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The Effects of Physical Forces on Cartilage Tissue Engineering

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Introduction

Despite long-held beliefs to the contrary, articular cartilage, which provides articulating joints with a nearly frictionless, weight-distributing surface for transferring forces between bones, does have a limited ability for self-repair (Cheung *et al.*, 1978; Mankin, 1982; Grande *et al.*, 1989). With age, repeated overuse, or injury, however, natural mechanisms may be inadequate for repairing the damage. Mechanical breakdown of the articulating surfaces within freely moving (diarthrodial or synovial) joints results in osteoarthritis, which afflicts over 30 million people in the U.S. alone (Mow *et al.*, 1992). Current treatment of severely damaged cartilage usually involves total replacement of affected joints with artificial prostheses or transplantation of donor tissue, each of which has its limitations. Artificial prostheses, because of their limited lifetime and need for replacement, are not the best option for younger patients. Donor tissue, on the other hand, is not always available, especially in the size and shape needed.

Promising new therapies already in clinical use or still under study include the development of replacement cartilage *in vivo*, either by injecting cells into the tissue (Brittberg *et al.*, 1994) or by implanting a matrix that was seeded with cells *in vitro* (Frenkel *et al.*, 1997). Another alternative is implantation of tissue constructs that have already been partially developed *in vitro*. A number of studies have shown that cartilage-like tissue can be regenerated *in vitro*, and that development of the tissue matrix is enhanced in culture systems simulating aspects of the native environment, ie that provide a compatible three-dimensional support structure, good mass transfer, and a physical (and/or chemical) stimulus. While progress has been made in growing tissue that has *biochemical* and even *histological* similarity, in most cases the

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Abbreviations: ECM, extracellular matrix; GAG, glycosaminoglycan; PGA, poly(glycolic acid); PLLA, poly(L-lactic acid).

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material and *mechanical* properties of the regenerated construct are not equivalent to those of native cartilage, especially over the long term. More study is needed to elucidate the structure-function relationships leading to the development of cartilage tissue that is biomechanically functional, ie has the ability to bear and distribute weight in a manner closely representative of native tissue.

Natural mechanisms of cartilage repair

Articular cartilage covers the ends of all synovial joints with a one-half to five millimetres thick organized tissue that provides articulating joints with a durable, weight-distributing surface. Cartilage is composed of chondrocytes, proteoglycans, collagen (primarily type II and smaller amounts of types IX and XI), and water (Mow *et al.*, 1992). Large, highly charged, aggregating proteoglycans, localized within the collagen network, keep the tissue hydrated and resistant to compression, while crosslinked collagen fibrils give the tissue its ability to resist shear and tension. During physical activity from walking to running, cartilage repeatedly delocalizes loads throughout this matrix and over the entire joint surface by means of its large and incompressible water content. These loads can range from zero at rest to several times body weight during dynamic loading resulting from normal daily activities. The unique mechanical properties of cartilage, which allow the joints to undergo these activities, result from the intermolecular associations and the arrangement of its components within the tissue (Mow *et al.*, 1992).

Inappropriate or overuse of the articulating joints, however, can result in damage to the cartilage, which may eventually lead to osteoarthritis. While limited, articular cartilage does have natural mechanisms of repair, which appear to differ among species (Cheung *et al.*, 1978; Grande *et al.*, 1989). One limitation is that traumatic injuries resulting in a tear or abrasion of the articulating surface are 'repaired' with fibrous tissue, which cannot withstand physical loading (Klompmaker *et al.*, 1992). Early experiments in which the cartilage repair process was monitored following mechanically-induced defects indicated that the new tissue resembled articular cartilage at two months, but appeared more like fibrous scar tissue at eight and twelve months (Mitchell and Shepard, 1976). In rabbits, however, repair cartilage was hyaline-like for up to twelve months in full-thickness defects (Furakawa *et al.*, 1980).

A number of studies have shown that joint compression or motion enhances production of normal hyaline cartilage at fracture sites (Mitchell and Shepard, 1976) and in full-thickness defects (Salter *et al.*, 1980; Todhunter *et al.*, 1993). In contrast to immobilization, continuous passive motion resulted in faster, more complete healing. Continuous passive motion also results in better repair of full-thickness defects filled with periosteal (O'Driscoll *et al.*, 1984, 1986), but not perichondrial (Woo *et al.*, 1987; Kwan *et al.*, 1989) grafts. While effective for some small defects, however, repair of large grafts does not appear to be improved by continuous passive motion (Athanasiou *et al.*, 1998).

