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Proteoglycan deposition around chondrocytes in agarose culture: Construction of a physical and biological interface for mechanotransduction in cartilage

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Abstract. With a view towards the development of methods for cartilage tissue engineering, matrix deposition around individual chondrocytes was studied during *de novo* matrix synthesis in agarose suspension culture. At a range of times in culture from 2 days to 1 month (long enough for cartilage-like material properties to begin to emerge), pericellular distributions of proteoglycan and matrix protein deposition were measured by quantitative autoradiography, while matrix accumulation and cell volumes were estimated by stereological methods. Consistent with previous work, tissue-average rates of matrix synthesis generally decreased asymptotically with time in culture, as *de novo* matrix accumulated. Cell-scale analysis revealed that this evolution was accompanied by a transition from predominantly pericellular matrix (within a few μm from the cell membrane) deposition early in culture towards proteoglycan and protein deposition patterns more similar to those observed in cartilage explants at later times. This finding may suggest a differential recruitment of different proteoglycan metabolic pools as matrix assembly progresses. Cell volumes increased with time in culture, suggestive of alterations in volume regulatory processes associated with changes in the microphysical environment. Results emphasize a pattern of *de novo* matrix construction which proceeds outward from the pericellular matrix in a progressive fashion. These findings provide cell-scale insight into the mechanisms of assembly of matrix proteins and proteoglycans in *de novo* matrix, and may aid in the development of tissue engineering methods for cartilage repair.

1. Introduction

Articular cartilage serves biomechanical roles in load bearing and joint lubrication in synovial joints. Its specialized extracellular matrix contains primarily cartilage-specific collagens and charged proteoglycans in a water-rich gel. A range of molecular properties which include electrostatic, electrokinetic, and transport phenomena allow proteoglycans to contribute to cartilage function in many different ways [10, 14]. Chondrocytes continually synthesize cartilage matrix, and can alter their rates [35] and spatial patterns [31] of pericellular matrix deposition in response to mechanical loads applied to the tissue. Although collagen deposition and remodelling is relatively slow in adult cartilage (requiring years [26]), proteoglycans are turned over much more rapidly (over the course of weeks [6]) and therefore represent a means of primary importance for the chondrocyte response to mechanical loads and matrix damage.

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Assembly of cell-associated proteoglycan-rich matrix and the chondrocyte metabolic response to mechanical compression are therefore of significant interest in cartilage physiology, biomechanics, and tissue engineering.

Chondrocytes in agarose represent a well-characterized model cell culture environment within which cell-associated matrix assembly can be observed during the progression from essentially no matrix (cells only) to the establishment of a biomechanically functional [3] "artificial tissue". The chondrocyte phenotype is well-preserved in agarose culture [1,17], making it valuable for the study of *de novo* matrix deposition by chondrocytes [21], and the influence of matrix accumulation on synthesis. Such information is of significant interest for the development of tissue engineering methods of cartilage repair. Indeed, agarose has even been used *in vivo* with some success as a "scaffold" for chondrocytes in an experimental cartilage repair application [34]. Additionally, since the cell response to compression has been characterized and shown to evolve as a function of time in agarose culture [2,9,20,22,27], more detailed study of matrix structure in this system could further elucidate relationships between mechanical compression, cell–matrix interactions, and pathways of the biological response.

Matrix proteoglycans (PGs) are abundant macromolecular constituents of the chondrocyte pericellular matrix which may play particularly important roles in mechanotransduction due to the wide range of physical phenomena in which they participate. PGs are the main source of resistance to solute transport and fluid flow in cartilage matrix [24] due to the dense polymeric meshwork they are believed to form *in situ* [28]. They are largely responsible for cartilage electrokinetic phenomena (such as streaming potentials) arising from flow-induced separation of electrical charges fixed to the PG polymeric structure from counter-ions dissolved in the matrix fluid phase [7,10]. They contribute significantly to cartilage mechanical stiffness, in large part due to double layer-mediated electrostatic repulsion between their constituent glycosaminoglycan (GAG) side chains [4]. Proteoglycans strongly influence cartilage mechanical function, and play central roles in the transduction of microphysical "signals" to chondrocytes during tissue compression. The pericellular distribution and evolution of matrix proteoglycan deposition during *de novo* matrix synthesis are therefore of significant interest for understanding the roles of proteoglycans in cartilage repair and the biological response to mechanical compression.

