Among the different connective tissues cartilage plays a special role because it has to withstand high pressure and absorb shock. The study of the development of this important skeletal tissue is essential not only because of its relevance to the developmental process but also is crucial if cartilage and bone replacement therapy is to become a reality. For this it is necessary that the mechanisms which regulate the differentiation of the cartilage cell, the chondrocyte, are understood.

The differentiation of a chondrocyte from an embryonic precursor cell is characterised by the secretion of a complex extracellular matrix, a process which involves not only a quantitative increase in matrix components but a qualitative and coordinated change in the pattern of gene expression. (1 for review).

The extracellular matrix in cartilage is characterised by collagens II, VI, IX, X and XI, chondroitin sulfate proteoglycans, noncollagenous proteins and water (2, 3, 4). Of these components, collagens type II, IX, X and XI and the hyaluronate-proteoglycan complexes are specific to cartilage and type II collagen is the major component of the extracellular matrix of this tissue.

In the embryo, the correct regulation of type II collagen is important not only for those structures that remain as cartilage but also for bone formation because hypertrophic cartilage forms the framework onto which the bone matrix is deposited during endochondral ossification (5). This occurs progressively in the foetus, and a general switch from type II to type I collagen is seen, as bone-forming cells functionally replace chondrocytes as producers of the extracellular matrix. However, the production of type II collagen by chondrocytes in the epiphyseal plate is important for the continuing growth of long bones, and a number of structures, such as the sternum, remain as cartilage into adult life.

To study the factors regulating type II collagen gene expression we have introduced a cosmid containing the human type II collagen gene (6), including 4.5 kilobases (kb) of 5' and 2.2 kb of 3' flanking DNA into embryonic stem (ES) cells in vitro (10). By taking advantage of the ability of ES cells to contribute to chimeric mice after injection into
host blastocysts (9, 10), we hoped then to be able to study the expression of the gene in vivo. Preliminary data had suggested that this approach was indeed feasible (11), and this is confirmed by the much more extensive analysis reported here. We present evidence for the tissue-cell- and stage-specific expression of the human type II collagen gene in the chimaeras. The human gene is shown to be correctly transcribed, and human type II collagen is synthesized in chimaeric mouse cartilage.

**Introduction of the human type II collagen gene into ES cells**

The cosmid clone, cosHcol1, carrying the complete human type II collagen gene was used to transform the ES cell line EK-HD14 by the calcium phosphate DNA precipitation method. Transformants containing the human gene were characterised further and 2 lines containing 5 (CN1) or 10 (CN15) copies were used to derive mouse chimaeras.

**Expression of cosHcol1.1 in chimaeras**

To determine the pattern of expression from cosHcol1.1, a number of chimeras were examined in detail for the presence of human α1(II) mRNA. The results obtained with CN1 and CN15 have been almost identical, so for simplicity we present mainly those for CN15.

Human type II collagen mRNA was detected using an RNase protection assay. In this assay radioactive [32P]-UTP-labelled RNA transcripts complementary to either the first or the last exon of cosHcol1.1 were synthesized in vitro and hybridised to total RNA from different chimaeric mouse tissues. In those tissues which express the human α1(II) gene, RNase treatment of the hybridised RNA duplexes yield fragments protected from degradation which correspond to the sizes of the first and last exons of the human type II gene. Bands of equivalent sizes are not seen when total RNA from mouse foetal sternal cartilage is used, demonstrating the specificity of these RNase protection assays. However, a number of smaller bands are seen that correspond to short regions of homology between the mouse and human type II collagen genes. These are useful as indicators of endogenous mouse gene transcription and help define appropriate tissue-specific expression. Significant levels of human α1(II) mRNA was seen in all tissues of mouse embryos which expressed endogenous mouse type II collagen (Table I).

Conversely, there was no evidence of any transcription of the exogenous gene in tissues that do not express mouse α1(II) mRNA. Expression was also not seen in adult chimeras. The RNase protection assays also show that correct initiation and termination of transcription of the human α1(II) gene had occurred. However levels of
expression were lower than the endogenous mouse gene.

Table I. Human α1(II) Transcripts in chimaeric mouse tissues

<table>
<thead>
<tr>
<th></th>
<th>14.5 pc</th>
<th>16.5 pc</th>
<th>18.5 pc</th>
<th>12 pp</th>
<th>&gt;42 pp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcass*</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soft tissues</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sternum*</td>
<td>+++</td>
<td>++</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribs*</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calvaria*</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eyes*</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tongue</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lu, Th, Ki, Li heart, Mu, Sp, blood</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Estimates of relative abundance were obtained from RNase protection assays. +++ Very abundant; ++ just detectable; + not detectable; pc, postcoitum; pp, postpartum. Lu: Lung; Th: thymus; Ki: kidney; Li: liver; Mu: muscle; Sp: spleen. *Positive also for mouse type II collagen mRNA.

Human type II collagen in chimaeric mice

In order to test if the human α1(II) mRNA was functional i.e. could be translated into type II collagen, a mouse monoclonal antibody specific to human type II collagen (gift of Dr. D. Hollister, 12) was used to test for the protein in fixed tissue sections from 14.5-day chimeric embryos. Normal mouse embryos of the same age and portions of 10-week human embryos were analyzed in the same way to provide controls. Intense labeling was seen in the extracellular matrix surrounding chondrocytes in the human material, and species specificity of the first antibody was shown by the absence of label over cartilage in control mouse embryos. However, there was clear labeling of
extracellular matrix in cartilage of chimeric embryos, from CN1 and CN15. Labeling was seen only in cartilage, in patches throughout the body as expected from the chimeric nature of the tissue.

Expression of cosHcoll.1 in mice is tissue-specific and stage specific

The data presented show that the DNA sequences within 4.5 kb upstream and 2.2 kb downstream of the human type II collagen gene were able to mediate the expression of the gene correctly from mRNA to the final protein product. Human a1(II) mRNA transcripts were detected only in tissue samples that contained endogenous mouse a1(II) mRNA, and antibody staining revealed the human protein to be localized to the matrix surrounding chondrocytes. Both observations suggest that expression of the exogenous gene in the chimaeras was tissue specific. The lack of detectable human a1(II) mRNA in adult chimeras is indicative that expression was also developmental stage-specific.

These studies now open the way for the further studies aimed at the identification of specific controlling elements which govern type II collagen expression during embryogenesis and cartilage differentiation.

References

Acknowledgements

We thank Dr. D. Hollister for his generous gift of the monoclonal antibody to type II collagen. This work was supported by the Medical Research Council, U.K.; Arthritis and Rheumatism Council, U.K.; The Croucher Foundation, Hong Kong and The Rockefeller Foundation, USA.