The inability of osteoarthritic and otherwise damaged tissue to regenerate and heal properly is due, in part, to the loss or death of chondrocytes, which are the cells that populate the tissue, albeit sparsely. Chondrocytes produce the sulphated GAG and collagen that are the primary constituents of the ECM. Only the chondrocytes of immature cartilage are capable of replicating after injury; mature chondrocytes have

very little, if any, ability to divide *in vivo* (DePalma *et al.*, 1966; Mankin, 1982). Many studies have shown, however, that chondrocytes of varying levels of maturity will divide readily *in vitro*. How effectively they can be induced to produce ECM, however, is less certain. Implantation of healthy chondrocytes into osteoarthritic tissue, in one manner or another, holds promise for limiting, or even reversing, the damage to articulating joints.

Neochondrogenesis

The phenotypic nature of the chondrocyte is a direct result of its environment. For example, without a three-dimensional support structure, the chondrocyte dedifferentiates into a flat, fibroblast-like cell and begins producing fibrous, rather than articular or weight-bearing, tissue (Horwitz and Dorfman, 1970; Sokoloff *et al.*, 1973; Benya and Shaffer, 1982). Mechanical stresses are also important to chondrocyte function: the repair of damaged cartilage *in situ* is improved in moving, rather than immobilized, joints (Salter *et al.*, 1980; Todhunter *et al.*, 1993). Cultured chondrocytes react favourably to intermittent compression (Palmoski and Brandt, 1984; van Kampen *et al.*, 1985; Veldhuijzen *et al.*, 1987) and/or fluid flow (Freed *et al.*, 1993a, 1994a; Vunjak-Novakovic *et al.*, 1996; Bursac *et al.*, 1996) by increasing production of ECM components. This response suggests that mechanical forces play an important role in development and structural organization of the ECM.

There have been many attempts to create new cartilage from implants of isolated, living chondrocytes since the late 1960s. These early experiments, however, resulted in the formation of fibrocartilage, or cartilage surrounded by fibrous tissue (Chesterman and Smith, 1968; Bentley and Greer, 1971; Bentley *et al.*, 1978). Because it is not equivalent to articular cartilage either biochemically or mechanically, fibrocartilage does not possess the same ability to bear weight (DePalma *et al.*, 1966; Meachim and Roberts, 1971; Furukawa *et al.*, 1980; Whipple *et al.*, 1985; Nelson *et al.*, 1988). Greater success has been achieved with transplantation of chondrocytes, expanded *in vitro* and immobilized in a bioresorbable matrix, into mechanically induced defects in chicks (Itay *et al.*, 1987) and as extraneous tissue in mice (Vacanti *et al.*, 1991). In some of the most promising results to date, articular cartilage defects have been repaired with cultured chondrocytes in humans (Brittberg *et al.*, 1994), in rabbits (Brittberg *et al.*, 1996), and in horses (Hendrickson *et al.*, 1994). In the human study, the cells were placed under a periosteal flap that was attached and sealed. Post-operative biopsies showed a predominance of hyaline-like cartilage, with some fibrocartilage. At eight months, defects filled with chondrocyte-fibrin matrix transplants showed increased levels of GAG and type II collagen, compared with ungrafted controls (Hendrickson *et al.*, 1994). While at least moderately successful in improving regeneration in humans and rabbits, autologous cell implantation in the canine model was not effective over the long term (Breinan *et al.*, 1997).

Support structures for tissue development

A variety of natural and synthetic materials has been used as a carrier or support structure for engineered tissues (see reviews by Hubbell, 1995; Kim and Mooney, 1998). *Table 19.1* contains a partial list of the materials that have been utilized as

Table 19.1. Materials used in supports for *de novo* chondrogenesis

Material	Applications	Reference
Agarose	*in vitro*	Buschmann *et al.*, 1995
	in vitro	Lee and Bader, 1997
Alginate-polysaccharide	*in vitro*	Loredo *et al.*, 1996
Collagen	*in vivo*	Sams *et al.*, 1995
	in vitro	Grande *et al.*, 1997
	in vitro	Nehrer *et al.*, 1998
	in vivo	Wakitani *et al.*, 1989
	in vivo	Noguchi *et al.*, 1994
	in vivo	Ben-Yishay *et al.*, 1995
	in vivo	Frenkel *et al.*, 1997
Fibrin	*in vivo*	Hendrickson *et al.*, 1994
Gelatin	*in vitro*	Stanton *et al.*, 1995
Nylon	*in vitro*	Grande *et al.*, 1997
PGA	*in vitro & in vivo*	Freed *et al.*, 1993b
	in vitro	Grande *et al.*, 1997
PGA coated with collagen	*in vitro*	Sittinger *et al.*, 1994
	in vitro	Grande *et al.*, 1997
PGA coated with PLLA	*in vitro*	Kim *et al.*, 1994
	in vitro & in vivo	Puelacher *et al.*, 1994
PLLA	*in vitro & in vivo*	Freed *et al.*, 1993b
PLLA/PGA copolymer	*in vitro*	Grande *et al.*, 1997