In an established chondrocyte-agarose culture system, we studied *de novo* matrix deposition around primary calf chondrocytes over a culture period long enough for cartilage-like material properties to begin to emerge. At a range of times in culture from 2 days to 1 month, spatial distributions of proteoglycan and matrix protein deposition around individual cells were measured by quantitative autoradiography, while matrix accumulation and cell volumes were estimated by stereological methods. Our objectives were to elucidate the roles of specific matrix components (particularly proteoglycans) in cartilage responses to compression and injury, insofar as *de novo* matrix synthesis in agarose culture represents a model for chondrocyte-mediated cartilage repair.

2. Methods

Under continual irrigation with phosphate buffered saline (PBS; Gibco) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin, articular cartilage was obtained from the distal femur (patellar groove) of freshly slaughtered 2-week old calves. Cartilage was sliced into small pieces of $\sim$1 mm characteristic size with a scalpel, and left overnight in culture medium (for each gram of tissue, 8 ml of DMEM (Gibco) high glucose, no HEPES, supplemented with 0.1 mM nonessential amino acids, 0.4 mM proline, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% fetal bovine serum

(Hyclone), and 50 μg/ml ascorbate (Sigma)) in a tissue culture incubator. The next day, the tissue matrix was digested by sequential treatment with 56 U/ml pronase E (Sigma) for 1–1.5 hrs and 752 U/ml collagenase II (Sigma) for 3–4.5 hrs. Liberated chondrocytes were then isolated by passing digestion products through a 100 μm and then a 20 μm pore-size filter (Millipore), with centrifugation ($\sim$10 min at 100 g) and washing (removal of supernatant and resuspension in $\sim$50 ml culture media) after filtrations to remove enzymes. Isolated cells were then left overnight in culture media at 4°C. The next day, chondrocytes were resuspended and an aliquot was counted in a Coulter counter. Cells were then mixed with low melting temperature agarose (SeaPlaque, Sigma) and cast in 1 mm sheets of 3% agarose at a density of 2×10^7 cells/ml. 3 mm diameter disks were punched from these sheets and maintained in culture for up to 32 days with daily changes of culture media (80 μl per cell-gel disk).

On days 2, 6, 11, and 32 after the initiation of cell-gel culture, disks were incubated for 1.5 hrs in medium containing (nominally) 50 μCi/ml of either ^{35}S-sulfate (for labelling of newly-synthesized proteoglycans) or ^{3}H-proline (for labelling of newly-synthesized collagen and matrix proteins). After a 1 hr wash in DMEM (to remove unincorporated radiolabel), disks were chemically fixed in PBS containing 5% glutaraldehyde and 0.05 M Na-cacodylate (Sigma) and equilibrated in graded series of (respectively) water/ethanol, ethanol/propylene oxide, propylene oxide/Epon 812. After embedding in Epon 812 (Fluka), ultrathin (1 μm) sections taken axially through a disk diameter were exposed to an autoradiographic emulsion (Kodak NTB-2) for $\sim$1 week. Following emulsion development, sections were stained with Toluidine Blue O.

