, and Kathrin Böhme*. Institute of Physiological Chemistry, Westfälische-Wilhelms-Universität, Münster, FRG, *Institut de Biologie et de Chimie des Protéines, Lyon, FRANCE, and *Department of Anatomy and Cell Biology, Harvard Medical School, Boston, USA.

Chondrocyte maturation consists of a cascade of distinct differentiation events that is initiated after chondrogenesis and eventually leads to hypertrophic cells. The process extensively occurs in cartilage undergoing endochondral bone formation in development, growth, and repair of long bones or in osteoarthritic joints. In permanent tissues, such as normal articular cartilage, chondrocyte maturation occurs to a much lesser extent and only in deep zones adjacent to subchondral bone. The stationary cellular environment as well as factors diffusing through the extracellular matrix, such as minerals, nutrients, growth factors, and hormones are thought to control chondrocyte differentiation.

In principle, the role of individual regulators can be investigated in cell culture-experiments, but the interpretation of the results is frequently complicated, if not compromised, by the choice of culture conditions. Undefined components of media (serum, tissue extracts, etc.) jeopardize the unequivocal assignment of regulatory roles to exogenously added factors.

The regulation of cell differentiation is rendered even more complex by the release of factors by the cells themselves which may or may not interfere with the action of signals added to the cultures. For example, cell viability is subject to a social control in that it depends on the presence of survival signals produced by nearby cells. In the absence of the factors, or if their receptors are not expressed, cells undergo programmed cell death as a default development. As a consequence, studies of cell-regulation must always take into account the concerted action of added regulators along with cellular products.

Finally, cellular products controlling differentiation may be diffusible molecules or matrix components. Newly synthesized matrix macromolecules exert their influence on cellular functions, their supramolecular organization being important. The state of aggregation of newly secreted matrix macromolecules depends, in turn, on the nature of the stationary environment experimentally provided. For example, maintenance of chondrocytes adhering to culture dishes leads to a loss of the cartilage phenotype.

Because of its complexity, the puzzle of environmental factors regulating chondrocyte maturation cannot be solved by a strictly reductionist approach. However, it may be possible to empirically unravel the importance of individual regulators in experimental systems that are defined in the utmost possible manner. For this reason, we have developed a serum-free cul-

ture system for chondrocytes to eliminate at least the complex, undefined, irreproducible and unnecessary cocktail of signals contained in sera. Employing this system, we have studied the influence of growth factors or hormones, and the role of the stationary extracellular environment on chondrocyte maturation in culture.

Cells were isolated from chick embryo sterna by collagenase digestion and were cultured in gels of agarose or acid-extracted and fibrillated bovine skin-collagen I. The medium was DMEM, supplemented with 1mM pyruvate, 1 mM cysteine, and 50 μ g/ml, each, of Na⁺-ascorbate and of 2-aminopropionitrile fumarate. Pepsin digested collagens and alkaline phosphatase activity extracted from the cultures served as molecular markers for different stages of maturation. In addition, the cells were morphologically characterized by light microscopy.

Cells from the cranial third of sterna from 14 to 18d-old chick embryos in agarose culture maintained the cartilage phenotype (expression of collagens II and XI) and uniformly and spontaneously underwent maturation. The latter process could be accelerated by anabolic signals (IGF-1, insulin, thyroid hormones, or serum). Markers for chondrocyte-hypertrophy (collagen X and alkaline phosphatase) were expressed after some time in culture. Addition of caudal cells prevented this development in cranial cells. This could also be effected by media conditioned by caudal cells (in part), or by a mixture of bFGF and TGF- β (entirely; 10 ng/ml, each) [1]. Both factors have been shown to be products secreted by chondrocytes.

When cells from the entire sterna were cultured in collagen I-gels under serum-free conditions, the cells dedifferentiated by morphological and biochemical criteria. They became fusiform and produced collagen I after a few days in culture. This development was not influenced by any of the above signals, but was prevented by serum. Consistently with earlier reports [2], the cells became hypertrophic chondrocytes under these conditions.

The data demonstrated that, both, diffusible signals and the nature of the experimental environment influenced chondrocyte differentiation. The precise mechanisms of the action of collagen I fibrils on chondrocytes remains to be elucidated.

[1] Böhme, K., Winterhalter, K.H., and Bruckner, P. (1994). Terminal differentiation of chondrocytes in culture is a spontaneous process and is arrested by transforming growth factor- β 2 and basic fibroblast growth factor in synergy. Submitted for publication.

[2] Gibson, G. J., Kielty, C. M., Garner, C., Schor, S. L., and Grant, M. E. 1983. Identification and partial characterization of three low-molecular-weight collagenous polypeptides synthesized by chondrocytes cultured within collagen gels in the absence and in the presence of fibronectin. *Biochem. J.* 211:417-426

CHONDROGENIC DIFFERENTIATION OF A MOUSE EC CELL-LINE, ATDC5

Chisa Shukunami, Chohei Shigeno*, Fujio Suzuki, and Yuji Hiraki. Department of Biochemistry, Osaka University Faculty of Dentistry, Osaka 565, JAPAN. *Calcium Laboratory, Department of Radiology & Nuclear Medicine, Kyoto University Faculty of Medicine, Kyoto 606, JAPAN.

Chondrogenesis is a key event in skeletal development of animals. During endochondral bone formation, undifferentiated mesenchymal cells condense to form a cartilaginous core, where chondrocytes undergo a series of changes including proliferation, maturation, and terminal differentiation prior to calcification. Actions of hormones and growth factors have been studied by simulation of these cellular events in primary culture of growth-plate chondrocytes *in vitro*. However, study of an early chondrogenesis has been hampered by a lack of a suitable chondrogenic cell-line.

Recently, Atsumi established a clonal cell-line, ATDC5, which was derived from mouse embryonal carcinoma AT805 [1]. Insulin induces chondrogenesis of the cells at a high incidence, as evidenced by formation of many cartilage-nodules. We report here that, upon stimulation by insulin, ATDC5 cells keep track of a differentiation pathway which is supposed to occur in epiphyseal growth-plate chondrocytes.

It has been known that chondrocytes respond to parathyroid hormone (PTH) through the PTH/PTHrP receptor which is indistinguishable from that expressed in bone (2,3). Although its physiological role in chondrogenesis is not fully understood, differentiation of ATDC5 cells *in vitro* clearly indicates that expression of the receptor gene is closely associated with early chondrogenesis.

[Methods] ATDC5 cells were maintained in DMEM/Ham's F12 (1/1) medium supplemented with 5% FBS, 10 μ g/ml human transferrin and 3 $\times$ 10⁻⁸ M sodium selenite. For induction of chondrogenic differentiation, bovine insulin (10 μ g/ml) was added to the above culture medium. Chondrogenesis and calcification were monitored by staining with 0.1% Alcian blue and 1% Alizarin red, respectively after fixation of cells with 95% ethanol. Expression of the marker genes in the cells was analyzed by Northern blot analysis. Quantitative analysis of mRNA levels in the cells were carried out by slot blot analysis of total cellular RNA. Hybridization probes were prepared by the random-primer method. Cell-surface binding of PTH was assessed by

the use of ¹²⁵I-labeled [Nle⁸, Nle¹⁸, Tyr³⁴] bovine PTH-(1-34) amide as described (3).

[Results] Figure 1 shows growth-curves of ATDC5 cells cultured in the presence or absence of insulin. Without insulin, the cells rapidly proliferated as characterized by a short doubling time (17 hr). The cells exhibited clear contact-inhibition of growth at confluency, while no expression of phenotype genes such as type II collagen and aggrecan genes was detectable. In contrast, insulin induced re-entry of the cells to a growing state with an interval of postconfluent growth-arrest for 3 days. This postconfluent growing stage was characterized by a longer cell doubling (48 hr). In association with postconfluent growth of the cells, Alcian blue positive cartilage-nodules appeared and differentiated chondrocytes dominated in the culture plate within 3 weeks postconfluency. Expression of type II collagen, type IX collagen, and aggrecan genes became detectable and progressively increased along with cartilage-nodule formation.

