Suppression subtractive hybridization is very effective to enrich differentially expressed genes in two different tissues or cells. We therefore used the technique to identify characteristic genes expressed in rat knee joint articular cartilage as compared to rat costal cartilage. In this study, we revealed that several genes were enriched in a subtracted articular cartilage cDNA library. The most enriched gene is lubricin that is a putative key molecule for joint lubrication. The second gene is milk fat globule epidermal growth factor (EGF) factor 8, MFG-E8 whose expression has never been observed in cartilage. Other enriched genes are known to be expressed in cartilage, however their differential expressions in cartilages have not been necessarily common. The preferential expression of characteristic genes in articular cartilage would provide unique properties to the tissue. Our findings will provide a new view of articular cartilage.

Key words: Articular Cartilage, Subtractive Hybridization, Gene Expression, Milk Fat Globule Epidermal Growth Factor (EGF) factor 8.
implications in understanding the differences between them and would be a prerequisite for understanding the physiological and pathological conditions of them.

In this study, we have performed a suppression subtractive hybridization analysis to clarify the feature of gene expressions in rat articular cartilage as compared to rat costal cartilage which primarily consists of a growth plate. Here we report that several characteristic genes including milk fat globule epidermal growth factor (EGF) factor 8, MFG-E8, an apoptosis related and a vascularization related factor², are preferentially expressed in articular cartilage. Our findings will provide a new view of articular cartilage.

Materials and Methods

Isolation of Rat Articular and Costal Chondrocytes

Rat articular chondrocytes were isolated from the knee condyles of 5-week-old Sprague-Dawley rats (Charles River Japan, Ltd., Yokohama, Japan). Briefly, articular cartilage layers were sliced and minced using a surgical blade. To remove the extracellular matrix, the cartilage pieces were digested with 3 mg/ml of collagenase D (Roche, Mannheim, Germany) in Dulbecco’s Modified Eagle Medium (DMEM; Gibco, Rockville, MD, USA) at 37 °C under 5% CO₂ for 6 h. The released chondrocytes were filtered through a nylon membrane, Nitex 100-μm mesh, twice with phosphate-buffered saline (PBS), pH 7.4.

Newborn rat costal chondrocytes were prepared from rib cartilages of 2-day-old Sprague-Dawley rats following the procedure of Lefebvre, et al.⁶. Briefly, the rib cages were isolated under sterile condition, and then were incubated in 3 mg/ml of collagenase D in DMEM at 37 °C under 5% CO₂ for 2 h until all soft tissue detached from cartilage. After careful washes with PBS, the cartilage pieces were further digested with collagenase D (3 mg/ml) for 4 h, and chondrocytes were collected as described above.

Subculture of Chondrocytes in Monolayer

Rat articular chondrocytes were plated in 100-mm tissue culture dishes at low density (2.5×10⁶ cells/cm²) in DMEM supplemented with penicillin (50 units/ml), streptomycin (50mg/ml), and 10% heat-inactivated fetal calf serum (Gibco). The cells were cultured at 37 °C under 5% CO₂ with medium changes at every 3 to 4 days. After confluence (about 1 week), the cells were serially passaged twice with replating at a 1:3 split after brief digestions with 0.05% trypsin-0.53 mM EDTA (Gibco).

RNA Preparation

Total RNA was isolated from rat costal cartilages, rat articular cartilages (C0) and cultured rat articular chondrocytes; primary cultured chondrocytes (P0), the first passaged chondrocytes (P1), and the second passaged chondrocytes (P2), using TRIzol® reagent (Life technologies, Inc., Rockville, MD, USA) following the manufacturer’s instructions. For the suppression subtractive hybridization experiments, total RNA was further purified using an RNasy® Mini Kit according to the manufacturer’s instructions (Qiagen, Valencia, CA, USA), and about 5 μg each of total RNA was recovered from articular and costal cartilages of a rat.

Construction of Subtractive cDNA Libraries and Analysis

Subtractive cDNA libraries were constructed by PCR-based suppression subtractive hybridization as described previously⁷. Briefly, total RNA (1 μg) from articular and costal cartilages was used to generate double-stranded cDNAs using a Super SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA). The generated cDNA products were then used for suppression subtractive hybridizations, which were performed using a PCR-Select cDNA Subtraction Kit (Clontech). The subtracted PCR products were then ligated into the pCR®2.1 (Invitrogen, Carlsbad, CA, USA) and transformed into TOP10 cells (Invitrogen). After transformation, white colonies were randomly picked for further analysis.