Cell volume and spatial patterns of cell-associated matrix deposition (autoradiography grain density as a function of distance from the cell membrane) were measured by image analysis as previously described [31]. Sections were systematically, randomly sampled [11] at $\sim$20 different locations and examined for cells with a well-defined nucleus–cytoplasm interface (indicating a section taken through a cell "diameter" such that distances from the cell membrane as seen on sections were representative of actual distances in three dimensions). Color images were centered on identified cells. Digitized high power light microscope (Olympus Vanox) images, 100 μm $\times$ 75 μm in total area at 6 pixels/μm resolution, were captured using a CCD color video camera (Sony), frame grabber (RasterOps XLTV) and microcomputer (Macintosh). Using an image processing program (IPLab Spectrum, Signal Analytics Corp.), the cell–matrix boundary was traced and autoradiography grains were identified by green intensity thresholding. The physical space in each image was then parameterized in terms of radial position relative to the traced cell–matrix boundary. Previously developed methods of calculating autoradiography grain density [5] were employed within regions of space defined by concentric annuli, 1 μm in breadth and conforming to individual cell shapes with an angular resolution of $\pi/12$ rad, at increasing distances from the cell membrane (cell-length scale grain distributions). Local "tissue-average" grain densities, calculated without regard for location relative to any cell membranes, were obtained using the entire 100 μm $\times$ 75 μm images. Traced cell–matrix interfaces from grain density measurements were used for estimation of cell volume with the nucleator sizing principle, using the average pixel location of the trace as reference point and sine-weighted directional probes for isotropic sampling within vertical sections [12]. Point counting of independently-acquired images was also used for the identification of volume fractions of cells and Toluidine Blue-staining matrix within cell–gel constructs. Although the Toluidine Blue stain appeared to function only above a certain threshold concentration of matrix PGs (data not shown) this measurement provided a useful indication of the accumulation of *de novo* matrix within cell–gel constructs during culture.

For each measured parameter, one-way ANOVA with post hoc Tukey testing was used to identify differences between radiolabelling times [8]. Results were considered to be significant for $p < 0.05$. Data are reported as mean $\pm$ sem.

3. Results

Spatial patterns of deposition of newly synthesized proteoglycans in the cell-associated matrix around chondrocytes were similar to those observed in calf cartilage explants [32], with the most rapid rates of deposition occurring in the immediately pericellular matrix (Fig. 1). In contrast, the visual impression of ^{3}H-proline histological autoradiography suggested that matrix protein deposition was somewhat more uniformly distributed throughout the matrix (Fig. 2), throughout the culture period.

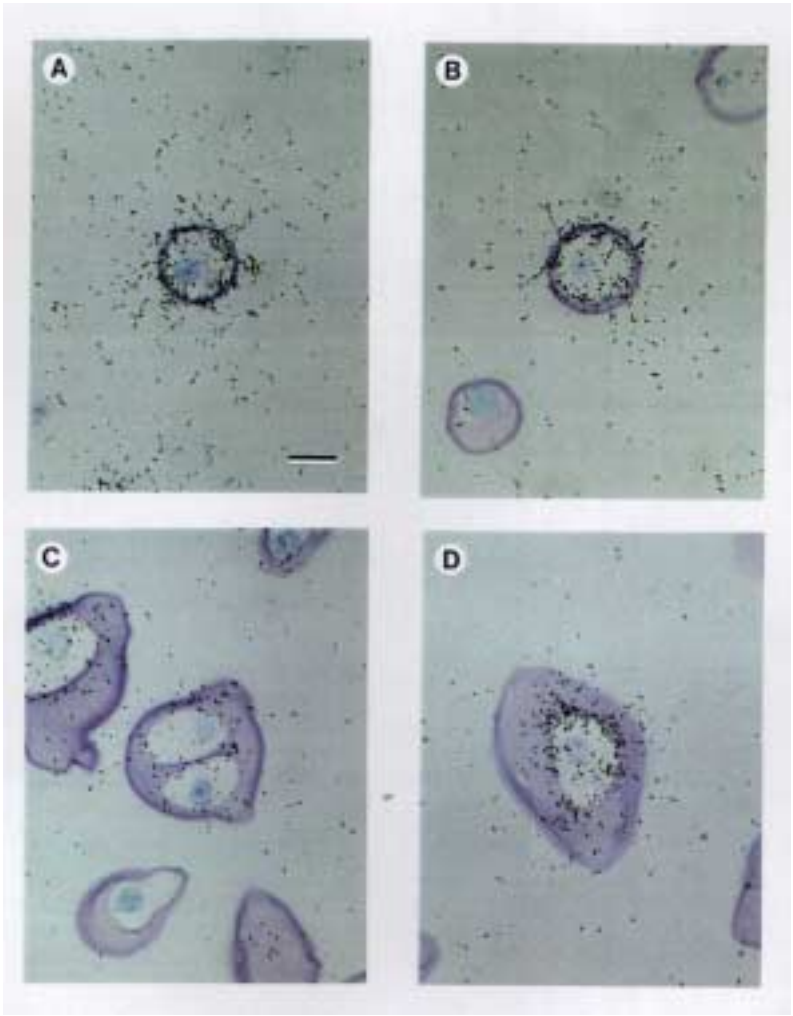

Fig. 1. Histological appearance of chondrocytes in agarose prepared for ^{35}S-sulfate autoradiography after (a) 2, (b) 6, (c) 11, and (d) 32 days of culture. Autoradiography grains represent proteoglycan deposition in the extracellular matrix.