Fig. 1. Growth curves of ATDC5 cells.

The cells were inoculated at a density of 3.5 $\times$ 10⁴ cells/well in a 24-well plate, and maintained in the presence or absence of insulin (10 μ g/ml).

After cessation of growth, expression of these genes reached a maximal level. Then, hypertrophic chondrocytes appeared in close association with dramatic elevation of alkaline phosphatase activity. Expression of type X collagen gene followed. Alizarin red staining suggested that calcification of cartilage-nodules markedly progressed after 5-7 weeks of culture.

Neither expression of PTH/PTHrP receptor gene nor specific cell-surface binding of PTH was detectable in undifferentiated ATDC5 cells

grown without insulin. Upon chondrogenic differentiation by insulin, expression of PTH/PTHrP receptor gene was dramatically induced in parallel with type II collagen gene expression and slightly precedes aggrecan gene expression (Fig. 2). Appearance of specific binding sites of PTH is also associated with chondrogenic differentiation of the cells.

Fig. 2. Expression of PTH/PTHrP receptor mRNA in insulin-treated ATDC5 cells.

[Discussion] A clonal cell-line, ATDC5, differentiates into chondrocytes at a very high incidence. It offers a reproducible culture system by which we can trace and analyze a course of multiple differentiation stages, from cartilage-nodule formation to calcification, characteristic of epiphyseal growth-plate chondrocytes.

PTH and PTH related peptide (PTHrP) share a common cell-surface receptor for action. PTH stimulates synthesis and maturation of aggrecans synthesized by chondrocytes, though its well-known target organs are kidney and bone (2). Recently ES cells were targeted at the PTHrP locus. Homozygous mice carrying the mutant PTHrP allele exhibited abnormalities in epiphyseal cartilage which resulted in shortened long bones (4). The present study clearly indicates that acquisition of PTH/PTHrP signaling is closely associated with early chondrogenesis. ATDC5 cells offer a further opportunity for molecular analysis of early chondrogenesis.

[References]

1. Atsumi, T., et al. (1990) *Cell Diff. Dev.* 30, 109-116.
2. Takano, T., et al. (1985) *Endocrinol.* 116, 2536-2542.
3. Hiraki, Y., et al. (1991) *J. Bone Min. Res.* 6, 1373-1385.
4. Karaplis, A.C., et al (1994) *Gene Dev.* 8, 277-289.

17P1-3

INDUCTION AND STABILITY OF THE CHONDROCYTE PHENOTYPE IN CELLS IN CULTURE

Arthur Veis, Denise Nebgen, Junehong Chen, Hiroyuki Inoue, Stanley Ciletti and Boris Sabsay. Division of Oral Biology, Northwestern University Dental School, Chicago, Illinois, 60611, USA.

Bone morphogenetic proteins (BMPs) implanted at ectopic sites *in vivo* act by inducing uncommitted fibroblast-like cells at the implant site to enter a chondrogenic pathway ultimately leading to the formation of bone. BMPs and other factors can also induce fibroblasts to express the chondrogenic phenotype *in vitro*. We have demonstrated this chondrogenic induction using one such factor that is derived from the dentin matrix (1,2,3). Fibroblasts induced to become chondrocyte-like by the dentin "chondrogenic agent (CIA)" retain the chondrogenic phenotype for many days in culture (3). On the other hand, bovine articular chondrocytes cultured on tissue culture plastic, spontaneously "dedifferentiate" within a few days and behave morphologically and biochemically as fibroblasts (4). These observations have led to the present study in which we have investigated the nature of bovine CIA (bCIA) and the mechanism of the fibroblast ↔ chondrocyte phenotypic transitions induced by CIA or *in vitro* culture.

[Methods] Bovine CIA was extracted and purified from bovine incisors, molars, and unerupted molars by the procedure of Amar et al. (3) with some modifications. The final reverse phase HPLC purification was carried out on a Vydac 218TP54 C-18 analytical column. The chondrogenic activity of each fraction was determined using a modified neonatal rat muscle fibroblast (RMF) ³⁵S-SO₄ incorporation assay (2). The purified fractions which demonstrated chondrogenic activity were subjected to amino acid analysis, NH₂-terminal amino acid sequence analysis and laser desorption (LD) and electrospray (ESI) mass spectrometry for molecular mass determination.

RMF were cultured in the presence of bCIA or bovine BMP (a gift from Dr. Marshall Urist). The presence of mRNA for type I (COL1) and type II (COL2) collagens was determined as a function of time, by Northern analysis. Similar cultures, in which the culture plates were coated with bCIA by the procedure of Iwata et al. (5), were also examined microscopically after fixation with methanol and staining with toluidine blue.

Bovine articular cartilage chondrocytes were isolated from metacarpophalangeal joints from hooves of 18-24 mo. cattle. Slices were carefully taken from the joint surfaces to avoid contamination with synovium. Cells were recovered from the slices following successive pronase and collagenase digestion. The cells were plated in 150 cm² flasks at a density of 2x10⁶ cells/cm² in Ham's F-12 media + 10% FBS and 0.1% gentamycin. Culture was terminated at various time periods and the cells were either stained for microscopic examination or the RNA was isolated. Northern and PCR analysis were used to determine the level of various chondrocyte and fibroblast phenotypic mRNAs. The technique of differential display (6) was used to follow overall changes in gene expression during the culture period.

[Results] Isolation of bCIA. Following 0.6 N HCl demineralization a 4 M Gu.HCl extract was fractionated on Sephacryl S-100. Fractions activity in the sulfate incorporation assay were combined and subject to reverse phase HPLC. The final chromatography and activity assay, Fig. 1, identified peak 5 as the active component. Peak 2 also showed activity significantly above background. Identical chromatography of recombinant hBMP2 and TGF-β indicated that TGF-β eluted in two peaks, one coincident with peak 2 and the other immediately after peak 5. BMP2 eluted much later than peak 5. Peak 5 was therefore subjected to amino acid analysis and NH₂-terminal amino acid sequencing. The

amino acid composition was interesting in that there was no detectable Cys. The principal NH₂-terminal sequence was: Leu-Pro-Ala-Gly-Pro-Gly-Leu-Pro-. Mass spectrometry showed that Peak 5 contained molecules of mass 5,805 and 22,315. Studies of the rat incisor CIA had suggested a M_r of 6,000-10,000. Thus, it is likely that the lower molecular mass component is the bCIA. Further separation of the two components of Peak 5, to be followed by activity analysis, sequencing and mass determination are in progress.

bCIA induction of the chondrocyte phenotype in RMF. The RNA was collected from RMF plated on plastic (Day 0) and after 6 days of treatment with bCIA. Northern analysis of the mRNA, Fig. 2, showed a down-regulation of COL1 mRNA and a marked up-regulation of COL2 mRNA, clearly confirming the ability of bCIA to alter the phenotype of the RMF. The expression of the COL2 mRNA was not detected until day 5 in culture. RMF cultured in the absence of CIA maintained the fibroblast phenotype. The cells cultured on bCIA aggregated within 10 days to form nodules that stained with toluidine blue, indicating the presence of a proteoglycan rich extracellular matrix. The nodules could be maintained in culture for at least 28 days.

Chondrocyte dedifferentiation. For the first 3-4 days primary chondrocytes plated on tissue culture plastic had a rounded appearance, but after 7 days in culture the majority of the cells became elongated and fibroblast-like (Fig. 3a,b). COL1 mRNA was detectable

at low levels on day zero using Northern blots and PCR, but no COL1 was detectable on Western blots. By day 5, both mRNA and protein were abundant. Over the same period, COL2 mRNA and expressed protein decreased, however, COL2 mRNA remained at a readily detectable level up to 15 days. A striking change took place in cell actin levels as well (Fig. 4). Actin mRNA increased dramatically as collagen II mRNA decreased. In fact, actin, fibronectin and glyceraldehyde-3-phosphate dehydrogenase mRNA

transcription were all up-regulated by day 1 in culture. Differential display, comparing days 0 and 15 in culture led to the identification of a number of up and down regulated mRNAs. Three of the sharply up-regulated mRNAs (as cDNAs) were selected for sequencing. One proved to be ribosomal protein S-27. Northern analysis also showed its up-regulation to be time dependent and strongly evident by day 1 in culture. The second was identified as a bovine mitochondrial gene, while the third sequence was not in the gene bank.