For DNA sequencing, plasmid DNA was amplified using TempliPhi DNA Amplification Kit according to the manufacture’s instructions (Amersham Biosciences, Piscataway, NJ, USA). Sequencing reactions were performed using DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) with either M13 reverse (5'-CACAGGAACACGCTATGACCACG-3') or M13 forward (5'-CGCCAGGGTTTTCCACCGACG-3') primer. The reaction products were then run on a DNA autosequencer, CEQ-2000 (Beckman Coulter). The resulting sequences were compared with sequences in GenBank using the NCBI BLAST program.

Northern Blot Analysis

Northern blot analysis was performed as described previously⁸. Briefly, 10 μg of total RNA was heat-denatured, separated by electrophoresis on a 1.2%
agarose gel, and transferred to a Hybond N membrane (Amersham Biosciences) by a vacuum blotter, VacuGene XL (Amersham Biosciences) as recommended by the manufacturer. Hybridizations were performed using cDNA fragments labeled with \([^{32}P]-dCTP\) (Amersham, Tokyo, Japan) in a solution containing 50% formamide, 5x SSPE, 5x Denhardt’s solution, 10% dextran sulfate, 0.1% SDS, 20 \(\mu g/ml\) denatured salmon sperm DNA. After hybridization, membranes were rinsed 3 x 20 min in 0.1x SSPE / 0.1% SDS at 65 \(^\circ\)C, and then exposed to x-ray film at –80 \(^\circ\)C. As control cDNA probes, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and 18S ribosomal RNA (18S) (Ambion, Inc., Austin, TX, USA) were used.

Expression of Fusion Proteins Encoding Mouse MFG-E8, Rat MFG-E8, and Mouse Developmental Endothelial Locus-1 (Del1)

Fragments of cDNA encoding mouse MFG-E8 (GenBank accession no. NM_008594 nucleotides 578-904) were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR). Briefly, crude RNA from mouse articular cartilage was reverse transcribed using specific primers for MFG-E8 and Del1 cDNAs that were generated served as templates for the amplification of MFG-E8 and Del1 cDNAs by PCR with a set of primers consisting of 5'-CGAGGCGGGAAATCTGT-3' and 5'-GGTCGGTTAGTAGTAGGCTCTG-3'. The single-strand cDNAs that were generated served as templates for the amplification of MFG-E8 and Del1 cDNAs by PCR with a set of primers consisting of 5'-GCCGATCCAGGC-CTTGTGACATTGAGACTG-3' and 5'-GGATCCGAGGATCACATGAGC-3' or 5'-GGCAGATCCAGGC-CTTGTGACATTGAGACTG-3' and 5'-GGATCCGAGGATCACATGAGC-3'. The generated cDNAs were ligated in frame into a bacterial expression vector pET15b (Merck Biosciences, Darmstadt, Germany), and successful ligation was confirmed by DNA sequencing. The generated plasmids were then transformed into bacterial strain BL21 (DE3). Expression of fusion proteins was induced by addition of 0.2 mM isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG) for 3 h, and then the bacteria were harvested by centrifugation.

SDS-PAGE and Western Blot Analyses of Fusion Proteins and Tissue Extracts

Fusion proteins with 6x His Tag were extracted by boiling the bacterial pellets for 10 min in a 1x SDS sample buffer (2% SDS, 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 130 mM dithiothreitol and 0.01% bromophenol blue). Fusion protein extracts were then subjected to electrophoresis on 18% SDS-polyacrylamide gels according to the method of Laemmli. Tissue extracts of mouse and rat articular cartilages were prepared as follows: Articular cartilages were isolated from 5-week-old rat and mouse femurs, and proteins were extracted for 24 h at 4 \(^\circ\)C by gentle stirring in 4 M guanidine hydrochloride, 0.05 M sodium acetate, pH 6.5, containing proteinase inhibitors (100 mM 6-aminohexanoic acid, 5 mM benzamidine hydrochloride, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). After dialysis against water, the extracts were freeze-dried and then dissolved completely in a 1x SDS sample buffer by boiling for 10 min. This crude extracts were subjected to electrophoresis on 10% SDS-polyacrylamide gels.

After electrophoresis, proteins were transferred to Hybond-P membranes (Amersham Biosciences) in a transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol). The membranes were blocked with non-fat milk (Block Ace; Dainippon Seiyaku, Osaka, Japan), and then treated for 2 h at room temperature with rabbit anti-mouse MFG-E8 antibody, rabbit anti-His-Tag antibody (MBL, Nagoya, Japan) or nonimmunized rabbit serum in 10% Block Ace containing 0.05% Tween 20, the membranes were incubated at room temperature for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Cell Signaling Technology, Beverly, MA, USA), followed by washing four times with 10% Block Ace containing 0.05% Tween 20. The specific protein bands were visualized with ECL Western Blotting Detection Reagents and Hyperfilm (Amersham Biosciences).