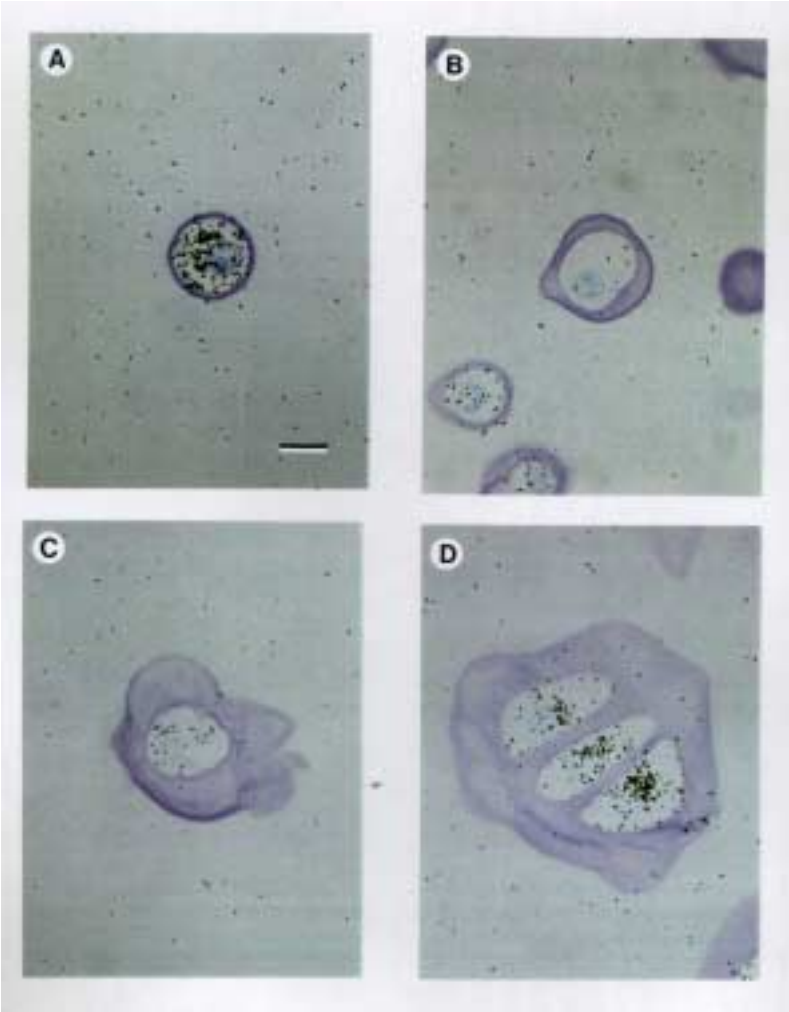

Fig. 2. Histological appearance of chondrocytes in agarose prepared for ^{3}H-proline autoradiography after (a) 2, (b) 6, (c) 11, and (d) 32 days of culture. Autoradiography grains represent deposition of collagen and matrix proteins in the extracellular matrix.

Over the culture period, chondrocyte volumes increased dramatically from values similar to those found in normal calf cartilage [32] on day 2 to almost double this at day 32 (Fig. 3). Therefore, isolation of chondrocytes from calf cartilage and casting in agarose appeared to have negligible effects on cell volumes, but subsequent culture for 32 days (during which the synthesis and deposition of *de novo* cartilaginous matrix proceeded rapidly) was associated with large increases in cell volumes.