Fig. 1. HPLC isolation of CIA, and assay of fractions.

Fig. 2. Northern analysis RMF + CIA, Days 0 & 6.

Fig. 3. Cultured Chondrocytes. A, Day 0. B, Day 7

Fig. 4. Chondrocytes, Northern Analysis

[Discussion] As previously reported (3) dentin matrix contains a low molecular weight protein (CIA) which can induce fibroblast-like cells in culture to shift to express secreted proteins phenotypic of chondrocytes. The composition, partial NH₂-terminal sequence and molecular mass data, as well as the chromatographic properties, all indicate that bCIA is not a member of the BMP superfamily. The cloning and characterization of the CIA is a priority of the laboratory.

As seen in *in vivo* studies (7) of bone induction by BMP, the fibroblast → chondrocyte transition must be the result of a series of steps. The data presented above indicate that the appearance of the chondrocyte-specific phenotypic products (e.g. COL2) is a late event. Some clues as to the early steps were provided by examination of the reverse process, the chondrocyte → fibroblast transition. Most significant were the up-regulation of actin, fibronectin and GAPDH by day 1, while production of COL2 was still high and COL1 was not expressed. Ribosomal protein S-27 was also upregulated prior to COL1 gene expression. These data suggest that changes in cell shape and cytoskeleton, in the mix of ribosomal proteins regulating translation, and in the amounts of enzymes involved in oxidative metabolism must all be established before the "dedifferentiated" chondrocyte can support the synthesis and secretion of products phenotypic of fibroblasts. The inverse series of stages must also apply to the induction of the chondrocyte phenotype in the RMF, and probably accounts for the five day period before COL2 gene expression was detected. The important question then is how the CIA interaction with the RMF cell, or chondrocyte interaction with the surface of a plastic culture dish, can so profoundly alter the cell housekeeping protein system? Is there a master gene, such as that proposed for the myoD system (8,9), which determines the cell phenotype? The fact that chondrocytes induced by CIA and cultured in the presence of CIA maintain their chondrogenic phenotype for a long period suggests that this may be the case. Present efforts to examine this hypothesis focus on the very early period of interaction between RMF and CIA.

[Acknowledgments] All of the above work has been supported by grants DE08525 and DE01374 from the National Institute of Dental Research, grant AR13921 from the National Institute of Arthritis, Metabolic and Skin Diseases, and Fellowship support from NIH-NIDR T32 DE07201 and the American Association of Oral and Maxillofacial Surgeons.

[References]

1. Veis, A., Sires, B. & Clohisey, J. (1989) Connective Tissue Research **23**, 137-144.
2. Veis, A., Sires, B., Clohisey, J., Sabsay, B. & Amar, S. (1990) Biomaterials **11**, 16-18.
3. Amar, S., Sires, B., Sabsay, B., Clohisey, J. and Veis, A. (1991) J. Biol. Chem. **266**, 8609-8618.
4. Benya, P.D. & Brown, P.D. (1986) In Articular Cartilage Biochemistry, (K.E. Kuettner, R. Schleyerbach and V. C. Hascall, Ed.) pp 219-233. Raven Press, New York.
5. Iwata, H., Satomi, O., Sato, K., Sato, T. and Kawamura, M. (1993) Clinical Orthoped. **296**, 295-300.
6. Liang, P. and Pardee, A.B. (1992) Science **257**, 967-971.
7. Reddi, A.H. In Biochemistry of Collagen, (G.N. Ramachandran & A.H. Reddi, Eds.) pp 449-478, Plenum Press, New York.
8. Weintraub, H. (1989) Proc. Natl. Acad. Sci. USA. **86**, 5434-5438.
9. Choi, J., Costa, M.L., Mermelstein, C.S., Chagas, C., Holtzer, S. & Holtzer, H. (1990) Proc. Natl. Acad. Sci. USA. **87**, 7988-7992.

17P2-1

MODULATION OF SKELETAL CELL PROLIFERATION AND DIFFERENTIATION BY RETROVIRUSES

P. Günter Strauss, Cornelia Speth, Wolfgang Gimbel, Jörg Schmidt, and Volker Erfle.
GSF-Institut für Molekulare Virologie, Neuherberg, GERMANY

A variety of retroviruses closely related to mouse endogenous ecotropic retroviruses have been examined for their effects on the skeleton of mice. In contrast to the osteosarcoma viruses FBR and FBJ, which carry a cellular homologue of the c-fos oncogene, murine leukemia-type retroviruses do not carry a cell derived oncogene homologue and do not cause osteogenic tumors. However, infection of newborn mice with some of the murine leukemia-type retroviruses leads to a massive distortion of the normal bone development (1,2). *In vitro*, osteogenic differentiation of the chondroprogenitor cells of mandibular condyles can be enforced by infection with osteopathogenic murine leukemia virus-type retroviruses (3). Together the data suggest that these retroviruses can influence the cell's phenotype by directly or indirectly interfering with the osteogenic differentiation pathway. One possibility is that the activity of the retroviral promoter and the retroviral genes may modulate the expression of cellular genes. The studies on the structure of osteopathogenic murine leukemia virus-type retroviruses and their interaction with cellular gene expression is expected to improve the understanding of the mechanisms by which regulatory molecules affect the physiology of osteogenic cells and subsequently influence the formation of the skeleton.

Methods

Newborn mice of the NMRI and CBA/Ca strains were intraperitoneally injected with infectious virus particles from cell free supernatants. Osteoma and osteopetrosis were diagnosed by X-ray analysis and histological examination. The molecular cloning of the RFB has been described elsewhere (2). The sequence analysis of the RFB was performed by standard methods.

The basic method of Differential Display PCR has been described previously (4). The method was modified in using an anchor primer sequence related to the loop region of the murine HLH-type gene myoD and primer sequences homologous to the murine Id gene in combination with random primers. RNAs from different time points of tissue cultures and cell cultures were reverse transcribed to cDNA. Differentially expressed RNAs were displayed as PCR products on nondenaturing polyacrylamid gels. Northern-blot and slot-blot analysis were performed by standard methods.

Results

The most potent of osteopathogenic murine leukemia virus-type retroviruses, the RFB (Reilly-Finkel-Biskis) virus, was injected into newborn CBA/Ca and NMRI mice. RFB

induced multiple osteomas in 100% of the CBA/Ca mice. In NMRI mice, RFB induced osteopetrosis and malignant lymphoma in each 100% of the animals, and osteoma in 60% of the animals. The RFB provirus was molecularly cloned and completely sequenced for structural analysis. In comparison to murine leukemia viruses that are not known to influence the skeleton, amino acid sequence differences were found clustered in distinct regions of the genes for the group specific antigen, the polymerase and the envelope. The biological relevance of these sequence differences is presently under investigation.

The effect of the RFB on the expression of cellular genes was tested using a modification of the previously published method of differential display PCR (4). The primers used in the reactions were designed to detect modulated expression of RNA sequences with homology to helix-loop-helix (HLH) - type transcription factors. Expression of HLH-related sequences was investigated during osteogenic differentiation in mandibular condyles and in cultures of progenitor cells derived from mandibular condyles. The results showed that sequences related to HLH genes are temporarily expressed, and that the pattern of expression is different in RFB-infected cultures from that in uninfected cultures. A 3 kb transcript, which showed increasing expression levels during osteogenic differentiation of mandibular condyles has been partially cloned and sequenced.

