Published in final edited form as:

Matrix Biol. 2016 March ; 50: 16–26. doi:10.1016/j.matbio.2015.10.003.

The development of a mature collagen network in cartilage from human bone marrow stem cells in Transwell culture

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Abstract

Damaged hyaline cartilage shows a limited capacity for innate repair. Potential sources of cells to augment the clinical repair of cartilage defects include autologous chondrocytes and mesenchymal stem cells. We have reported that culture of human bone marrow mesenchymal stem cells with specific growth and differentiation factors as shallow multilayers on Transwell permeable membranes provided ideal conditions for chondrogenesis. Rigid translucent cartilaginous disks formed and expressed cartilage-specific structural proteins aggrecan and type II collagen. We report here the analysis of the collagen network assembled in these cartilage constructs and identify key features of the network as it became mature during 28 days of culture. The type II collagen was co-polymerized with types XI and IX collagens in a fibrillar network stabilized by hydroxylysyl pyridinoline cross-links as in epiphyseal and hyaline cartilages. Tandem ion-trap mass-spectrometry identified 3-hydroxylation of Proline 986 and Proline 944 of the $\alpha 1(\text{II})$ chains, a post-translational feature of human epiphyseal cartilage type II collagen. The formation of a type II collagen based hydroxylysyl pyridinoline cross-linked network typical of cartilage in 28 days shows that the Transwell system not only produces, secretes and assembles cartilage collagens, but also provides all the extracellular mechanisms to modify and generate covalent cross-links that determine a robust collagen network. This organized assembly explains the stiff, flexible nature of the cartilage constructs developed from hMSCs in this culture system.

Keywords

Bone marrow stem cell; Cartilage; Type II collagen; Extracellular matrix; Pyridinoline cross-link; 3-Hydroxyproline

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Author contributions

ADM: Conception of project, experimental design, laboratory effort, manuscript writing.

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Introduction

Chondrocytes synthesize and deposit three tissue-specific collagen molecules, types II, IX and XI that copolymerize to form the nascent fibrillar framework of hyaline cartilage matrix (Fig. 1). The three collagen types become cross-linked by covalent bonds to form heteropolymeric fibrils [1]. Trivalent hydroxylysyl pyridinoline (HP) cross-links between amino-(N) or carboxy-(C) telopeptides and helical sites are the most prevalent intermolecular bonds stabilizing the mature cartilage collagen [2]. These cross-links together with their divalent precursors bond type II to type II and type II to type XI in the interior and type II to type IX collagen molecules on the surface of the hybrid polymer [1,3]. The type XI collagen molecules are cross-linked head-to-tail to each other exclusively by divalent ketoamine cross-links and laterally to C-telopeptides of type II collagen [4]. This cross-linked collagen framework counterbalances the swelling pressure imparted by the osmotic properties of aggrecan and enables epiphyseal and articular cartilage to withstand mechanical stresses.

The post-translational quality of type II collagen molecules with respect to prolyl 3-hydroxylation at specific sites has been shown to vary between tissue types [5]. Mutations in prolyl 3-hydroxylase isoenzymes, associated helper proteins that affect proline 3-hydroxylation [6–14] and mutations that alter the primary structure of the $\alpha 1(\text{II})$ collagen chains can disrupt fibrillogenesis and result in a disorganized cartilage extracellular matrix during development and growth [15–19]. Pro-986 has been shown to be highly 3-hydroxylated in $\alpha 1(\text{IIA/B})$ chains in mouse cartilage [20] and in the Swarm rat chondrosarcoma (RCS-LTC) cell line pN- $\alpha 1(\text{IIB})$ chains [21]. Pro-944 and Pro-707 are also 3-hydroxylated and positioned within 3 residues of the collagen D-period molecular stagger (234 amino acids) suggesting a supplementary role in collagen fibril assembly [5,21,22]. The variation in the degree of 3-hydroxylation at the Pro-944 in $\alpha 1(\text{II})$ collagen is highly tissue-specific, ranging from 10% in bovine articular cartilage, 40% in the nucleus pulposus of the intervertebral disk to 90% in the vitreous of the eye.

