Reconstruction of the Anterior Cruciate Ligament;

Alternative Strategies

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The anterior cruciate ligament (ACL) is a short intraarticular, extrasynovial structure which acts to control rotational movements and anterior translation of the tibia upon the fixed femur. Rupture of the ACL is a traumatic event, often as a consequence of twisting a weight-bearing limb. The ACL has no intrinsic healing capacity, and thus rupture can lead to instability of the knee. Replacement using a graft is indicated to reduce instability complaints and limit the number of recurring pivotal moments with subsequent damage. The ideal ligament replacement should be readily available and have no donor-site morbidity; it should be of sufficient length and diameter; it should have mechanical properties similar to the ligament it replaces; it should not disturb normal structures and it should retain or develop a vascular supply.

Long-term studies evaluating the subjective and objective outcomes of most commonly used autograft and allograft replacements demonstrate greater than 90% success rates[125,160,207]. However reports on ACL reconstruction surgery with a follow-up of more than ten years are rare[161,195,222]. It is unknown if the high success rates described in literature will also be applicable after more than ten years. Will the stability rates and clinical results continue to be good after more than ten years or will they decline? To make any statements about success rates, it would be interesting to study what the stability rates, and clinical outcomes would be, when the reconstructed patient becomes older and less in demand of a highly stable knee. With long-term follow-up studies of more than ten years one could also study if there is a preventive role of ACL reconstruction in the development of other intra-articular damages, such as meniscal or cartilage injuries.

ACL reconstruction is not a universally successful procedure. Harvest of autologous tissue from the patellar tendon for ACL reconstruction for example has been associated with anterior knee pain, decreased range-of-motion, and loss of strength[141]. The risk of disease transmission and immunologic reaction with the use of allografts are still serious concerns[17,39,218,250]. Although the clinical importance of such an immune response is currently unknown, it may affect

graft incorporation, revascularization, and graft remodeling. Also the commonly used grafts still have long-term mechanical properties that are far from those of a normal, non-injured ACL[42,44].

The limitations in currently used techniques in ACL reconstruction surgery have prompted ongoing research aimed at developing a tissue-engineered ligament graft that mimics the biologic and mechanical properties of the native ACL. Regenerative medicine or tissue engineering is a field of multidisciplinary interactions aimed at incorporating the principles of biochemistry, engineering, clinical knowhow and materials science to develop substitutes for replacing injured or diseased tissues. Researchers in several disciplines have expanded the scope of this technology and are developing engineered replacements for a variety of tissues, including nerve, skin, cardiac valves, bone, cartilage and menisci[118,153,212,246,247]. The concept of regenerating a functional ligament requires a system that combines appropriate differentiated cells that have the capability to proliferate and produce relevant matrix. Furthermore it needs a biocompatible carrier or scaffold with high mechanical properties that guides these cells and provides initial strength. The cell seeded scaffold needs to be cultured in an environment that provides sufficient nutrient transport and appropriate regulatory stimuli. The appropriate growth factors and mechanical stimulation are essential for successful ligamental tissue engineering.

Current strategies for ACL reconstruction surgery have improved tremendously the past decades, however several limitations still exist and the long term effects of this intervention have not been explored extensively yet. The current strategies that surround the regeneration of an ACL substitute are in full development and some report promising results. However many determinants remain to be explored. In every aspect of the process from selecting the donor cells and seeding them on a scaffold in the laboratory, to a functional ligament reconstructed in the knee, a myriad of questions await investigation.

The aims of this thesis are as follows:

1. To study the long-term outcome following arthroscopic assisted anterior cruciate ligament reconstruction using allografts and to investigate the clinical relevance of immunologic reactions against the donor;
2. To investigate the optimal cell source for ligament tissue engineering;
3. To study the effect of different growth factors on proliferation and differentiation of bone marrow stromal cells seeded onto poly(L-lactide/glycolide) scaffolds;
4. To study the effect of static load on proliferation and differentiation of bone marrow stromal cells seeded onto poly(L-lactide/glycolide) scaffolds;
5. To develop a method to study the survival and functionality of bone marrow stromal cells in vivo.

In **chapter 2** a review is presented on the ACL, where we discuss properties and reconstructive strategies throughout history. Furthermore we give a review on the alternative strategies for ACL reconstruction surgery; namely the research performed on tissue engineering of ligaments.

Chapter 3 describes a patient series with clinical and immunological outcome of arthroscopic anterior cruciate ligament reconstruction using fresh frozen bone-patellar tendon-bone allografts with a 15-year follow-up. In this chapter the **first aim** was addressed.

In **Chapter 4** we aimed to find the optimal cell type for our tissue-engineered ligament (**aim 2**). The optimal cell type should be easily harvested and contain optimal properties to proliferate and to differentiate into a fibroblast which produces collagen typical for ligaments. What is the appropriate cell type to use? Based on literature we analyzed the behavior of bone marrow stromal cells (BMSCs), ACL and skin fibroblasts.

In **chapter 5** we studied the effect of different growth factors on proliferation and differentiation of BMSCs seeded onto poly(L-lactide/glycolide) scaffolds (**aim 3**). Little is known about the regulatory signals involved in ligament formation. Research has shown a possible role for growth and differentiation factors (GDFs) of the transforming growth factor-beta gene superfamily in the induction of neotendon/ligament formation[84,258]. It will be interesting to know if these growth factors can stimulate the seeded cells in vitro to increase proliferation and differentiation, in order to predetermine the cells' fate towards the ligament lineage.

In **Chapter 6** we studied the effect of static load on proliferation and differentiation of BMSCs seeded onto poly(L-lactide/glycolide) scaffolds. The importance of physiologic strain in maintaining ligament strength has already been demonstrated in vivo[260], and therefore the role of mechanical stimulation in the development of a tissue-engineered ligament could be an important factor to study (**aim 4**).

If we will be able to design a biological and mechanical stimulated construct with productive cells in vitro, how can we prove that these cells survive in vivo, differentiate and thus contribute to graft formation? A method to show that the cells not only survive but also differentiate and produce ECM in vivo has not been described before. Before a cell seeded scaffold is reconstructed into the knee, it is essential to first prove the viability and functionality of the cells in an in vivo ectopic setting. In **Chapter 7** we developed a method to study the survival and functionality of BMSCs in vivo (**aim 5**). We applied retroviral genetic marking to trace implanted cells and studied their differentiation by species-specific immunolabelling of the extracellular matrix produced.

Chapter 8 summarizes the studies described in this thesis and final conclusions are given.

2

THE ANTERIOR CRUCIATE LIGAMENT – A REVIEW ON FUNCTION AND RECONSTRUCTIVE AND REGENERATIVE STRATEGIES

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Anatomy

The anterior cruciate ligament is composed of longitudinally oriented bundles of collagen fibers arranged in fascicular subunits within larger functional bands. The ligament is surrounded by synovium, thus making it extrasynovial. The ACL originates from the medial wall of the lateral femoral condyle posterior to the intercondylar notch and inserts on the tibial plateau, medial to the insertion of the anterior horn of the lateral meniscus in a depressed area anterolateral to the anterior tibial spine. In the ACL we can identify an anteromedial and a posterolateral bundle.

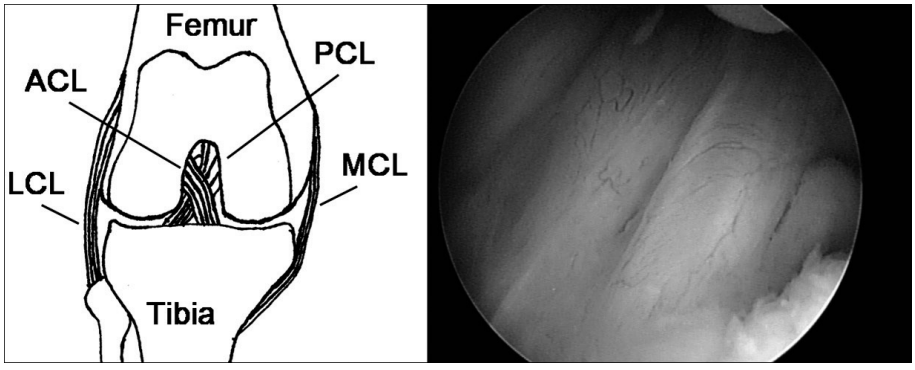

Figure 1. *Left: anatomy of the ligaments in the knee. Right: arthroscopic view of the anteromedial and posterolateral bundle of the ACL. (See full colour section page 175)*

The tibial attachment site is larger and more secure than the femoral site. The ligament is 31 to 35 mm in length and on average 31.3 mm^2 in cross section[88,198]. The primary blood supply to the ligament is from the middle geniculate artery, which penetrates the posterior capsule and enters the intercondylar notch near the femoral attachment. Additional supply comes from the retropatellar fat pad via the inferior medial and lateral geniculate arteries. This source plays an important role when the ligament is injured, because blood vessels contributing to the revascularization originate from the fat pad[166]. The osseous attachments of

the ACL contribute little to its vascularity. The posterior articular nerve, a branch of the tibial nerve, innervates the ACL. The paucity of free nerve endings might explain the absence of pain at the moment of injury and the development of severe pain only after the joint capsule becomes distended with blood. The type I and II nerve endings, which are found in histological studies of the ACL, might be responsible for the sensation of instability[11].

Biochemistry and microstructure

Ligaments in general contain approximately two-thirds of water by weight; nearly 75% of their dry mass is made up of collagen. More than 90% of this collagen is type I, with a small percentage of type III. Smaller portions such as elastin, glycosaminoglycans and proteoglycans compose the final matrix, which provide elasticity and lubrication[81]. Ligaments are composed of densely aligned fascicles. These fascicles contain collagen fibrils that are longitudinally aligned and surrounded by a collagenous sheath. Collagen fibrils in adult human tendons/ligaments have an average diameter of 60 to 175 nm[69]. Ligaments have a crimped, waveform appearance when seen under a polarized light. This pattern has been shown to play an important role in its mechanical properties. It is thought that the crimp pattern straightens out during initial loading of the tendon/ligament[142], providing a spring mechanism and a barrier to failure loading.

In ligament healing fibroblasts from surrounding tissue migrate to the repair site and start to produce collagen, with a higher proportion of Type III collagen. In the reparative phase, the fibroblasts remain the predominant cell type and Type III collagen comprises a relatively larger percentage of the scar tissue. In the last phase of the healing process, cellularity decreases, the collagen density and the cross-linking increases and collagen fibers are aligned with the axis of the ligament. There will be a higher percentage of collagen type III in a repaired ligament as compared to the normal non-injured ligament[166].

Biomechanics and function

The ACL is the primary restraint to anterior tibial displacement, accounting for approximately 85% of the resistance to the anterior drawer test when the knee is at 90 degrees of flexion and neutral rotation[225]. Selective transection of the ACL has shown that the anteromedial band is tight in flexion, providing the primary restraint, whereas the posterolateral bulky portion of this ligament is tight in extension. The posterolateral bundle provides the principal resistance for hyperextension. Tension in the ACL is least at 30 to 40 degrees of knee flexion. The ACL also functions as a secondary restraint on tibial rotation and varus-valgus angulation at full extension.

The complexity of the arrangement of the ligament fibers and their response to load has important implications regarding the results of tensile tests. Tensile testing of the ACL depends on age of the specimen, angle of knee flexion, direction of tensile loading with respect to the ACL, and rate of the applied load. In other words, the maximal strength of the ACL should not be assumed to have one fixed value[260].Notwithstanding this qualification, investigators have studied the mechanical properties of the ACL. Noyes determined the ultimate load to be 1725 ± 269 N; the stiffness, 182 ± 33 N/mm; and the energy absorbed to failure, 12.8 ± 2.2 N-m[198]. Woo et al., in younger specimens, found the ultimate load to be 2160 ± 157 N and the stiffness 242 ± 28 N/mm[261] (see table 1). The tensile strength is defined as the ultimate load which is needed to cause rupture of the ligament. Stiffness is defined as the resistance of the ligament to deformation by an applied force.

The stabilizing role of the ACL is particularly important in athletes, such as football players, performing activities involving cutting (i.e. sudden change in direction), pivoting, and kicking.

Unlike extra-articular ligaments that heal after injury, the ACL does not have intrinsic healing properties. After rupture, a layer of synovial tissue is formed over the ruptured surface and the ends retract[192]. These events may play a role in the lack of healing of the ruptured ACL.

Table 1. mechanical properties of the ACL and its commonly used grafts

Source	Tensile strength (N)	Stiffness (N/mm)
Human*	1725-2160[198,261]	182-242
BPTB	2300-2900[198,236]	620-685
DLSTG	4213-4590[106,244]	861-954
Quadriceps**	2352[236]	
Goat***	1547-2023[197]	275-306
Dog***	1129[95]	
Rabbit***	369[113]	130

young adults, ** quadriceps tendon with a bone block from te superior pole of the patella, * mature animals, BPTB=Bone-Patella Tendon-Bone, DLSTG=Double-Looped Semi-Tendinosus and Gracilis graft.*

Epidemiology and morbidity of ACL ruptures

Today there is an increasing number of people who participate in sports related activities at all ages. This consequently results in an increasing number of injuries and accidents. An acute rupture of the ACL is a common sports related injury. In the US 95.000 people annually are diagnosed with a torn ACL. This is comparable with one out of 3000 people[79]. In the US more than half of the people with a torn ACL undergo a reconstruction[82]. The main causes for ACL ruptures in Europe are soccer and skiing. The ACL is most frequently ruptured in a non-contact decelerating valgus angulation with external rotation. Other mechanisms include hyperextension with torsion, valgus angulation caused by an external force and hyperflexion[75].

Depending on the pattern of the mechanism and force of the trauma causing the ACL rupture, associated injuries to menisci, other knee ligaments and the cartilage are often observed. ACL injury predisposes the knee to chronic instability, further meniscal and chondral damage and an impaired quality of life[127,187]. It may also predispose to osteoarthritis[66,228]. However there is no evidence in literature to show that reconstruction of the ACL can provide protection against damage of the cartilage[168]. It is thought that the development of osteoarthritis in the injured knee is caused by a pathogenic process that is initiated at the time of the injury

combined with the long-term changes in dynamic joint loading[168].

The most common symptom of patients with a torn ACL is instability, also called the 'giving way' sign. The functional instability of a patient with a torn ACL can vary depending on his or her activity level and muscle strength. While ACL reconstruction is a clinically accepted intervention, non-operative management is often indicated for people who are less active, have minimal instability symptoms, and who are unable or unwilling to follow the demanding post-surgical rehabilitation protocols. It is noteworthy that a Cochrane review comparing surgical versus non-surgical intervention for ACL injuries found no evidence from randomized controlled trials to inform current practice which treatment is superior[165]. Conservative treatment, including muscle strength exercises and bracing, has been considered to have a good outcome in the general population[54]. Nevertheless, ACL surgery has become very popular especially among surgeons treating sports injuries and it is becoming common practice for young and active people with ACL rupture to be treated operatively.

Operative treatment

Throughout the past century many techniques have been developed to repair or reconstruct the torn ACL in patients. In 1895, A.W. Mayo Robson (Leeds, UK) performed the first cruciate ligament repair in a man by stitching his two torn cruciate ligaments in position at their femoral attachments[182]. Hey Groves presented in 1917 the first report of an intraarticular procedure to reconstruct the torn ACL[116]. He used a tethered fascia lata graft through anatomically placed drill holes in the femur and the tibia (figure 2).

However the poor mechanical properties of the fascia lata led to its demise. Primary repair of the torn ACL was advocated by some authors in the 1950s, and short-term results were encouraging; however, long-term retrospective and prospective studies showed that as many as 40% to 50% failed within 5 years based on the presence of clinical instability[73,103,253]. Previous experience

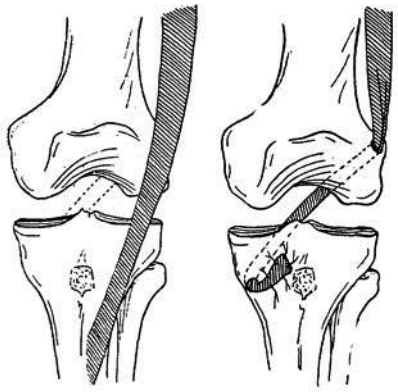

Figure 2. *First intraarticular reconstruction with the fascia lata (described by Hey Groves).*

with intraarticular reconstruction procedures was discouraging because of the frequent postoperative stiffness and persistent avulsion laxity. Extra-articular procedures were developed to circumvent these problems. These procedures generally create a restraining band on the lateral side of the knee. They avoid the problem of a lack of blood supply to the intraarticular reconstructions. Most lateral extra-articular procedures use the iliotibial band connecting the lateral femoral epicondyle to the Gerdy tubercle. When used alone, extra-articular techniques have been associated with high failure rates[214].

In 1963, Kenneth G. Jones revived the idea of using a central one-third of patellar tendon graft with an attached patellar bone block[136]. However, the technique described in his paper differed from the one used nowadays. The tendon was left attached to the tibia; there was no tibial tunnel; and because of the shortness of the graft, the author had to drill the femoral tunnel from the anterior margin of the notch. The ligament was secured to the periosteum at the superolateral exit site on the femur. With this technique the femoral tunnel was in the wrong place; however, the technique was simple and caused minimal operative trauma, which made it a distinct improvement.

The advances made in arthroscopy in the past decades have led to the development of arthroscopic techniques for ACL reconstruction. Simultaneously, the increased

understanding of technical issues of graft selection, placement, tensioning, and fixation as well as of postoperative rehabilitation led to dramatically improved results compared with previous intraarticular reconstructions.

Different kinds of grafts as well as various graft fixation methods have and are being used for intraarticular reconstruction of the ACL. The materials used for ACL replacement can broadly be classified as artificial, autologous and heterologous ligaments.