Discussion

Chondrogenic cells from mandibular condyles and xiphoids from newborn mice can be "reprogrammed" during *in vitro* cultivation and are able to differentiate to the osteogenic or adipogenic lineage, respectively (5, 6, 7). The cause underlying this switch of the lineage program is not yet understood, however, it illustrates the versatility of the lineages of skeletal cell types. In mandibular condyles, the switch to the osteogenic lineage is further enforced by infection with RFB. After infection of the host cell, the proviral DNA becomes an integral part of the host genome. The activity of the proviral promoter (LTR, long terminal repeat) and/or proviral genes changes the normal physiology of the infected cells. This results in alterations of the normal skeletal growth, materializing in osteomas and osteopetrosis. The retrovirus-induced change of cell physiology is, at least in part, due to modulation of the expression of cellular genes. The results suggest that, in infected osteogenic cells, RFB modulates the expression of regulatory genes including transcription factors of the helix-loop-helix type. HLH genes are known to direct the differentiation of myogenic cells to myoblasts. It is intriguing to speculate that HLH-type transcription factors may also play a central role in osteogenic differentiation. Therefore, the *in vitro* and *in vivo* data indicate that the RFB retrovirus influences the basic lineage program of the infected skeletal cell.

Acknowledgement

This work was partly supported by the Deutsche Forschungsgemeinschaft (DFG grant STR 226).

References

1. Pedersen, L., P.G. Strauss, J. Schmidt, A. Luz, V. Erfle, P. Jørgensen, N.O. Kjeldgaard, and F.S. Pedersen. Pathogenicity of BALB/c derived N-tropic murine leukemia viruses. *Virology* 179, 931-935, 1990
2. Pedersen, L., Behnisch, W., Schmidt, J., Luz, A., Pedersen, F.S., Erfle, V., Strauss, P. G. Molecular cloning of osteoma-inducing replication-competent murine leukemia viruses from the RFB osteoma virus stock. *J.Virol.* 66, 6186-6190, 1992
3. Schmidt, J., M. Casser-Bette, A. B. Murray, A. Luz, and V. Erfle. Retrovirus-induced osteopetrosis in mice. Effects of viral infection on osteogenic differentiation in skeletoblast cell cultures. *Am.J.Pathol.* 129:503 - 510, 1987.
4. Liang, P. and A. B. Pardee. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 257, 967-971, 1992.
5. Strauss, P.G., E.I. Closs, J. Schmidt, and V. Erfle. Gene expression during osteogenic differentiation in mandibular condyles *in vitro*. *J. Cell Biol.* 110, 1369-1378, 1990.
6. Closs, E.I., A.B. Murray, J. Schmidt, A. Schöna, V. Erfle, and P.G. Strauss. C-fos expression precedes osteogenic differentiation of cartilage cells *in vitro*. *J. Cell Biol.* 111, 1313-1323, 1990.
7. Hoermeier, K, Strauss, P. G., Erfle, V. and Schmidt, J. Adipose differentiation of cartilage *in vitro*. *Differentiation* 56, 45-53, 1994

17P2-2

EXPRESSION OF FOS AND JUN FAMILY PROTEINS IN CULTURED CHICKEN CHONDROCYTES.

Hideo Iba, Takashi Kameda, Martha Harumi Sonobe, Masao Murakami and Hirotaka Watanabe.
Department of Tumor Virus Research, Institute of Medical Science, University of Tokyo, Tokyo, 108 JAPAN

The *v-fos* gene was first isolated in the genome of FBJ-MuSV, which causes osteosarcoma in mouse. Its cellular counterpart, *c-fos* and its related genes form a gene family. Their gene products and Jun proteins are the components of transcriptional factor AP-1 and believed to be involved in cellular growth, differentiation and malignant transformation.

While *c-fos* as well as other *fos* family members is immediate early gene, which is transiently inducible by several extracellular stimuli in a variety of cell types, it is noteworthy that *c-fos* gene is reported to be constitutively expressed in fetal cartilage¹ or hypertrophic chondrocytes². To understand the biological function of AP-1 in chondrocytes, we have here analyzed the expression levels of all the components of AP-1 in chicken chondrocytes. We also designed and constructed some transdominant mutants of Fos³ or Jun⁴ and further tested whether they can function as "a general inhibitor of AP-1", considering the future application to chondrocytes.

[Methods] Chondrocytes were isolated from the caudal or cephalic portion of sterna of 18 days old chicken embryo as described previously⁵ with slight modifications. Cell cultures were pulse-labeled with ³⁵S-methionine for 1 hour and cell lysates were prepared in denaturing conditions as described previously³. Immunoprecipitation was performed using anti-Fos pep1 (cross-reactive to all the Fos family proteins)⁶, anti-Fra-2 pep2 (Fra-2 specific)⁶ and anti-Jun antiserum (cross reactive to all the Jun family proteins). Replication-competent avian retrovirus vectors^{3,6}, infection protocols and colony formation assays were described previously^{3,4}.

Fig. 1. Structure of the transdominant mutants.

1.0 indicates no suppression at all, while 0 indicates the complete suppression.

[Results and Discussion] Chicken chondrocytes that were prepared from either caudal or cephalic portion of sterna were kept in monolayer cultures. They were pulse-labeled with ³⁵S-methionine at various cultural stages (4, 11 and 18 days after preparation) and disrupted in denaturing conditions to prepare cell lysates for immunoprecipitation. In all the cultures, we have detected a significant level of c-Fos expression, which apparent molecular weight was lower than that of highly phosphorylated forms of c-Fos (62K) that is transiently detectable in growth-stimulated CEF⁶. This result is quite different from CEF, where c-Fos expression is detectable neither in logarithmically growing nor in resting cells⁶.

We also detected moderate levels of expression of Fra-2 which formed broad band of 40-47K. This migration pattern represents lower phosphorylation forms compared with Fra-2 (46-47K) detected in growth-induced CEF⁶. As for Jun proteins, high level expression of c-Jun as well as low level expression of JunD were detected in all the cultures.

For the analysis of the biological function of constitutively expressed Fos or other components of AP-1 in chondrocytes, development of a general inhibitor of AP-1 would be useful. For

this purpose, we have designed two putative transdominant mutants (Fig.1). supFos-1³ is derived from v-Fos and in vitro experiments have showed that it has full activity to form complex with c-Jun while the formed complex has completely lost DNA binding activity because of the insertion between the basic resion and the leucine zipper. c-Jun was deleted in its transforming region to generate another mutant, supJun-1⁴. It can, however, form dimers with Fos or Jun proteins and the resultant dimers retain full DNA binding activity.

Using retrovirus vectors, either of these mutants were introduced into CEF, which were subsequently superinfected with *c-jun*, *c-fos*, or *fra-2*-carrying viruses. As judged from colony forming activity in soft agar (Table 1), expression of either supFos-1 or supJun-1 strongly suppressed the transformation by these oncogenes. This result indicate that both of the mutant functioned as transdominant inhibitors of AP-1-mediated transcriptional regulation. To introduce these mutants into chondrocytes, we are now in the process of screening the conditions for efficient infection to these cells.

[Acknowledgements] We thank Dr. Y. Kato and M. Iwamoto for introducing the preparation protocols for chicken chondrocytes. This work was partly supported by Grants-in-aid from Ministry of Education, Science and Culture of Japan.