support structures for cartilage development. PGA, which, along with PLLA and their co-polymers, has been approved by the FDA for a variety of *in vivo* uses, is probably the material that has been used most often in the engineering of cartilage (Kimura *et al.*, 1984; Cima *et al.*, 1991; Freed *et al.*, 1993b). Non-woven meshes of PGA have been used successfully to support chondrocytes, as demonstrated by cell densities equivalent to those found in native tissues and by production of type II collagen and GAG (Freed *et al.*, 1993b). PLLA and co-polymers of PGA and PLLA have also been used for this purpose. Altering the ratio of lactic to glycolic acids in the co-polymer allows tailoring of the degradation rate to fit the application. Improved cell attachment and greater retention of the spherical morphology typical of cells in native tissue have been achieved by coating the PGA matrices with collagen (Sittinger *et al.*, 1994). While matrices of collagen alone result in very poor GAG production, collagen production is high (Grande *et al.*, 1997). The type of collagen used for the matrix is important since the amounts and types of collagen produced have been found to vary with the chemistry of the matrix (Nehrer *et al.*, 1998). Matrices formed from both collagen and PGA, which supports high GAG production, may yield constructs with high levels of both GAG and collagen (Grande *et al.*, 1997).

Effects of mechanical loading on native tissue and cultured cells

As has been observed clinically, joint loading and motion can affect both the maintenance and repair of cartilage *in vivo*. Through mechanical conditioning, the application of mechanical stress can help strengthen articular cartilage (Yao and Seedham, 1993). Some biomechanical properties of articular cartilage at specific joint surfaces are altered by exercise in horses (Palmer *et al.*, 1995). Joint motion during the repair process (ie post-operative exercise) results in improved chondrogenesis compared to that in immobilized joints (Todhunter *et al.*, 1993; Athanasiou *et*

al., 1998). During repair, the concentration and production of GAG are reduced with load reduction or immobilization of a joint, whereas they are increased with greater dynamic loading (Kiviranta *et al.*, 1988; Caterson and Lowther, 1978). When normal levels of loading are exceeded, however, cartilage degradation may follow (Gritzka *et al.*, 1973; Radin *et al.*, 1984). How the cartilage actually responds to loading is not clear as the response could be mediated by hydrostatic pressure, cell and tissue deformation, fluid flow and/or nutrient concentration (Hall *et al.*, 1991). Because cartilage is avascular, joint motion aids nutrition by circulation of the synovial fluid and interstitial fluid flow.

The effects of mechanical loading on the synthesis of ECM components in both isolated chondrocytes and cartilage explants have been explored, but in most cases, especially in the earlier studies, only for time periods of 48 hours or less. While static, compressive loading inhibits GAG synthesis (Glowacki *et al.*, 1983; Schneiderman *et al.*, 1986; Gray *et al.*, 1988), cyclical loading inhibits cell division (Veldhuijzen *et al.*, 1979, 1987; van Kampen *et al.*, 1985) and promotes GAG synthesis and overall matrix production (Palmoski and Brandt, 1984; van Kampen *et al.*, 1985; Veldhuijzen *et al.*, 1987; Bacharach *et al.*, 1995; Burton-Wurster *et al.*, 1993; Farquhar *et al.*, 1996; Torzilli *et al.*, 1997).

Whether the force is applied as physical compression or as a hydrostatic pressure is also important. Physical compression, in contrast to hydrostatic pressure, results in loss of newly synthesized proteoglycans from the matrix (Sah *et al.*, 1989) and inhibition of matrix production (Burton-Wurster *et al.*, 1993). Interestingly, negative intermittent pressure also increases synthesis of proteoglycan in cylindrical plugs of cartilage, suggesting that chondrocyte biosynthesis can be stimulated with either positive or negative loading (Suh *et al.*, 1995). There appear to be limits, however, for both magnitude and frequency of the pressure cycle, outside of which these effects may be detrimental (Parkkinen *et al.* 1993; Bacharach *et al.*, 1995; Burton-Wurster *et al.*, 1993; Farquhar *et al.*, 1996). There is also evidence of a 'rebound' effect with increased cellular metabolism following release of pressure (Lippiello *et al.*, 1985). Generally speaking, application of stress below normal physiological level appears to stimulate catabolic activity, whereas that within the physiological range leads to maintenance of explants (Burger *et al.*, 1991). Stresses higher than physiological are likely to result in tissue damage (Farquhar *et al.*, 1996).