Artificial ligaments

The simplest solution to regain the stability of torn ACL, would be to find a biocompatible strong rope-like structure to replace the torn ACL. Artificial ligaments offer a number of theoretical advantages compared with reconstruction with the use of autologous tissues; off-the-shelf availability, no donor-site morbidity, simpler and easier reconstructive technique, and more rapid rehabilitation because they do not become weak during tissue revascularization and reorganization.

Artificial ligaments can function as a prosthetic ligament alone, as a stent temporarily protecting or augmenting an autologous graft; or as a scaffold providing support for and stimulating the ingrowth of collagen tissue.

The use of a synthetic material for ACL reconstruction was first described in 1918 by Alwyn-Smith who attempted to use silk sutures as a replacement[4]. These failed within three months. In 1977 Jenkins reported the use of pure carbon in a filamentous structure and showed that it induced the formation of a new tendon[132]. Later he reported that the carbon fiber slowly breaks up at the implantation site and was found in regional lymph nodes[131]. Experience with other fully prosthetic grafts has also been disappointing. Graft substitutes have been too abrasion-sensitive, resulting in rupture and osteolysis. These results apply to the artificial ligaments Dacron[93,177], Gore-tex[94,223] and the Leeds-Keio prosthesis[210].

The Kennedy ligament augmentation device (LAD) was introduced in 1980 and consists of a 6 mm cylindrical prosthesis of diamond-braided poly-propylene and was designed to protect the biological graft while it undergoes its phase of degeneration and weakness. Several studies have compared the efficacy of the LAD with either hamstring or patellar tendon grafts, however none has shown superior results[103,231]. Fixation of the LAD at both ends can lead to failure of the biological graft as a result of stress shielding.

The limitations of the synthetic grafts described above have led to the development of more biologically based grafts consisting of collagen-based materials. These grafts have been used alone or seeded with fibroblasts in an attempt to create neoligaments[68,129]. However these collagen ligaments are usually of animal origin and have poor tensile strength, with limited flexibility for modification.

Interest in the use of artificial ligaments for treatment of symptomatic knees with ACL deficiency remains, despite the above described disappointing clinical results, probably because of the theoretical advantages as compared to autologous and heterogenous substitutes. However until now no synthetic graft has been shown to be superior to autologous grafts.

Autologous ligaments

Autologous grafts for ACL reconstruction are the most commonly used grafts at this moment. Autografts have the advantages of low risk of adverse immune reactions and virtually no risk of disease transmission. About every structure around the knee has been used as a substitute; patellar tendon, hamstrings, quadriceps, iliotibial tract.

After an initial phase of acellular and avascular necrosis[127]an autograft undergoes repopulation of cells[148], revascularization and finally ligament maturation[12], but initially a 50% loss of graft strength occurs after implantation[42,44]. Early graft incorporation and remodeling of ACL grafts are essential to the success of ACL reconstruction. This process is dependent upon the cellular response to the mechanical forces applied to the graft during the healing process and the

amount of graft motion within the bone tunnel. Graft fixation is the weakest link in the initial 6- to 12-week period, during which healing of the graft to in the host occurs[216,256]. Studies have demonstrated that the time for complete graft incorporation differs significantly between different interfaces, i.e. bone to bone or tendon to bone interfaces[101,255]. ACL reconstructions in a goat model using bone patellar tendon grafts offer the ability to study bone to bone healing and soft-tissue to bone healing in the same animal. Histological evaluations from 3 to 6 weeks revealed progressive and complete incorporation of the bone block in the femoral tunnel, but only partial incorporation of the tendinous part of the graft in the tibial tunnel[202]. In recent years, studies have also aimed to enhance the rate of integration of tendon-bone interfaces during early graft incorporation that would permit an earlier and more aggressive postoperative rehabilitation. The use of bone morphogenic protein-2 (BMP-2) has shown some potential in a rabbit model[179].

Widening of both femur and tibia tunnels after reconstruction has been reported in literature. More anteriorly placed tibial tunnels resulted in more tunnel enlargement, although the widening seemed to decrease at a longer follow-up period[268]. There seems to be a greater risk of tunnel widening when using hamstring grafts compared with patellar tendon bone graft[57,156]. Explanations for the widening are the windshield-wiper effect and the bungy cord effect[156]. The "windshield-wiper effect" is thought to take place when the tunnel is relative larger than the graft, which may allow greater synovial fluid entrance into the bone tunnel and may allow motion of the tendinous portion of the graft within the tunnel, potentially leading to tunnel expansion. It is believed that fixation of the graft at a certain distance from the intraarticular surface, will result in longitudinal pistoning of the graft, resulting in micromotion at the graft-bone tunnel interface, resulting in a "bungy cord effect".

In a prospective randomized study, a significant reduction of tunnel widening was found in both femur and tibia when the graft was fixed close to the joint compared to a system where the graft is fixed extra-articularly or with more distance to the joint[77].

The most common current graft choices are the bone–patellar tendon–bone graft (BPTB) and the quadrupled hamstring tendon graft. A BPTB graft involves surgically harvesting the central one third of the patellar tendon with the attached bone from the patella and tibia. Proponents of the BPTB autograft cite superior graft strength, secure fixation and ease of harvest. Concern over patellar tendonitis and rupture, patellar fracture and anterior knee pain exists with use of the BPTB graft[141].

The hamstring graft involves harvesting the tendinous portion of the patient's semitendinosus and/or gracilis muscles. Those favoring the hamstring autograft cite smaller incisions, decreased donor site morbidity, multi-bundled structure with higher tensile strength and larger surface area for incorporation[97]. However this technique is associated with increased graft incorporation time[202] and possible hamstring weakness[1].

Anatomic and biomechanical studies suggest a novel strategy using a double-bundle instead of a single-bundle surgical reconstruction technique because of a more native footprint recreation and restoration of mechanical functions of the ACL[40,87]. In a recently published prospective randomized trial with a follow-up of 19 months, a significant advantage in anterior and rotational stability was shown for the double bundle technique compared to the single bundle technique[230]. In this study no differences in subjective outcome were seen.

At present ten randomized trials are reported which compare the bone patellar tendon bone technique with the hamstrings technique. The two most recent randomized trials with a follow-up of five years report no difference in functional outcome and knee discomfort[110,221]. Several meta-analyses have been studying the difference in outcome of the patellar tendon and hamstring technique[30,97,115], but no conclusions to state which graft is superior could be made.

Many aspects of the surgical technique have been improved, for example in location of the portals and fixation techniques. But despite all these advances, donor-site morbidity of both patellar tendon and hamstring grafts remain a relevant drawback with the use of these autografts.

Heterogenous ligaments

Free allografts for ligament reconstruction have received growing attention during the past 15 years. They have several advantages above autografts, such as elimination of donor site morbidity, a shorter surgery time and possibly even lower costs[59]. Furthermore there is no size limitation and an allograft may be more appropriate for revision surgery and multiligament reconstruction.

Obviously there are also important disadvantages associated with the use of the allografts; one of these disadvantages is the risk of disease transmission. The main concern is the transmission of viral pathogens such as HIV. In 1989 it was estimated that the risk of using an allograft screened with the available methods from a unrecognized HIV donor was 1:1.667.000[17,38]. Since that time the polymerase chain reaction for HIV has been added to the laboratory tests for HIV. In comparison the risk of HIV transmission in a unit of blood is one in 200.000-800.000[39].

Allografts can also evoke an immune response by the recipient. Allografts have been shown to generate a more vigorous host immunologic response compared to autografts in a goat model[127]. Experimental and clinical studies have shown evidence of donor-specific antibodies in the synovial fluid, as well as in the serum of individuals receiving fresh-frozen bone-patellar tendon-bone allografts[218,250]. The clinical importance of such an immune response is currently unknown, but it may affect graft incorporation, revascularization, and graft remodelling.

The host immune response to ligament allografts is elicited by the major histocompatibility complex class I and class II antigens that are present on donor cells within the ligament and bone components. Freezing the allografts during graft preparation kills donor cells and may eliminate cell-surface histocompatibility antigens and thus result in decreased graft immunogenicity[237]. Until now, research has focused on the basic science of allografts and on early clinical results. During remodelling, autograft and allograft tendons both undergo conversion from large-diameter fibers to smaller fibers[126]. Compared with autografts, allografts lose more of their time zero strength during remodeling; however this difference has not been associated with poorer prognosis[126].

The use of allografts has shown good stability rates and functional results in studies with a follow-up between two and five years[111,149,226]. However there are also several studies describing a higher instability rate with the use of allografts as well as higher number of ruptures in allografts as compared to autografts[24,99,205,227,238]. In a recently published randomized controlled trial, laxity was not increased in allograft tendons compared with autografts and clinical outcome scores 3 to 6 years after surgery were similar[71]. In a meta-analysis on the stability of allografts compared to autografts after ACL reconstruction surgery, the authors found significantly lower stability rates compared to autografts[211]. Irradiation is thought to have a negative effect on mechanical properties of the reconstructed allograft[224], leading to increased failure rate[239]. One report with a follow-up of 10 years after allograft reconstruction found that knee stability was maintained after reconstruction of the ACL with a fresh-frozen bone-plug-free allograft[195].

As mentioned, allografts have the advantage of bypassing the problems of donor-site morbidity found with the use of autografts. Moreover, reports in literature cannot unambiguously prove lower stability rates as compared to autografts. However, long-term randomized trials are not described in literature. The effect of an immune response evoked by allografts and the actual risk of disease transmission remains an unsolved issue. Furthermore an allograft may not always be ready available.

Alternative strategies for ACL reconstruction

Long-term studies evaluating the subjective and objective outcomes of both autograft and allograft replacements have greater than 90% success rates in terms of restoration of knee stability, patient satisfaction, and return to full athletic activity[256]. However, as mentioned before, reports on ACL reconstruction surgery with a follow-up of more than ten years are rare[161,195,222]. Despite the described outcomes serious drawbacks are associated with the use of the

autografts and allografts, as mentioned in the above paragraphs. Furthermore the commonly used grafts still have long-term mechanical properties that are far from those of a normal non-injured ACL[106,198,236]. Studies have proven that in reconstructed autografts and allografts cells die and that the graft is repopulated by host cells and replaced by scar-like tissue[8,106,128,148,198]. These limitations have prompted ongoing research aimed at developing a ligament graft to address the deficiencies of existing therapies. Ongoing research includes studies on the application of growth factors in the ligament and gene transfer of the transplanted cells[171,179,188,190,215]. Another alternative strategy could be tissue engineering of the ligament, in which autologous cells are seeded onto resorbable scaffolds.

Tissue engineering

Tissue engineering is a multidisciplinary field that was first described in the late 1980s by Langer et al[248]. It includes the potential use of biodegradable polymers combined with autologous cells in culture to transplant into the human body to replace deficient tissues and organs. Researchers have expanded this concept a for variety of tissues and also for different orthopaedic applications, namely bone, cartilage, meniscus, tendon and ligaments[119,153,212].

The development of a functional tissue-engineered ligament is based on a biodegradable structure that harbors living functioning cells, that induces neoligament formation, and that eventually, after being reconstructed in the knee, will have the mechanical and biochemical properties required for physiologic loading and active use.

This biodegradable structure, also called scaffold, may be designed for a system in which it is seeded with autograft cells and implanted into the host and encourage tissue ingrowth and neoligament formation in vivo. Conversely the scaffold may be seeded with cells and stimulated to develop a ligament in an in vitro system that mimics the physiologic milieu present during normal ligament development and repair. Exogenous stimuli are applied to induce seeded cells to synthesize collagen and extracellular matrix (ECM). This ex vivo system will

allow for immediate implantation of a functional ligament that undergoes physiologic remodeling in the host knee. Successful implication of both systems requires that the seeded cells produce collagen in an organized fashion like the native ligament and that this neoligament has the mechanical and biochemical properties required for loading after implantation. Current research has focused on defining the optimal constitutions of in vitro and in vivo systems for human ligament synthesis.

Cell source

The ideal cell source for use in tissue engineering of ligaments should be ready available, show good proliferation and possess the capability to differentiate into fibroblasts which produce organized ECM typical for ligaments. However choice of the cell source may also be mediated by its location in the body. In other words, is the cell type easily harvested? The use of various cell types have been studied in literature[3,25,47]. Specialized and therefore more differentiated cells like tenocytes and ligament cells were initially used in early engineering of tendon and ligaments[25,47,68,164]. These cells theoretically would produce tissue that resembles the native tissue more closely. Patellar tendon fibroblasts have been shown to proliferate more rapidly than ACL fibroblasts when cultured on collagen ligament analogs[68]. In this study it was also shown that production of collagen by both cell types was dependent on the surface the cells were cultured on; both cell types synthesized 10-fold greater collagen on the ligament analogue as compared to the tissue culture plate. ACL and skin fibroblasts seeded onto a collagen ligament analogue remained viable in the rabbit knee for 4 weeks following implantation[25]. More viable ACL cells were found after 6 weeks in a subcutaneous pouch as compared to the intraarticular location. Human ACL and medial collateral ligament (MCL) cells were compared when seeded onto a synthetic biodegradable polymer fiber scaffold[164]. Both cells showed rapid proliferation that was enhanced with mechanical mixing and the addition of transforming growth factor (TGF)-β_1.

BMSCs are also an attractive candidate for ligament engineering. These cells have been shown to be capable of differentiating toward various types of connective tissue, such as bone and cartilage[21,83,105] and also ligament fibroblasts[208]. BMSCs can be easily harvested from the patient without requiring significant additional surgery and can be rapidly expanded in cell culture media. BMSCs cultured in a bioreactor in which controlled application of longitudinal and torsional strain was applied, showed an upregulation of ligament fibroblast markers and resulted in the formation of oriented collagen fibers[3]. Modifications of the scaffold and addition of growth factors to the culture media had a positive effect on directing the BMSCs towards the fibroblast phenotype[56,190]. Although further characterization of the role of BMSCs and their capacity for proliferation and differentiation compared to more differentiated fibroblasts has yet to be established, these cells show promising results for use in ACL tissue engineering.

Scaffolds

Whereas defining the ideal primary cell source in ACL tissue engineering has still to be defined, the properties and the design of the scaffold is also an essential component in the success of ACL tissue engineering. The ideal scaffold must be biocompatible with the cells and recipient and it must allow for cell adhesion and proliferation. Additionally it must have sufficient initial strength to withstand mechanical stress and subsequently degrade over time. Its structure will also influence the transport of nutrients and metabolites to and from the cells. Collagen as a scaffold has been shown to establish fibroblast proliferation in vitro and in vivo[25,68]. However the scaffold has shown unsatisfactory results as regard to tissue ingrowth and mechanical strength[52,100]. Furthermore the use of collagen scaffolds are also limited by their allogenicity[98]. Although the use of collagen scaffolds is still being explored, the mechanical and immunogenic limitations have led investigators to study alternative scaffold materials.

The use of a processed silk scaffold has shown promising results as a ligament replacement solution[2,74]. Silk, like collagen, has also been used in suture materials

and is inexpensive and shows predictable biodegradation. When woven into a 'wire-rope' like structure, it shows mechanical properties comparable to those of the native ACL[2,3].

Although the clinical results of synthetic ACL replacement surgery have been disappointing, the use of synthetic materials for ligament tissue engineering has shown significant potential. These polymers are biocompatible and are gradually reabsorbed, reducing the risk of an inflammatory reaction. The characteristics of these polymers can be altered with specific surface and structure modifications, providing flexibility to the overall scaffold design. The most commonly used polymers are poly(glycolic acid) (PGA), poly(L-lactid acid) (PLA) and Poly(lactid-co-glycolic acid) (PLGA) [61,172]. PGA sutures coated with polycaprolactone were unbraided and seeded with ACL and MCL fibroblasts; the cells proliferated, produced ECM and responded to chemical and mechanical stimulation[164]. More recently a 3D braided PLGA scaffold was designed for ACL engineering[61,172]. With a 3-D braided structure discrete pore sizes can be achieved which promotes tissue ingrowth. In vitro evaluation of these 3D constructs showed proliferation of ACL fibroblasts and confirmation of these cells with the geometry of the scaffold.

Investigators are also using novel fabrication strategies to produce nanofibers for tissue engineering. Through the process of electrospinning, fibers with diameters ranging from less than 3 nm to over 1 μm can be constructed. These structures have a high surface area to volume ratio, and they can provide ample space for cell attachment. It has been shown that on electrospun scaffolds in which the nanofibers are longitudinally oriented, fibroblasts take on a spindle-shape morphology and synthesize more collagen than cells that were seeded on a randomly oriented scaffold when subjected to uniaxial strain[162].

More recently, researchers have studied more biologic materials again as potential scaffold sources. Molecules such as hyaluronic acid, chitosan and alginate, which are biocompatible and have cell-adhesion properties, have been modified to make them more appropriate for ligament engineering applications[64,176]. Preliminary results have been promising in terms of cell proliferation and differentiation.

Growth factors

Many studies have attempted to determine the effects of various growth factors on a ligament engineering system with regard to cell proliferation, ECM synthesis and mechanical properties[96]. However, little is known about the regulatory signals involved in ligament development or repair. Fibroblast growth factor (FGF), transforming growth factor (TGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), and growth and differentiation factor (GDF) have all shown to improve cell proliferation and or matrix synthesis in ligament constructs[96]. One study found that bFGF with TGF-β supported the greatest ingrowth of human BMSCs in a silk matrix after 14 days of culture in addition to the greatest cumulative collagen type I expression[190]. The precise mechanism of action and physiologic concentrations of these agents will be the topics of future research, as well as the incorporation of these agents into the scaffolds to achieve slow release in vivo.