[References]

1. Dony, C., & Gruss, P. (1987) Nature 328,711-714.
2. Smeyne, R. J., Schilling K., Robertson, L., Luk D., Oberdick J., Curran, T., & Morgan, J. I. (1992) Neuron 8, 13-23
3. Okuno, H., Suzuki, T., Yoshida, T., Hashimoto, Y., Curran, T., & Iba, H. (1991) Oncogene 6, 1491-1497.
4. Suzuki, T., Murakami, M., Onai, N., Fukuda, E., Hashimoto, Y., Sonobe, M. H., Kameda, T., Ichinose, M., Kazumasa, M., & Iba, H., (1994) J. Virol 68, 3527-3535.
5. Iwamoto, M., Golden, E. B., Adams, S. L., Noji, S., & Pacifici, M. (1993) Exp. Cell Res. 205, 213-224.
6. Nishina, H., Sato, H., Suzuki, T., Sato, M., & Iba, H. (1990) Proc. Natl. Acad. Sci. 87, 3619-3623

17P2-3

HOMEBOX-CONTAINING GENES AND GROWTH FACTORS IN REGULATION OF LIMB OUTGROWTH, PATTERN FORMATION, AND CARTILAGE DIFFERENTIATION

Robert A. Koshier, Caroline N. Dealy, and William B. Upholt. Department of Anatomy, University of Connecticut Health Center, Farmington, Connecticut 06030, USA.

The outgrowth, patterning, and differentiation of the cartilaginous skeletal elements of the developing vertebrate limb involves a complex series of regulatory interactions among various homeobox-containing (hox) genes, growth factors, and other secreted signalling molecules. The apical ectodermal ridge (AER) at the distal tip of the limb bud maintains the mesenchymal cells directly subjacent to it in a rapidly proliferating, undifferentiated state. The roles and relationships among the hox genes *Msx-1*, *Msx-2*, and *Dlx-5*, and the growth factors IGF-I, insulin, and FGFs in the AER/mesoderm interactions required for limb outgrowth and patterning will be discussed. When the subridge mesenchymal cells of the developing limb bud leave the influence of the AER, those located in the proximal central core of the limb bud initiate cartilage differentiation. The onset of chondrogenesis is characterized by a transient cellular condensation process during which intimate cell-cell and/or cell-matrix interactions occur that trigger chondrogenesis and the expression of genes for cartilage-specific matrix proteins including type II collagen, aggrecan, and type IX collagen. The roles of TGF-βs, BMPs, and the integral membrane heparan sulfate proteoglycan syndecan-3 in regulating the onset of condensation and cartilage differentiation will be discussed. Studies on the possible role of the hox gene *Dlx-5*, a member of the *Distal-less* family, in the regulation of cartilage-specific gene expression will be presented.

18A-1
ROLE OF TIMPs AS GROWTH FACTORS IN THE
PROLIFERATION OF ARTICULAR CHONDROCYTES

Kyoko Yamashita, Akira Shinagawa* and Taro Hayakawa. Department of Biochemistry, School of Dentistry, Aichi-Gakuin University, Nagoya 464, JAPAN and * Research Laboratories 1, Fuji Chemical Industries, Ltd., Toyama 933, JAPAN.

It has been well recognized that various tissue explants and cells synthesize and release matrix metalloproteinases (MMPs) and their intrinsic inhibitors called tissue inhibitors of metalloproteinases, TIMP-1 and TIMP-2. The ability of these TIMPs to inhibit MMPs suggest that they play an important role in the regulation of MMP activity, effectively controlling the amount of connective-tissue breakdown.

A number of reports have shown that cartilage contains collagenase inhibitors, and it has been suggested that these inhibitors could be responsible for the resistance of cartilage to vascularization and tumor invasion.

We recently reported that both TIMP-1 and -2 have potent cell growth-promoting activity for a wide range of cells (1, 2) and that this activity is independent of their MMP inhibitory activity. In this paper, we report the growth-promoting activity of both TIMPs toward bovine articular chondrocytes (BAC) and discuss their mechanism of action.

[Methods] Human recombinant TIMPs were expressed in the CHO-K1 cell line and purified as described (2). BAC were isolated from slices of bovine ankle articular cartilage as reported earlier (3). TIMP-1 and TIMP-2 were determined by the corresponding one-step sandwich enzyme immunoassay (4, 5). TIMP-1- and TIMP-2-free FCS were prepared by the passage of FCS through anti-TIMP-1 (6) and anti-TIMP-2 (5) monoclonal antibody-affinity columns, respectively. Stationary phase BAC were incubated at 37°C in D-MEM containing various amounts of either TIMP. Following incubation for various lengths of time, 0.8 µCi/ml of [³H]thymidine (³H-TdR) was added, and the cells were further incubated for 5 hrs as described (2).

[Results] Both TIMP-1 and TIMP-2 contents in bovine articular cartilage were found to be 51.1 ± 29.0 and 56.5 ± 16.8 ng/g wet tissue

(mean ± SD, n=6), respectively. BAC at the stationary phase and incubated for 48 hrs at 37°C secreted both TIMP-1 (37.0 ± 6.6 ng/ml, mean ± SD, n=3) and TIMP-2 (36.3 ± 4.0 ng/ml) into their culture medium.

BAC were not able to grow in D-MEM alone. However, they grew significantly in D-MEM supplemented with either TIMP-1 or TIMP-2, with the maximal effect being at 500 ng/ml of TIMP-1 and 50 ng/ml of TIMP-2 (Table 1). We examined also the dose-dependent stimulation of DNA synthesis following the addition of either TIMP-1 or TIMP-2 to BAC at confluence, and obtained the maximal stimulation at either 50 ng/ml of TIMP-1 or 1.0 ng/ml of TIMP-2. The cells showed a significant increase in ³H-TdR incorporation at as early as 3 hrs after the addition of either TIMP, and the incorporation was maximal at 20 hrs. Either TIMP-1- or TIMP-2-dependent stimulation of DNA synthesis was dose-dependently suppressed in the presence of tyrosine kinase inhibitors such as genistein and herbimycin A, but not in the presence of protein kinase inhibitors such as H-7 and K-252 b (Fig. 1). The proliferation of BAC was markedly suppressed in medium containing either TIMP-1- or TIMP-2-free FCS. The growth was, however, significantly restored by the addition of TIMPs.

[Discussion] ³H-TdR incorporation study suggested that both TIMPs seemed to directly stimulate the DNA synthesis of chondrocytes as new mitogens via the participation of receptor(s) possibly having tyrosine kinase activity. As it has been found that both TIMPs are constitutive components of human serum and FCS (2, 5), cell proliferation in non-vascular tissues such as articular cartilage seems to be more dependent on both TIMPs that are produced by the cartilage cells themselves.

[References]

1. Hayakawa, T., et al. (1992) FEBS Lett. 298, 29-32.
2. Hayakawa, T., et al. (1994) J. Cell Sci., in press.
3. Yasui, T., et al. (1992) Biomed. Res. 13, 343-348.
4. Kodama, S., et al. (1990) J. Immunol. Meth. 127, 103-108.
5. Fujimoto, N, et al. (1993) Clin. Chim. Acta 220, 31-45.
6. Kodama, S. et al. (1987) Collagen Rel. Res. 7, 341-350.

Table 1. Effect of the addition of different concentrations of TIMP-1 and TIMP-2 on the proliferation of bovine articular chondrocytes in serum-free basal medium

	concentration (ng/ml)	DNA content (ng/dish)	% of control
day 0		165	7.3
D-MEM alone		218 ± 9	9.6 ± 0.4
+ 10 % FCS		2268 ± 196	100
+ TIMP-1	5	1265 ± 68	55.8 ± 3.0
	50	1343 ± 54	59.2 ± 2.4
	100	1400 ± 100	61.7 ± 4.4
	500	1600 ± 99	70.6 ± 4.4
	1000	1234 ± 97	54.4 ± 4.3
+ TIMP-2	1	1261 ± 62	55.6 ± 2.7
	5	1241 ± 83	54.7 ± 3.7
	10	1373 ± 51	60.5 ± 2.2
	50	1509 ± 75	66.5 ± 3.3
	100	1295 ± 58	57.1 ± 2.6

DNA content was determined at day 4. Results are the means of three experiments, along with SD.

