Master thesis

Is collagen type II antiangiogenic in printed scaffolds for cartilage tissue engineering?

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Introduction

Arthritis commonly occurs in individuals over the age of 65. In the U.S. alone, 46 million people have been diagnosed with arthritis. There are many different forms of arthritis, the most common one being osteoarthritis. Symptoms include pain, inflammation, stiffness and loss of mobility in a joint.[26] One of the first characteristics of osteoarthritis is degradation of the cartilage. Cartilage consists mostly of collagen type II (almost 60%), the remainder contains other collagens such as collagens type VI, IX, and XI, proteoglycans and chondrocytes, the cartilage cells.[9] When a small amount of cartilage has been degraded, focal stress around the joint can lead to further cartilage loss.[22] Moreover, this loss of cartilage can result in malalignment of the joint, which worsens the degree of focal loading and so increases the cartilage loss.[22] When collagen type II degrades, mechanisms are set in motion to restore the tissue, but the regenerative capacity of the cartilage is insufficient. One of these mechanisms, is to vascularize the normally avascular cartilage, in an attempt to provide the necessary nutrients and oxygen needed for the chondrocytes to produce matrix, and compensate for the degeneration.[59]

Tissue engineered cartilage used for therapy against arthritis should be antiangiogenic, to prevent vessel ingrowth. This review will focus on the antiangiogenic properties of collagen type II, the main component of cartilage, and the printability of a collagen type II gel, for tissue engineering purposes. We hypothesize that collagen type II is antiangiogenic, and responsible for normally avascular cartilage. During arthritis collagen type II is degraded and vessel ingrowth cannot be prevented. Questions that arise are: does the matrix contain antiangiogenic factors preventing endothelial cells from entering? Or do the endothelial cells not have the ability to break down collagen type II, either because they do not express the correct integrins to attach themselves to the collagen, or because they do not produce MMPs that can disintegrate the collagen?

Secondly, for tissue engineering purposes, we want to print a collagen type II gel using a fiber deposition system. Collagen type II is considered a better model for cartilage research as cartilage consists mostly of collagen type II and printing gives us a unique way of making cartilage grafts. With organ tissue printing, the cells are mixed with a collagen type II gel, and organized into layered hybrid constructs, with a controlled architecture and defined cellular placement.[21] We want to know if it is possible to print collagen type II using a fiber deposition system.

Angiogenesis, vasculogenesis and endochondral bone formation

Angiogenesis and vasculogenesis depend on multitudinous regulatory factors and are very complex multistep processes.[7] The difference between them is that angiogenesis forms vessels from pre-existing vasculature and vasculogenesis is *de novo* capillary formation from endothelial precursor cells (EPCs), in embryonic development as well as in the adult (Figure 1).[12] Angiogenesis occurs during endochondral bone formation and during arthritis.[70] Angiogenesis begins with the endothelial basement membrane being broken down by MMPs. This releases matrix-bound angiogenic factors that excite endothelial cell migration and proliferation. The endothelial cells, in turn,