The volume fraction of Toluidine Blue-staining matrix increased monotonically with time in culture (Fig. 4a), indicating the steady accumulation of *de novo* matrix. Tissue-average rates of proteoglycan and collagen/matrix protein deposition, as evidenced by ^{35}S-sulfate and ^{3}H-proline autoradiography, were highest during the first week of culture (Fig. 4b), consistent with previous observations [3]. In general, rates of matrix synthesis were highest on day 2 and then decreased monotonically to day 11, after which a steady-state or slightly increasing rate of deposition was evident between days 11 and 32 (Fig. 4b). These

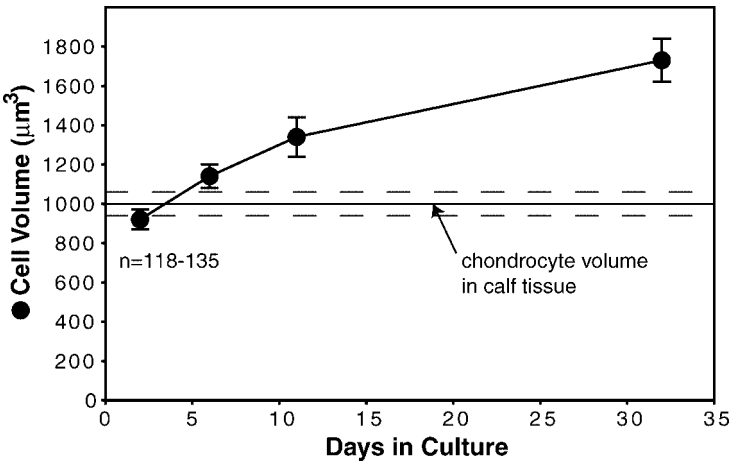

Fig. 3. Chondrocyte volume versus time in agarose culture. Horizontal lines represent cell volume in calf articular cartilage from which chondrocytes were isolated prior to agarose suspension culture (mean $\pm$ sem, $n = 142$ [32]).

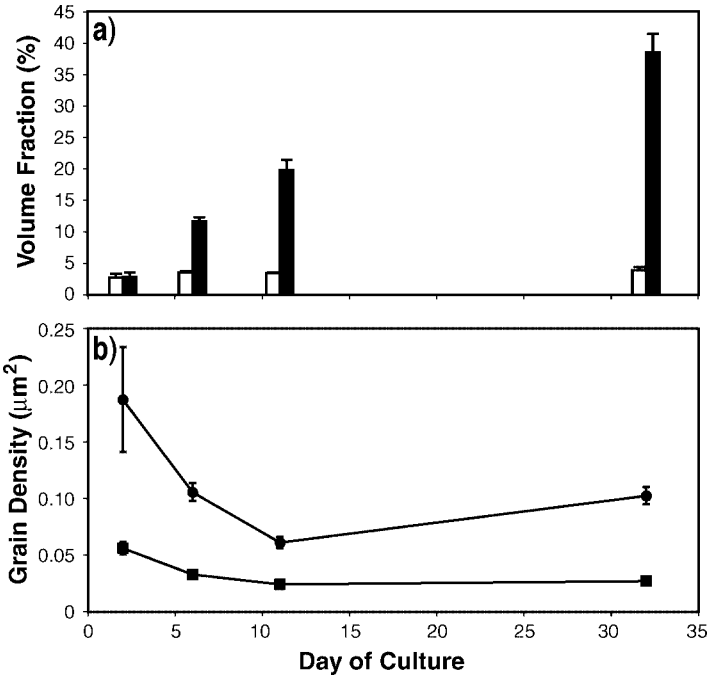

Fig. 4. (a) Volume fractions of chondrocytes (□) and Toluidine Blue-staining matrix (■) versus time in agarose culture. (b) Tissue-average densities of ^{35}S-sulfate (●) and ^{3}H-proline (■) autoradiography grains (representing proteoglycan and matrix protein deposition, respectively) appearing on histological sections.

"steady-state" deposition rates were comparable to those observed in calf articular cartilage explants under similar culture conditions [31,32].