Smith *et al.* (1995) investigated the effects of fluid-induced shear on articular chondrocytes. High-density, mono-layer cultures of adult human and bovine chondrocytes were exposed to different durations of fluid-induced shear in a cone viscometer. After 48 hours, the chondrocytes were elongated. While the bovine chondrocytes responded to fluid-induced shear by making larger GAG, the human chondrocytes responded by synthesizing both more and longer GAG. Smith *et al.* (1995) postulated that the metabolic effects might have been mediated by changes in cell morphology (ie shape and alignment).

While tension is not typically a significant force for cartilage *in vivo*, its effects on chondrocyte metabolism have been studied in a few experiments. Lee *et al.* (1982) have cyclically stretched chondrocytes adhered to a supportive elastin membrane. Although protein and collagen production was decreased, GAG synthesis increased 2–3-fold. Increased GAG synthesis by chondrocytes as a result of exposure to cyclic tensile stretch was also reported by DeWitt *et al.* (1984) and Fukuda *et al.* (1997).

Effects of physical forces on neochondrogenesis in tissue constructs

Cells in tissue constructs appear to respond positively to application of physical forces (compression, fluid-induced shear, and pure shear) in much the same manner as do explants, ie by altering production of the ECM.

INTERMITTENT PRESSURE

Motion in articulating joints consists of various mechanical stresses on an intermittent (but not necessarily regular) basis, with the primary loading mode in articular joints being compression. While physical deformation may be an appropriate means of stressing explants or fully formed tissue, hydrostatic pressure is probably the best (or only) means of safely exposing tissue constructs, particularly at the early stages of development, to compressive forces. Some polymeric scaffolds, eg non-woven meshes of PGA, which serve as a temporary matrix for the developing tissue, are also incapable of resisting large compressive loads.

With a few exceptions (Hall *et al.*, 1991; Buschmann *et al.* 1995), prior experiments to study the relationship between compressive force and cartilage development were done at pressures less than physiological and/or for very short time periods. While design of novel culture systems allowed longer-term studies, early experiments were still limited by pressures below the physiological range (Heath and Magari, 1996). Even at low pressures, however, intermittent compression for several weeks resulted in a qualitatively stronger tissue construct compared to non-pressurized samples.

More recently, the effects of physiological levels of intermittent compression on developing tissue constructs were investigated using a custom-made compression/perfusion system that can be operated with fluid flow, which is a critical element for cells immobilized in polymeric supports (Carver and Heath, 1999a–c). The compression/perfusion system was designed to operate with application of both intermittent compression and medium perfusion to developing constructs (*Figure 19.1*). Pressurized air is delivered to or removed from twelve air cylinder/piston arrangements, each of which forms the cap of a fluid-filled reactor or culture vessel. Inside each reactor is a three-tiered support cage containing cell/polymer constructs. The fluid in the culture vessels (containing the cell-polymer composites) can be pressurized up to 2,000 psi (~ 13.5 MPa), which is near the high end of pressures normally experienced by the human hip (Macirowski *et al.*, 1994). Normal, daily contact stresses on human joints range from 3 to 10 MPa (Mow *et al.*, 1992).

When the pressure in the chambers is atmospheric, air-actuated valves open and the constructs are perfused with medium that is pumped through the reactors connected to external reservoirs. The vessels are filled with culture medium for the duration of each experiment to avoid an unreasonably large deflection of the piston and a high, potentially toxic, dissolved oxygen concentration in the culture medium. The reactors are housed inside an incubation chamber that is maintained at 37°C.

In one set of experiments, cartilage was harvested from the stifle joints of healthy foals, no older than 24 months, and adult horses. The horse served as the model system for these experiments both because of the intrinsic merit of and need for cartilage repair in horses and because the compositions of equine and human articular cartilage are similar (Vachon *et al.*, 1990). Chondrocytes were isolated from the tissue