A reliable, reproducible method to extract donor bone marrow from humans is well established making bone marrow stem cells an attractive first choice for regenerative medicine [23]. The potential for bone marrow mesenchymal cells to renew, repair or develop into tissues is now being actively pursued. Human bone marrow stem cells (hMSCs) have been shown to have chondrogenic potential and strategies to regenerate cartilage are being explored [24–29]. Under suitable culture conditions hMSCs produce a cartilage-like tissue with a matrix based on type II collagen and aggrecan [30,31]. Transwell culture of hMSCs on flexible porous membranous supports has been shown to improve chondrogenic matrix properties. [31,32]. However, to what extent the fibrillar matrix is well developed, in terms of the post-translational biochemistry, assembly and cross-linking of type II collagen and its co-assembly with (types IX and XI collagens), has not been characterized. We have developed methods to screen for normal collagen co-polymeric assembly in order to monitor the quality of the matrix deposited by chondrocytes in culture and in cartilages affected by heritable or acquired disease states [20,33,34]. This study investigated the ability of human bone marrow stem cells undergoing chondrogenesis to deposit collagen in the neo-matrix and to assemble a type II collagen-based cross-linked network characteristic of cartilage.

Results

Human MSCs were initially seeded as a shallow 8–9 cell deep multilayer on the Transwell membrane. By day 3 the cell layer had a translucent appearance but little structure and when unfixed it collapsed into a spheroidal mass following removal from the membrane. In contrast, by day 7 a thin but coherent tissue layer formed, which remained intact on removal from the membrane and by day 14 the disk-like tissue had a glistening, firm cartilage-like appearance, which by day 28 was a firm, rigid disk having a cartilage-like consistency (Fig. 2A). This was as previously reported [31] and is shown in the example histology of fixed sections (Fig. 2B). The pattern of chondrogenesis was very uniform in these constructs and expression of genes encoding cartilage matrix proteins, collagens, and proteoglycans was greatly increased particularly in the first 7 days of culture. [31]. To characterize the collagen deposited during the initial phase of chondrogenesis, the collagen network laid down in the matrix by day 3 and day 7 was depolymerized using pepsin. The pepsin resistant collagen chains in these extracts were resolved by SDS-PAGE and identified by mass spectrometry of in-gel trypsin digests (Fig. 2C). At day 3 only the $\alpha 1(I)$ chain was detected in Band 1, which was most likely a product of undifferentiated hMSC. However, by day 7 Band 1 was primarily the $\alpha 1(II)$ chain with less $\alpha 1(I)$ chain detected. At day 7 Band 2 was identified as an $\alpha 1(XI)$ chain, consistent with type XI collagen being co-expressed with type II and forming the template for type II collagen fibrils. Abundant type II collagen and also type XI collagen were therefore deposited in the matrix by the end of the first 7 days in culture.

The collagen content of representative cartilage constructs (Table 1) increased continuously from day 7 to day 28. The collagen content by day 28 (1% of wet weight) was about a third that of 15-week human fetal epiphyseal cartilage (3% of wet weight). Mature trivalent hydroxyllysyl-pyridinoline (HP) cross-links were minimal at 7 days, but had increased by 14 days to the level in 15-week fetal human cartilage and remained constant to 28 days. Lysyl-pyridinoline (LP) was too low to quantify in the constructs, or in fetal cartilage. It is very likely that this mature collagen network could be conducive to the rigidity of the cultured cartilage constructs.

To assess more details of the homo- and hetero-polymer collagen cross-links formed during the 28 days, pepsin resistant collagen fragments from the matrix at days 7, 14 and 28 were screened using specific antibodies after separation by SDS-PAGE. To examine if a heteropolymer of type IX collagen to type II collagen had formed in the constructs, the blot was probed with monoclonal antibody 2B4 that identifies the C-terminal sequence, KGPDP, of the $\alpha 1(IX)$ chain, which in cartilage is cross-linked to $\alpha 1(II)$ collagen chains [35]. As seen in Fig. 3; panel A, the antibody reacted strongly with the $\alpha 1(II)$ chain in extracts at days 14 & 28 of culture indicating that pepsin-generated C-terminal stubs of the $\alpha 1(IX)$ collagen chain were cross-linked to $\alpha 1(II)$ and hence heteropolymeric type IX–type II cross-links had formed as early as 14 days, subsequent to the type XI–type II cross-links (see antibody 5890 below) and persisted as the construct developed. The reactive band migrating slightly slower in the control cartilage and in day 28 digests is an incomplete cleavage product of the $\alpha 1(IX)$ collagen chain cross-linked to an $\alpha 1(II)$ chain and has been previously characterized [35].