Fig. 1. Effect of protein kinase inhibitors on TIMP-1- or TIMP-2-dependent stimulation of [³H]thymidine incorporation into bovine articular chondrocytes

THE ROLE OF HGF/SF IN CARTILAGE DEVELOPMENT

Masahiro Iwamoto¹, Akitoshi Jikko¹, Toshiaki Takebayashi², Motomi Enomoto-Iwamoto³, Kunio Matsumoto⁴, Toshikazu Nakamura⁴, Kojiro Kuriu and Sumihare Noji⁵. Departments of Anatomy & Cell Biology, ¹Radiology and ³Biochemistry, Osaka University Faculty of Dentistry, Osaka, ²Okayama University Dental School, Okayama, ⁴Division of Biochemistry, Biomedical Research Center, Osaka University Medical School and ⁵Department of Bioengineering, Tokushima University Faculty of Engineering, Tokushima, JAPAN

Hepatocyte growth factor/ scatter factor (HGF/SF) is a multifunctional growth factor which promotes proliferation, motility, and morphogenesis of epithelial cells (1). Recently a HGF receptor, the c-met proto-oncogene product, has been reported to be expressed in developing limb buds (2), indicating that HGF/SF may play a role in limb development. In this study, we investigated the possibility that HGF/SF is involved in the early skeletogenesis. We found that chondrocytes expressed a HGF/SF receptor and that HGF/SF stimulated the motility, proliferation, and proteoglycan synthesis in chondrocytes. Further, in situ hybridization analysis showed that HGF/SF mRNA was restrictively expressed in the areas of the future joint regions in developing limb buds and in intercostal spaces near the tips of developing costal cartilages. These findings suggest that HGF/SF plays important roles in cartilage development by its multiple stimulatory actions on motility, proliferation and maturation of chondrocytes.

[Materials and Methods]

Cells and cell culture: Chondrocytes were isolated from 4-week-old New Zealand white rabbits or 4-week-old Wistar rats. Synovial fibroblasts were isolated from synovial tissue of the knee joints. Embryonic mesenchymal cells were isolated from the limb bud muscle tissue of 20-day rat embryos. Cells were maintained in DMEM supplemented with 10 % FBS, 60 µg/ml of kanamycin and fed every other day.

Measurement of DNA and proteoglycan synthesis: The rate of DNA synthesis was estimated by measuring the incorporation of [³H]-thymidine into 5 % trichloroacetic acid-insoluble cell precipitate. Proteoglycan synthesis was determined by measuring incorporation of [³⁵S]-sulfate into material precipitated with cetylpyridinium chloride after protease digestion.

In situ hybridization: A 1.4 kb EcoRI fragment of a rat HGF/SF cDNA (RBC1 clone) was subcloned into the pGEM7 vector to synthesize both antisense and sense run-off transcripts labeled with [α -³⁵S]UTP (3). In situ hybridization was performed as described previously (3).

[RESULT S]

HGF/SF has mitogenic and motogenic activities on chondrocytes, but not on other mesenchymal cells.

We added HGF/SF exogenously in various cell cultures and observed changes in cell morphology. A typical culture of HGF/SF-treated articular chondrocytes is shown in Fig. 1. Without HGF/SF, polygonal chondrocytes proliferated and formed clusters (Fig 1 Left), while no such clusters were observed in the presence of HGF/SF (Fig.1 Right). In contrast to chondrocytes, HGF/SF scattered neither fibroblasts nor synovial cells.

Fig.1 HGF/SF scatters cultured articular chondrocytes.

Control

HGF/SF 3 ng/ml

To examine the effects of HGF/SF on proliferation of chondrocytes, the confluent cultures were serum-starved for 24 h and treated with various doses of HGF/SF. A dose-dependent increase in [³H]-thymidine incorporation into DNA was observed in chondrocytes isolated from 4-week old rabbits and 23-day rabbit embryos. A maximum three-fold stimulation of DNA synthesis was observed in the presence of 1 ng/ml HGF/SF, while fibroblasts showed no response. Treatment with HGF (10 ng/ml) also increased cell number of articular chondrocytes about 1.8-fold. Further, HGF/SF stimulated [³⁵S]-sulfate incorporation in chondrocytes in a dose dependent manner with a maximal stimulation at 1 ng/ml.

Expression of the HGF/SF receptor gene (c-met) in chondrocytes.

The expression of c-met in chondrocytes *in vivo* and *in vitro* was examined by RT-PCR. Articular and rib cartilages were dissected out from 4-week old rats and total RNA was extracted. The extracted RNA was reverse transcribed, and amplified using c-met primers. We detected trace amounts of c-met expression in both articular and rib cartilages after 40 cycles of PCR amplifications. In contrast, significant expression of c-met gene were detected in cultured chondrocytes after 35 cycles of PCR amplifications, indicating that cultured chondrocytes expressed higher levels of c-met mRNA than cartilage *in vivo*.

Expression of HGF/SF mRNA in the developing limb.

We examined expression of HGF/SF mRNA by in situ hybridization analysis. In 11-day embryos, diffuse expression of HGF/SF mRNA was detected around the proximal region of the limb. At this stage, no cartilaginous condensation occurred in the limb. As cartilaginous condensation proceeded, the expression of the HGF/SF gene became more restricted as shown in Fig. 2. At 12.5-14-days p.c.,

when stylopodial, zeugopodial and autopodial elements are formed, expression of the HGF/SF gene was observed at the future wrist/ankle and elbow/knee joint regions (Fig. 2). At later stage (16-days p. c.), HGF/SF transcripts were detected in the restricted mesenchymal cells adjacent to the cartilaginous condensations at the same wrist/ankle and elbow/knee regions. The level of HGF/SF gene expression in the limb decreased with development. Throughout the experiment, no significant signals for HGF/SF mRNA were detected in the hand or foot plates. No positive hybridization signal was detected in the precartilaginous condensations.

Fig. 2 Expression of HGF/SF in the developing limb. Sagittal sections of the hind limb of 14-days p.c. mouse embryos were hybridized with a ³⁵S-labeled rat HGF/SF riboprobe. HGF/SF was expressed in the future knee and ankle joints.

[Discussion]

It is generally accepted that mesenchymal cells are sources of HGF/SF, but that they are not its target cells. The results of the present study, however, clearly demonstrated that chondrocytes express HGF/SF receptor and have the ability to respond to this factor. HGF/SF stimulates both proliferation and proteoglycan synthesis in chondrocytes. HGF/SF also functions as a motogen in these cells. No growth factors have been previously identified which modulate migration, proliferation, and proteoglycan synthesis by chondrocytes. In this study, HGF/SF was shown to be a unique multifunctional factor for chondrocytes and is likely to be involved in chondrogenesis or chondrocyte differentiation. Further, the expression pattern of the HGF/SF gene in the developing limb and costal regions suggests physiological roles of HGF/SF in the control of skeletogenesis.

[Acknowledgements] This work was supported by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan and by supports from Chugai Pharmaceutical Co. LTD., and Sumitomo Metal Industries, LTD.

[References]

1. Matsumoto, K. And Nakamura, T. (1992) *Oncogene* **3**, 27-54.
2. Sonnenberg, E. D., et al. (1993) *J. Cell Biol.* **123**, 223-235.
3. Noji S., et al. (1990) *Biochem. Biophys. Res. Comm.* **173**, 42-47.

18A-3

INTERDEPENDENT REGULATION OF CHONDROCYTE
DIFFERENTIATION BY TGF- β AND VITAMIN D₃ METABOLITES

Zvi Schwartz, David Dean*, and Barbara Boyan*. Hebrew Univ., Jerusalem, Israel 91-010; *The Univ. of Texas Health Science Center, San Antonio, Texas 78284 USA

Introduction

Transforming growth factor- β (TGF- β) has been shown to modulate chondrocyte metabolism (1), but its precise role in chondrogenic differentiation is still uncertain due to the fact that different assay systems and cell types have been used. Some possibilities that need to be considered in reconciling the prior studies include the fact that chondrocytes of varying stages of maturation were used (2,3), that TGF- β has direct effects on chondrocyte differentiation (4), and that TGF- β is produced by these cells and released in latent form and then activated in the matrix (5).