Normalizing the spatial distributions of ^{35}S-autoradiography grains to their values just outside the cell membrane revealed that PG deposition was relatively more restricted to the pericellular matrix on day 2 than on day 32 (Fig. 5a). Similarly, collagen and matrix protein deposition as evidenced by ^{3}H-proline

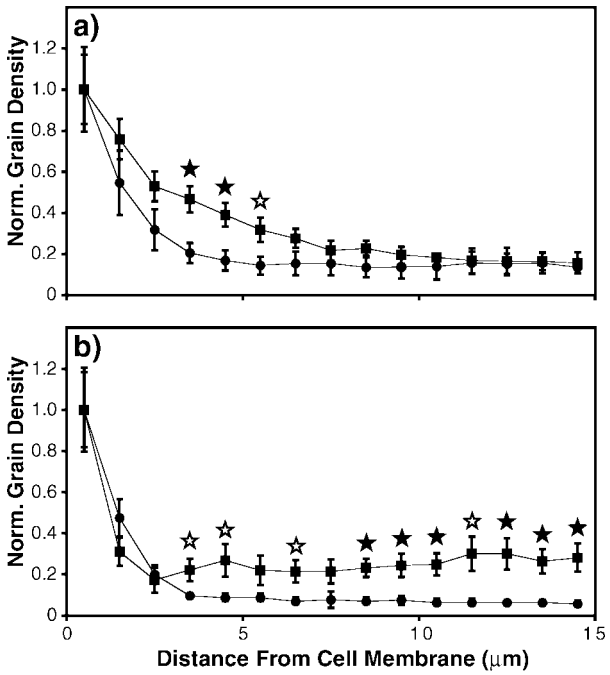

Fig. 5. Patterns of cell-associated matrix deposition as determined by image analysis of histological autoradiography. Extracellular grain densities (measured as a function of "radial" distance from the cell membrane) on days 2 (●) and 32 (■) have been normalized to those just outside the cell membrane. (a) ^{35}S-sulfate autoradiography grains reflect patterns of pericellular PG deposition ($n = 21$). (b) ^{3}H-proline autoradiography grains reflect patterns of deposition of collagen and other matrix proteins ($n = 21$). ★: $p < 0.05$; ★: $p < 0.01$.

autoradiography was emphasized in the pericellular matrix on day 2 (Fig. 5b). However, with time in culture, the spatial distribution of matrix protein deposition included relatively more of the further-removed matrix by day 32. At this later time in culture, patterns of matrix protein deposition therefore appeared to be similar to cell-associated ^{3}H-proline deposition in calf cartilage, where deposition rates are greatest outside of the pericellular matrix [32].

4. Discussion

Consistent with previous findings, results indicate that assembly of *de novo* cell-associated matrix around chondrocytes in agarose gel culture progresses from the pericellular matrix outward. This appears to be consistent with understanding of cartilage matrix assembly and the role of cell–matrix interactions in "anchoring" both the proteoglycan and collagen network constituents of the extracellular matrix [14, 16]. Combined with previous results indicating rapidly turning over proteoglycan pools in the pericellular matrix [23], these observations suggest that pericellular matrix deposition and remodelling may represent a leading priority activity of chondrocytes, particularly within a damaged extracellular matrix.

Assuming a constant density (2×10^7 ml^{-1}) of spherical cells in a cubic lattice arrangement, the time in culture at which Toluidine Blue-staining cell-associated matrices around adjacent cells "contacted" one another was likely between days 6 and 11 (Figs 3 and 4a). This contiguity of deposited matrix would seem to mark an important point in the transition from a collection of individual cells in suspension culture to the establishment of a functional cartilage-like tissue. Previous studies have shown that the

chondrocyte biosynthetic response to mechanical compression strengthens over a similar time period [2], suggesting a mediating role for the cartilaginous extracellular matrix in the transduction of tissue compression to cell activities. However, cartilage-like material properties evolve within chondrocyte–agarose cultures over somewhat longer times (more then 70 days [3]), indicating that *de novo* matrix deposition and remodelling may be a relatively long-term process requiring months before a biomechanically static, cartilage-like tissue is "regenerated". Over the one month period of the present study, cell-associated proteoglycan and protein deposition patterns evolved (Figs 1, 2 and 5) to become similar to those observed in calf tissue [32]. The present study therefore highlights patterns of cell-associated matrix deposition which depend heavily upon the structure and composition of the "substrate" to which *de novo* matrix is added. Interestingly, results suggest that the different metabolic pools of matrix proteoglycans may have been differentially affected: the rapidly turning-over "pericellular" pool appeared to be preferentially deposited at early times within a sparse matrix, while the more slowly turning over "further-removed" pool [15] formed a greater proportion of the deposited PG at later times in culture (Fig. 5a). These associations were consistent with the "tissue-scale" rates of matrix metabolism (Fig. 4b) which were relatively rapid early in culture compared to later times.