Stripping and re-probing the blot using the monoclonal antibody 10F2 revealed evidence of a cross-linked polymer of the type II collagen formed in the matrix by 7 days. As seen in Fig. 3; panel B, the antibody recognizes the $\alpha 1(\text{II})$ collagen chains in pepsin extracts of day 7, 14 and 28 day constructs indicating that the C-telopeptide $\alpha 1(\text{II})$ stubs were linked to $\alpha 1(\text{II})$ and that a cross-linked type II–type II collagen network was progressively assembled in the cultures [33]. An $\alpha 1(\text{XI})$ band was also faintly detected in the control fetal cartilage and on higher exposures in the day 28 extracts (data not shown) indicating that $\alpha 1(\text{II})$ -C-telopeptide was also linked to $\alpha 1(\text{XI})$ chains.

To determine if type XI–type XI collagen cross-links had also formed in the cartilage constructs, the same blot was stripped and re-probed with polyclonal antibody 5890. This antibody recognizes an epitope in the N-telopeptide of the $\alpha 1(\text{XI})$ chain, even when cleaved from $\alpha 1(\text{XI})$, but cross-linked laterally to a different chain. Fig. 3; panel C, shows that this antibody strongly recognizes the $\alpha 1(\text{II})$ band in extracts from days 7, 14 and 28 indicating cross-linking of the N-telopeptide of $\alpha 1(\text{XI})$ to $\alpha 1(\text{II})$ chains and hence that type XI–type II cross-links had formed by day 7. A band migrating slightly slower than $\alpha 1(\text{II})$ was also detected at day 28, which has been previously identified as the $\alpha 3(\text{XI})$ chain of type XI collagen cross-linked to an N-telopeptide of the $\alpha 1(\text{XI})$ chain from another molecule and thus a homopolymer of type XI collagen had also formed [4,20]. Finally to confirm that collagen chains were still present on the blot following the extensive stripping method, the monoclonal antibody 1C10 specific to residues 934–945 in the $\alpha 1(\text{II})$ chain of type II collagen was used to probe the blot. As Fig. 3; panel D shows, the major reactive band was the $\alpha 1(\text{II})$ chain confirming that type II collagen was still abundantly present. The molecular interpretations of the western blots are diagrammed for clarity in Fig. 4.

Since post-translational modifications of $\alpha 1(\text{II})$ chains are turning out to be important for the stability and assembly of type II collagen molecules into fibrils [12,21,22], the 3-hydroxylation of specific proline residues in the $\alpha 1(\text{II})$ collagen chains following day 3 was evaluated by SDS-PAGE and mass spectrometry. Fig. 5A clearly showed a major pepsin-resistant Coomassie blue stained protein band in developing cartilages, increasing in intensity from days 7 to 28 that migrated identically to the $\alpha 1(\text{II})$ collagen chain from human fetal cartilage. Mass spectrometry (Fig. 5B) showed 91% 3-hydroxylation at Pro-986 in the $\alpha 1(\text{II})$ chains when compared to 100% 3-hydroxylation in the fetal control. The Pro-944 site in the $\alpha 1(\text{II})$ chains was highly 3-hydroxylated and showed 84% occupancy that decreased to 62% by day 28. 15-week fetal cartilage revealed only 3% 3-hydroxylation at this site. Candidate 3-hydroxyproline residues in the (GPP)₄-repeat containing peptide at the C-terminus of the $\alpha 1(\text{II})$ chain [21] were below detection limits. It was noticeable that no significant Coomassie blue stained protein bands were seen in the range (45 kDa) expected for pepsin treated type X collagen [36,37] (data not shown), suggesting that there was little type X collagen assembled into the matrix.

Discussion

Over many years, a focused attempt to generate cartilage in culture from chondrocytes and stem cells has led to progress in the field of cartilage tissue engineering [24,25,27,28]. However, biochemical and mechanobiological limitations have also become obvious [27,38].