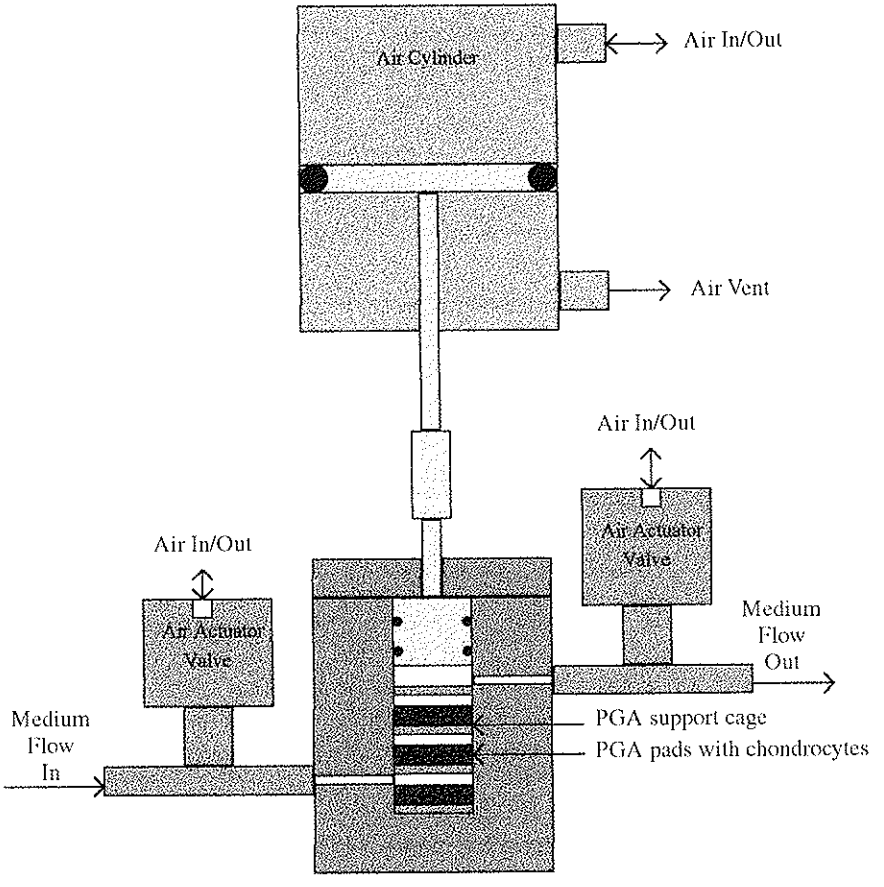

Figure 19.1. A compression/perfusion vessel (not drawn to scale; used with permission from Carver and Heath (1999a)).

(Klagsbrun, 1979) and expanded in tissue culture flasks (approx. three passages). The chondrocytes were dynamically seeded into PGA pads at a density of 5–7.5 × 10^6 cells/pad in spinner flasks for one week (Vunjak-Novakovic *et al.*, 1996) and then placed in the compression/perfusion vessels, where they remained for an additional five weeks until harvest. Half of the reactors were intermittently pressurized and the other half served as non-pressurized controls. Pressure was applied intermittently (five seconds pressurized and fifteen seconds depressurized) at two levels (500 and 1,000 psi). This pressure regime was applied every four hours for five weeks. Medium was recirculated semi-continuously through the vessels when they were not pressurized. The tissue matrix formed by the chondrocytes, as well as native tissue from foals and adult horses, was assayed for cell density (Kim *et al.*, 1988), GAG content (Farndale *et al.*, 1986), and collagen content (Woessner, 1961).

Intermittent compression resulted in more matrix formation than non-pressurized controls (Carver and Heath, 1999b). The concentration of GAG in the tissue constructs was affected both by the level of pressure and by the age of the donor cells (*Table 19.2*). Intermittent compression caused a greater GAG concentration in

Table 19.2. GAG concentration in cell/polymer constructs after 6 weeks of culture with and without intermittent pressure (mean ± pooled standard deviation)[a]

Intermittent Pressure (psi)	GAG Concentration (mg/g tissue)	
	Foal	Adult
Control (no pressure)	26.0 ± 24.4	2.0 ± 1.0
500	89.3 ± 31.4	5.7 ± 1.0
1,000	133.7 ± 38.5	3.5 ± 1.4

[a]from Carver and Heath (1999b)

constructs derived from foal cells, with higher concentrations resulting at the higher level of pressure (1,000 psi). This observation is consistent with animal studies showing higher levels of GAG in areas of frequently loaded cartilage (Caterson and Lowther, 1978; Salter *et al.*, 1980; Kiviranta *et al.*, 1988). Foal cells produced higher concentrations of GAG than adult cells and at native levels, which are 40–120 and 80–120 mg/g tissue for foal and adult tissues, respectively. All adult constructs had GAG concentrations well below native levels indicating that, while adult chondrocytes do respond to intermittent pressure, they are poor candidates for neochondrogenesis. GAG concentration was also positively correlated with the compressive modulus in the pressurized, but not control, constructs, suggesting that intermittent pressure may also affect tissue structure, perhaps by increasing GAG size and/or aggregation.

In contrast to GAG, collagen concentration in foal cell constructs was no different between control constructs and those pressurized at 500 psi (*Table 19.3*). At an intermittent pressure of 1,000 psi, however, collagen concentrations were significantly greater than the control in both adult and foal constructs, suggesting that there may be a minimum level of pressure needed to stimulate collagen formation. One question raised from these results is whether different receptors are activated at higher levels of intermittent compression. While neither adult nor foal constructs reached native levels (100–150 and 120–180 mg/g tissue for foal and adult cartilage, respectively), collagen concentrations in the foal samples were significantly higher than those in the adult constructs, again indicating that cells from young donors have a higher regenerative capacity. Coating the PGA with collagen may stimulate increased production of collagen (Grande *et al.*, 1997).