Immunohistochemistry

Knee joints of 5-week-old mice were fixed in 10% buffered formalin for two days, and decalcified at room temperature in 0.5 M EDTA for at least one week. The decalcified tissues were then dehydrated with ascending alcohol concentrations, and embedded in paraffin. Serial sections of 5 \(\mu m\) were made and
stained with rabbit anti-mouse MFG-E8 antibody that was purified by Protein A Sepharose CL-4B (Amersham Biosciences). Briefly, after quenching of endogenous peroxidase with 0.3% H₂O₂, nonspecific binding sites for antibodies were blocked with 10% normal rabbit serum in PBS. Then the sections were incubated with rabbit anti-MFG-E8 antibody diluted to 20 μg/ml for 1 h at room temperature. After rinsing four times with PBS, the sections were incubated with HRP-conjugated antibody against rabbit IgG for 1 h. Immunoreaction was visualized with 3, 3′-diaminobenzidine (DAB) after five PBS washes.

**Results**

**Screening of Subtracted cDNA Libraries**

To identify genes preferentially expressed in articular cartilage, we first generated subtractive cDNA libraries. For this purpose, articular cartilages were isolated from rat knee joints taking care to avoid, as much as possible, contamination by non-cartilagenous tissues. Total RNA was then isolated from the tissues and used to synthesize double-stranded cDNA. Costal cartilage cDNA was also prepared in a similar way.

cDNA libraries enriched for genes unique to articular cartilage and costal cartilage were constructed by subtractive hybridization of cDNA, first using articular cartilage as the test with costal cartilage as the driver to get cDNA unique to articular cartilage and then with costal cartilage as the test cDNA and articular cartilage cDNA as the driver to obtain sequences unique to costal cartilage. A total of 192 clones were randomly isolated from each library and their cDNA sequences were compared with sequences in GenBank using the BLAST algorithm. Sequence analysis revealed that every clone was found to correspond to known mRNA sequences, and that 9 genes expressed by articular cartilage and 19 genes by costal cartilage were identified multiple times. Genes represented in the library by more than 2 clones were shown in Table I. Of these genes, we selected six genes with high hit number and subjected them to further analysis by Northern hybridization (Fig. 1) to verify the efficacy of the sub-

**Table I.** Summary of clones identified from the subtractive libraries of (a) articular and (b) costal cartilages.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Accession no.</th>
<th>Hit no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Articular cartilage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lubricin</td>
<td>NM_021400</td>
<td>69</td>
</tr>
<tr>
<td>MFG-E8</td>
<td>NM_012811</td>
<td>48</td>
</tr>
<tr>
<td>Osteoprotegerin</td>
<td>NM_012870</td>
<td>10</td>
</tr>
<tr>
<td>α1 (I) collagen</td>
<td>AJ224879</td>
<td>10</td>
</tr>
<tr>
<td>Osteonectin / SPARC</td>
<td>NM_012656</td>
<td>5</td>
</tr>
<tr>
<td>C-type lectin superfamily</td>
<td>AF317204</td>
<td>4</td>
</tr>
<tr>
<td>Matrix Gla protein</td>
<td>NM_012862</td>
<td>3</td>
</tr>
<tr>
<td>TNF-stimulated gene-6 (TSG-6)</td>
<td>U85903</td>
<td>3</td>
</tr>
<tr>
<td>(b) Costal cartilage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Periostin</td>
<td>NM_015784</td>
<td>22</td>
</tr>
<tr>
<td>Phospholipase A2, group II A</td>
<td>NM_031598</td>
<td>18</td>
</tr>
<tr>
<td>α1 (XI) collagen</td>
<td>XM_215687</td>
<td>11</td>
</tr>
<tr>
<td>α1 (II) collagen</td>
<td>AJ224879</td>
<td>10</td>
</tr>
<tr>
<td>α2 (I) collagen</td>
<td>AK075707</td>
<td>5</td>
</tr>
<tr>
<td>Chondromodulin-I</td>
<td>AF051425</td>
<td>4</td>
</tr>
<tr>
<td>Small inducible cytokine A2</td>
<td>NM_031550</td>
<td>4</td>
</tr>
<tr>
<td>Synaptic vesicle glycoprotein 2b</td>
<td>NM_097207</td>
<td>4</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>NM_019143</td>
<td>3</td>
</tr>
<tr>
<td>Matrix metalloproteinase 13</td>
<td>XM_217083</td>
<td>3</td>
</tr>
</tbody>
</table>