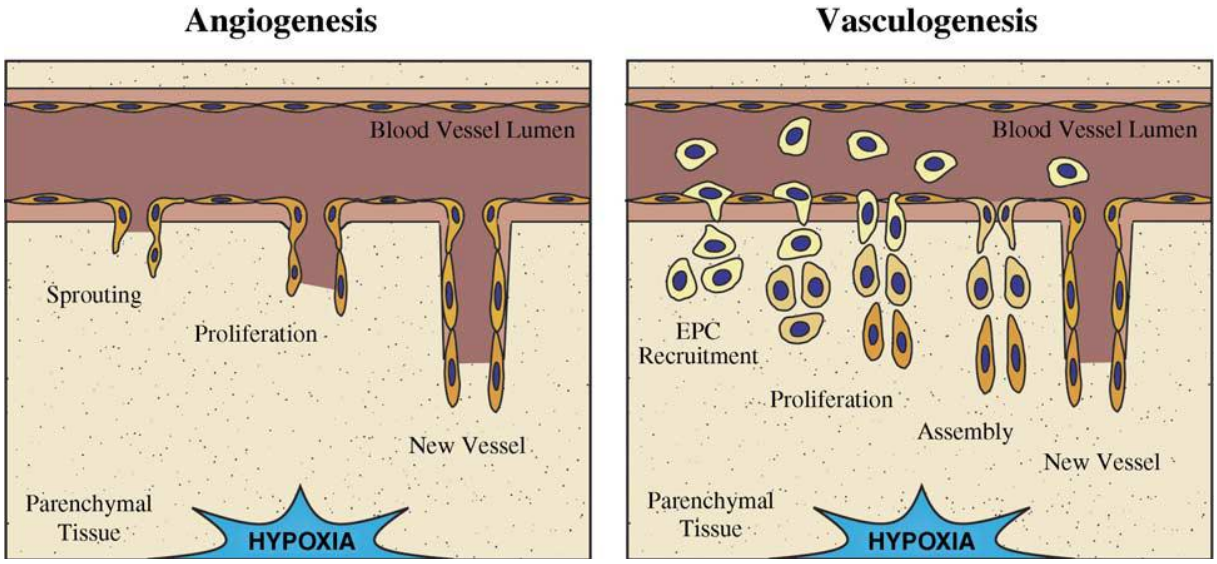

Figure 1. Angiogenesis and vasculogenesis. In angiogenesis, mature endothelial cells sprout from existing vascular networks to form new blood vessels. In contrast, vasculogenesis is an ordered process whereby circulating bone marrow-derived EPCs home to ischemic tissue to form new vessels in response to a hypoxic stimulus.[12]

produce factors such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), angiopoietins, cytokines (TNF-α, interleukin-1) and various other factors.[7] Some factors, such as PDGF, engage supporting cells, while other factors stabilize new vessels or induce apoptosis in redundant vessels.[7] Many cells can produce angiogenic factors directly or indirectly, such as macrophages, neutrophils, hypertrophic chondrocytes, lymphocytes and endothelial cells, increasing the new vasculature.[1]

Cartilage is usually avascular, but exceptions are made during endochondral ossification (bone formation) and arthritis. The mechanisms for both processes are thought to be similar.[59] First, the chondrocytes undergo rapid mitosis, followed by hypertrophy, during this process, matrix metalloproteinases (MMPs) are produced to degrade the cartilage extracellular matrix (ECM), and to form cartilage canals (Figure 2).[6] MMPs 1, 2, 3, 9, 10, 13 and 14 are involved in endochondral ossification. MMP 13 is the main player in the formation of the cartilage canals, as its function is to cleave collagen type II. MMP 13 is upregulated in terminal hypertrophic chondrocytes in the growth plate and in osteoblasts.[69] Hypertrophy of the chondrocytes is followed by mineralization and apoptosis succeeded by *de novo* bone matrix formation by osteoblasts.[6] To remove cartilage debris in the canals, macrophages are enlisted and Tartrate-resistant acid phosphatase (TRAP) is released by osteoclasts and macrophages to mediate cartilage matrix disintegration. Finally pro-angiogenic factors, such as VEGF and connective tissue growth factor (CTGF) are produced by the hypertrophic chondrocytes, which induce vessel formation.[6]

Antiangiogenic factors released by chondrocytes

In normal articular cartilage chondrocytes excrete antiangiogenic factors, preventing vessel formation by endothelial cells. These factors are chondromodulin1 (ChM1), thrombospondin1 and 2 (TSP1 and 2), troponin1 (Tn1) and tissue inhibitor of matrix metalloptoteinases2 and 3 (TIMP2 and 3).[16, 25, 30, 46, 64] ChM1 is expressed by chondrocytes.

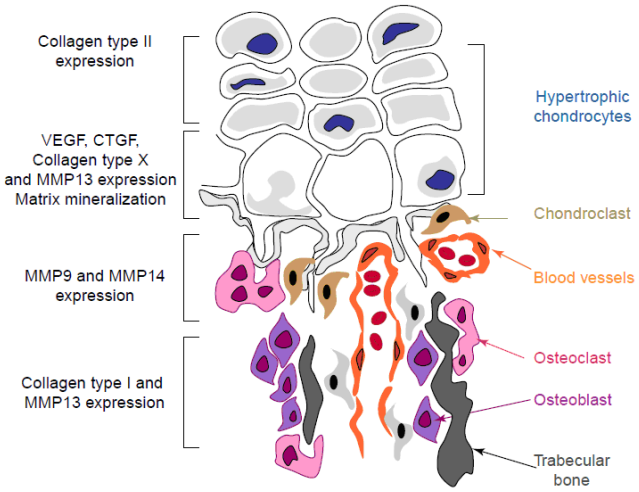

Figure 2. "The process of endochondral ossification is dependent upon neovascularization, during which cartilage is replaced by bone. The differentiation of chondrocytes into hypertrophic chondrocytes is accompanied by the expression of angiogenic growth factors, notably VEGF, allowing vascular invasion of the cartilage. Proliferating chondrocytes express collagen type II, whereas hypertrophic chondrocytes express collagen type X, MMP 13 and high levels of angiogenic growth factors: VEGF and CTGF."[54]