The active metabolites of vitamin D, 1,25-(OH)₂D₃ (1,25) and 24,25-(OH)₂D₃ (24,25), also play a critical role in chondrocyte metabolism (6). Boyan et al. (7) have shown that chondrocytes at distinct stages of endochondral maturation respond differentially to vitamin D₃ metabolites. Vitamin D₃ metabolites are also produced by the chondrocytes, themselves, in response to various hormones and growth factors (8).

The combination of TGF- β with other growth factors, hormones, or cytokines probably changes the net response of the target cell. Recently, we observed that 1,25 and TGF- β synergistically enhanced alkaline phosphatase activity in osteoblasts *in vitro* (9). The present study was designed to evaluate the combined effect of vitamin D₃ metabolites and TGF- β on chondrocyte differentiation. Moreover, this study examined the regulation of latent TGF- β (LTGF- β) production by 1,25 and 24,25, as well as the regulation of vitamin D₃ metabolite production by active TGF- β .

Methods

Chondrocytes were isolated from the resting zone (RC) and growth zone (GC) of rat costochondral cartilage (10). Cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 1% antibiotics, and 50 μ g/ml ascorbic acid. Fourth passage cells were used in all experiments, since previous studies had shown retention of phenotype at this passage. Recombinant human TGF- β 1 (0.03-0.88 ng/ml), 1,25 (10⁻¹⁰-10⁻⁸M), and 24,25 (10⁻⁹-10⁻⁷M) were added to confluent cultures for 24 hours. Alkaline phosphatase specific activity (ALPase) was measured in the cell layer. In some experiments, addition of both TGF- β and vitamin D₃ metabolites to the cultures was examined at 3, 6, 12, 24, and 48 hours. For assay of [³H]-thymidine incorporation, cells were made quiescent and the appropriate concentration of hormone or vehicle added. After 20 hours, 1 μ Ci/ml of [³H]-thymidine was added and radiolabel incorporation into DNA measured four hours later.

The release of latent and active TGF- β by chondrocytes treated with 1,25 or 24,25 was examined. Conditioned media were assayed for active and latent TGF- β content by measuring growth factor-dependent increases in ALPase of ROS 17/2.8 osteoblast-like cells before and after acid activation. The specificity of this assay was confirmed with pan-neutralizing antibodies to TGF- β . The synthesis of 1,25 and 24,25 from [³H]-25-(OH)₂D₃ by cultures treated with TGF- β was measured by a previously described HPLC technique using radioisotope detection (8).

Results

Addition of 1,25 to GC cells resulted in a dose-dependent increase in ALPase, while TGF- β stimulated ALPase in a biphasic manner at 0.06-0.22 ng/ml of TGF- β . Addition of both 1,25 and TGF- β increased ALPase to a level similar to that observed with TGF- β alone. These effects were also observed after treatment for 3 to 48 h. The addition of 24,25 to GC cells had no effect on ALPase; the effect of TGF- β in combination with 24,25 was identical to that observed with TGF- β alone.

1,25 dose-dependently inhibited [³H]-thymidine incorporation by GC cells, while TGF- β produced a dose-dependent increase in [³H]-thymidine incorporation. The addition of both 1,25 and TGF- β significantly inhibited [³H]-thymidine incorporation at low TGF- β concentrations (0.03-0.11 ng/ml).

No change in ALPase was detected in RC cell layers after incubation with either 1,25 or 24,25; however, biphasic stimulation of ALPase was observed after addition of 0.06-0.22 ng/ml TGF- β . The combination of 24,25 and TGF- β significantly increased ALPase to more than twice that found with TGF- β alone. These effects were also observed after treatment for 3 to 48 h.

The effect of TGF- β and 24,25 on [³H]-thymidine incorporation by RC cells was similar to that observed with GC cells treated with 1,25 and TGF- β . Lower concentrations of TGF- β inhibited [³H]-thymidine incorporation, while higher ones had no effect or stimulated DNA synthesis.

The addition of 1,25 to either GC or RC cultures inhibited release of LTGF- β into the medium; no active TGF- β was detected. In contrast, the addition of 24,25 to GC or RC cultures had no effect on LTGF- β production. Treatment of RC cells with active TGF- β for 24 h dose-dependently increased synthesis of 24,25, while it inhibited synthesis of 1,25 by RC cells. Synthesis of vitamin D metabolites by GC cells was unaffected by incubation with TGF- β .

Discussion

Vitamin D metabolite inhibition of TGF- β -induced stimulation of [³H]-thymidine incorporation suggests that growth factor and hormone promote differentiation of both GC and RC cells, but only when coupled with the metabolite appropriate to the target cell. In GC cells, TGF- β appears to modulate 1,25-dependent effects by maintaining or even decreasing ALPase. This differs from the synergistic increases in ALPase seen in osteoblast-like ROS 17/2.8 cells (9), but is similar to the effect of TGF- β and 24,25 on RC cells. This data supports the hypothesis that TGF- β

negatively regulates terminal differentiation of GC cells to a calcifying phenotype, but enhances differentiation of less mature RC cells. Further, the regulation of chondrocytes by TGF- β and vitamin D₃ metabolites is also dependent on their local production; that is, vitamin D or TGF- β have pronounced effects on TGF- β , 1,25, and 24,25 production by the chondrocytes. This suggests that regulation of chondrocytes *in vivo* is complex, involving interrelationships among hormones and growth factors.

References

1. Centrella et al.: *FASEB J* 2:3066-3073, 1988.
2. Rosier et al.: *Connect Tissue Res* 20:295-301, 1989.
3. Carrington et al.: *Exp Cell Res* 186:368-373, 1990.
4. Schwartz et al.: *Endocrinology* 132:1544-1552, 1993.
5. Wakefield et al.: *J Biol Chem* 263:7646-7654, 1988.
6. Norman AW: *Contrib Nephrol* 18:1-11, 1980.
7. Boyan et al.: *Endocrinology* 122:2851-2860, 1988.
8. Schwartz et al.: *Endocrinology* 130:2495-2504, 1992.
9. Bonewald et al.: *J Biol Chem* 267:8943-8949, 1992.
10. Boyan et al.: *Bone* 3:185-194, 1988.

NONGENOMIC MECHANISMS OF VITAMIN D ACTION IN GROWTH PLATE CHONDROGENESIS

Barbara D. Boyan, David D. Dean, Victor Sylvia and Zvi Schwartz*. University of Texas Health Science Center at San Antonio, Texas 78284 USA; *Hebrew University Hadassah Faculty of Dental Medicine, Jerusalem, Israel

Introduction

Chondrocyte proliferation, metabolism, and differentiation are regulated by vitamin D *in vivo* and *in vitro*. Cellular response is dependent on the state of chondrogenic maturation. While cells derived from the resting zone (reserve zone) respond primarily to 24,25-(OH)₂D₃, cells from the growth zone (prehypertrophic and upper hypertrophic zones) respond primarily to 1,25-(OH)₂D₃. This phenotypic difference in vitamin D metabolite preference is observed *in vivo* as well. Rachitic rat tibias can be healed by direct injection of 24,25-(OH)₂D₃ into the resting zone or 1,25-(OH)₂D₃ into the hypertrophic zone (1,2).

Many of the effects of vitamin D metabolites involve genomic events. Receptors for both 1,25-(OH)₂D₃ and 24,25-(OH)₂D₃ have been found in cartilage (3-5) and several studies have shown that new gene expression and protein synthesis occur. It is becoming increasingly clear, however, that vitamin D metabolites can also exert their effects through nongenomic mechanisms; that is, changes in gene transcription, mRNA stabilization, or protein synthesis do not occur. Examples of nongenomic actions of vitamin D metabolites in cultures of resting zone and growth zone chondrocytes include changes in calcium flux, arachidonic acid turnover, membrane fluidity, and protein kinase C activity [PKC] (6-9).