Mechanical load

Fibroblasts within a ligament are exposed to dynamic stress, and the structural properties of the ligament are influenced by these forces. The beneficial effect of exercise and dynamic loading on ligament strength and the detrimental effect of prolonged immobilization on ultimate failure strength was earlier demonstrated[260]. This finding has resulted in further research on the effect of mechanical stimulation in the development of a tissue-engineered ligament. The effect of uniaxial, biaxial, and nonuniform strain on neonatal and adult dermal fibroblasts on a synthetic monolayer was studied earlier[26]. The response of the cells in terms of proliferation and alignment was highly dependent on the applied strain profile. In another study it was shown that cellular proliferation and ECM production was enhanced by the application of cyclic strain[27]. Literature suggests that mechanotransduction signals are sensed by deformation of the cytoskeleton that is transmitted from the surrounding matrix via transmembrane cellular adhesion molecules, resulting in reorganization of the cytoskeleton and

initiating up-regulation of ECM synthesis[80,104]. Differentiation of BMSCs towards a fibroblast-like cell lineage was enhanced by multidimensional mechanical strain in the absence of growth factors[3]. Factors such as the magnitude and frequency of the applied strain can have an effect on the cellular response. Further research will have to study the precise mechanism of mechanical stimulation and to analyze the optimal stimulus for tissue engineering of ligaments.

The current strategies that surround the regeneration of an ACL substitute are in full development and some report promising results. However, the goal of synthesizing a functional ligament has yet to be realized. Published data are promising but many obstacles still exist. To date, no engineered construct has the appropriate biologic and mechanical strength for in vivo implantation.

3

CLINICAL AND IMMUNOLOGIC OUTCOME OF ARTHROSCOPIC ANTERIOR CRUCIATE LIGAMENT RECONSTRUCTION USING FRESH FROZEN BONE-PATELLAR TENDON-BONE ALLOGRAFTS; 15-YEAR FOLLOW-UP

ABSTRACT

Purpose: To evaluate the long-term outcome following arthroscopic assisted anterior cruciate ligament reconstruction using deep frozen bone-patellar-tendon-bone allografts and to study the clinical relevance of immunologic reactions against the donor.

Methods: Thirty-eight patients underwent an allograft ACL reconstruction. Thirty-five patients were available for examination after a mean follow-up of 15 years. Examination included a detailed history, the KOOS-questionnaire, physical examination, KT-1000 arthrometer testing, the IKDC standard evaluation form, and radiographic evaluation. Clinical failure was defined as a KT-1000 left-right difference of more than 5mm, IKDC group C or D or a revision of the ACL. HLA-typing of the patients was done pre-operatively. Three months, 3-4 years and 6-7 years after the reconstruction blood samples were taken to assess HLA antibody reactivity against the donor.

Results: The average age at the time of the reconstruction was 29 years. Clinical failure occurred in 21 of the 35 patients (60%). Failure of the allograft occurred in 6 patients of which 4 had a traumatic rupture. The IKDC score deteriorated between the 4 and 15 year follow-up period (p= 0.002). Patients with a (severely) abnormal IKDC score showed statistical significant lower scores for KOOS Symptoms and Quality of life (p=0.045 and p=0.021). The Lachman and the KT-1000 test improved significantly at the 15 year follow-up period as compared to preoperative (p=0.000). In patients with a clinical failure of the graft more degenerative changes were found on X-rays (p=0.021). Donor specific HLA antibodies were detected in 8 (25%) patients, but no association with failure of the graft was found.

Conclusion: This study indicates that clinical outcome of allografts for ACL reconstruction deteriorates at the long-term follow-up. In this small patient series no significant association was found between specific antibody reactivity against the HLA-antigens of the donors and clinical outcome.

INTRODUCTION

Although autologous tendinous tissue is still the first choice in anterior cruciate ligament (ACL) reconstruction surgery, the use of allografts in ACL reconstruction surgery has risen tremendously the past decade. It's use has several advantages above autografts, such as elimination of donor site morbidity, a shorter surgery time and possibly lower costs[59]. Furthermore there is no size limitation and an allograft may be more appropriate for revision surgery and multiligament reconstruction.

There are however important disadvantages associated with the use of the allografts, of which the most important is the risk of disease transmission. The main concern is the transmission of viral pathogens such as HIV[17,38].

Allografts can also evoke an immune response by the recipient. Host immune response to ligament allografts is elicited by major histocompatibility complex class I and class II antigens that are present on donor cells within the ligament and bone components. Experimental and clinical studies have shown evidence of donor-specific antibodies in the synovial fluid, as well as in the serum of individuals receiving fresh-frozen bone-patellar tendon-bone allografts[218,250]. Freezing the allografts during graft preparation kills donor cells and may denaturate cell-surface histocompatibility antigens and thus resulting in decreased graft immunogenicity[237]. Allografts also have been shown to generate more vigorous host immunologic response than autografts in a goat model[127]. The clinical importance of such an immune response is currently unknown, but it may affect graft incorporation, revascularization, and graft remodelling.

The use of allografts has shown good stability rates and functional results in studies with a follow-up between two and six years[22,71,112,149,209]. However there are also several studies describing a higher instability rate with the use of allografts[24,205,226] as well as higher number of ruptures in allografts as compared to autografts[99,238]. In a meta-analysis on the stability of autografts compared to allografts after ACL reconstruction surgery, the authors found significantly lower

stability rates compared to autografts[211]. Reports on ACL reconstruction surgery with a follow-up of more than ten years are rare[161,195,222], especially for allografts. The purpose of the present study was to evaluate the long-term outcome following arthroscopic assisted ACL reconstruction using fresh frozen bone-patellar-tendon-bone allografts; furthermore we studied the clinical relevance of the induction of donor specific HLA antibodies measured in the blood until six years after the reconstruction.

METHODS

Materials

Between 1991 and 1992 38 arthroscopic ACL reconstructions with an allograft were performed by one experienced arthroscopic surgeon. The choice of the graft depended on the availability of the allografts. All patients were asked to participate in the study. Patient demographics are presented in table 1. Sporting activities were the cause of injury in 90% of the cases. Soccer and skiing were the most common injury-causing sports. 19 patients had never undergone surgery of the index knee after the trauma. In the remaining 19 patients, 14 partial or complete medial and/or lateral meniscectomies and 4 medial meniscus repairs were performed before the reconstruction. In 4 patients the reconstruction of the ACL was a revision: 1 patient had a primary repair before, one had a reconstruction with an autograft and two with a synthetic graft.

All patients were preoperatively screened; a pre-trauma and a preoperative Tegner score was assessed, and clinical as well as KT-1000 instrumented laxity tests were conducted.

Allografts

The fresh frozen (-112ºF) bone-patellar tendon-bone allografts were thawed in an antibiotic solution before preparation to an average width of 10mm. The

mean age of the donors was 40 years (range 17-51 years). All allografts were extensively screened and processed according to the standards of tissue banking (BIS Eurotransplant, Leiden, The Netherlands)[149]; No irradiation was used.

HLA antibody screening

Preoperatively HLA-typing for the A-, B-, and DR- locus of the 37 patients as well as of the donors was performed with a complement-dependent lymphocytotoxicity (CDC) test. 28 Patients were preoperatively as well as 3 months postoperatively screened for HLA-antibodies. Screening for the presence and specificity of anti-HLA class I and II antibodies was performed with two selected panels of 24 and 54 donors. 33 patients were also screened for HLA-antibodies 3-4 years post-operatively (8 patients of this group were not tested for anti-bodies at the 3 month follow-up period). Furthermore, 6-7 years after the reconstruction, of 24 patients blood samples had been taken again (one patient of these 24 was not tested after 3-4 years and 4 patients were not tested three months post-operatively). Pregnancies and blood transfusions were listed.

Surgical technique

The operative technique consisted of standard arthroscopic ACL reconstruction with a one-incision technique performed by a single surgeon. All associated intra-articular injuries were addressed at the time of the index operation and a notchplasty was performed. The tibial tunnel was drilled using a drill guide (Acufex Microsurgical inc.), aiming for the centre of the footprint of the ACL. The femoral tunnel was drilled through the tibial tunnel, after the place of entry was marked with an isometry meter. Laxity of up to 3 mm with the isometer was allowed. The proximal bone block was placed flush with the intercondylar wall and fixed with an interference screw at the anterior portion of the tunnel. The distal bone block was fixed with an interference screw under approximately 5 kg of tension with the knee in 20° of flexion, after taking the knee at least 5 times through a full range of motion. If the distal part protruded too much, a trough was cut, and the bone block was fixed with two staples.

Rehabilitation

Rehabilitation was standard for all patients and started with continuous passive motion on day 1, through a range of 0° to 90°. Isometric quadriceps exercises were encouraged within this range. Partial weight-bearing was started on the second postoperative day. Patients were discharged once they achieved a comfortable range of motion of 0° through 90°. Until 6 weeks a removable splint or a hinged brace was worn, with partial weight-bearing continued until 4 weeks and after swelling of the knee had subsided. During this period active exercises like quadriceps strengthening and hamstring curls were performed. Only after 6 weeks passive mobilization over 90° was allowed. Normal running was permitted at 4 months and a gradual return to activity was noted at 6 months. Return to full activity was delayed until 12 months.

Follow-up Examination

The follow-up examination took place at a mean follow-up of 4 years (2,5-5,3 years) and at a mean follow-up of 15 years (13,8-16,5 years). At each time-point the patients were re-examined by a different independent examiner. After 4 years data of 27 patients were available. 35 patients could be re-examined after 15 years (Three patients were lost to follow-up because they changed to an unknown address).

The standard knee ligament evaluation form of the International Knee Documentation Committee (IKDC) with the 4 categories A-D, was used as a general outcome measure, including effusion, passive motion deficit and ligament examination. A detailed physical examination to assess the Lachman sign (grade 0 (1-2mm), grade I (3-5mm), grade II (6-10mm) and grade III (>10mm)), pivot shift sign (grade 0, grade 1 (glide), grade 2 (clunck), grade 3 (gross)), anterior posterior drawer test, range of motion, and varus and valgus instability was performed. Loss of motion exceeding 5° in both flexion and extension compared with the contralateral limb was classified as loss of motion. Only the patients with a non-injured contralateral extremity were included for the KT-1000 arthrometer test. All additional surgery until final follow-up was noted.

At the follow-up of 15 years all patients also completed the questionnaire of the Knee injury and Osteoarthritis Outcome Score (KOOS) and the Tegner activity level was assessed. The KOOS questionnaire is a 42-item self-administered knee specific questionnaire, assessing pain, symptoms, activities of daily living, sport and recreation function and knee-related quality of life in five separate subscales[219]. At this follow-up period the radiographs were analyzed for degenerative signs such as joint space narrowing by 2mm or more, subchondral bone sclerosis or osteophyte formation and classified according to the IKDC knee evaluation form as (a) none, (b) mild, (c) moderate or (d) severe. Position of the graft was measured on radiographs using the Amis circle and Taylor score[10,121]. With the Amis score, we measured the femoral position of the graft. A proper position of the graft is located for at least at 60% in this circle (good >59%, not good ≤59%). The Taylor score was used for locating the tibial placement of the graft. The Taylor score provides an indication of possible impingement of the graft by the roof of the intercondylar notch of the graft (0 = absent, 1=moderate, 2=severe impingement).

Statistical analysis

We used SPSS for Windows (version 15.0; SPSS, Chicago, IL) for statistical analysis. A level of significance of $P<0.05$ was used. The Friedman test for categoral variables and the Mann-Whitney U test for continual variables were used for the comparison of nonparametric data. Parametric data are presented as means and non-parametric data are presented as median with the inter quartile range (iqr). Odds ratio's (OR) with confidence intervals (CI) were used to test the significance of the association between the induction of donor specific HLA antibodies and clinical failure or failure of the allograft. Because of the explorative character of this study no correction for multiple testing was done.

regel 1
regel 2
regel 3
regel 4
regel 5
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regel 31

RESULTS

Pre- and per-operative findings

Demographics and clinical parameters are listed in table 1.

The interventions at the time of the reconstruction are listed in table 2. Subsequent interventions were performed in 18 patients (47%). Sixty percent of the patients showed no or hardly any signs of arthritis (grade 0 and 1).

Follow-up

No general complications like infection or thrombosis were seen.

Clinical failure of the allograft, defined as a KT-1000 left-right difference of more than 5mm, IKDC group C or D, or a revision of the ACL, occurred in 21 of the 35 patients (60%). Of these 21 patients two patients were subjected to an early failure of which one consisted of loosening of the femoral block (identified within three months after the reconstruction). This patient did not receive any subsequent surgery. The other patient had early instability complaints and underwent a revision of her iliotibial band tenodesis, which she had had 2 years before the reconstruction. Of the 21 clinical failures 6 were failures of the allograft: One patient underwent the above mentioned re-iliotibial band tenodesis. Four patients had a traumatic rupture, one patient 5 years after the allograft reconstruction and 3 patients more than ten years after the reconstruction. In another patient the allograft had resolved and underwent a reconstruction of the ACL after four years.

Reoperations

Between the ACL reconstruction and the final follow-up, in total 29 reoperations were performed in 19 (54%) patients. Seven patients underwent 2 or 3 reoperations and one patient four reoperations on the index knee. The additional surgeries after the ACL reconstruction were: removal of staples (n=13), meniscal lesions (n=5; two had a failed meniscopexy within a year, one patient had a

Table 1. Baseline demographic and clinical parameters (n=38)

Mean age at time of reconstruction, years	29 (13-46)
Male-female	47%-53%
Median trauma-operation interval, months (iqr)	35 (8-98)
No of patients with previous operations after trauma	
0	19
1	9
2	8
3	1
4	1
Previous operative interventions	34
Revision ACL reconstruction	4
Partial or complete medial meniscectomy	16
Partial or complete lateral meniscectomy	3
Medial meniscus repair	4 (1 failed)
High tibial osteotomy	2
Perichondriumplasty	2
Lateral reconstruction	3
Repair of posterior oblique ligament	1
Adhesiolysis	1
Mean Tegner score pre-trauma (min-max)	8 (6-10)
Mean Tegner score pre-reconstruction (min-max)	4 (0-7)
Lachman pre-operative, n=36	**No of patients**
0-5mm	2
6-10mm	15
>10mm	19
KT-1000 pre-operative, n=26 (R/L difference)	
0-2mm	1
3-5mm	14
>5mm	21

Iqr= inter quartile range

meniscectomy within a year and two patients after more than ten years), cyclops lesion (two times in one patient), revision of the iliotibial band tenodesis (n=1), revision of the ACL (n=4), high tibial osteotomy (n=2, 2 and 7 years after reconstruction), and one total knee prosthesis (n=1). After 15 years 16 patients (46%) had undergone a partial or total medial and/or lateral meniscectomy before, during or after the index surgery.

Table 2. Peroperative findings and subsequent intervention at the time of reconstruction (n=38)

Articular cartilage injury – Outerbridge	No of patients
Grade 0	15
Grade 1	8
Grade 2	6
Grade 3	3
Grade 4	5
Missing data	1
Subsequent intervention	
No	20
Medial meniscectomy	6 (3 re-meniscectomies)
Lateral meniscectomy	2 (1 re-meniscectomy)
Medial meniscus repair	7
Lateral meniscus repair	3
Repair of the medial collateral ligament and posterior oblique ligament	1

Subjective and functional results

The 15 years subjective and functional results are presented in table 3. Four patients (three patients with an ACL revision and one with a total knee prosthesis) were excluded from the subjective and functional analysis performed at the last follow-up, leaving 31 patients.

Patients who were classified as (C) or (D) showed a lower outcome of all KOOS scores, except for KOOS ADL, as compared to the patients who were classified as (A) and (B) (p=0.045 for KOOS Symptoms and p=0.021 for KOOS QOL, Table 4). All patients with a clinical failure of the allograft also showed clearly lower KOOS scores for all subscales, except for KOOS ADL (p=0.026 for KOOS Symptom, table 4). The IKDC score at final follow-up deteriorated as compared to the IKDC score after a mean follow-up of 4 years (Figure 1, p=0.002). The Lachman significantly improved at the final follow-up as compared to the pre-operative Lachman (p=0.000), and did not deteriorate as compared to the Lachman at the 4 years follow-up (p=0.248).

Of the 31 patients, 27 patients had a normal contra-lateral knee and were tested with the KT-1000 arthrometer. A side-to-side difference of 0 to 2 mm was seen in 19 patients (70%), a 3 to 5mm difference in 4 patients (15%) and a difference

Table 3. Patient subjective and functional results at final follow-up (N=31)

KOOS	**Median**	
Pain	97,2	Iqr 92-100
Symptoms	85,7	Iqr 68-96
ADL	98,5	Iqr 94-100
Sport	90,0	Iqr 65-95
QOL	81,3	Iqr 56-94
IKDC	**No of patients**	
Normal (A)	3	10%
Nearly normal (B)	12	39%
Abnormal (C)	12	39%
Severely abnormal (D)	4	13%
Tegner score	4 (0-7)	
Lachman test		
1-2mm	10	32%
3-5mm	14	45%
6-10mm	6	19%
>10mm	1	3%
Loss of motion (extension)	5	16%
Loss of motion (flexion)	9	29%
Radiographic signs of arthritis (IKDC)		
None	19	61%
Mild	6	19%
Moderate	2	6%
Severe	4	13%

Iqr= inter quartile range

of more than 5 mm in 4 patients (15%). The side-to-side difference after 4 years and at final follow-up was statistical significant lower as compared to the pre-operative side-to-side differences (p=0.000). No difference could be found in the means of the side-to-side differences between the 4 years and 15 years follow-up (Figure 1).

Table 4. Comparison of KOOS results for patients with a (nearly) normal IKDC (A+B) with patients with a (severely) abnormal IKDC (C+D), and for patients with or without a clinical failure.