It inhibits tube morphogenesis of bovine aortic endothelial cells (BAEC) but stimulates DNA and proteoglycan synthesis of cultured growth plate chondrocytes.[30] Furthermore, ChM1 production decreases when chondrocytes become hypertrophic and collagen type X is produced, the early symptoms of endochondral ossification. Therefore, ChM1 can be called an antiangiogenic factor that prevents vascular invasion in healthy mature articular cartilage.[64] TSP1 has been shown to reduce microvessel density, macrophage infiltration and interkleukin-1b levels, which leads to less severe osteoarthritis.[31] TSP2 functions the same way as TSP1. It has been shown that if TSP2 is overexpressed in SCID mice implanted with human inflammatory infiltrates, vascularization of the lesions is inhibited. Moreover, in rheumatoid arthritis, inflammation is suppressed by production of interferon and tumor necrosis factor and a decrease of tissue-residing T- cells.[56] Another antiangiogenic factor found in cartilage matrix is Tn1. Tn1 has been introduced to mouse corneas, where reduced neovascularization was observed.[46] TIMPs inhibit matrix metalloproteinases that degrade cartilage, such as MMP 1, MMP 2, MMP 3, MMP 9, MMP 10 and MMP 13.[1] If these MMPs are inhibited by TIMPs no cartilage canals are formed and vessel ingrowth is prevented.

Integrins

The lack of vascularization in normal cartilage could be because the endothelial cells lack receptors to bind to collagen type II. Integrins and discoidin-domain receptors (DDRs) can bind to collagen type II. DDRs are not found on endothelial cells, so they cannot bind to collagen type II via that way. Integrins, however, are expressed on endothelial cells. They can

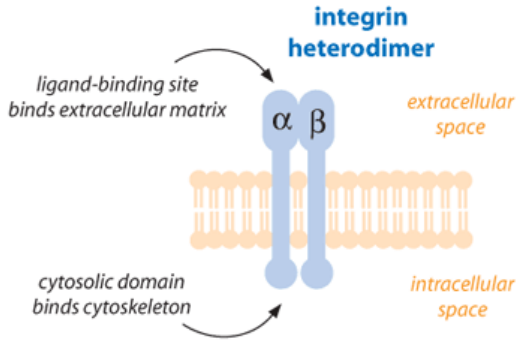

Figure 3. The basic structure of an integrin, containing an alpha and beta domain [19].

be part of focal adhesion points, which are large, dynamic protein complexes through which both mechanical force and regulatory signals are transmitted.[13] Integrins are a family of transmembrane glycoproteins containing a large globular alpha and beta domain (Figure 3), which bind to growth factors, cytokines, matrix-degrading proteases and specific extracellular matrix proteins, such as fibronectin, laminins, tenascin, thrombospondin, and collagens.[3, 29, 43] At the moment, 24 integrin heterodimers have been found by combining 18 α-subunits and 8 β-subunits. Besides the extracellular domains, integrins have cytoplasmic domains which interact with cytoskeletal proteins and signaling proteins inside the cell. Integrin mediated signaling pathways can activate mechanisms of cell survival, such as preventing apoptosis. Loss of adhesion or unligated integrins can initiate programmed cell death, called anoikis.[18]

We want to know if endothelial cells display integrins that can bind to collagen type II. Of course the various types of endothelial cells display different integrins. Moreover, integrin expression is dynamic and can change with age, differentiation status and respond to microenvironmental conditions.[3]

Endothelial cells express a multitude of integrins, such as: α5β5, α5β3, α5β1, α5β8[62], α1β1, α2β1, α3β1, α6β1, αvβ1, α6β4, αvβ3, αvβ5[33], α1, α2, α6 (β subunit not investigated).[3] The number of integrins present on a given cell correlates with the extent to which that cell will adhere to and migrate into the matrix.[18]

We are investigating if integrins are responsible for endothelial cell binding to collagen type II, the main component of cartilage. Integrins that can bind to collagen type II are: α1β1 together with other β1-integrins capable of binding type II collagen, α2β1 in combination with α1β1[35, 42], α10β1 which has a very high affinity with collagen type II, shown by affinity chromotography [11, 35] and α3β1.[45, 61] Of these integrins, α1β1, α2β1 and α3β1 are expressed by endothelial cells.[33] It is therefore plausible that endothelial cells are able to attach to collagen type II, and that the antiangiogenic activity of the chondrocytes is the restraining factor, and not the matrix.