The different responses of collagen and GAG to intermittent pressure might indicate that their signalling mechanisms differ, ie that they are uncoupled, as has been suggested by Lee and Bader (1997). The response may also be frequency dependent; the frequency that was used may not have been effective in stimulating production of both GAG and collagen.

Table 19.3. Collagen concentration in cell/polymer constructs after 6 weeks of culture with and without intermittent pressure (mean ± pooled standard deviation)[a]

Intermittent Pressure (psi)	Collagen Concentration (mg/g tissue)	
	Foal	Adult
Control (no pressure)	6.3 ± 1.6	0.5 ± 0.3
500	6.7 ± 1.9	3.0 ± 0.3
1,000	11.9 ± 2.7	7.3 ± 0.5

[a]from Carver and Heath (1999b)

How the cells sense hydrostatic pressure (or other forces) and signal intracellular events is not clear. One possibility is that changes in cytoskeletal structure may be responsible for changes in phenotypic expression (Brown and Benya, 1988; Mallein-Gerin *et al.*, 1990; Wang *et al.*, 1993). The presence of ECM also appears to be important as previous studies have shown that stimulation of biosynthesis was enhanced following dynamic compression of chondrocytes immobilized in agarose gel when more matrix was present around the cells (Buschmann *et al.*, 1995). Either, or both, of these events (ie changes in cytoskeletal structure or interactions between the cell and the ECM) may involve activation of cell-surface receptors or cell-signalling molecules.

FLUID FLOW AND MIXING

While the primary loading mode is compression, interstitial fluid flow resulting from joint motion is responsible for lubrication and nutritional transport in the tissue (Mow *et al.*, 1992). Because cartilage is avascular, interstitial fluid flow enhances exchange of nutrients and metabolites to and from cells within the tissue.

Compared to static cultures, studies with stirred reactors have shown that tissue development in cell/polymer constructs can be improved by mixing (Freed *et al.*, 1993a, 1994a,b; Bursac *et al.*, 1996; Vunjak-Novakovic *et al.*, 1996). *In vitro*, fluid flow and turbulent mixing can impact tissue morphogenesis by both hydrodynamic and mass transfer effects (Vunjak-Novakovic *et al.*, 1996). Fluid flow can induce changes in cell shape and function and can improve mass transfer in tissues, especially in large constructs where diffusion is inadequate. Rabbit chondrocytes seeded into porous PGA scaffolds that were cultured in bioreactors under forced fluid flow produced constructs that were firmer and thicker than those cultured statically (Dunkelman *et al.*, 1995). The effects of mixing on tissue-engineered constructs were further examined by Vunjak-Novakovic *et al.* (1996). Bovine chondrocytes were seeded into porous PGA scaffolds and cultured under static and mixed culture conditions in petri dishes and spinner flasks. Turbulent mixing improved the composition and size of the constructs. Compared to those from static cultures, constructs from mixed cultures contained more cells, GAG and collagen. Constructs from the mixed cultures were also surrounded by an outer capsule, which resembled the outer, superficial tangential layer of articular cartilage.

COMBINED INTERMITTENT COMPRESSION AND FLUID FLOW

While the preceding paragraphs have shown that intermittent compression and fluid-induced shear are independently capable of eliciting an increase in the production of ECM, actual joint motion consists of simultaneously (or nearly so) applied forces. Applied at the same time, do these forces still act independently, or is there a synergistic relationship perhaps resulting in a better-than-linear combination of their separate effects?

To partially address this question, cell/polymer constructs were cultured with various combinations of intermittent pressure, fluid flow, and mixing (Carver and Heath, 1999c). The cell/polymer constructs were cultured first in spinner flasks for one, two, or four weeks, and then in the custom-made compression/perfusion system

Table 19.4. GAG and collagen concentrations in cell/polymer constructs after 6 weeks of culture under different durations of fluid mixing and intermittent pressure (mean ± pooled standard deviation)[a]

Condition[b]	GAG Concentration (mg/g tissue)	Collagen Concentration (mg/g tissue)
1S 5C	15.8 ± 16.1	1.1 ± 0.7
1S 5P	56.3 ± 18.6	5.4 ± 0.8
2S 4P	137.7 ± 16.1	8.9 ± 0.7
4S 2P	83.5 ± 22.7	8.5 ± 1.0
6S	72.9 ± 22.7	5.8 ± 1.0

[a]from Carver and Heath (1999c)
[b]Conditions are indicated by weeks in a given environment, ie S = spinner, C = control (no mixing or pressure), and P = intermittent pressure with perfusion

with intermittent pressure and fluid flow (low fluid-induced shear) for a total of up to six weeks. Control constructs were either cultured for all six weeks in the spinner flasks (high fluid-induced shear), or for one week in spinners, followed by five weeks in the perfusion system without intermittent compression. Unfortunately, it was not possible to culture the constructs under the condition of simultaneous, intermittent compression and high fluid-induced shear.