	IKDC	A +B	C+D	P-value
KOOS (iqr)	**Pain**	100 (97-100)	94 (75-100)	NS
	Symptoms	89 (86-96)	75 (55-89)	0.045
	ADL	99 (96-100)	99 (79-100)	NS
	Sport	90 (80-100)	70 (30-93)	NS
	QOL	94 (75-100)	78 (45-88)	0.021
	Clin. failure	**No**	**Yes**	**P-value**
KOOS (iqr)	**Pain**	100 (97-100)	94 (75-100)	NS
	Symptoms	91 (86-97)	82 (55-89)	0.026
	ADL	99 (97-100)	97 (79-100)	NS
	Sport	93 (77-100)	70 (30-93)	NS
	QOL	91 (75-100)	81 (47-91)	NS

Evaluation of KOOS subscales. Data are presented as median (iqr)
NS= not significant

Radiologic findings

Radiologic findings were analyzed in all patients without a revision surgery of the allograft reconstruction (n=31). According to the IKDC criteria 6 patients (19%) developed moderate or severe degenerative changes (Table 3). In the patients with a clinical failure of the allograft a statistically significant higher amount of degenerative changes was found (Figure 2, p=0.021). No significant difference was seen in osteoarthritis between the patients with or without a history of a meniscectomy (Figure 2, p=0.059).

Position of the graft was measured with weight bearing lateral radiographs. Of the 31 patients 27 (87%) patients had an Amis score of at least 60% and 2 patients had an Amis score of <60% (7%). Of two patients the femoral tunnel could not be identified. The position of the tibial tunnel was not visible on the x-ray in 4 patients. Of the remaining patients, 4 patients (13%) had a Taylor score of 0, 10 patients (31%) had a Taylor score of 1 and 13 patients (42%) had a Taylor score of 2.

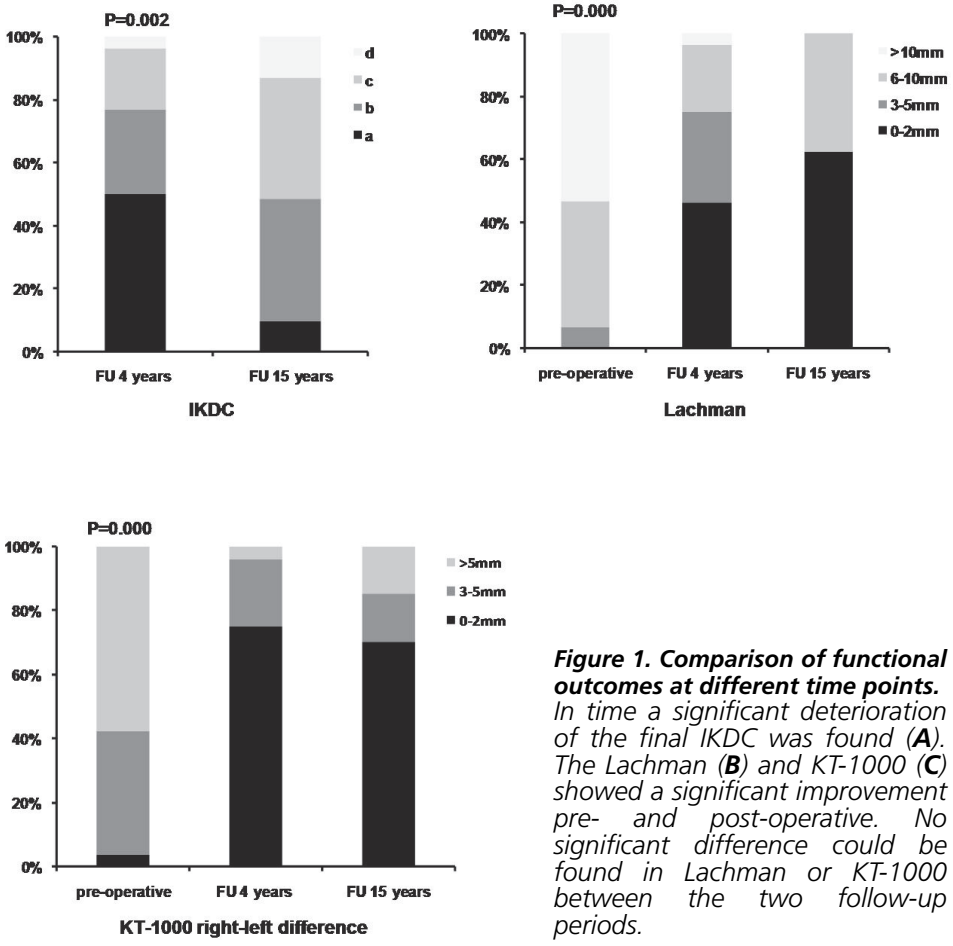

Figure 1. Comparison of functional outcomes at different time points. *In time a significant deterioration of the final IKDC was found (**A**). The Lachman (**B**) and KT-1000 (**C**) showed a significant improvement pre- and post-operative. No significant difference could be found in Lachman or KT-1000 between the two follow-up periods.*

Immune reactivity

Induction of donor specific HLA antibodies was detected in 8 patients (table 5). One of the eight patients who tested positive had three pregnancies in the six years after the transplantation. Two of the patients who showed positive reaction in the follow-up period, received the allograft of the same donor of which the HLA typing was not known. They both showed reactivity against HLA-A1 and HLA-B8, which are common alleles. We concluded that this reaction was most probably against the donor.

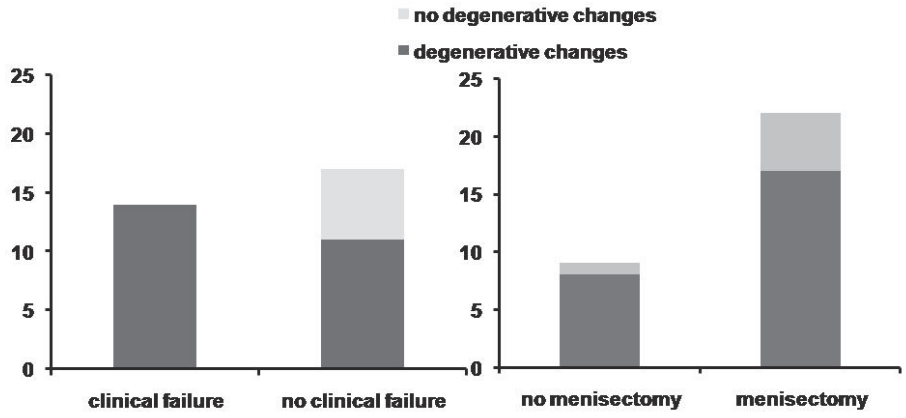

Figure 2. Comparison of degenerative changes between patients with or without a clinical failure of the allograft and between patients with or without a history of a meniscectomy.
** In patients with a clinical failure of the graft more degenerative changes were found as compared to patients without clinical failure of the graft (p=0.021).*
*** No statistical difference could be found in degenerative changes between patients with or without a history of a meniscectomy (p=0.059).*

In two of the eight patients the antibodies were only detectable at three months after the operation. After 3 years these antibodies could not be detected anymore. None of the patients with a specific reaction showed any clinical signs of rejection, like synovitis or effusion. Five of the 8 patients (63%) had a clinical failure of the allograft, of which one showed a failure of the allograft due to resorption. No significant association could be found between a specific reaction and clinical failure (OR 1,4 with CI 0,3-7,3) or failure of the allograft (OR 0,6 with CI 0,1-6,8).

DISCUSSION

The use of allografts in ACL reconstruction surgery is increasing, but unfortunately there are still limited data on the long-term results of these grafts. The clinical failure rate of 60% (21 patients) and an allograft failure of 17 % (6 patients) described in current study is relatively high as compared to what is described

Table 5. Immunoreactivity against the donor

Patient	Follow-up 3 months	3-4 years	6-7 years	Clinical failure	
1 ♀	+	-	-	Yes	IKDC severely abnormal
2 ♀	+	-	-	Yes	IKDC abnormal
3 ♀[1]	-	+	+	No	
4 ♂	-	+	+	No	
5 ♀	-	+	+	Yes	Kt-1000 >5mm
6 ♂	±[2]	+	+	Yes	IKDC abnormal
7 ♀[3]	+	+	-	No	
8 ♀[3,4]	-	+	+	Yes	Allograft resorption
Patients tested	28	32	23		

In eight patients immunoreactivity against the donor was found; no patient received a blood transfusion within the 6 years after the transplantation.
[1] This patient had 3 pregnancies in the 6 years after the transplantation
[2] Antigen coming up
[3] The HLA typing of the donor of these patients was unknown. In both patients reactivity against HLA-A1 and HLA-B8 was found, presuming specific reactivity against the donor.
[4] Allograft: this patient showed resorption of the graft four years after the reconstruction.

in literature. However a few considerations have to be mentioned. Our patient population also included patients who already had a history of knee surgery of the index knee, including cartilage repair procedures, lateral reconstruction, and even ACL reconstruction. Also 22% of the patients were classified as having articular cartilage injury grade 3 or 4 at the time of the reconstruction. This makes comparisons with other series difficult. The clinical failure rate of 60% can also partly be explained by the surgical technique used in this study. Although no statistical correlation between tibial tunnel placement and clinical failure of the allograft could be found, 31% of all patients had moderate and 42% had severe impingement as assessed by the Taylor score on radiographs. An anterior position of the tibial tunnel of the ACL graft is correlated with instability[120].
In a series of 61 patients with a ten year follow-up after ACL reconstruction with bone plug-free allografts only one patient (1,6%) showed a KT-2000 side-to-side difference of 5mm and 3 out of 61 patients (4.6%) had a traumatic rupture and

underwent revision ACL reconstruction[195]. Three of the four traumatic ruptures found in our study occurred after more than ten years after surgery, which could have been missed with a mean follow-up of ten years. Furthermore in the bone-plug free study only 55% of the patients were examined at ten years follow-up. In a study using bone-patellar tendon-bone autografts for ACL reconstruction 9 of 101 (9%) graft ruptures occurred after ten years and the final IKDC score of normal or nearly normal was 90%[161]. In time a slight increase in number of patients scoring abnormal for the final IKDC was seen in the autograft study. In current study a significant deterioration in final IKDC was seen in time. It is unknown whether the difference in length of final follow-up (10 versus 15 years in current study) can at least partially explain the differences in final IKDC outcome between this autograft study and our study.

Another study on ACL reconstruction with bone-patellar tendon-bone autografts reported a rupture rate of 13% after thirteen years[222]. The total number of patients with an abnormal or severely abnormal IKDC score in this study was 26%, and interestingly also a significant deterioration in final IKDC was seen between the mid- and long-term follow-up.

This study is to our knowledge the first describing correlations between subjective KOOS scores and functional IKDC scores. The KOOS questionnaire is a self administered evaluation tool and in this study gave comparable information as the IKDC score, when the groups were divided into final IKDC A+B and C+D (except for the KOOS ADL). Therefore we think that implementation of the KOOS questionnaire might be useful in long term follow-up for ACL reconstruction surgery.

None of the 8 patients tested with a positive reaction against donor-specific HLA antigens post-operatively had clinical signs of rejection. No new specific reaction was identified at the 6-7 follow-up period. Only one of six patients with an allograft failure showed a specific reaction against the donor. In this patient the allograft had resolved; we can only speculate if this resorption was caused by an immune response.

In a study on 18 patients receiving freeze dried ethylene oxide sterilized bone-patella tendon-bone allografts, 3 patients showed a specific reaction until 12 months post surgery[218]. Today HLA antibody analysis is best performed by ELISA or luminex assays, which are much more sensitive than CDC. In organ transplantation the presence of donor specific antibodies detected in CDC before transplantation is a contra-indication for transplantation. The clinical relevance of non complement fixing antibodies detectable in ELISA and luminex is still a point of discussion, although they are considered a risk factor for complications. As we did not see a clinical effect of donor specific antibodies detected in CDC, it is not likely that the more sensitive assays will change the picture. In this study we focused on antibodies. However, T- cells may also play a role in graft rejection. The presence of T-cells in the blood was already shown after transplantation of bone allografts[67] and in the tissue itself after meniscal allograft transplantation[217]. The clinical importance of the different immunological reactions after allograft transplantations remains to be established.

The limitation of this study is that it is a patient series. Furthermore our patient population also included patients who already had a wide history of knee surgery of the index knee, making it difficult to compare the results with other series described in literature.

CONCLUSION

This patient series indicates that clinical outcome of allografts for ACL reconstruction may deteriorate at the long-term follow-up. Twenty-five percent of the patients showed a specific antibody reactivity against the HLA-antigens of the donors, but in this small patient series no significant association between reactivity and clinical outcome could be found. Randomized controlled trials are needed to investigate long-term clinical outcome of allografts for ACL reconstruction.

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4

TISSUE ENGINEERING OF LIGAMENTS: A COMPARISON OF BONE MARROW STROMAL CELLS, ANTERIOR CRUCIATE LIGAMENT AND SKIN FIBROBLASTS AS CELL SOURCE

ABSTRACT

Anterior cruciate ligament (ACL) reconstruction surgery still has important problems to overcome, such as "donor site morbidity" and the limited choice of grafts in revision surgery. Tissue engineering of ligaments may provide a solution for these problems. Little is known about the optimal cell source for tissue engineering of ligaments. The aim of this study is to determine the optimal cell source for tissue engineering of the anterior cruciate ligament. Bone marrow stromal cells (BMSCs), ACL, and skin fibroblasts were seeded onto a resorbable suture material (poly(L-lactide/glycolide) multifilaments) at five different seeding densities, and cultured for up to 12 days. All cell types tested attached to the suture material, proliferated, and synthesized extracellular matrix rich in collagen type I. On day 12 the scaffolds seeded with BMSCs showed the highest DNA content ($p<0.01$) and the highest collagen production ($p<0.05$ for the two highest seeding densities). Scaffolds seeded with ACL fibroblasts showed the lowest DNA content and collagen production. Accordingly, BMSCs appear to be the most suitable cells for further study and development of a tissue-engineered ligament.

INTRODUCTION

Rupture of the anterior cruciate ligament (ACL) is one of the most common sports-related injuries, with approximately 95,000 cases per year in the United States[134]. It is estimated that more than 50,000 ACL reconstructions are performed each year in the United States[134], making it one of the most commonly performed surgical procedures in sports medicine. Because of the poor long-term results of synthetic grafts[82,177], biologic grafts are most frequently used for ACL reconstructions. However, biological grafts still have considerable drawbacks, such as donor-site morbidity (autografts) and the risk of disease transmission (allografts)[126,141]. Tissue engineering could potentially provide a solution for the limitations described. The basic principle of this technology has been firmly established[157,158] and its application would dramatically improve clinical practice in ligament reconstructive surgery.

The aim in tissue engineering of ligaments is to provide an implant that results in a fully regenerated living autologous ligament with long-term viability. Our goal is to reconstruct a ligament by providing a resorbable scaffold seeded with cells, that induces neoligament formation that adequately meets the required biological and mechanical properties.

To date, as compared with bone or cartilage tissue engineering, limited information is available on tissue engineering of tendons or ligaments[2,3,18,19,25,47,68,164,266]. Various biodegradable materials are used as a scaffold (poly(glycolic acid) (PGA), silk, and collagen). Also the use of various cell lines is described, such as ACL and medial collateral ligament fibroblasts, bone marrow stromal cells (BMSCs), and tenocytes. All these studies show promising results in the attachment, proliferation, and differentiation of these cells when seeded onto the scaffolds. The appropriate cell type for ligamental tissue engineering must show enhanced proliferation and production of an appropriate extracellular matrix and must be able to survive in an intraarticular environment in the patient's knee. ACL fibroblasts are cells specialized in producing all ligament constituents and maintaining the ligament tissue in the appropriate conformation. However, it

is well known that the anterior cruciate ligament has poor healing capacities in contrast to other ligaments, such as the medial collateral ligament. This deficient repair could be due to several intrinsic properties of the cells, as responsivity to growth factors[181,234], adhesion strength[240,241,265], proliferation, and migration rate were reported to be lower compared with other cell types[9,92,151,163,193,240,241,265]. Bellincampi *et al*. demonstrated the survival of skin fibroblasts in an intraarticular environment to be at least 4 weeks[25], making this cell source also a potential donor cell for our engineered ligament. BMSCs are cells that have been shown to be capable of differentiating toward various types of connective tissue, such as bone and cartilage[21,83,105] and also ligament fibroblasts[48,49,208]. Although BMSCs are used in in vivo experiments for tendon repair[18,266], no data are available on the behavior of BMSCs in ligament repair in an intraarticular synovial environment. The purpose of this study was to compare ACL and skin fibroblasts and BMSCs with regard to cell proliferation and differentiation (matrix synthesis) on three-dimensional bipolymer scaffolds, in order to determine the optimal cell source for tissue engineering of ligaments. The cells were seeded at various densities onto braided poly(L-lactide/glycolide) scaffolds to study the effect of initial seeding density on proliferation and matrix synthesis.

MATERIALS AND METHODS

Design

BMSCs, skin fibroblasts, and ACL fibroblasts were all isolated from one adult Dutch milk goat. After culture expansion, the three different cell types were seeded onto a scaffold made of braided Panacryl. For each cell type, five different seeding densities were used: 1×10^4, 3×10^4, 1×10^5, 3×10^5, and 1×10^6 cells per scaffold. All samples were analyzed for various proliferation and differentiation parameters on day 1, day 6, and day 12 (block staining, environmental scanning electronic microscopy, DNA fluorometry, total collagen biochemistry, histology, and immunohistochemistry). Each quantative analysis was performed in triplicate.

Cell isolation and expansion

Skin fibroblasts. The skin of the proximal left hind leg was retrieved, using a dermatome, after shaving the skin. The skin was subsequently rinsed three times with phosphate-buffered saline (PBS; GIBCO, Grand Island, NY), transferred to a petri dish containing a PBS solution with 0.25% dispase II (Roche, Mannheim, Germany), and stored at 4°C for 17 h. Next, the dermis was split from the epidermis, using a forceps. The dermis was minced and transferred to 15 mL of 0.25% dispase II–0.75% collagenase A solution (Roche) and incubated on an XYZ shaker (Polymax; Heidolph Instruments, Schwabach, Germany) at 37°C for 3 h. Subsequently the suspension was passed through a filter chamber (200-µm pore size; NTBI, Emmercompascuum, The Netherlands).