Matrix metalloproteinases

Another explanation for the lack of vascularization in cartilage can be that the cells can bind to collagen type II, but they cannot break it down to form vascularized cartilage canals. MMPs are enzymes involved in precise ECM degradation. The human genome contains 23 different MMPs that can be grouped into collagenases, gelatinases, stromelysins, matrilysins, membrane-type (MT)-MMPs and others (Table 1).[48] Collagenases can hydrolyze collagen type II to form gelatine, which is degraded even further by other MMPs (gelatinases).[5] There are three collagenases, MMP 1, 8 and 13. MMP 13 has the highest affinity for cleaving collagen type II, followed by MMP 8[55] and then followed by MMP 1.[34, 75]

Cleaved collagen type II has been described to upregulate mRNA and protein levels of MMP 2, MMP 3, MMP 9 and MMP 13 in bovine chondrocytes, resulting in a vicious circle of cartilage degradation.[67] Further investigation into these MMPs shows that MMP 2, a gelatinase, can cleave collagen type II, but has a higher affinity for already cleaved collagen fragments of ¼ or ¾ length[57]. Another MMP capable of cleaving collagen type II is stromelysin-1 also called MMP 3.[5, 17, 74] Some report that MMPs 7, 14 and 15 can degrade collagen type II[68] and others add MMP 10 to the list (Table 1).[24] All in all, MMP 13 cleaves collagen type II the most.

Of course we want to know which of these MMPs are expressed by endothelial cells, and can endothelial cells alone create cartilage canals into collagen type II? Endothelial cells produce MMPs 9 and 10 during endochondral ossification.[54] MMP 9 is a gelatinase that can break up collagen type IV and V and MMP 10 degrades collagens, including collagen type II, proteoglycans and fibronectin.[54, 68] Additionally, evidence suggests that MMP 2 and membrane type 1-MMP (MMP 14) are also produced by endothelial cells.[28] MMP 1, 2, 10, 13 and 14 are likewise described to be expressed by endothelial cells.[52, 77] Furthermore, in tubulogenesis human umbilical vein endothelial cell (HUVEC) production of MMPs 2, 14, 15 and 16 is upregulated[36] and if basic fibroblast growth factor (bFGF) is added to endothelial cells, MMP 3 is increased.[17] MMP 7 is weakly expressed in endothelial cells, but upregulated in tumors.[49] In conclusion, collagen type II is cleaved by MMPs 1, 2, 3, 7, 8, 10, 13, 14 and 15, of these MMPs endothelial cells can produce MMPs 1, 2, 3, 7, 9, 10, 13, 14, 15 and 16, meaning that endothelial cells have ample opportunity of degrading collagen type II using MMPs 1, 2, 3, 7, 10, 13, 14 and 15. (Table 1)

Table 1. Members of the MMP family

Subgroup	MMP	Name	Substrate	Expressed by endothelial cells
1. Collagenases	MMP-1	Collagenase-1	Col I, II, III, VII, VIII, X, gelatin	yes
	MMP-8	Collagenase-2	Col I, II, III, VII, VIII, X, aggrecan, gelatin	no
	MMP-13	Collagenase-3	Col I, II, III, IV, IX, X, XIV, gelatin	yes
2. Gelatinases	MMP-2	Gelatinase A	Gelatin, Col I, II, III, IV, VII, X	yes
	MMP-9	Gelatinase B	Gelatin, Col IV, V	yes
3. Stromelysins	MMP-3	Stromelysin-1	Col II, IV, IX, X, XI, gelatin	yes
	MMP-10	Stromelysin-2	Col II, III, IV, V, IV, laminin, fibronectin, elastin	yes
	MMP-11	Stromelysin-3	Col IV, fibronectin, laminin, aggrecan	nk
4. Matrilysins	MMP-7	Matrilysin-1	collagen types II, III, IV, V, X, fibronectin, laminin, Col IV, gelatin	yes
	MMP-26	Matrilysin-2	Fibrinogen, fibronectin, gelatin	nk
5. MT-MMP	MMP-14	MT1-MMP	Collagen types I, II, III gelatin, fibronectin, laminin	yes
	MMP-15	MT2-MMP	Collagen types I, II, III gelatin, fibronectin, laminin	yes
	MMP-16	MT3-MMP	Gelatin, fibronectin, laminin	yes
	MMP-17	MT4-MMP	Fibrinogen, fibrin	nk
	MMP-24	MT5-MMP	Gelatin, fibronectin, laminin	nk
	MMP-25	MT6-MMP	Gelatin	nk
6. Others	MMP-12	Macrophage metalloelastase	Elastin, fibronectin, Col IV	nk
	MMP-19		Aggrecan, elastin, fibrillin, Col IV, gelatin	nk
	MMP-20	Enamelysin	Aggrecan	nk
	MMP-21	XMMP	Aggrecan	nk
	MMP-23		Gelatin, casein, fibronectin	nk
	MMP-27	CMMP	Unknown	nk
	MMP-28	Epilysin	Unknown	nk