The chondrocyte-agarose culture system may be regarded as a model environment within which events important to cell-mediated cartilage repair and tissue engineering may be studied. Present results emphasize that chondrocyte-mediated repair of damaged cartilaginous matrix may follow a "scar-remodelling" temporal sequence similar to bone or skin repair: disorganized matrix is initially deposited rapidly, and then longer-term remodelling may continue at slower metabolic rates. Therefore, the high level of structural organization of adult articular cartilage might best be regarded as a long-term goal of cartilage tissue engineering, with the establishment of a minimally-functional, less organized, immature cartilage-like tissue a potentially necessary intermediate step. Indeed, it is possible that the fully organized structure of adult articular cartilage may only be attained after an extended period under *in vivo* biochemical and biomechanical conditions.

Pericellular matrix synthesis appears to be a "high priority" for chondrocytes synthesizing *de novo* matrix in agarose since it is the first matrix zone reconstructed and its assembly is associated with the highest metabolic rates (Figs 4b and 5). Combined with observations indicating that the chondrocyte response to compression changes with matrix assembly [2,20], these results suggest that the pericellular matrix plays an important role in mechanotransduction. This specialized matrix [29], adjacent to the cell membrane, is particularly rich in proteoglycans, poor in large-diameter collagen fibrils, and includes macromolecular constituents which differentiate it from the further-removed matrix. Its physical location implies that it must play a major role in cell–matrix interactions. Therefore, it seems likely that physical factors which may play a role in the chondrocyte response to compression, including solid deformations, physicochemical changes, alterations in transport properties of bioactive solutes, fluid flows, and electrical streaming potentials, are ultimately communicated to chondrocytes via the pericellular matrix. This matrix might therefore be usefully regarded as an extension of the cell itself which is essential to the expression of the chondrocyte phenotype, including matrix synthesis, remodelling, and the response to compression.

Concomitant with rapid, but steadily decreasing rates of matrix synthesis (Fig. 4b) during agarose culture, chondrocytes exhibited steadily increasing cell volumes (Fig. 3). This suggested alterations to cell volume regulatory processes secondary to changes in the cell microphysical environment due to matrix damage. This was also consistent with the interpretation that cellular hypertrophy can indicate rapid metabolic activity and an attempted repair response. At the same time, these observations emphasize that