ACL fibroblasts. Left and right anterior cruciate ligaments were excized under sterile conditions and minced. The pieces were suspended in a 0.15% collagenase II solution (Worthington Biochemical, Lakewood, NJ) and incubated on an XYZ shaker at 37°C for 20h. Subsequently the suspension was filtered through a cell strainer (Falcon, 100-µm pore size; BD Biosciences Discovery Labware, Bedford, MA). The ACL and dermis suspensions were expanded in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS) (Sigma, St. Louis, MO) penicillin–streptomycin (100 U/mL each), and L-glutamine (580 mg/L), a culture medium that has been found to be optimal for cells of the fibroblast lineage. Cells were cultured until confluence, with medium changes performed three times per week.

Bone marrow stromal cells. Bone marrow was aspirated from the distal and proximal left femur under sterile conditions. The aspirate was cultured until confluence in expansion medium. Although previously no significant differences between α-MEM and DMEM were observed with respect to proliferation and differentiation of subcultured human BMSCs[58], we routinely used α-MEM (GIBCO) for expansion of BMSCs, supplemented with 10% fetal bovine serum

(GIBCO), L-glutamine (2 mM; GIBCO), ascorbic acid (0.2 mM; Sigma), and penicillin–streptomycin (each 100 U/mL; GIBCO). The culture medium was renewed three times per week, during which nonadherent cells were removed from the culture flask.

Scaffolds

The degradable scaffolds were made of braided Panacryl (size 0; Ethicon, Somerville, NJ). Panacryl is composed of 11-µm poly(L-lactide/glycolide) fibers, braided in bundles to a multifilament of ± 450 µm and coated with 90% caprolactone and 10% glycolide. Five strands were braided together and pieces approximately 1 cm in length were knotted at the ends with Panacryl. For resterilization after handling, the braided scaffolds were autoclaved at 121°C for 20 min. On autoclaving, the scaffolds appeared to have slightly diminished in size. The scaffolds were subsequently incubated in medium supplemented with 10% FBS the night before seeding.

Seeding and three-dimensional culture

As proliferation and differentiation have been demonstrated not to differ between passage 3 and 4 of human BMSCs[37] and skin fibroblasts[186], BMSCs were cultured to passage 3 and the skin and the ACL fibroblasts were seeded at passage 4 in order to be able to seed all cells at the same time. The cultured cells were trypsinized, counted, and resuspended. ACL and skin fibroblasts were resuspended in DMEM, to which, in addition to the supplements of the expansion mix, ascorbic acid (0.2 mM) and 10% FBS had been added. BMSCs were resuspended in the same medium as used for expansion, α-MEM containing 10% FBS and 0.2 mM ascorbic acid. Aliquots of 2 mL containing 1 x 10^4, 3 x 10^4, 1 x 10^5, 3 x 10^5, or 1 x 10^6 cells were added to 2-mL tubes (Eppendorf, Hamburg, Germany), each containing one scaffold. The tubes were sealed with a hydrophilic filter (Eppendorf) and placed on a roller bank (1 rpm, low profile roller; New Brunswick Scientific, Edison, NJ) at 37°C and 5% CO_2. After 24 h, the scaffolds were removed from the tubes and transferred to replica well plates

(Sterilin, Stone, Staffordshire, UK) containing 2 mL of medium in each well. These plates were cultured on an XYZ shaker at 30 rpm (37°C, 5% CO_2). Medium was changed three times per week.

Outcome parameters

Cell attachment. Methylene blue block staining was performed to visualize cell attachment. The samples were carefully rinsed with PBS and fixed with 1.5% glutaraldehyde in 0.14 M cacodylate buffer overnight. The scaffolds were stained with methylene blue and subsequently placed under a stereomicroscope (SM2-10A; Nikon, Tokyo, Japan).

Matrix structure. Environmental scanning electron microscopy (ESEM; Philips, Eindhoven, The Netherlands) was used to qualitatively evaluate matrix synthesis. Using this technique, the structure and the produced matrix can be visualized. To prepare for ESEM, the scaffolds that were previously used for block staining were dehydrated, critical point dried, and gold sputter coated.

Cell proliferation. Cell proliferation was assessed by fluorometric quantification of DNA, using the CyQuant cell proliferation assay kit (Molecular Probes, Eugene, OR). After rinsing with PBS, all samples were digested in a 1.0-mg/mL proteinase K solution (Sigma) for 16 h at 56°C, after which the samples were treated according to the manufacturer's instructions. The fluorometric analysis was done at 560 nm with a luminescence spectrometer (PerkinElmer, Buckinghamshire, UK).

Collagen quantification. As a parameter for matrix synthesis, we determined the total amount of collagen, using the hydroxyproline assay described by Brown *et al*[36]. Briefly, aliquots of 100 µL of the proteinase K digests used for the DNA assay were hydrolyzed overnight with 100 µL of 12 M HCl at 110°C in a heating block (Techne, Cambridge, UK). Fluid was removed by nitrogen

evaporation (evaporator; Pierce, Rockford, IL). The hydrolysates were dissolved in demineralized water. The solutions were oxidized with Chloramine-T (Sigma) followed by the Ehrlich reaction, adding *p*-methyl-amino-benzaldehyde (Sigma) dissolved in a propanol–perchloric acid solution (Merck, Darmstadt, Germany). Absorbance was measured at 570 nm with a microplate reader (Biokinetics reader; Bio-Tek Instruments, Winooski, VT). Collagen content was calculated by assuming 14% as the content of hydroxyproline in collagen of ligaments[213].

Histology and immunohistochemistry. For histology, the scaffolds were carefully rinsed with PBS, fixed with 1.5% glutaraldehyde in 0.14 M cacodylate buffer overnight, dehydrated and embedded in glycol methacrylate (GMA), followed by cutting with a microtome and hematoxylin and eosin (H&E) staining. For immunohistochemistry of collagen type I, the scaffolds were rinsed with PBS, embedded in Tissue-Tek O.T.C. compound (Sakura Finetek USA, Torrance, CA), and stored at -80°C. The samples were sectioned and stained for collagen type I (Oncogene Research Products/EMD Biosciences, San Diego, CA), using a 1:200 dilution and pre-treatment with proteinase K solution for 10 min (Dako Cytomation, Carpinteria, CA). Counterstaining was done with hematoxylin. Both immunosections and histological sections were analyzed by light microscopy (Eclipse E600; Nikon).

Statistical analysis

Differences in cell proliferation and collagen synthesis were assessed by analysis of variance (ANOVA). Multiple paired comparisons of the factors cell type, seeding density, and day were calculated with a Bonferroni correction. Analyses were carried out with SPSS version 10.0 (SPSS, Chicago, IL). As univariate analysis revealed that there was no interaction between cell type and seeding density, data derived from scaffolds seeded at various cell densities were pooled per cell type to analyze the overall difference in collagen and DNA content between the cell types. A p value of <0.05 was considered statistically significant.

RESULTS

Cell attachment, proliferation, and matrix formation

All cell types readily attached to the neoligament scaffold as observed by methylene blue block staining on day 1 (Fig. 1). At this time, the cells flattened and began to proliferate. As illustrated by ESEM (Fig. 2D), the cells were aligned along the axis of the monofilaments. By day 6, the cells fully surrounded the multifilaments, and the BMSCs and skin fibroblasts seeded at 1 x 10^6 even bridged the gaps between the individual multifilaments. This bridging was present for all cell types on day 12.

Also by light microscopy, cells and matrix were shown to be present on all outer surfaces of the scaffolds (Fig. 3). On these outer surfaces of the scaffolds, the cells were mainly elongated in shape and densely packed in layers, whereas more toward the middle of the scaffold the cells were more ovoid in shape and the tissue was less dense. Hardly any cell infiltration and matrix production were seen in the center of the multifilaments, but in areas with a more open structure between the monofilaments, such as in the tip of the sutures, cells and tissue were present inside the scaffold (Fig. 3B and D).

The produced extracellular matrix contained fibrous structures (diameters between 15 and 130 nm, measured by ESEM) and the amount increased in time for each cell type, as indicated by ESEM (data not shown). Immunohistochemistry demonstrated the presence of collagen type I for each cell type on day 6 and day 12 (Fig. 4).

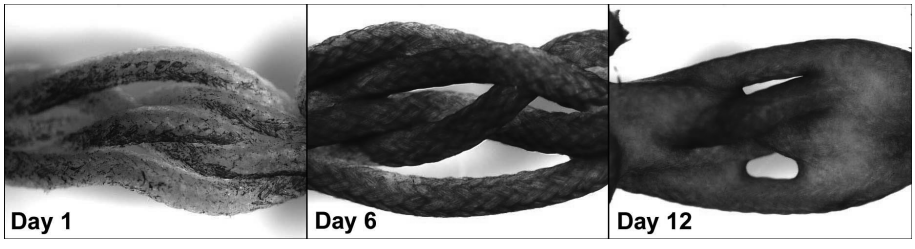

Figure 1. *Effect of culture period on cell content and matrix deposition. Methylene blue staining of scaffolds seeded with 100,000 BMSCs (original magnification, x5). Increased accumulation of cells and matrix is observed with time. Similar effects of culture period were observed for scaffolds seeded with skin and ACL fibroblasts. (See full colour section page 175)*

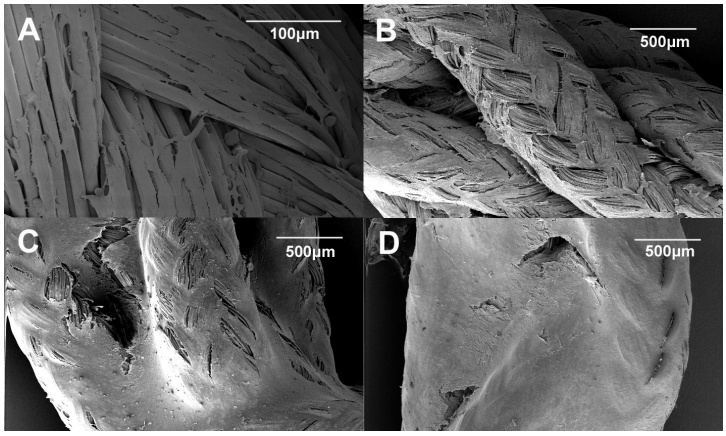

Figure 2. *Matrix deposition by BMSCs, ACL, and skin fibroblasts. (**A**) Alignment of BMSCs with monofilaments on day 1. Scanning electron micrograph of scaffolds seeded and cultured for 12 days with ACL fibroblasts (**B**), skin fibroblasts (arrow) (**C**), and (**D**) BMSCs. Covering of scaffolds with cells and matrix is visibly most complete with BMSCs (scaffolds all seeded with 100,000 cells).*

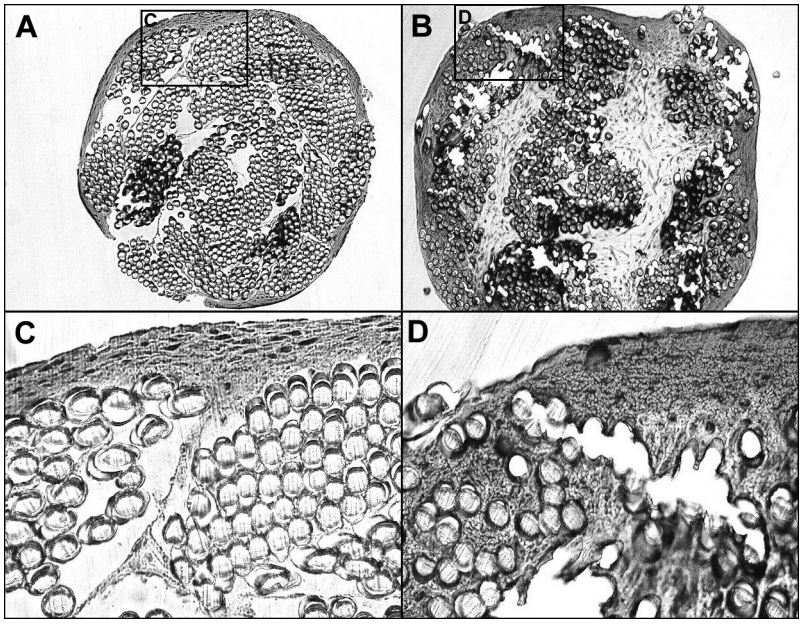

Figure 3.
*Histology of scaffolds seeded with 300,000 BMSCs on day 12. H&E staining of cross sections showing (**A** and **C**) cells and tissue visible only on the outside of the scaffold and (**B** and **D**) cells and tissue present both on the outside and at the center of the scaffold in areas with a more open structure between the monofilaments. (See full colour section page 176)*

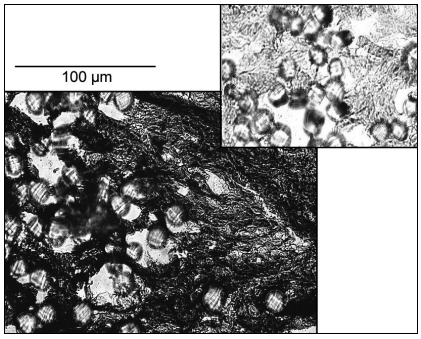

Figure 4. *Immunohistochemistry for collagen type I. Collagen type I staining was demonstrated for all cell-seeded scaffolds independent of the cell type seeded (shown: scaffold seeded with 100,000 skin fibroblasts on day 12.* Inset: *Negative control). (See full colour section page 176)*

Effect of cell type on proliferation and matrix production

Proliferation. Analysis of DNA content revealed no significant interaction of cell type with seeding density, and therefore all seeding densities were pooled to analyze the overall difference between cell types. The increase in cell number over time for each cell type, as suggested by methylene blue staining, was confirmed by the DNA assay (Fig. 5). On day 12, a statistically significant difference was observed, the scaffolds seeded with BMSCs displaying the highest DNA content (p <0.01; Table 1). The increase in DNA for the BMSCs was highest between day 6 and day 12. In contrast, the DNA content of scaffolds seeded with ACL and skin fibroblasts displayed the highest increase between day 1 and day 6 (Fig. 5).

Table 1. TOTAL DNA, COLLAGEN, AND COLLAGEN/DNA ON DAY 12[a]

	DNA (µg)		Total collagen (µg)		Collagen/DNA	
Cell type	*Mean*	*Std.dev*	*Mean*	*Std.dev*	*Mean*	*Std.dev*
ACL	12.97	3.49	18.02	5.33	1.43	0.40
Skin	20.57	6.56	74.99	46.93	3.38	1.68
BMSCs	25.40	5.77	114.01	59.45	4.26	1.80

[a]*The scaffolds seeded with BMSCs showed a higher DNA content as compared with the other cell types (*p < *0.01; pooled seeding densities,* n = *15). Differences between the various cell types were statistically significant (*p < *0.01).*

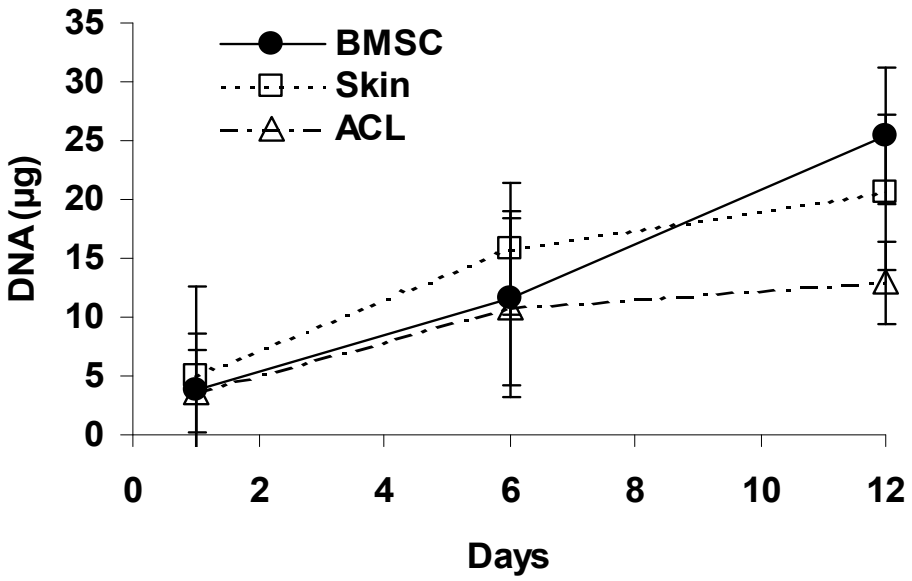

Figure 5. *Increase in DNA content during culture. During the culture period of 12 days, an increase in total DNA per scaffold was seen for all cell types (pooled seeding densities,* n = *15). On day 12, the scaffolds seeded with BMSCs showed the highest DNA content (p < 0.01).*

Matrix production. ESEM evaluation indicated that the BMSCs had produced the highest amount of matrix on day 12 (Fig. 2B–D). This was confirmed by measuring the hydroxyproline content of scaffolds as a parameter for collagen (matrix) production. As the analysis of collagen content and collagen per DNA after 12 days of culture showed a significant interaction of cell type with seeding density, seeding densities were not pooled. Statistical analysis of the difference between the cell types with respect to collagen production and collagen per DNA after 12 days was performed per seeding density. For all cell types, collagen content of the scaffolds increased with culture time (Fig. 6). The increase in collagen content over time was statistically significant for each cell type ($p < 0.05$, except for the seeding density of 1×10^4 cells per scaffold for both the BMSCs and the skin fibroblasts), with the increase in BMSCs and skin fibroblasts being higher than

the increase in ACL fibroblasts. On day 12, collagen content was highest for the scaffolds seeded with BMSCs as compared with other cell types (Fig. 6 and Table 2). This difference was significant for the two highest seeding densities ($p < 0.05$; Table 2). When expressed in terms of collagen produced per microgram of DNA, the scaffolds seeded with BMSCs and skin fibroblasts showed a higher level of collagen production per microgram of DNA on day 12 as compared with ACL fibroblasts (Fig. 7, statistically significant only for the three highest seeding densities, $p < 0.01$; Table 2). Between day 6 and day 12, an increase in collagen production per microgram of DNA was found, which was clearly higher for the BMSCs and the skin fibroblasts as compared with the ACL fibroblasts ($p < 0.05$, except for a seeding density of 10,000 per scaffold for the BMSCs).