24, 36, 49, 52, 64, 66, 68

Antiangiogenic properties of the extracellular matrix

Perhaps there are other factors that prevent endothelial cells from forming vessels in collagen type II. Recently, various polypeptides in shark cartilage were discovered and proven to possess antiangiogenic abilities.[15] Although little is known about the mechanism or the amino acid sequence of these polypeptides, they have proven to be antiangiogenic *in vitro* and *in vivo.*[60] One proposed mechanism is that shark cartilage reorganizes focal adhesion points between the endothelial cells and ECM and reduces stress fiber formation, resulting in less adhesion to the cartilage.[14] However, clinical studies using purified antiangiogenic factors from shark cartilage, Neovastat (AE-941), have not shown improvement for patients with advanced cancer. Nevertheless, this may be due to crude extracts that vary in composition because of seasonal and environmental factors and isolation methods. Moreover, oral administration might not be the most effective way of administering the extract.[78] In any case, the antiangiogenic effect of these polypeptides may prevent endothelial cells from migrating into normal articular cartilage. It was not mentioned if these polypeptides were matrix derived or tissue derived. Therefore, it cannot be concluded that cartilage matrix is antiangiogenic, only that shark cartilage matrix together with chondrocytes are.

Bayless et al.[4] has tested *in vitro* vascularization in a collagen type II gel. Here a collagen type II gel (cat. no. C7806, Sigma-Aldrich), from bovine nasal septum, is mixed with M199, NaOH and sphingosine 1-phosphate (S1P), a lipid mediator, stimulating vascular invasion. This gel thermo-polymerizes at 37°C, after which an endothelial monolayer is seeded on top. To stimulate rapid sprouting, culture medium with VEGF and bFGF is added, also mimicking a luminal compartment. Similar sprouting responses into the gel were shown for HUVECs, human dermal microvascular cells and retinal endothelial cells. S1P was responsible for a dose-dependent sprouting effect (Figure 4a,b). HUVEC and retinal endothelial cell invasion was α2β1 integrin dependent, shown by adding several specific integrin blocking antibodies to the cells (Figure 4c,d).[4] This article confirms that endothelial cells can infiltrate collagen type II, because they express the necessary integrins and possibly produce several MMPs that can degrade collagen type II. This means that normal cartilage is avascular because antiangiogenic factors are produced by chondrocytes and not that collagen type II itself is antiangiogenic. For tissue engineering purposes, antiangiogenic factors should be added to collagen type II to prevent vessel ingrowth.

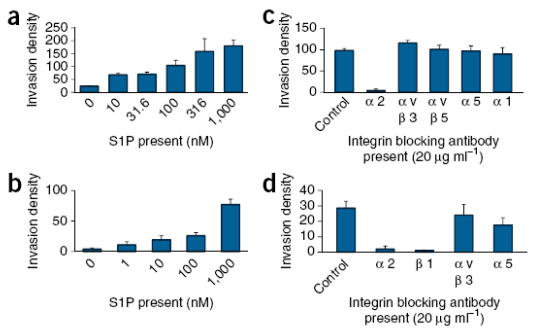

Figure 4. **"**Endothelial cell invasion in type II collagen matrices is driven by S1P and growth factors and is integrin dependent. (**a** and **b**) Collagen matrices were prepared with increasing concentrations of S1P and allowed to invade for 48 h or (**c** and **d**) 1 μM S1P and allowed to invade for 24 h. (**c** and **d**) Integrin blockade was accomplished by adding 20 μg/ml − 1 of integrin blocking antibodies directed to the α 2, α v β 3, α v β 5, α 1 or β 1 subunits. (**a** and **c**) Wells were seeded (40,000 cells per well) with HUVECs or (**b** and **d**) retinal endothelial cells. In all panels, invasion density was quantified following glutaraldehyde fixation and toluidine blue staining. Quantification of invasion density was carried out using a ×10 objective (1 mm^2) and data presented are average numbers of invading cells per field (± s.d.)."[4]