One shortcoming is the low concentration of collagen in the constructs synthesized by chondrocytes or hMSCs in culture compared with native cartilage. Also, simply increasing collagen production does not necessarily produce mechanically sound neo-cartilage, as it is all the elements of extracellular processing that convert fibrillar collagen into a cross-linked heteropolymeric unit that underpins essential cartilage mechanical properties. We show here for the first time evidence that hMSCs in a suitable chondrogenic environment elaborate a collagen network with all the features of a rapidly maturing covalently cross-linked matrix.

The culture of hMSC in Transwells begins as a shallow multilayer of cells and the first week is accompanied by cell division, (8-fold increase in DNA), whereas thereafter there is little increase in cell number [31]. This first phase of chondrogenesis is also characterized by transient Notch signaling through Jagged 1 (days 1–3), which has to be switched off for chondrogenesis to proceed [39]. This clearly coincides with sparse matrix collagen production and predominantly type I collagen on days 0–3 of culture. However, by 7 days expression of the major chondrocyte matrix collagen gene COL2A1 increased more than 100-fold [31] and a coherent type II collagen matrix was established. Over the following culture to day 28 (Fig. 3A), collagen increased to 1% of wet weight and in other hMSC Transwell cultures has approached 2% [31]. This is still much below mature cartilage, but it is comparable to 4-month-old human fetal cartilage (3% collagen by wet weight). Of potentially greater importance was the further evidence of the rapid extracellular processing of the collagen to form the essential components of a highly organized cross-linked collagen network combining the 3 major cartilage collagens.

Most of the collagen present at 7 days was due to an increase in type II collagen protein ($\alpha 1(\text{II})$ chains) (Fig. 5A). A transition from type IIA to type IIB mRNA transcripts was observed in the first 5 days of chondrogenesis in Transwell cultures [31]. Whether this transition plays a part in chondrogenesis, or occurs during a stage when the splicing machinery for the type IIB isoform is not completely expressed is still debatable. We have recently shown no effect on chondrogenesis and skeletal development in a transgenic mouse expressing only the type IIA collagen isoform [20]. In the cartilage constructs at the protein level we could not determine whether there was a type IIA in the matrix since both forms of type II N-propeptides were likely cleaved either by the N-propeptidase ADAM-TS3 [40–42], or by the pepsin required to solubilize the collagen network for analysis. However, over the major phase of matrix production, days 5 to 28, type IIB was the mRNA transcript expressed [31].

Despite the continued increase in proteoglycan content, the rate of gain in wet mass was less from day 14 to day 28 (Table 1, [31]). During the same period collagen content also increased, principally type II and type XI (Fig. 5A) and the collagen trivalent cross-link content approached levels present in 15-week human fetal cartilage (Table 1). This is consistent with a robust collagen network having been formed by day 14 and continued to develop until day 28. This network appears to have restricted further swelling of the matrix by the increasing proteoglycan content [43,44] forming a more rigid cartilage-like tissue. As the formation of mature collagen cross-links requires extracellular processing by lysyl oxidase of specific lysine residues in collagen fibrils, the generation of pyridinoline cross-links by day 7 is entirely compatible with the reported 5-fold up-regulation of LOX gene

expression in comparable hMSC Transwell cultures between days 3 and 6 and continued expression thereafter (Barter and Young, University of Newcastle, UK; personal communication). In an earlier comparison of the Transwell format with cell pellet cultures [31], the Transwell disk lost much less aggrecan to the medium over 28 days of culture, compared to a spherical pellet (24% compared to 36%), although the surface area of the disk was much larger. The proportion of collagen lost was not measured at this time, but as aggrecan is a diffusible secreted product its retention in the nascent construct is very dependent on the developing integrity of the collagen matrix. It was also noticeable that less aggrecan was lost as the culture matured [31], which correlates well with the increase of 63% in collagen content between days 14 and 28 and the maturation of cross-links revealed in our new analysis. The expression of lysyl oxidase from day 7 as noted above may be an important factor facilitating this rapid maturation. Different forms of mechanical stimulation have been shown to boost cartilage matrix formation [45] and it would therefore be important to investigate if these factors could further enhance the maturation of the collagen network in these Transwell cultures.