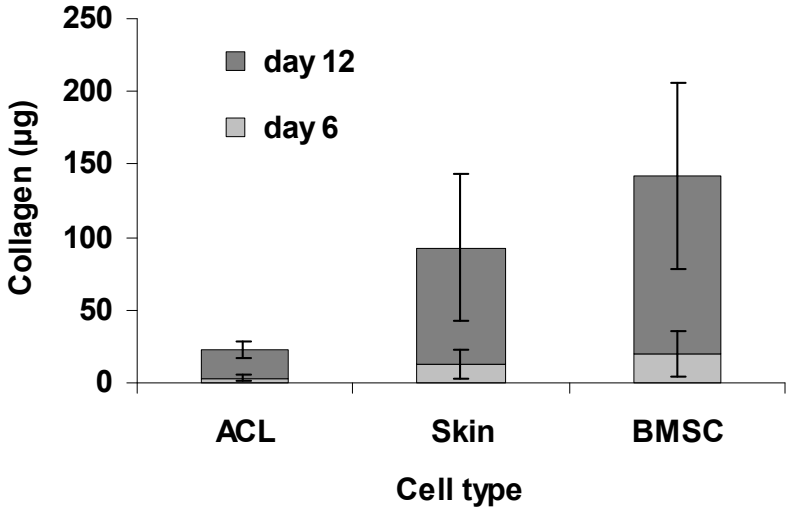

Figure 6. *Collagen production on days 6 and 12. On day 12, total collagen content (in micrograms) was highest for the scaffolds seeded with BMSCs as compared with the other cell types (seeding densities are pooled to illustrate the effect of cell type on collagen production,* n = *15). The increase in collagen content was statistically significant between day 6 and day 12 for each cell type.*

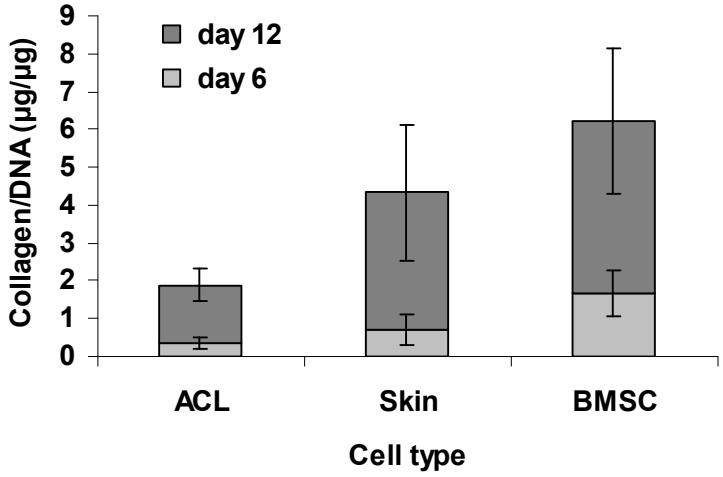

Figure 7. *Total collagen per microgram of DNA on days 6 and 12. Collagen production per microgram of DNA on day 12 was highest for the scaffolds seeded with BMSCs when compared with ACL and skin fibroblasts (seeding densities are pooled to illustrate the effect of cell type on collagen production per microgram of DNA,* n = *15).*

Table 2. COLLAGEN AND COLLAGEN/DNA, PER SEEDING DENSITY ON DAY 12[a]

Seeding density	Comparison	Collagen (µg)	Collagen/DNA
		Mean ±stdev	*Mean ±stdev*
10,000	ACL - Skin	13.0 ±4.2 < 18.6 ±1.4	1.5 ±0.8 > 1.3 ±0.1
	ACL - BMSCs	13.0 ±4.2 < 32.5 ±3.7	1.5 ±0.8 < 1.9 ±0.1
	Skin - BMSCs	18.6 ±1.4 < 32.5 ±3.7	1.3 ±0.1 < 1.9 ±0.1
30,000	ACL - Skin	17.7 ±3.9 < 28.3 ±9.8	1,5 ±0,2 < 2,1 ±0,9
	ACL - BMSCs	17.7 ±3.9 < 69.0 ±7.4 *	1.5 ±0.2 < 3.0 ±0.5
	Skin - BMSCs	28.3 ±9.8 < 69.0 ±7.4 †	2.1 ±0.9 < 3.0 ±0.5
100,000	ACL - Skin	17.6 ±8.3 < 86.8 ±17.5 *	1.5 ±0.3 < 4.6 ±1.5 *
	ACL - BMSCs	17.6 ±8.3 < 128.9 ±19.7	1.5 ±0.3 < 4.8 ±0.6 *
	Skin - BMSCs	86.8 ±17.5 < 128.9 ±19.7 †	4.6 ±1.5 < 4.8 ±0.6
300,000	ACL - Skin	23.6 ±0.4 < 122.9 ±22.6 *	1.5 ±0.3 < 4.9 ±1.1 *
	ACL - BMSCs	23.6 ±0.4 < 161.0 ±64.4 *	1.5 ±0.3 < 5.6 ±1.2 *
	Skin - BMSCs	122.9 ±22.6 < 161.0 ±64.4 ‡	4.9 ±1.1 < 5.6 ±1.2
1,000,000	ACL - Skin	18.2 ±3.7 < 118.3 ±1.0 *	1.1 ±0.3 < 4.0 ±0.4 *
	ACL - BMSCs	18.2 ±3.7 < 178.7 ±19.4 *	1.1 ±0.3 < 6.0 ±1.4 *
	Skin - BMSCs	118.3 ±1.0 < 178.7 ±19.4 *	4.0 ±0.4 < 6.0 ±1.4

[a]*Comparison of collagen content for the various cell types and seeding densities demonstrated a statistically significant higher collagen content for scaffolds seeded with BMSCs as compared with the other cell types at seeding densities of 300,000 and 1,000,000. Scaffolds seeded with BMSCs and skin fibroblasts yielded statistically significantly higher values of collagen per DNA at the three highest seeding densities.*
*p= *0.01,* †p= *0.02,* ‡p = *0.05*

Effect of seeding density on proliferation and matrix synthesis

The number of cells that attached to the scaffold increased with increasing seeding density (Fig. 8). For the ACL fibroblasts, there were no statistically significant differences found in DNA and collagen production between the various seeding densities on day 12 (Fig. 9A–C). On day 12, DNA content showed a more pronounced increase with increasing seeding density for the scaffolds seeded with BMSCs and skin fibroblasts (Fig. 9A). Collagen content and collagen per DNA also showed an increase with increasing seeding density (Fig. 9B and C), with the three highest seeding densities showing the highest amounts.

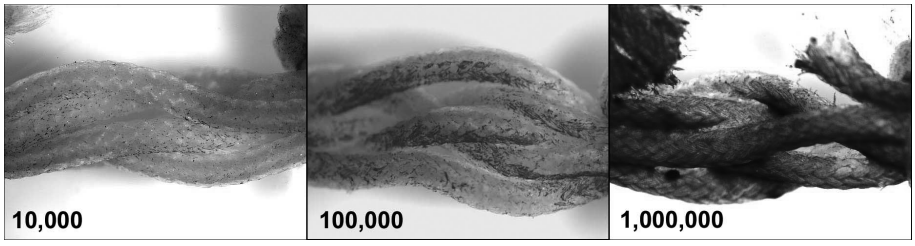

Figure 8. *Effect of seeding density on cell content on day 1. Methylene blue staining of scaffolds seeded with BMSCs (original magnification, x5). Increased accumulation of cells is observed with increasing seeding densities. Similar effects of seeding density were observed for scaffolds seeded with skin and ACL fibroblasts. (See full colour section page 177)*

DISCUSSION

In the current study, several cell types were compared for their capacity to enhance ligament formation on braided biodegradable scaffolds. Previous studies on tissue engineering of ligaments describe the use of various cell lines, such as ACL fibroblasts, medial collateral ligament (MCL) fibroblasts, skin fibroblasts, BMSCs, and tenocytes[2,3,18,19,25,43,47,68,164,266]. The present study is, to the best of our knowledge, the first to compare three different cell types with regard to two important parameters in ligament tissue engineering: in vitro proliferation and matrix production.

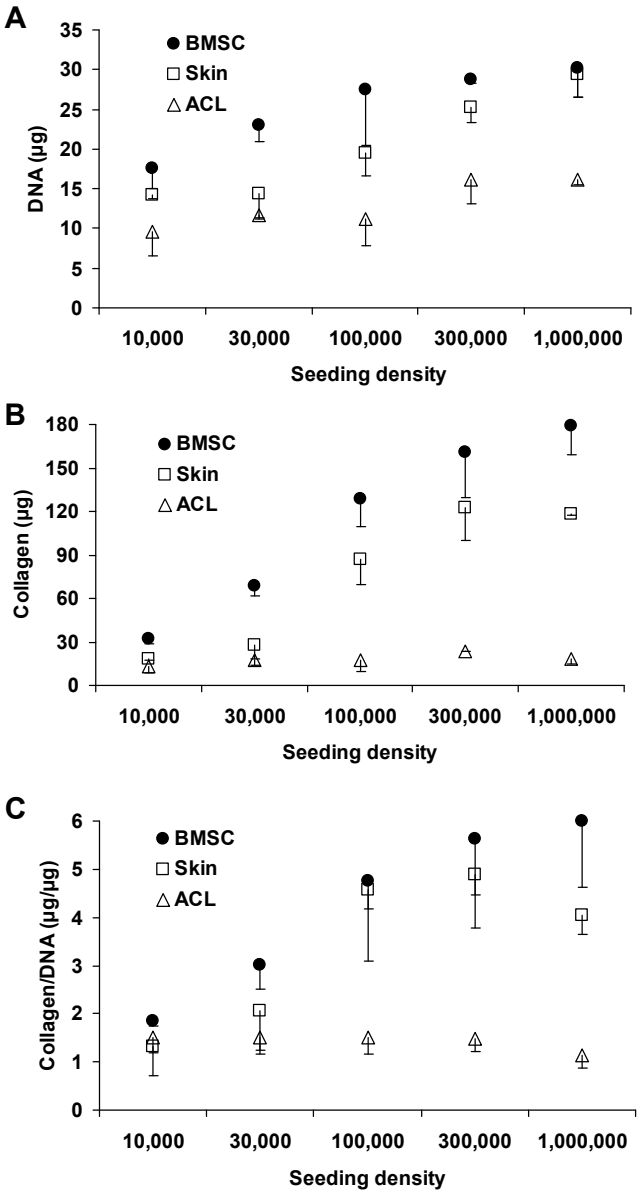

Figure 9. *Effect of seeding density on DNA content (**A**), collagen production (**B**), and collagen/DNA (**C**) on day 12. For the scaffolds seeded with ACL cells, the yield of DNA and collagen on day 12 did not significantly differ between seeding densities. For the scaffolds seeded with BMSCs and skin fibroblasts, an increase in DNA, collagen, and collagen/DNA was found with increasing seeding density (n = 3). The three highest seeding densities showed the highest amounts of collagen and collagen per DNA for the BMSCs and the skin fibroblasts (n = 3).*

Seeding of the Panacryl scaffolds with ACL, skin, and bone marrow stromal stem cells resulted in attachment and alignment along the longitudinal axis of the scaffolds. With time a distinct proliferation and matrix synthesis, as reflected by the final collagen content, was shown for all cell types. However, clear differences were noted between the cell types with respect to proliferation, total collagen production, and collagen production per cell, as reflected by DNA content. Proliferative activity of the BMSCs was highest, whereas skin fibroblasts proliferated more readily than ACL cells. Also, the final collagen content was highest for the scaffolds seeded with BMSCs and lowest in those seeded with ACL fibroblasts. When collagen production was expressed per cell, there was no difference between skin fibroblasts and BMSCs, but the ACL cells had clearly generated less collagen, thus indicating that the difference between skin fibroblasts and BMSCs in final collagen content was mainly due to a higher cell number at the end of the culture period. Both for skin fibroblasts and BMSCs, seeding density correlated positively with final collagen content, although densities beyond 3 x 10^5 did not seem to bring about more collagen deposition. For ACL fibroblasts an increase was indicated, but was so low that no statistical significance could be attained.

The presence of collagen type I in the produced matrix was an important finding. Native ligaments and tendons typically consist of 90% collagen type I and less than 10% collagen type III. The fibrous structures observed with ESEM probably are collagen fibrils that were formed out of collagen microfibrils produced by the cells. The collagen dimensions measured in this study are in the range of those described elsewhere (between 20 and 500 nm)[232].

For cell-based tissue engineering, the choice of cell type is crucial and is influenced by various considerations. For ligament tissue engineering, ACL fibroblasts initially would seem the optimal candidate, as they are highly specialized in the production of ligament tissue. However, in line with previous findings[68,181,193], the present study shows that ACL proliferation and matrix production lagged far behind the other two cell types, even when seeded at higher densities. In

contrast, both skin cells and BMSCs readily proliferated and deposited collagen onto the scaffolds. As the proliferative activity of the BMSCs was in general higher than that of skin fibroblasts, total matrix production was optimal with the former cell type. It is noteworthy that, in contrast to ACL fibroblasts, both skin fibroblasts and BMSCs display an increase in collagen production per cell with increasing seeding density, indicating that the balance between proliferation and matrix production might be dependent on the amount of cells already present. A similar effect is reported by Carrier *et al.*, who describe an increase in the fraction of differentiated cardiac myocytes with the number of cells seeded[50]. In contrast, the ACL cells produced the same amount of collagen per cell irrespective of initial seeding density, possibly because of their highly differentiated state. Despite the fact that the ACL and skin cells are in theory the same cell type, there is clearly a significant difference in their potency to adapt to different circumstances. Skin fibroblasts are known to have great healing potential and have already been shown to survive in an intraarticular environment[25].

From the present data it appears that both BMSCs and possibly to a lesser extent skin fibroblasts possess the appropriate characteristics for application in ligament tissue engineering, whereas ACL fibroblasts seem of limited use. Moreover, in a clinical setting, BMSCs and skin fibroblasts are easily retrieved by bone marrow biopsy and a split skin graft, respectively, conferring an additional advantage to the use of these cells over ACL fibroblasts. ACL fibroblasts are more difficult to obtain, because this requires an arthroscopy in the already injured knee. As the data are based on one donor, a broad generalization is not yet in view. However, our experiences and those of other researchers with goat cells indicate that interdonor variation is small in terms of cell proliferation[153] and cartilage formation and osteogenesis in vivo[212]. We conclude that bone marrow stromal cells, and possibly skin fibroblasts, promise to be the cell type of choice in ligament tissue engineering and warrant further investigations focusing on other relevant parameters, such as the survival of the cells in an intraarticular environment and the mechanical properties of the produced matrix.

5

EFFECT OF TRANSFORMING GROWTH FACTOR-BETA AND GROWTH DIFFERENTIATION FACTOR-5 ON PROLIFERATION AND MATRIX PRODUCTION BY HUMAN BONE MARROW STROMAL CELLS CULTURED ON BRAIDED POLY LACTIC-CO-GLYCOLIC ACID SCAFFOLDS FOR LIGAMENT TISSUE ENGINEERING

ABSTRACT

Tissue engineering of ligaments based on biomechanically suitable biomaterials combined with autologous cells may provide a solution for the drawbacks associated with conventional graft material. The aim of the present study was to investigate the contribution of recombinant human transforming growth factor beta 1 (rhTGF-β1) and growth differentiation factor (GDF)-5, known for their role in connective tissue regeneration, to proliferation and matrix production by human bone marrow stromal cells (BMSCs) cultured onto woven, bioabsorbable, 3-dimensional (3D) poly(lactic-co-glycolic acid) scaffolds. Cells were cultured for 12 days in the presence or absence of these growth factors at different concentrations. Human BMSCs attached to the suture material, proliferated, and synthesized extracellular matrix rich in collagen type I and collagen III. No differentiation was demonstrated toward cartilage or bone tissue. The addition of rhTGF-β1 (1–10 ng/mL) and GDF-5 (10–100 ng/mL) increased cell content ($p < 0.05$), but only TGF-β1 also increased total collagen production ($p < 0.05$) and collagen production per cell, which is a parameter indicating differentiation. In conclusion, stimulation with rhTGF-β1, and to a lesser extent with GDF-5, can modulate human BMSCs toward collagenous soft tissue when applied to a 3D hybrid construct. The use of growth factors could play an important role in the improvement of ligament tissue engineering.

INTRODUCTION

Ruptures oof the anterior cruciate ligament have a fairly high incidence, with approximately 250,000 new cases per year in the United States[102]. The natural course of these ACL lesions entails joint instability, altered load bearing, ensuing articular cartilage damage, and ultimately osteoarthritis, for which reason 30% to 40% of the ruptures are treated with surgical reconstruction[135,175].

Although biological grafts are the graft of choice for operative reconstruction, autografts and allografts have some major drawbacks, such as donor-site morbidity, immunological responses, disease transmission, and high cost[23,28,141] as a result of which there is a need for alternative approaches. In vitro tissue engineered constructs provide a realistic alternative for future use in ligament reconstruction[16,87,89,140]. Tissue engineering of ligaments commonly departs from a so-called hybrid construct containing cells with biodegradable scaffolds, the latter contributing to the immediate mechanical support required for ligament constructs[118,124,158]. Cells shown to be most suitable for tissue engineering in terms of matrix production and proliferation are bone marrow stromal cells (BMSCs)[34,68,72,185]. As scaffold materials, ideally, compounds are used that approach, as much as possible, the biomechanical properties of ligament tissue. A well documented biomaterial suitable for this purpose would be poly(L-lactide/glycolide) acid (PLGA). Although the use of PLGA has been documented mostly for cartilage and bone tissue engineering[55,62,123,204,245,251], it has been shown to be suitable for tendon and ligament regeneration too, produced in yarns to knit scaffolds of variable thickness and strength[72].