Requirements for a collagen type II gel

As cartilage consists mainly of collagen type II, it is generally thought that collagen type II is a better molecular environment for chondrocyte culture than collagen type I.[23] Therefore, for tissue engineering purposes, we want to use a collagen type II gel, to print highly organized grafts using a bioprinter. The bioprinter is a machine that allows layer-by-layer deposition of cell laden hydrogel to create heterogeneous/organized tissue constructs.[21]

One of the current challenges of printing is finding a printable gel. Gel requirements are that the gel must be cytocompatible and proliferation of the cells possible. Additionally, the viscosity of the hydrogel must allow extrudation through a needle, whilst solidifying rapidly to keep construct shape.[20] There are several types of hydrogel solidification methods. Firstly, a gel can be thermosensitive, which means that crosslinking is temperature dependent and can be achieved by lowering or increasing the temperature, for example by cooling the printer cartridge and heating the plate you print on.[72] Secondly, some gels are photosensitive, meaning they contain a photoinitiator that will crosslink the gel once it is exposed to (UV) light. The third way is to chemically crosslink the construct by using a crosslinking-reagent such as $CaCl_2$.[21] A fourth type is a pH sensitive gel, where the gel solidifies when the pH is increased.[20]

Additional requirements for a printable collagen type II gel include, not eliciting an immunologic response in animal models, and it is preferable if the gel is biodegradable, releasing non toxic degradation products, and that the degradation rate is the same as the tissue regeneration rate of the cells.

Printing collagen type I and II gel

So far various natural and synthetic hydrogels have been used for bone and cartilage tissue engineering. Collagen type II is considered a better molecular environment for chondrocytes, as cartilage consists mainly of collagen type II, it is surprising that it has not been used more often. This could be explained by some major drawbacks of a collagen type II gel: that it is expensive and that it has potential arthritogenic properties.[2, 23] Collagen type I is also easier to handle, as acid-extracted type I collagen can organize into a fibrillar network *in vitro* more easily than papain treated type II collagen.[23] Another consideration is that chondrocytes in collagen type II gel do not proliferate or excrete matrix as much as chondrocytes in a collagen type I gel, because in normal cartilage chondrocytes are senescent. Nevertheless, for these reasons alone collagen type II should not be dismissed.

There are many different types of bioprinters/three-dimensional bioassembly tools for tissue engineering (Figure 5). They use various hydrogels such as alginate, matrigel, pluronic F-127, or collagen type I, but printing of collagen type II gels has not been described to the author's knowledge. For example, a three-dimensional, direct-write bioassembly system was used to deposit collagen type I mixed with BAECs in a controlled three-dimensional pattern (Figure 5B).[65] Briefly, a 3.0 mg/mL collagen type I solution was prepared with Dulbecco's modified Eagle's medium (DMEM). The pH was brought to 7.0-7.4 and the solution was kept at 10°C. It was possible to print 5 layers on top of each other with a 1.5mm strand distance and the gel was crosslinked by raising the temperature to 37°C.[65] Another article describes a bioprinter where an ultrasonic transducer deposits a fine mist of collagen