No significant amount of type X collagen was detected in the cultures up to day 28. Although this appears to contrast with the significant expression of COL10A1 mRNA during hMSC chondrogenesis in pellet cultures [30], it is consistent with barely detectable levels of type X collagen by immunohistochemistry in Transwell cultures [31]. Chondrogenesis to a permanent hyaline cartilage phenotype rather than a transient endochondral phenotype was thus maintained up to day 28 of culture.

The present findings show that as hMSCs undergo chondrogenesis, type II collagen molecules rapidly become inter-molecularly cross-linked to other type II molecules (Fig. 3, 10F2), to type XI (Fig. 3, 5890) and type IX collagen molecules (Figs. 3 and 2B4), as we have previously shown for epiphyseal and articular cartilage *in vivo* [2,4,46]. We have used this western blotting methodology to demonstrate the deposition of cross-linked, nascent type II N-procollagen fibrils in the matrix of the Swarm rat chondrosarcoma cell line, RCS-LTC [33], and characterize type IIA–XI fibril heteropolymers in transgenic mice expressing exclusively the type IIA isoform [20]. Under polarized light microscopy, the matrix of the hMSC constructs revealed a diffuse barely discernible birefringence typical of thin randomly ordered fibrils and electron microscopy confirmed a network of thin fibrils [31] very similar to that seen in native human epiphyseal cartilage [15]. The present data suggest that by day 28 (Figs. 3 and 4) the nascent collagen was assembled into a heteropolymeric fibril network typical of hyaline cartilage.

The post-translational quality of the type II collagen in the cartilage constructs was also examined. The degree of 3-hydroxylation of specific proline residues (Pro-944 and Pro-986) suspected to be involved in supramolecular assembly of collagen molecules was also informative. As in native cartilage Pro-986 was 90–97% 3-hydroxylated (Fig. 5) at all stages. However Pro-944 was highly 3-hydroxylated in the early days of culture and decreased to 62% 3-hydroxylation by day 28 in contrast to 3% 3-hydroxylation in fetal cartilage *in vivo*. The (GPP)₄-repeat at the C-terminus of $\alpha 1(\text{II})$ although a candidate for proline 3-hydroxylation [21] showed negligible hydroxylation. It is notable that type II collagen fibrils in the nucleus pulposus of the intervertebral disk, vitreous of the eye and

Swarm rat chondrosarcoma (RCS-LTC cell line) matrix are especially thin, ranging in diameter from 6 nm–20 nm [33,47]). The degree of 3-hydroxylation of $\alpha 1(\text{II})$ Pro-944 in all these collagens is high, ranging from 40% in the nucleus pulposus, 57% in the RCS-LTC matrix [21] to 90% in the vitreous [5]. To speculate, perhaps thin fibrils of type II collagen deposited in the early stages of chondrogenesis are in part regulated by increased 3-hydroxylation of P944 due to increased prolyl 3-hydroxylase 2 (P3H2) expression [21]. From a tissue engineering perspective this could be significant as the ability to modulate the thickness of the collagen fibrils by selectively inhibiting or stimulating P3H2, is an attractive hypothesis. Further studies are required to test this concept.

Experimental procedures

Mesenchymal stem cell culture, chondrogenic differentiation and histology

Human bone marrow mesenchymal stem cells were isolated by adherence to tissue culture plastic from the mononuclear cell fraction of human bone marrow (Stem Cell Technologies, Cambridge, UK) and expanded in monolayer culture in MSC Growth Medium supplemented with FGF2 [31]. Cells (at passages 2–5) were dissociated with trypsin and transferred to serum-free chondrogenic medium containing TGF β 3, dexamethasone, ascorbic acid 2-phosphate, sodium pyruvate, proline and ITS +1 as previously described [32]. Aliquots of cells (5×10^5) were centrifuged in 6.5 mm diameter Corning Trans-well™ filter units. Cultures were maintained at 37 °C, 5% CO₂ for 3, 7, 14, and 28 days for cartilage formation. Growth media were replenished on a two-day interval. Cartilage constructs were frozen for future biochemical analysis.