Nongenomic mechanisms are particularly important in growth plate chondrogenesis where cells not only need to synthesize extracellular matrix, but must also modify it to accommodate cell hypertrophy and facilitate calcification. Cartilage cells use matrix vesicles as a means of conveying information to the extracellular matrix. The composition of these discrete organelles is under genomic control. Thus, matrix vesicles produced by resting zone cells differ from those produced by growth zone cells. Matrix vesicles are unique membrane bound entities since they have a distinct phospholipid composition and independent phospholipid metabolism, and are enriched in alkaline phosphatase specific activity when compared with the plasma membrane of the cell from which they were derived. In addition, they contain enzymes associated with calcification [such as alkaline phosphatase, phospholipase A₂, NTP pyrophosphohydrolase] and matrix processing [such as acid and neutral metalloproteinases, gelatinase, and plasminogen activator] (10-11).

The studies described in this paper examined whether chondrocytes can regulate matrix vesicles via nongenomic interactions with vitamin D metabolites. To do this, we addressed the following questions. Can chondrocytes produce vitamin D metabolites? Is metabolite production regulated? Can matrix vesicle enzyme activity be directly regulated by vitamin D metabolites? Are the direct effects of 1,25-(OH)₂D₃ and 24,25-(OH)₂D₃ cell maturation-dependent? Do the vitamin D metabolites alter matrix vesicle membrane fluidity? Are the vitamin D effects mediated through PKC? Can the direct action of 1,25-(OH)₂D₃ or 24,25-(OH)₂D₃ on matrix vesicles result in feedback of information to the cell?

Methods

Resting zone (RC) and growth zone (GC) chondrocytes were isolated from rat costochondral cartilage and cultured in DMEM containing 10% FBS, 50µg/ml ascorbic acid, and 1% antibiotics in an atmosphere of 5% CO₂ and 100% humidity at 37°C. Effects of exogenous 1,25-(OH)₂D₃, 24,25-(OH)₂D₃, TGFβ, and dexamethasone on vitamin D metabolite production were assessed by using [³H]-25-(OH)D₃ as a substrate; the resultant vitamin D metabolites synthesized and released into the medium were separated by HPLC and detected by a radioisotope method (12).

Alkaline phosphatase and phospholipase A₂ specific activities in matrix vesicles were assayed as described before (10). Activity in matrix vesicles isolated from cultures that had been treated with hormone were compared to those incubated directly with 1,25-(OH)₂D₃ or 24,25-(OH)₂D₃. The effect of 1,25-(OH)₂D₃ and 24,25-(OH)₂D₃ on matrix vesicle membrane fluidity was assessed by measuring the anisotropy of membranes pre-labeled with the fluorophore, TMA-DPH (8). The PKC specific activity of isolated matrix vesicles and plasma membranes was assayed by measuring the phosphorylation of myelin basic protein with ³²P (9). The identity of specific PKC isoforms was determined by use of specific antibodies. The ability of 1,25-(OH)₂D₃ or 24,25-(OH)₂D₃ to stimulate the activation of latent TGFβ by isolated GC and RC matrix vesicles, respectively, was also examined.

Results

When GC cells were exposed to 1,25-(OH)₂D₃, there was a dose-dependent increase in matrix vesicle alkaline phosphatase and phospholipase A₂, whereas 24,25-(OH)₂D₃ had no effect. Similarly, 1,25-(OH)₂D₃ stimulated alkaline phosphatase and phospholipase A₂ when incubated directly with isolated GC matrix vesicles, and 24,25-(OH)₂D₃ had no effect. When RC cells were exposed to 24,25-(OH)₂D₃, there was a dose-dependent increase in matrix vesicle alkaline phosphatase, but a decrease on phospholipase A₂. When isolated RC matrix vesicles were incubated directly with 24,25-(OH)₂D₃, similar effects on enzyme activity were observed; as expected, 1,25-(OH)₂D₃ had no effect.

1,25-(OH)₂D₃ increased GC matrix vesicle membrane fluidity, while 24,25-(OH)₂D₃ had a similar effect on RC matrix vesicle membrane fluidity. The observed effects were metabolite-specific to each cell type.

Matrix vesicles contained PKC activity that was distinct from that of the plasma membrane. By use of isoform-specific antibodies, matrix vesicles were shown to contain predominantly PKC_ζ, while plasma membranes contained PKC_α. When GC and RC matrix vesicles were incubated directly with 1,25-(OH)₂D₃ and 24,25-(OH)₂D₃, respectively, PKC_ζ was inhibited. In contrast, plasma membrane PKC_α was stimulated by the vitamin D metabolites.

Both RC and GC chondrocytes produce 1,25-(OH)₂D₃ and 24,25-(OH)₂D₃ constitutively, although the amounts vary with cell type. In general, production of 1,25-(OH)₂D₃ is down-regulated by 1,25-(OH)₂D₃ and up-regulated by 24,25-(OH)₂D₃. Conversely, production of 24,25-(OH)₂D₃ is down-regulated by 24,25-(OH)₂D₃ and up-regulated by 1,25-(OH)₂D₃. Metabolite production is also regulated by TGF-β and dexamethasone in a cell maturation- and time-dependent manner.

1,25-(OH)₂D₃ stimulated activation of latent TGFβ by GC matrix vesicles, but had no effect on RC matrix vesicles. Further, 24,25-(OH)₂D₃ had no effect on activation of latent TGFβ by matrix vesicles isolated from either cell type.

Discussion and Conclusion

These results show that both 1,25-(OH)₂D₃ and 24,25-(OH)₂D₃ can directly affect matrix vesicles via nongenomic pathways. Two mechanisms which appear to play a role are changes in membrane fluidity and inhibition of PKC. The specific effects of each hormone are conferred by cell maturation-specific differences in matrix vesicle composition. In addition, the nongenomic effects on matrix vesicles can be differentiated from effects on plasma membranes by both phospholipid composition and general enzyme distribution, as well as by isoforms of PKC. The ability of matrix vesicles to activate latent TGFβ is both cell maturation-dependent and metabolite-specific, indicating that nongenomic effects of vitamin D can have direct physiologic consequences. Activated TGFβ may act back on the chondrocytes to regulate differentiation, as well as vitamin D metabolite production.

Acknowledgments

These studies were supported by PHS grants DE08603 and DE05937.

References

- Atkin I, Pita JC, Ornoy A, Agundez A, Castiglione G, and Howell DS. Bone 6:113-123, 1985.
- Lidor C, Atkin I, Ornoy A, Dekel S, and Edelstein S. J Bone Min Res 2:91-98, 1987.
- Corvol M, Ulmann A, and Garabedian M. FEBS Lett, 116:273-278, 1980.
- Balmain N, Hauchecorne M, Pike JW, Cuisinier-Gleizes P, and Matlieu H. Cell Molec Biol 39:339-350, 1993.
- Fine N, Binderman I, Somjen D, Earon Y, Edelstein S, and Weisman Y. Bone 6:99-106, 1985.
- Schwartz Z, Langston GG, Swain LD, and Boyan BD. J Bone Min Res 6:709-718, 1991.
- Swain LD, Schwartz Z, and Boyan BD. Biochim Biophys Acta 1136:45-51, 1992.
- Swain LD, Schwartz Z, Caulfield K, Brooks BP, and Boyan BD. Bone 14:609-617, 1993.
- Sylvia VI, Schwartz Z, Shuman L, Morgan RT, Mackey S, Gomez R, and Boyan BD. J Cell Physiol 175:271-278, 1993.
- Schwartz Z, Schlader DL, Swain LD, and Boyan BD. Endocrinology 123:2878-2884, 1988.
- Dean DD, Schwartz Z, Muniz OE, Gomez R, Swain LD, Howell DS, and Boyan BD. Calcif Tissue Int 50:342-349, 1992.
- Schwartz Z, Brooks BP, Swain LD, Del Toro F, Norman A, and Boyan BD. Endocrinology 130:2495-2504, 1992.