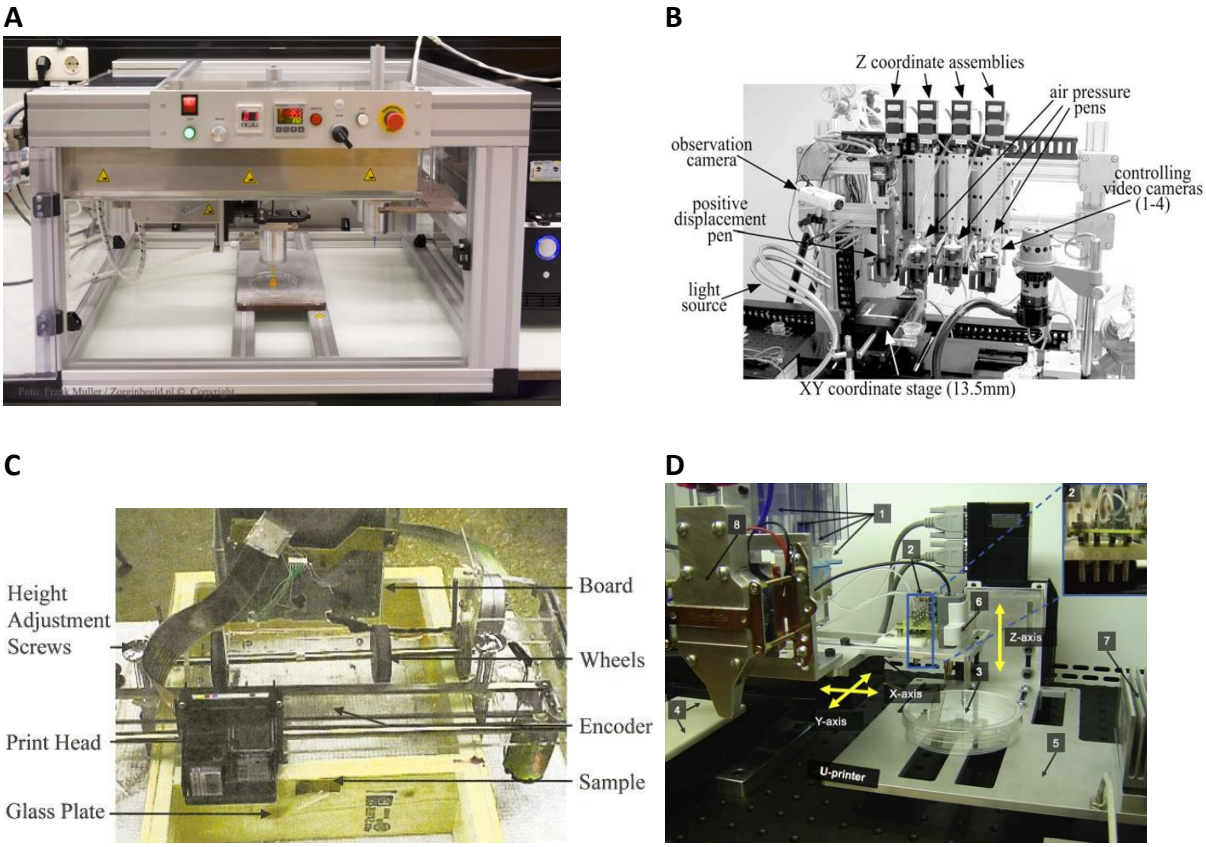

Figure 5. Examples of bioprinters. **A** Bioprinter, department of Orthopedics, UMC Utrecht, Netherlands. **B** Three-dimensional bioassembly tool, division of Biomedical Engineering, University of Arizona, Tucson, Arizona[65]. **C** Protein printer, department of Bioengineering, Clemson University, Clemson, South Carolina[73]. **D** Tissue printing platform, department of Radiology, Harvard Medical School, Boston USA.[39]

type I on a sodium bicarbonate ($NaHCO_3$) coating, partially crosslinking the collagen. Then a cell suspension with embryonic rat neural cells was applied to the collagen layer followed by another coating of nebulized $NaHCO_3$ to fully crosslink the collagen (Figure 5D).[39] The collagen type I solution was made with rat tail collagen, acetic acid (0.02N) and phosphate buffered saline (PBS) (volume ratio: 1:1:2, pH 4.5) to a concentration of 1.12 mg/ml.[39] Jakab, Xu and Wilson tried a different approach by first printing a collagen type I bio-paper and then printing cardiac and endothelial cells into the bio-paper using a bioprinter (Figure 5C).[32, 73, 76] The rat tail collagen was dissolved in 1M acetic acid and adjusted to pH 7.2 with 2M sodium hydroxide, HEPES buffer and DMEM-F12K to 1mg/mL. Before polymerization, VEGF was added (20ng/mL) to stimulate cell growth.[32] A fourth printing method makes use of fluidic channels. A bio-printer prints a block containing collagen type I and gelatin layers. Upon heating the block to 37°C, the gelatin melts and the fluidic channels appear which presumably can be used for vascularization studies.[40] The collagen hydrogel precursor was diluted with PBS (2.23mg/mL) and kept at a pH of 4.5 in an ice bath. Nebulized $NaHCO_3$ was used for crosslinking.[40] All in all, as collagen type I and II do not vary greatly in chemical properties, printing a collagen type II gel in a similar fashion seems feasible.