**Fig. 1.** Northern blot analysis of six representative clones that are preferentially expressed in rat articular or costal cartilages. An equal amount of total RNA (10 μg) prepared from articular cartilage (lane 1) and costal cartilage (lane 2) was subjected to Northern blot analysis. Hybridization was performed using cDNA inserts from each of the following clones: lubricin (LUB), milk fat globule epidermal growth factor (EGF) factor 8 (MFG-E8), osteoprotegerin (OPG), periostin (PERI), α1 (XI) collagen (COLXI), chondromodulin-I (ChM-I), and glyceroldehydes-3-phosphate dehydrogenase (GAPDH) as a control.
tractive hybridization procedure. The results clearly confirmed that genes selected by the subtractive hybridization procedure and found in high numbers of clones were selectively expressed by either articular or costal cartilage. In this study, we have chosen to focus on the genes preferentially expressed in articular cartilage, so the genes in costal cartilage were not analyzed further.

Specific Expression of MFG-E8 mRNA in Articular Cartilage

Lubricin, also known as superficial zone protein, is specifically expressed by chondrocytes located in the superficial zone of articular cartilage\textsuperscript{11,12}. Osteoprotegerin, known as a decoy receptor for the receptor activator of nuclear factor \( \kappa \)B ligand (RANKL) was also observed in normal articular cartilage\textsuperscript{13}. Although other genes such as osteonectin\textsuperscript{14}, C-type lectin superfamily 1\textsuperscript{15}, matrix Gla protein\textsuperscript{16}, and tumor necrosis factor-stimulated gene-6\textsuperscript{17} have been also observed to be expressed in articular cartilage, the expression of milk fat globule epidermal growth factor (EGF) factor 8, MFG-E8\textsuperscript{4,18}, has never been reported in cartilage. To see if the expression of MFG-E8 might be associated with the differentiated state of chondrocytes, we compared its expression to the expression of the type II collagen gene, a hallmark of differentiated cartilage.

It has been shown that normal chondrocytes maintained at low cell-density in monolayer culture easily dedifferentiate to fibroblast-like cells\textsuperscript{19}. As shown in Fig. 2, the level of type II collagen mRNA decreased with subculture, indicating a loss of the chondrogenic phenotype. Interestingly, lubricin and osteoprotegerin mRNAs were barely detectable in the cultured chondrocytes. MFG-E8 mRNA, on the other hand, was readily detectable in initial culture and although was still present in culture at passage 3, its level decreased with each passage as did type II collagen. These data indicated that the expression of MFG-E8 might be related to the expression of the chondrogenic phenotype.
Expression of MFG-E8 Protein in Articular Cartilage

In order to examine the expression of MFG-E8 protein in articular cartilage, we intended to use a specific antibody for detecting it by Western blotting. Although an antibody that specifically recognizes MFG-E8 was available, the antibody was developed against a synthetic peptide derived from the second EGF-like domain of mouse MFG-E8 that contains an integrin-binding Arg-Gly-Asp (RGD) sequence motif\(^2\). The amino acid sequence of the peptide is almost identical between mouse and rat except for one amino acid residue. Therefore, we first analyzed whether the antibody could recognize both mouse and rat MFG-E8. As shown in Fig. 3A, Western blot analysis clearly indicated that fusion proteins of mouse and rat MFG-E8 were specifically recognized by this antibody. The specificity of the antibody was further supported by Western blot analysis on crude extracts of mouse and rat articular cartilage (Fig. 3B). We could not detect any specific band except for the 51-kDa band in mouse and for the 47-kDa band in rat. These results clearly indicated that the antibody specifically recognizes both mouse and rat MFG-E8. Moreover, they also indicated that MFG-E8 protein is certainly expressed in articular cartilage.

Immunohistochemical Analysis of MFG-E8

To examine the localization of MFG-E8 in articular cartilage, we used affinity-purified anti-mouse MFG-E8 antibody to stain normal mouse tissue by immunohistochemistry. As shown in Fig. 4 A and B, MFG-E8 protein expression was detected ubiquitously in articular cartilage, but was less prominent in growth plate cartilage (Fig. 4 A, C, and D), especially, in the middle zone of the growth plate where its immunoreactivity was not observed (Fig. 4 C). In articular cartilage, the antibody stained primarily the pericellular matrix and cytoplasmic region of chondrocytes, although it also faintly stained interstitial matrix. It is of interest to note that cytoplasmic structures of prehypertrophic chondrocytes in growth plate cartilage were specifically stained in spite of the negative staining of mature hypertrophic chondrocytes. Preimmune rabbit serum has no staining at all.