A promising way of enhancing in vitro tissue regeneration is the application of bioactive proteins and growth factors, both in vitro and in vivo. Various growth factors have been used to enhance connective tissue generation. In particular, TGF-β1 is a potent inductor of extracellular matrix (ECM) production and promotes the generation of bone, cartilage, and soft connective tissues like ligaments[20,199]. Also BMSCs respond to TGF-β by increasing proliferation and

collagen production[85,196]; thus, this might be a valuable factor in enhancing in vitro tissue engineering of ligaments. However, TGFβ can direct cells along many connective tissue lineages, including cartilage, necessitating careful monitoring of tissue differentiation markers during its application for tissue engineering. Another, more-specific, growth factor suitable for enhancing soft connective tissue differentiation might be growth differentiation factor (GDF)-5. Ectopic application of GDF-5 has been shown to induce differentiation along the ligament pathway[114,258], and this factor is involved in healing of ligamentous tissues. Moreover, addition of GDF improves the biomechanical properties of healing tendon lesions[15,171,215,233,235].

The aim of the current study was to compare the effects of TGF-β1 and GDF-5 on the in vitro generation of ligament-like soft connective tissue. Proliferation and ECM production were determined for BMSCs seeded and cultured on knitted, bioabsorbable, 3-dimensional (3D) PLGA scaffolds in the presence and absence of different concentrations of TGF-β and GDF-5.

METHODS AND MATERIALS

BMSC expansion

Pelvic bone marrow aspirates were acquired from 2 patients undergoing orthopedic surgery after informed consent was obtained. BMSCs from the bone marrow aspirates were selected using differential adhesion, as described previously[72], and cultured to 80% to 90% confluence in culture medium containing alpha-minimum essential medium (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (Gibco), 2mM L-glutamine (Invitrogen, San Diego, CA), 0.2mM ascorbic acid (Sigma, St. Louis, MO), and 100U/mL penicillin/streptomycin (Gibco). The culture medium was renewed 3 times a week. BMSCs were cryopreserved at passage 1 and seeded at passage 3.

Scaffolds

The degradable 3D scaffolds were manually prepared, using a handmade template, by braiding the inner core of commercially available suture material (Panacryl 2.0, Ethicon, Sommerville, NJ), consisting of poly(L-lactide/glycolide) fibers. The 1-cm-long woven scaffolds were sterilized using repeated immersion in 70% ethanol and after air-drying were washed with sterile phosphate buffered saline (PBS). Before the BMSCs were seeded, scaffolds were incubated overnight at 37°C in the aforementioned culture medium supplemented with 1 ng/mL basic fibroblast growth factor (bFGF).

Seeding and culturing of BMSCs

The isolated and cryopreserved BMSCs were subcultured, and at passage 3, 1mL of 1 x 10^5 viable BMSCs/mL was seeded on scaffolds by incubation for 24 h in 1.5-mL vials on a roller bank (1 rpm). After 24 h, the seeded scaffolds were transferred to 25-well plates (Sterilin, Stone, Staffs, UK) and cultured on an XYZ shaker (30 rpm) in 2mL of alpha-minimum essential medium supplemented with 2% FBS, 2mM L-glutamine, 0.2mM ascorbic acid, and 100 U/mL penicillin/streptomycin. Recombinant human TGF-β1 (R&D Systems, Minneapolis, MN) and GDF-5 (Biopharm, Heidelberg, Germany) were added at 1, 5, and 10 ng/mL and 10, and 100 ng/mL, respectively. Culture media were changed every 3 days. The hybrid constructs were harvested on days 0, 1, 3, 6, and 12 for stereomicroscopy, conventional light microscopy, electron microscopy, and determination of deoxyribonucleic acid (DNA) and collagen content.

Stereomicroscopy

Scaffolds were rinsed in PBS and fixed in 10% buffered formalin at days 0, 1, 3, 6, and 12. Fixed scaffolds were stained with methylene blue to visualize cell attachment using a stereomicroscope (Nikon SM2-10A, Tokyo, Japan).

Picrosirius-Red staining

After overnight fixation with 10% buffered formalin, scaffolds were embedded in paraffin. Five-µm transverse sections of the scaffolds were deparaffinated, hydrated to water, and stained for 1 h in a 0.2% solution of Sirius Red (Direct Red 80, Aldrich, Milwaukee, WI) in aqueous saturated picric acid and counterstained with fresh Harris' hematoxylin for 6 min. The presence of a collagenous matrix was visualized using polarization microscopy[138,139,189]. Collagen type I appears as thick, bright (strongly birefringent) yellow or red filamentous structures shining against a dark background, whereas collagen type III highlights as thin filamentous structures with a weak birefringence of a greenish color. Collagen type II typically would appear as a loose mesh with a weak birefringence of varying color[189].

Immunohistochemistry

Five-µm sections of scaffolds embedded in paraffin were incubated in PBS containing 5% bovine serum albumin to block aspecific binding. After blocking endogenous peroxidase with 3% hydrogen peroxide in PBS, and for collagen II immunolocalization, antigen retrieval for 10 min at 95°C in citrate buffer, sections were incubated with rabbit anti-collagen I (1:60, Biogenesis, Poole, UK) or mouse anti-collagen II (1:200, DSHB, Iowa City, IA). Sections were washed and incubated with anti-rabbit PowerVision (ImmunoVision Technologies, Brisbane, UK) according to the manufacturer's protocols or with goat anti-mouse polyclonal horseradish peroxidase (Dako, Glostrup, Denmark), respectively. After washing, sections were developed with 0.3 mg/mL 3,3'-diaminobenzidine and 0.01% hydrogen peroxide in 0.04 M citric acid/0.12 M sodium phosphate and counterstained with Mayer's hematoxylin. Negative controls were rabbit isotype control (Sigma) and mouse isotype control (Dako), respectively.

Electron microscopy

For electron microscopy, scaffolds were fixed in 0.05 M sodium cacodylate containing 50mM potassium chloride, 2.5 mM magnesium chloride, and 2.5%

glutaraldehyde (pH 7.2) overnight. The fixed scaffolds were post-fixed for 2 to 3 h in 2% osmium tetroxide. After dehydration in a graded ethanol series and acetone and infiltration with Eponacetone mixture (1:1) overnight, the scaffolds were embedded in Epon 812, ultrathin sectioned (80 nm), and stained with uranyl acetate (5% methanolic) and lead citrate. Ultrathin sections were examined at 60 kV in an electron microscope (JEM 1200EX, JEOL USA, Peabody, MA).

Cell proliferation and collagen synthesis

Cells and surrounding ECM were detached from the scaffolds through digestion with 1 mg/mL proteinase K (Sigma) for 16 h at 56°C. Quantification of the cell number was performed on aliquots of the digested samples using a fluorometric assay[130] for the detection of double-stranded DNA (PicoGreen, Molecular Probes, Eugene, OR), (CytoFluor; Applied Biosystems, Foster City, CA). To determine the amount of collagen synthesized, we used a hydroxyproline assay, described previously[36,72]. Briefly, 100-µL aliquots were taken from the proteinase K digests and hydrolyzed in 6 M hydrochloric acid at 110°C. After hydrolyzation, samples were desiccated using nitrogen evaporation (Evaporator, Pierce, Rockford, IL), and the hydrolyzates were diluted in demineralized water. After oxidation with Chloramine T solution (Sigma) and reaction with the addition of p-methylamino-benzaldehyde (Sigma) in a propanol/perchloric acid solution (Merck), absorbance was measured at 570 nm in a microplate reader (Biokinetics reader, Bio-Tek Instruments, Winooski, VT). The total amount of collagen was calculated based on the assumption that the hydroxyproline content of collagen is 14%[70].

Statistics

Data are representative of 2 independent experiments and donors, with 3 scaffolds per time point and growth factor concentration. Differences in gene expression, DNA, and hydroxyproline content were assessed using analysis of variance using Dunnet's test for post hoc testing, with univariate comparisons between growth factors and time points performed with a Bonferroni correction. The level of statistical significance was established at $p<0.05$.

RESULTS

Stereomicroscopy

As observed using stereomicroscopy of methylene blue-stained scaffolds on day 1, human BMSCs attached readily to the scaffold. Cells of spindle-shaped morphology were spread evenly over the surface of the braided fibers and were found throughout the 3D hybrid construct. An increase in cell number could clearly be observed during the culture period for all growth factor concentrations (Fig. 1A, B). Moreover, scaffolds cultured for 12 days displayed the deposition of ECM, which could be observed throughout the scaffold.

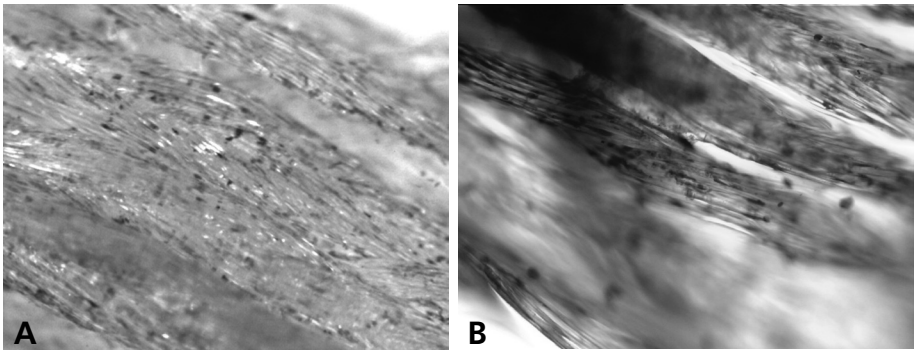

Figure 1. *(**A**) Cell attachment throughout the 3-dimensional (3D) scaffold with deposition of matrix (day 1, transforming growth factor beta 1 (TGF-β1) at 5 ng/mL). Methylene blue staining of 3D scaffolds, imaged with stereomicroscopy (magnification, 125x). (**B**) Cell attachment throughout the 3D scaffold with deposition of matrix (day 3, TGF-β1 at 5 ng/mL). Methylene blue staining of 3D scaffolds imaged with stereomicroscopy (magnification, 100x). (See full colour section page 177)*

Histology and immunohistochemistry

In general, sectioning of the paraffin-embedded scaffolds resulted in some dissociation of the constructs and the tissue deposited, probably due to the elastic nature of the PLGA fibers. Picrosirius Red–stained paraffin sections of scaffolds cultured for 12 days using polarization microscopy demonstrated a collagen

matrix that seemed to be deposited in bundles. Abundant red and clear-green staining revealed the presence of collagen type I and III (Fig. 2). Collagenous material was most prominent in the scaffolds cultured in the presence of growth factors and was found at the periphery and inside the scaffolds. No loose mesh of thin fibrils typical for collagen type II was observed. Immunohistochemistry of scaffolds harvested after 12 days of culturing confirmed the presence of collagen type I in the synthesized matrix at the end of the culture period (Fig. 3A). Control sections incubated with isotype immunoglobulin G were negative. Immunostaining for collagen type II was negative (Fig. 3B), whereas intervertebral disc tissue sections stained strongly for collagen II around the nucleus pulposus cells (Fig. 3C).

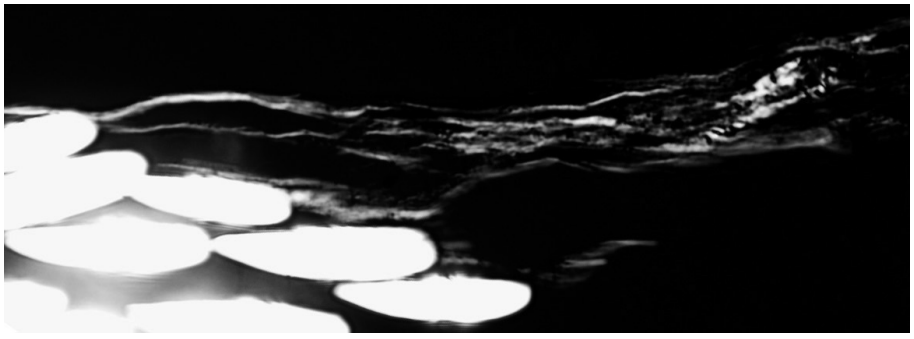

Figure 2. *Picrosirius Red staining of collagen (day 12, transforming growth factor beta 1 at 1 ng/mL) observed using polarization microscopy. Red and green birefringence is seen, demonstrating the presence of collagen type I and type III, respectively (magnification, 400x). (See full colour section page 178)*

Electron microscopy

At the ultrastructural level, cells were shown to have a typical elongated shape. Cells and cell extensions were found to be intimately associated with the scaffold fibers (Fig. 4A). Deposition of ECM demonstrated cross-banding, with a periodicity typical for collagen type I (Fig. 4B). No dot-like aggregates typical of proteoglycans were seen.

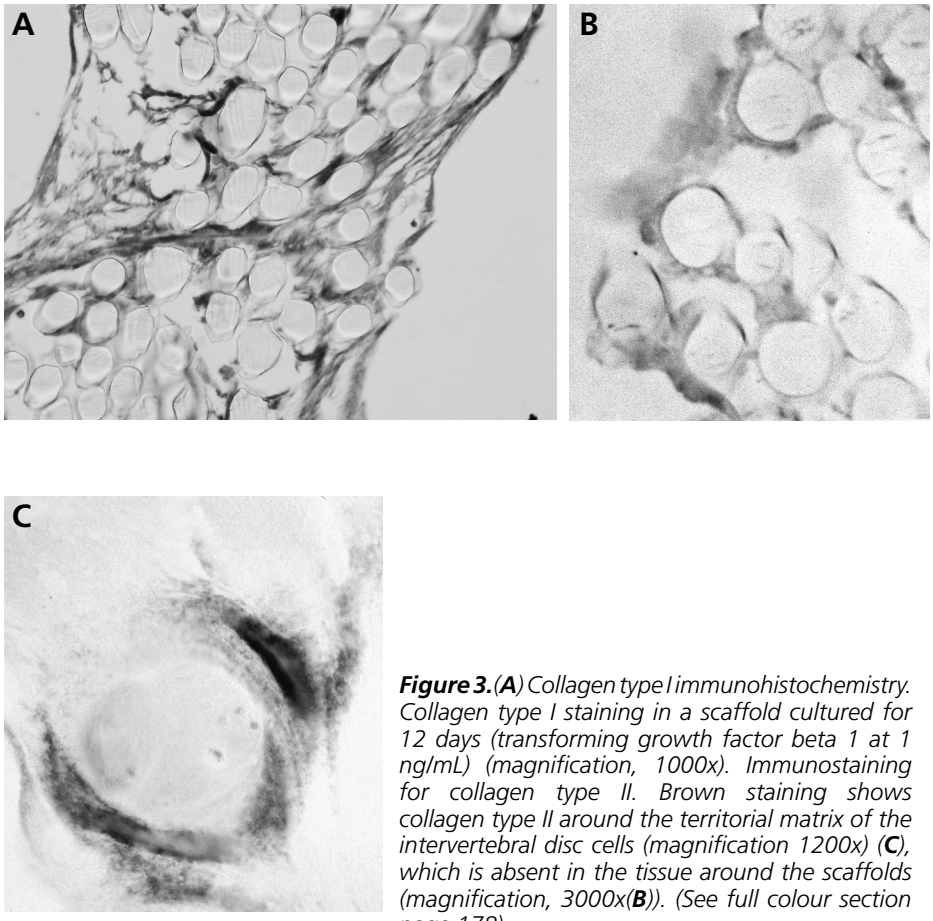

Figure 3. *(**A**) Collagen type I immunohistochemistry. Collagen type I staining in a scaffold cultured for 12 days (transforming growth factor beta 1 at 1 ng/mL) (magnification, 1000x). Immunostaining for collagen type II. Brown staining shows collagen type II around the territorial matrix of the intervertebral disc cells (magnification 1200x) (**C**), which is absent in the tissue around the scaffolds (magnification, 3000x(**B**)). (See full colour section page 178)*

Seeding efficiency and cell proliferation

Seeding efficiency was determined for scaffolds harvested immediately after seeding by assessing the DNA content and expressing this as the percentage of total DNA content of the applied cell suspension. The seeding efficiency of up to 30% was comparable in the 2 independent experiments.

During culturing, cell numbers in all scaffolds increased. Addition of recombinant human TGF-β1 to the culture medium resulted in significantly greater proliferation of BMSCs than with untreated controls in a dose-dependent way. The increase was not consistently significant at day 3 but was at day 6 at the concentration

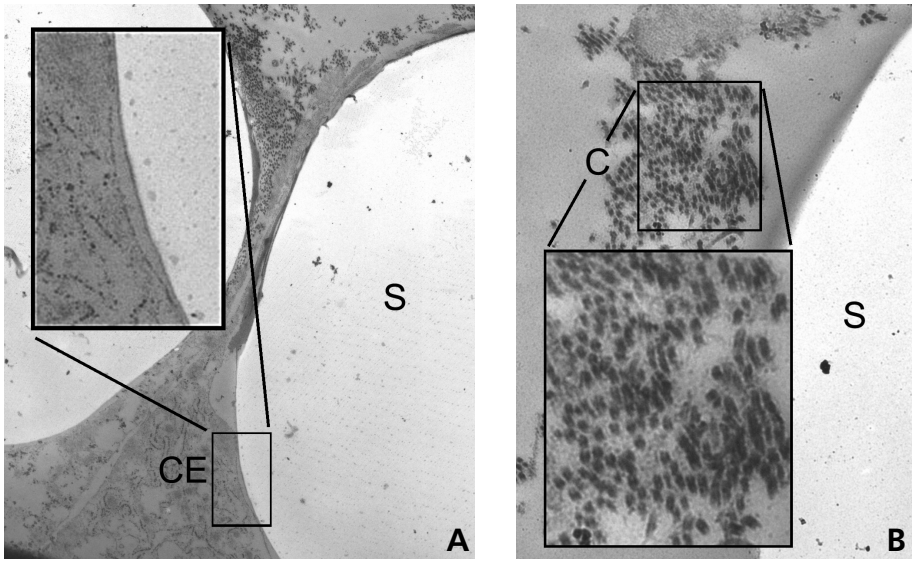

Figure 4. *(**A**) Electron micrograph of a part of the scaffold (S) with cells and cell extensions (CE) closely related to the scaffold (magnification, 2x10⁶x). Cells are closely associated with scaffold (day 12, transforming growth factor beta 1 (TGF-β1) at 1 ng/mL). (**B**) Electron-microscopic detail of fibrils with cross-banding (**C**) typical for collagen (magnification, 5x10⁶x).*

of 5 ng/mL TGF-β1 ($p<0.01$). The stimulatory effects of the concentrations 1 and 10 ng/mL were inconsistently significant at this time point (Fig. 5). At day 12, the presence of TGFβ1 resulted in an increase in proliferation in all concentrations ($p<0.01$) in 1 experiment. GDF-5 had a less profound effect on the proliferation of BMSCs. A significant increase in cell proliferation could only be established on day 12 at the concentration of 100 ng/mL ($p<0.05$).