A few reports in literature describe fabrication methods of collagen type II sponges/matrices, which are not printed.

One article outlines that primary chondrocytes remain phenotypically unaltered on collagen type II sponges.[47, 50] Sponges made from collagen type II seeded with chondrocytes, show a cartilaginous-like layer on the surface with clusters of chondrocytes within.[58] After implanting these collagen type II matrices in rabbits, they displayed an increase in the amount of superficial cartilage like tissue and the cells kept their chondrocyte phenotype, even though cell migration was low.[10]

One method of producing these sponges/matrices is by freeze-drying reconstituted cartilage before UV light exposure.[47, 50, 51, 71] Another method is to treat the collagen with Tris-HCl, EDTA and pepsin and precipitate it with hyaluronic acid (HAc). Finally, atelocollagen (water soluble form of collagen) can be aggregated by dialysis against a phosphate buffer and then lyophilized.[10, 58]

Readymade collagen type II gels (MCK-K-41, Cosmo bio co.) or lyophilized collagen powder (MCK-K-42, Cosmo bio co), is also available. To summarize, chondrocytes seeded on collagen type II sponges have kept their phenotype, proving that collagen type II is a suitable material for chondrocyte culture and possibly tissue engineering.

Future experiment

Is it possible to print collagen type II using a fiber deposition system? As collagen type I has been printed before, a similar experiment could be designed. When checking the requirements of the gel, eliciting an immunologic response seems unlikely as Bayless et al. has already described that collagen type II is cytocompatible and it naturally resides in humans and mice.[4] The main problem will lie with the viscosity of the gel and the speed at which it gellifies. First of all, it is very important to adjust the pH for cytocompatibility reasons and gellifying of the gel. The gel should be slightly basic and not too acidic, a pH between 7.0 and 7.4 is preferred.[65] To let the gel polymerize faster, more NaOH should be added.[4] To test the cytocompatibility, make several solutions with various NaOH concentrations mixed with cells, then perform a live/dead assay after 1, 3 and 7 days, for example.

For printable collagen type I gels only the pH was adjusted to 4.5 and the gel was diluted to 1.12mg/mL in 0.02N acetic acid and PBS (1:1:2 ratio).[41] For the biopaper, the collagen type I pH was adjusted to 7.2 with NaOH and HEPES buffer and then diluted to 1 mg/mL with DMEM/F12K.[32]

If the viscosity of the gel is still not high enough, alginate could be added. This worked for a collagen type I gel, where a minimal percentage of 35% alginate was used with collagen type I, making it more viscous.[8] Another option is mixing the gel with fibrinogen, a component of fibrin glue, which has been tried before with collagen type I, and improves collagen type II and proteoglycan production in chondrocytes.[44] Fibrin glue has also been mixed with alginate and printed using a 3D plotter.[37, 38] A third option is mixing hyaluronan hydrogel with collagen type II, this has been used for constructs and an *in vivo* injectable gel and is viscous enough to print.[53, 63]

Other aspects to keep in mind are that nutrient and oxygen diffusion range from 100µm to 200µm, subsequently, a porous scaffold is preferred over a solid one.[27] Secondly, growth factors such as TGFβ2 added to the medium can improve chondrocyte morphology.

Conclusion

In conclusion, cartilage matrix in itself is not antiangiogenic, but the chondrocytes secrete antiangiogenic factors resulting in avascular cartilage. Endothelial cells are

able to migrate and proliferate in collagen type II gel, which is shown by Bayless et al.[4] Endothelial cells are able to produce MMP 1, 2, 3, 7, 10, 13, 14 and 15, which can all cleave collagen type II. This enables them to degrade collagen type II matrix and form canals where vasculogenesis can take place. Migration into these canals is mediated by integrins, integrin α1β1, α2β1 and α3β1 are expressed by endothelial cells and can bind to collagen type II. Our second question deals with the feasibility of printing collagen type II using a fiber deposition system. Collagen type I gels have been printed simply diluted with DMEM or by adding thickeners such as alginate, fibrinogen or hyaluronic acid. As collagen type I and II are similar, we suggest trying to print collagen type II mixed with chondrocytes and if necessary the thickeners. We also suggest adding growth factors and perhaps antiangiogenic factors, as collagen type II is not antiangiogenic, but the factors excreted by the chondrocytes are.

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