For histology cultures were fixed in 4% paraformaldehyde and processed into paraffin wax. Sections were cut at 5 μ m and distribution of polyanions revealed by staining with Safranin O/Fast Green.

Total collagen and collagen cross-link analysis

Samples were thawed, drained of excess liquid and weighed. They were hydrolyzed in 6 M HCl at 110 °C for 24 h. An aliquot of the hydrolysate was colorimetrically assayed for hydroxyproline as a measure of total collagen content [48]. Collagen content was expressed as percentage of collagen by mass of wet tissue. An acid hydrolysate of human fetal cartilage (15 weeks *in utero*) was used as a comparative control. Quantitation of pyridinoline cross-linking residues was carried out by reverse-phase HPLC [49,50]. Pyridinoline content was expressed as moles per mole of collagen.

Analysis of post-translational modifications of type II collagen chains

Following SDS-PAGE and Coomassie blue staining of collagen extracted from hMSC-derived engineered cartilage, in-gel trypsin digests of collagen chains were analyzed on an LCQ Deca XP ion-trap mass spectrometer (MS) with an in-line a C8 column (Thermo Finnigan). Tryptic peptides were identified by calculating the theoretical MS ion profile and matching these to the experimentally determined mass spectrometric peptides [21]. The percentage 3-hydroxylation at a particular proline site was determined from the relative

abundance of the 3Hyp-containing ion as a fraction of the sum of both 3Hyp- and Pro-containing tryptic peptide ions [21].

Collagen heteropolymer analysis

The collagen network laid down by differentiating hMSCs was depolymerized and extracted using 500 µg/mL pepsin in 3% acetic acid. A pepsin extract of type II collagen from fetal cartilage (15 weeks *in utero*) was used as a standard. Collagen chains and chain fragments were resolved by SDS-PAGE [51] and transferred to PVDF membrane for western blot analysis using a series of primary antibodies by probing, stripping and reprobing between each blot. [34]. The monoclonal antibody (mAb) 10F2 recognizes a cleavage site (neoepitope) generated on cross-linked forms of the type II collagen C-telopeptide [20,33]. The monoclonal antibody 2B4 recognizes an amino acid sequence in the $\alpha 1(\text{IX})$ chain that in normal cartilage tissue is cross-linked to the $\alpha 1(\text{II})$ chain [35]. Extracts were additionally probed with the polyclonal antibody 5890, that recognizes a sequence in the N-terminal region of the $\alpha 1(\text{XI})$ chain that is cross-linked to other chains of type XI collagen and type II collagen [20]. The blots were subsequently probed with mAb 1C10, which recognizes an epitope in the helical region of type II collagen chains [40]. A chemiluminescence reporting system was used to detect protein bands. The blots were stripped in 62.5 mM Tris-HCl buffer, pH 6.8 containing 2% SDS, and 100 mM 2-mercaptoethanol for 40 min at 50 C.

Acknowledgments

This work was supported in whole or in part by NIH grants AR057025 (R.J.F.) and AR037318 (D.R.E.) and a UK Research Council grant to the UK Centre for Tissue Engineering with support from The Wellcome Trust to Wellcome Trust Centre for Cell-Matrix Research, University of Manchester. The authors would like to thank Sara E. Funk, Geoffrey R. Traeger and Mary Ann Weis for their technical expertise. R.J.F. would like to gratefully acknowledge Dr. Audrey McAlinden for encouragement and helpful discussions.

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Fig. 1.

Model of the architecture of the type II/IX/XI collagen heterofibril characteristic of native articular cartilage. Collagen type II (blue) is the predominant molecule in fibrillar articular cartilage, and it forms a covalent crosslinking network with collagen types XI (yellow) and IX (red). Type XI collagen forms a filamentous template within the type II collagen fibril. Type IX collagen decorates the surface of the fibril.

Fig. 2.