Discussion

We have found several genes preferentially expressed in articular cartilage and in costal cartilage by suppression subtractive hybridization. This technique is very efficient to identify differentially expressed genes in two different tissues, though the detection of a gene depends partly on its original abundance\(^2\). Most of genes listed in Table I have been already observed in cartilage. However, the expression levels of each gene in articular cartilage and in costal cartilage were clearly different as shown in Fig. 1. Especially the most highly represented gene, lubricin was only observed in articular cartilage and this is consistent with previous observations that lubricin is synthesized and deposited in the extracellular matrix by the chondrocytes located in the superficial layer of articular cartilage\(^1\,^2\). Therefore, these data strongly support the validity of our subtraction methodology, even though type II collagen gene was found to be differentially expressed both in the articular and costal cartilage libraries.
Fig. 2. Localization of MFG-E8 protein in the distal femur of the mouse. Immunohistochemical staining was carried out on a section of distal femur from a 5-week-old mouse as described in Materials and methods (A). Boxed regions are enlarged. MFG-E8 protein expression was observed mainly in articular cartilage and in and around blood vessels at secondary ossification center indicated by asterisks (A). In articular cartilage (B), the antibody stained primarily the pericellular matrix (indicated by arrowheads) and cytoplasmic region of chondrocytes, although it also faintly stained interstitial matrix. In growth plate cartilage (C), the immunoreactivity was absent except for its epiphyseal and diaphyseal (D) regions.
In addition to known genes in cartilage, we found a high level expression of MFG-E8 gene in articular cartilage that has never been reported. MFG-E8 was originally identified as one of the major lipoproteins associated with milk fat globule membrane. Recently, the function of this molecule was identified as a factor that mediated uptake of apoptotic cells by macrophages. Activated macrophage-derived MFG-E8 protein binds specifically to apoptotic cells, and then this complex is recognized by the macrophage. However, cartilage is avascular and contains no macrophages, therefore MFG-E8 must have some other specific function in articular cartilage. Furthermore, MFG-E8 deficient mice are viable without any apparent skeletal abnormality. Therefore, MFG-E8 might have a possible role in maintaining the structure of articular cartilage rather than in the regulation of skeletal development. MFG-E8 is a secreted molecule and contains an integrin-binding RGD (Arg-Gly-Asp) sequence motif and two repeated discoidin-like domains homologous to discoidin-1, a lectin in *Dictyostelium discoideum*. Therefore, these motif and domains of MFG-E8 might facilitate and stabilize chondrocyte adhesion to the surrounding extracellular matrix, but further investigation is required.

The latest draft sequences of mouse and rat genomes indicated that MFG-E8 genes are located near the genes for aggrecan and link protein 3 which are cartilage characteristic molecules. Interestingly, these genes form a gene cluster having paralogous gene pairs, MFG-E8/De1, aggrecan/versican, and link protein 3/cartilage link protein 1. Therefore, the two paralogous gene products, MFG-E8 and developmental endothelial locus-1/EGF-like repeats and discoidin I-like domains 3 (Del1/Edil3) show some structural similarity as described by Hanayama et al. However, the anti MFG-E8 antibody did not show any cross-reactivity to Del1 (Fig. 3A, lane 1 and 4). Although the genes in each cluster are not necessary co-regulated, it seems that paralogous gene pairs have some close relationship in their expression. For instance, in contrast to the ubiquitous expression of MFG-E8 in articular cartilage, a paralogous gene, Del1 is expressed in a restricted manner, essentially restricted to the superficial layer. Similarly, the expression of versican, a paralogous gene to aggrecan is also restricted to the superficial layer of articular cartilage in the chicken (Shinomura, T., unpublished observation).

In this study, we compared gene expression in articular cartilage and costal cartilage. However, we have not emphasized importance of genes that are highly expressed in costal cartilage, because the separation of cartilage and bone in this case was so difficult that we could not exclude the possibility of bone tissue contamination in the preparation of costal cartilage. Thus, some genes, such as peristin might be derived from bone tissue, and not from cartilage. Further work will be necessary to unequivocally identify genes preferentially expressed in costal cartilage.

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