Of all the combinations tested, high fluid-induced shear (in the spinner flasks) followed by intermittent compression (500 psi) resulted in constructs most like native tissue. The improvement in regeneration with the combined (but not simultaneous) conditions was manifested primarily in increased construct size (4 x). Tissue constructs cultivated for two or four weeks in spinner flasks, followed by low fluid-induced shear with intermittent pressure, had significantly higher concentrations of both GAG and collagen than constructs cultured with high fluid-induced shear or intermittent pressure alone (*Table 19.4*). Initial cultivation in the spinner flasks, with turbulent mixing, enhanced both cell attachment and early development of the ECM. Subsequent culture with perfusion and intermittent compression appeared to accelerate ECM formation. Constructs that were not stressed beyond the one week seeding period lost mechanical integrity upon harvest, suggesting that application of physical forces, particularly compression, to tissue constructs during their development may be an important determinant of their ultimate biomechanical functionality.

Although the fluid velocity in the compression/perfusion system was low compared to that in spinner flasks, these results agree with those of earlier studies (Freed *et al.*, 1995). More specifically, fluid flow enhances tissue development, either by increasing mass transfer to and within the constructs and/or by providing a mechanical stimulus for ECM production. The cell flattening and increased production of collagen relative to GAG near the surface of constructs in environments with fluid flow (Vunjak-Novakovic *et al.*, 1996) suggest that fluid flow may do more than increase nutrient transport by convection. Rather, as with many other cells, chondrocytes may respond to fluid flow by altering their phenotypic expression. Higher levels of fluid flow, with intermittent compression, may yield even larger, and possibly stronger, tissue constructs.

Mechanotransduction

While the different types and levels of force that influence production of the ECM are

now partially understood, little is known about how the cells actually process these extracellular physical signals into intracellular events. Unlike many other tissues, cartilage is avascular, aneural, and alymphatic. While the exact mechanisms are not yet known, changes in phenotypic expression occur by transduction of mechanical signals into metabolic events and structural adaptations (Salter *et al.*, 1980; Sah *et al.*, 1989; Suh *et al.*, 1995). For example, *in vivo* studies have shown that joint immobilization results in degenerative changes characterized mainly by the loss of GAG production (van Kampen and van de Stadt, 1987). Application of mechanical force, however, influences cartilage metabolism (Caterson and Lowther, 1978; Kiviranta *et al.*, 1988), and can improve cartilage repair following induced injury (Todhunter *et al.*, 1993).

Cell-surface integrin receptors are likely to be responsible, in a large part, for the transfer of information (chemical, mechanical, electrical, etc.) between the intracellular compartment and the ECM. Integrins are heterodimeric glycoproteins consisting of α and β sub-units, each of which spans the cell membrane (Loeser *et al.*, 1995). They mediate cell attachment and physically link the ECM with the intracellular cytoskeleton (Ingber, 1991; Hynes, 1992). Integrins, in particular the $\beta1$ sub-unit, may serve as mechanoreceptors and transmit mechanical signals from the ECM to the cytoskeleton, which may then mediate activation of signalling pathways (Wang *et al.*, 1993; Wright *et al.*, 1997). Protein kinase C is a key enzyme for signal transduction and is involved in the regulation of chondrocyte proliferation and synthesis of proteoglycan (Choi *et al.*, 1995; Fukuda *et al.*, 1997). Integrin-signalling pathways are also involved in regulating the expression of genes whose products are involved in tissue repair (Arner and Tortorella, 1995). To take advantage of these integrin-mediated molecular interactions, biodegradable polymer surfaces have been engineered to present cell adhesion ligands in specific patterns for tissue engineering applications (Patel *et al.*, 1998). The sequences promoting cell adhesion can be patterned in ways that can spatially influence cell attachment and mobility, providing some control over the regeneration process.