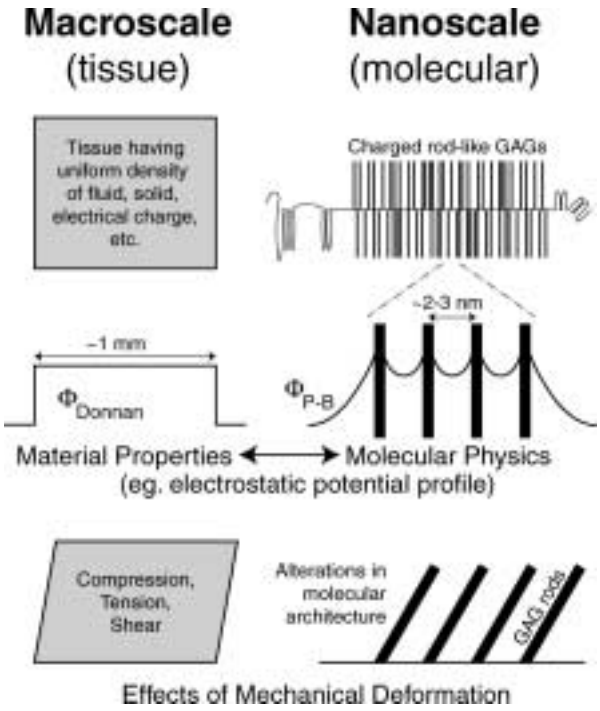

Fig. 6. Schematic representation of relationships between tissue-scale properties of cartilage and molecular-scale behavior of matrix proteoglycans (adapted from [4]). For example, the macroscopic Donnan electrical potential profile representing the charged GAG-filled tissue as a medium of uniform fixed charge density may be compared to the space-varying potential profile derived from the Poisson–Boltzmann model, pictured as surrounding each rod-like charged GAG chain of the aggrecan proteoglycan molecule. The Donnan model assumes a constant potential Φ_{Donnan} while the P–B model accounts for the space-varying potential between GAG chains. These models are each cast at different length scales (macroscopic versus molecular) and can thereby give significantly different predictions of the swelling stress and stiffness of the charged, poroelastic tissue. Consideration of effects of tissue mechanical deformations on proteoglycan architecture may further refine understanding of these relationships.

cell volume alone may be a relatively poor indicator of metabolic activity: the chondrocyte response to static compression exhibits opposite trends, whereby cell volume and metabolism correlate [5].

Proteoglycans play a central governing role in the poroelastic mechanics and biochemistry of the cartilage extracellular matrix. Double-layer mediated electrostatic interactions between glycosaminoglycan molecules [10] (Fig. 6) imbue the “proteoglycan gel” with swelling forces that contribute directly to the tissue compressive modulus, and indirectly to the tensile modulus due to pre-stress of the collagen network [25]. Due in part to their association with the Poisson–Boltzmann equation (Fig. 6), these electrically mediated swelling forces are highly nonlinear functions of PG gel fluid volume fraction [4], contributing to a marked stiffening of the matrix as it is compressed. Fluid flow in cartilage is also governed largely by PG gel density [24]. Classical models for flow through arrays of cylindrical fibers [13] have been shown to provide useful models for cartilage hydraulic permeability [7] when GAG molecules are treated as rod-like particles. Solute transport within the cartilage extracellular matrix can also be strongly dependent upon PG gel density [24], particularly for solutes which interact with PGs electrically, sterically (due to large physical size), or via biochemical binding. Since adult articular cartilage is avascular, transport limitations of bioactive solutes through the PG gel may play an important role in

mediating the cell response to compression [19,33], and changes in cell metabolic activity with time in agarose culture (Figs 4b and 5).

Some recent work has aimed at developing a model for GAG molecular rearrangements during cartilage compression [30] (Fig. 6). Using a "unit cell" approach wherein GAG rods were approximated as solid cylinders surrounded by a sheath of fluid, the unit cell geometry was required to deform during tissue compression in a way which was consistent with macroscopic PG gel deformations and some of the basic constraints of molecular microstructure. Results indicated that anisotropic deformations of an initially isotropic PG gel could give rise to anisotropic hydraulic permeability due to selective orientation of GAGs and nonuniform dehydration of their surrounding fluid sheaths [30]. Predictions of the model were consistent with data acquired from compressed cartilage and cartilage-like materials under physiological levels of compression. Other recent work has suggested that similar phenomena may underlie a direct contribution by glycosaminoglycans to the shear modulus of cartilage. The dependence of the shear modulus on bath salt concentration [18] may be related to changes in the electrostatic energy density of GAGs (and associated "unit cells") at constant fluid volume fraction [18]. Therefore, the contributions of PGs to cartilage material and transport properties may be sensitively dependent upon the details of the molecular response to tissue deformations, beyond relatively simple changes in fluid volume fraction.

Proteoglycans play a ubiquitous role in determining the mechanical and transport properties of cartilage. In agarose culture, rapid deposition of pericellular proteoglycans is among the earliest events in the establishment of *de novo* matrix. In mature tissue, chondrocytes respond to physical stimuli from their microenvironment, in ways which tend to maintain matrix homeostasis during cartilage use, overuse, and even damage. It seems inescapable that matrix proteoglycans mediate the "flow of physical information" during mechanical compression, from the scale of the tissue to that of the cell. Proteoglycans therefore represent the source of a rich array of physicochemical information for chondrocytes, as well as one of their most important means of biological response to such stimuli.

Acknowledgements

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