A. Scaffold-free hMSC cartilage constructs. Cartilage constructs expanded and differentiated into a firm tissue by day 14. A rigid, glassy, smooth cartilage-like disk developed by day 28. Left to right: Days 3, 7, 14, and 28. B: Histological distribution of sulfated polyanion in sections of disk cultures. Staining with Safranin O/Fast Green at 1, 7, 14, and 28 days of culture. Modified from [31]. C. SDS-PAGE showing collagen chains identified by in-gel trypsin digestion and tandem mass spectrometry. Representative sequences of tryptic peptides from SDS-PAGE collagen bands 1 and 2 seen in gel are shown in table. P* = 3-hydroxyproline; P# = 4-hydroxyproline. Collagen types II and XI are synthesized and deposited in the extracellular matrix as early as day 7 in culture. Type I collagen is also present at day 3, potentially from undifferentiated hMSCs prior to chondrogenesis.

Fig. 3.

Type II, type IX and type XI collagen heteropolymer formation in cartilage constructs. A type II collagen based chondrogenic phenotype was observed in the constructs. Equivalent sample loads were electrophoresed and probed in Western blots. A. The mAb 2B4 recognized the pepsin cleavage stub in the C-telopeptide domain $\alpha 1$ (IX) chain when it is cross-linked to $\alpha 1$ (II) chains. Reactivity was observed only after days 14 and 28 in culture. B. The mAb 10F2 reacted with cross-linked $\alpha 1$ (II) chains in all lanes. This antibody specifically recognizes the C-telopeptide domain of type II collagen when it is cross-linked to $\alpha 1$ (II) collagen chains as observed for fetal human type II collagen. C. pAb 5890 specifically recognizes N-telopeptide domain of $\alpha 1$ (XI) collagen when cross-linked to chains of $\alpha 1$ (II) chains in all lanes. This antibody also reacted with the slower moving $\alpha 3$ (XI) chains at day 28. D. The monoclonal antibody (mAb) 1C10 confirmed the presence of type II collagen chains in cultures from all days. In all blots, signal increased in intensity with time in culture indicating a temporal increase in type II collagen deposition and heteropolymer formation. 15-week human fetal cartilage collagen was used as a control.

Fig. 4.

Molecular interpretations of collagen heteropolymer assembly from Western blot analysis.

A. Antibody 2B4 reacted with the $\alpha 1(\text{II})$ chains, showing that the C-telopeptide domains of $\alpha 1(\text{IX})$ (KGPDP epitope) had become cross-linked to type II collagen chains from a different molecule and thus a heteropolymer of type IX collagen and type II collagen had formed. B. Antibody 10F2 reacted with the $\alpha 1(\text{II})$ chains, showing that the C-telopeptide domains of $\alpha 1(\text{II})$ (EKGPDP LQ epitope) had become cross-linked to type II collagen chains from a different molecule and thus a homopolymer of type II collagen had formed. C. Antibody 5890 reacted with the $\alpha 1(\text{II})$ chains and the $\alpha 3(\text{XI})$ chains, indicating that the N-telopeptide of $\alpha 1(\text{XI})$ (DGSKGPTISA epitope) had become cross-linked to $\alpha 3(\text{XI})$ chains or $\alpha 1(\text{II})$ chains from a different molecule and thus a homopolymer of type XI collagen and a heteropolymer of type XI and type II had formed. These heterotypic cross-linking reactions have been demonstrated for type IIA/IIB collagen heteropolymers that are present in the matrix of developing cartilage [3,4,20,34,35].

Fig. 5.

A. SDS-PAGE showing collagen chains with time in culture. The most prevalent Coomassie blue stained chain was $\alpha 1(\text{II})$ chain. The amount of type II collagen progressively increased with time in culture. Two other faintly stained chains moving slightly slower than the $\alpha 1(\text{II})$ chain were identified as $\alpha 2(\text{XI})$ and $\alpha 1(\text{XI})$ chains. B. Occupancy of 3-hydroxyproline at Pro 986 and Pro 944. Degree (%) of 3-hydroxylation of the proline residues at 986 and 944 sites were determined by using tandem mass spectrometry and quantitated as in [21].

Table 1

Collagen and pyridinoline cross-link content of hMSC cartilage constructs.

Days in culture	Wet wt. tissue analyzed (mg)	µg hydroxyproline	% collagen (wet wt.)	Pyridinoline moles/ mol collagen	
				HP	LP
7	3.45	1.95	0.42	0.05	–
14	9.4	6.7	0.54	0.23	–
28	9.7	11.57	0.90	0.22	–
Human fetal cartilage control (15 weeks)	13	3.98	2.98	0.23	–