Chondrocytes express a variety of integrins, including $\alpha1$, $\alpha3$, $\alpha5$, and $\beta1$ (Loeser *et al.*, 1995; Hirsch *et al.*, 1997; Shakibaei *et al.*, 1997; Wright *et al.*, 1997). Studies have shown that the expression of all three α sub-units increased during cell culture (Loeser *et al.*, 1995), and that the response of cultured chondrocytes following pressure-induced strain involved the $\alpha5\beta1$ integrin (Wright *et al.*, 1997). While the exact molecular mechanism is poorly understood, $\beta1$ integrins are involved in interactions between chondrocytes and type II collagen, which result in maintenance of the differentiated phenotype (Hirsch *et al.*, 1997; Shakibaei *et al.*, 1997; Wright *et al.*, 1997). This observation suggests that pre-coating the polymeric supports with collagen might alter the rate, and possibly the mechanism, of ECM formation.

A cell's sensitivity to a particular stimulus can vary as integrin receptors are known to change in number, density, and location (Ingber, 1991), as well as in specificity and affinity (Hynes, 1992). This raises the question of whether differential expression of integrins contributes to the dissimilar regenerative capacities of old and young cells described earlier (Carver and Heath, 1999b).

Mechanical properties of regenerated tissue

The mechanical properties of articular cartilage are a critical component of its

functionality. In turn, the ability of tissue constructs to function as native cartilage depends on the composition and structure of the developed matrix (Mak *et al.*, 1987). Measurement of one or more key biomechanical properties can provide valuable information on both the composition and structure of the developing construct. The aggregate modulus of the matrix, a measure of the compressive stiffness, correlates directly with the GAG content and inversely with the water content of the tissue. The shear stiffness of the matrix depends mainly on the collagen content and orientation (Mow *et al.*, 1992). The concentration and orientation of collagen fibrils primarily determine the lateral tensile stiffness (Jurvelin *et al.*, 1997). GAG concentration can strongly influence both hydraulic permeability and axial compressive stiffness through generation of repulsive electrostatic swelling forces between the GAG chains (Jurvelin *et al.*, 1997). Therefore, compressive, tensile and shear moduli can provide important indicators of both the interactions between collagen and GAG in regenerated constructs and how these components affect the mechanical properties.

While most measurements of mechanical properties have been done on cartilage explants, there have been some recent studies of the mechanical properties of tissue constructs. Cell-polymer constructs developed for six weeks under intermittent pressure demonstrated compressive strengths that were directly proportional to the concentration of GAG, but one to two orders of magnitude less than that of native tissue (Carver and Heath, 1999c). Culturing the constructs for a longer period of time appears to be important to the development of mechanical properties. Following initial decreases, both the compressive and aggregate moduli of cell-polymer constructs increased, and achieved the same order of magnitude as those of native tissue at 9–12 weeks (Ma *et al.*, 1995). Using dynamic, non-destructive measurements in shear, Stading and Langer (1999) found that the shear modulus, while significantly less than that of native cartilage, correlated well with both collagen and GAG content in the tissue constructs. The difference in the shear modulus between that of constructs and native tissue was shown to depend on both the biochemical composition and the microstructure of the constructs, making measurement of the shear modulus a good indicator of ECM development.

Despite achieving near-native composition, mechanical testing of tissue constructs clearly shows that success in composition is not sufficient for success in biomechanical function. Unless tissue constructs can be developed with the same macro- and micro-structure as native tissue, use of tissue-engineered constructs will not result in return of functionality. Inclusion of mechanical testing as a regular component of tissue construct assessment will inevitably lead to improved methods of tissue development *in vitro*, and may ultimately result in constructs that can be implanted *in vivo*.

Conclusions

Both external forces and/or deformations have been effective in altering production of the ECM. While joint immobilization or high forces may inhibit normal function or even result in tissue degradation, normal stresses and deformation actually enhance maintenance and repair of cartilage. The mechanisms by which cells sense and respond to these stimuli, however, are not completely understood. Transmembrane receptors called integrins are involved in the process by which cells sense mechanical force and transduce this signal into differentiated function. These receptors, because

they communicate bi-directionally between the intra- and extra-cellular environment, mediate actions such as gene expression, cell adhesion, and organization of the ECM. These molecules are capable, by a variety of mechanisms, of initiating intracellular events, which could include up-regulation of proteoglycan and collagen type II expression. Because mechanical stress affects the expression of proteoglycan and collagen type II differently, the signal transduction pathways for proteoglycan and collagen stimulation are probably not the same. Strategies such as pre-coating of polymer supports with exogenous collagen (type II), which could activate these receptors, might initiate earlier matrix production and organization by the cells, enhancing the regeneration process. Differential expression of these receptors may help to explain how cells respond to different types and levels of mechanical force, and why cells from young and old animals have dissimilar capacities for regenerating tissue. Understanding the relationship between extracellular events and intracellular actions may provide new opportunities for influencing *de novo* chondrogenesis, resulting in development of cartilage that is both biochemically and biomechanically equivalent to native tissue.

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