Collagen synthesis

During the first 6 days of the culture period, little production of ECM was shown, independent of growth factor concentration. On day 12, a clear enhancement of collagen production was demonstrated. The addition of TGF-β1, at the concentration of 5 ng/mL and 10 ng/mL ($p<0.05$) resulted in distinctly greater collagen synthesis than with controls (Fig. 6). GDF-5 induced only a moderate increase that was not statistically significant.

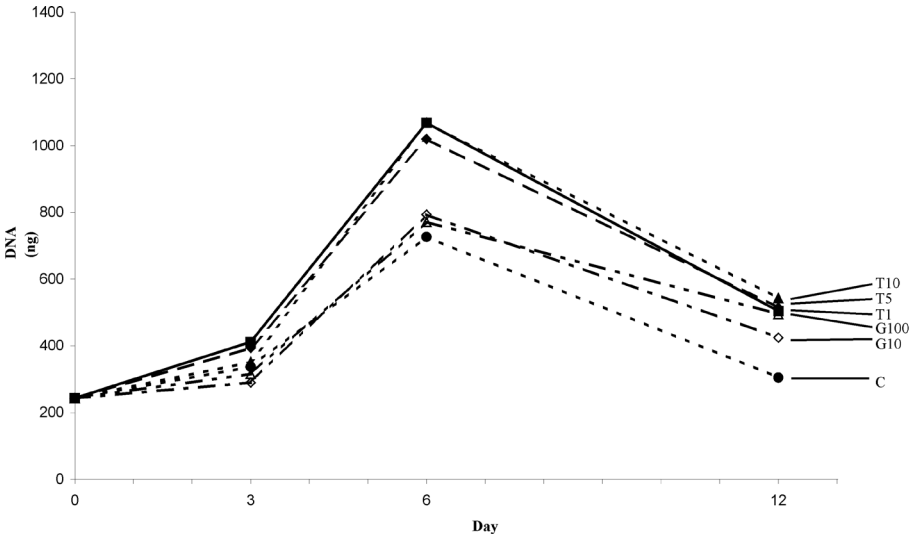

Figure 5. *Graph showing an increase in deoxyribonucleic acid (DNA) content (in ng) in the presence of growth factors (transforming growth factor beta 1 at 1 (T1), 5(T5), and 10 (T10) ng/mL, respectively; growth differentiation factor-5 at 10 (G1) and 100 (G100) ng/mL, respectively). C, control.*

Collagen synthesis/cell

When looking at the total collagen production per cell, as a derivative definition of differentiation of BMSCs, the effect of TGF-β1 was even more pronounced. Total collagen synthesis per cell was greater using TGF-β1 at 5 ng/mL than with controls ($p<0.05$). The increase of synthesis found at 1 ng/mL of TGF-β1 was statistically significant ($p<0.05$) in 1 experiment only (Fig. 7). In contrast, there was no effect of GDF-5 on the amount of collagen produced per cell.

DISCUSSION

The current study is, to the best of our knowledge, the first to investigate the effect of GDF-5 and TGF-β1 on proliferation and matrix production of human BMSCs cultured on PLGA. The 3D PLGA braided scaffolds allowed for cell attachment and proliferation over time, as found previously[72]. As initially performed,

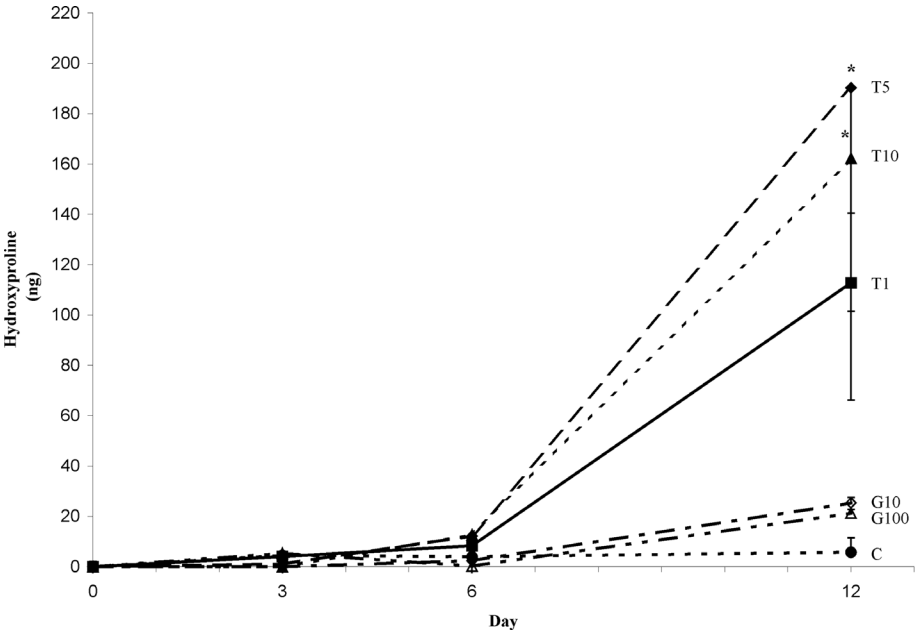

Figure 6. *Graph showing an increase in collagen production (in ng) in the presence of growth factors, starting from day 6, in comparison with the control. Asterixes denoting statistical significance as mentioned in the Results and standard errors of the mean for time points 1–6 days, are not demonstrated for easy reference (transforming growth factor beta 1 at 1 (T1), 5 (T5), and 10 (T10) ng/mL, respectively; growth differentiation factor-5 at 10 (G1) and 100 (G100) ng/mL, respectively). C, control.*

semiquantitative polymerase chain reaction for tenascin-C and collagen type I did not reveal statistically significant differences between effects of various growth factors; because of high standard deviations (data not shown), analyses in this study were restricted to biochemical parameters. The first days of culture were characterized mainly by proliferation, after which cell numbers declined and collagen production increased, indicating differentiation and concomitant senescence. At the end of the culture period, cells were aligned along the scaffold material and had deposited ECM throughout the scaffold. Collagen types I and III were found in the ECM, whereas collagen type II was absent. Transmission electron microscopy revealed an intimate association between fibroblast-like cells

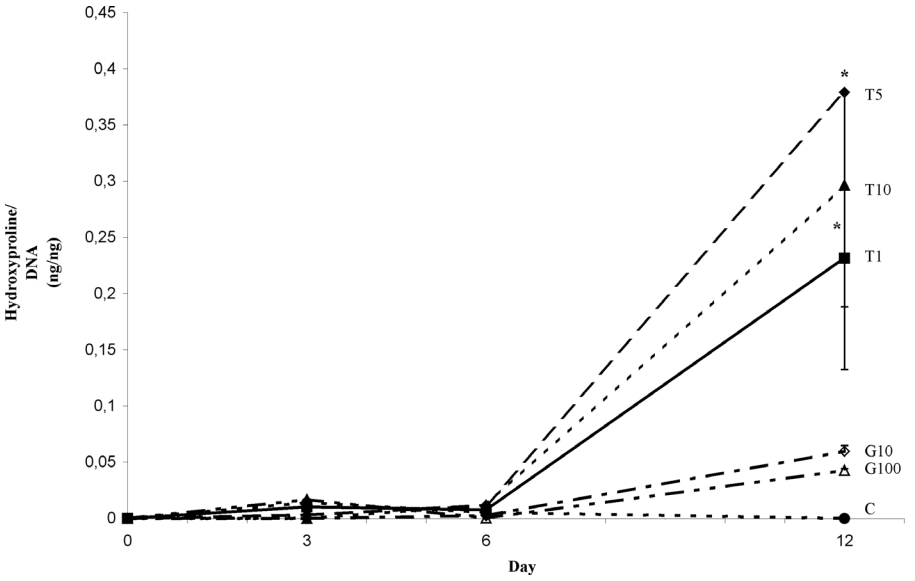

Figure 7. *Hydroxyproline per deoxyribonucleic acid (DNA); total collagen synthesis per cell. Graph showing an increase in collagen production per cell in the presence of growth factors, starting from day 6, in comparison with the control. Asterixes denoting statistical significance as mentioned in the Results and standard errors of the mean for time points 1-6 days are not demonstrated for easy reference (transforming growth factor beta 1 at 1 (T1), 5 (T5), and 10 (T10) ng/mL, respectively; growth differentiation factor-5 at 10 (G1) and 100 (G100) ng/mL, respectively). C, control.*

and the scaffold fibers and an ECM containing typical cross-banded collagen fibrils without any discernible proteoglycan aggregates or mineralization foci. GDF-5 and TGF-β1 increased total cell numbers during culture, as reflected by DNA content, but only TGF-β1 also significantly increased collagen production, both in absolute amounts and on a per cell basis, the latter reflecting synthetic activity of the cells. No statistically significant dose dependency could be demonstrated. This might be because the concentrations used were too close to each other to have significantly different effects. Alternatively, the occurrence of feedback mechanisms might explain the seemingly stronger effect of 1 and 5 ng/mL of TGF-β than of 10 ng/mL. Protein and cytokine production after TGF-β receptor activation are known to suppress the cellular response to TGF-β[35,85].

In the current study, chondrogenesis did not seem to be induced, given the absence of collagen II, even in the presence of TGF-β1, although this growth factor has been applied previously to enhance in vitro cartilage neoformation by mesenchymal stem cells[76,257,262]. The relatively short culture period, leaving the constructs with relatively immature tissue that can still be directed in other pathways might explain the absence of cartilage-like matrix components.

However, chondrogenic differentiation by mesenchymal stem cells, in terms of collagen II and proteoglycan production, has been reported already after 3 days of culturing[32]. More likely, the culture medium used might have played a role in the lack of chondrogenic markers found. Chondrogenic differentiation is usually achieved in the absence of serum[55,169] and requires factors such as dexamethasone, insulin, transferrin, and selenium[203,252], which were all absent in the culture medium used here, which on the other hand contained fetal calf serum. It should be borne in mind that TGF-β1 not only can induce chondrogenesis, but is also involved in, for example, cardiac fibrosis characterized by excess collagen deposition[143]. Various other factors are likely to codetermine the effect of TGF-β on the final pathways of differentiation.

Concerning the lack of evidence for osteogenic differentiation on the scaffolds, the commonly added dexamethasone and b-glycerophosphate were also lacking in the culture medium[144]. In addition, PLGA has been shown to exert an intrinsic inhibitory effect on osteogenesis of BMSCs[251], which also holds true for TGF-β1[53,174].

The effects of GDF-5 on growth and differentiation of BMSCs on the PLGA constructs in vitro turned out to be limited to a small increase in proliferation, as found previously for periodontal ligament cells, where the addition of GDF-5 left collagen I expression unchanged but did increase cell proliferation[194]. Thus, although it has been shown that this factor can induce ectopic tendon formation[114,258] and enhance tendon healing in vivo[15,171,215,233,235], effects in vitro seem to be limited. Concomitant cues may also be needed for GDF-5 to induce optimal enhancement of connective tissue formation.

Thus, within the time span of the culture period, and irrespective of the growth factor added, TGF-β1 could enhance the formation of soft connective tissue. To what extent the matrix formed was similar to ligament tissue is not clear. Until now, no specific markers for ligament tissue have been available. Proteins suggested to be ligament and tendon specific are fibronectin and tenascin-C, but these are also being produced in cartilage and dermis[242], and collagen I and III are found in bone and in soft connective tissues like skin and are the major ECM components of tendon and ligaments[166]. The ratios of collagen I vs III might have provided some insight into the nature of the tissue formed. The tissue formation demonstrated here may partly reflect a wound healing process. Wound healing in general is associated with relatively higher contents of type III collagen[7,150,170], and TGF-β is a crucial mediator in this process, as well as in tendinous tissue[122]. Although this growth factor is also responsible for scar formation, during the culture period investigated, we found no signs of scarring such as deposition of a disorganized and densely packed collagen matrix and the accompanying paucity of cells[29]. Nevertheless, caution may be warranted in using higher concentrations or longer exposure times.

However, even if the tissue generated is not identical to mature ligament tissue, it might not be necessary to specifically regenerate this type of tissue in vitro. In vivo conditioning by the surrounding ECM and biomechanical cues may be sufficient to induce further ligament differentiation. In this respect, it is important to note that dermal fibroblasts were found to be as effective as tenocytes in their capacity to repair a defect of the flexor superficial tendon seeded and implanted on a PLGA scaffold. Eventually, the fibroblasts on the PLGA constructs appeared to have adopted a tenocyte phenotype after implantation[167]. Thus, constructs of knitted PLGA, shown previously to have biomechanical properties comparable with tendon and ligament[200,201], on which atypical soft connective tissue production is enhanced, may provide the initial biomechanical stability required immediately. More ligament-like tissue may then slowly replace them in vivo through cues from its local environment.

In conclusion, in vitro exposure to recombinant human TGF-β1 significantly stimulates soft connective tissue formation by human BMSCs cultured on a 3D PLGA hybrid construct, whereas GDF-5 has a limited effect on proliferation only. The use of TGF-β1 might prove useful for enhancement of ligament tissue engineering. Further studies combining growth factors or growth factors and biomechanical stimuli and subsequent implantation in vivo may enhance understanding of connective tissue, in particular ligament, differentiation and thus in vivo regeneration.

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6

THE EFFECT OF TIMING OF MECHANICAL STIMULATION ON PROLIFERATION AND DIFFERENTIATION OF GOAT BONE MARROW STEM CELLS CULTURED ON BRAIDED PLGA SCAFFOLDS

ABSTRACT

Bone marrow stromal cells (BMSCs) have been shown to proliferate and produce matrix when seeded onto braided poly(L-lactide/glycolide) acid (PLGA) scaffolds. Mechanical stimulation may be applied to stimulate tissue formation during ligament tissue engineering. This study describes for the first time the effect of constant load on BMSCs seeded onto a braided PLGA scaffold. The seeded scaffolds were subjected to four different loading regimes: Scaffolds were unloaded, loaded during seeding, immediately after seeding, or 2 days after seeding. During the first 5 days, changing the mechanical environment seemed to inhibit proliferation, because cells on scaffolds loaded immediately after seeding or after a 2-day delay, contained fewer cells than on unloaded scaffolds or scaffolds loaded during seeding ($p < 0.01$ for scaffolds loaded after 2 days). During this period, differentiation increased with the period of load applied. After day 5, differences in cell content and collagen production leveled off. After day 11, cell number decreased, whereas collagen production continued to increase. Cell number and differentiation at day 23 were independent of the timing of the mechanical stimulation applied. In conclusion, static load applied to BMSCs cultured on PLGA scaffolds allows for proliferation and differentiation, with loading during seeding yielding the most rapid response. Future research should be aimed at elucidating the biomechanical and biochemical characteristics of tissue formed by BMSCs on PLGA under mechanical stimulation.

INTRODUCTION

Injuries to the anterior cruciate ligament (ACL) are common, particularly in young and active people. The prevalence of ACL ruptures continues to increase, due to a more active lifestyle. The current treatment options to reconstruct the ACL, such as with the use of autografts and allografts, have relevant drawbacks. Problems such as donor site morbidity with the use of autografts and risk of disease transmission with the use of allografts are well-known problems to overcome[63,126,141]. Tissue engineering (TE) could provide a solution for the limitations described. Various studies have focused on one or more aspects of this multifactorial process to tissue engineer an ACL with autologous cells and a degradable scaffold[3,19,25,33,45-47,61,162,164,172,220]. Because scaffolds should not only be biodegradable and biocompatible, but also exhibit sufficient mechanical strength, braided poly(L-lactide/glycolide) acid (PLGA) represents a potentially good material for ACL TE. Various braided structures allow for attachment of cells throughout the whole scaffold and provide an environment for cells to proliferate and produce extracellular matrix (ECM)[2,61,172,220]. In particular, the design of the braided scaffold should be optimized in such a way that cells and surrounding matrix can be deposited throughout the scaffold[190,220].

Initially, specialized and therefore more-differentiated cells such as tenocytes and ligament cells were used in engineering of tendon and ligaments[25,47,68,164] because these cells would theoretically produce tissue that resembles native tissue more closely. Recently it was shown that bone marrow stromal cells (BMSCs) seeded and cultured on PLGA scaffolds proliferate more readily and produce more matrix than more-differentiated cell types[72] and therefore may be more suitable for TE of the ACL. However, because BMSCs do not intrinsically produce ligament specific protein profiles and have a multilineage potential, their in vitro and in vivo differentiation should be monitored carefully. Among several options to direct differentiation of BMSCs, such as the addition of biochemical factors to culture medium or in vivo gene transfer, application of biomechanical

stimulation may represent an efficient cue. For differentiated ligament and dermal cells, alignment, proliferation, and matrix production have been shown to be dependent on load profile[26,162]. At least part of these effects are mitigated through interactions between the ECM and the cells[41,107]. Not much is known about the effect of mechanical stimulation of undifferentiated cells such as BMSCs in ACL TE. BMSCs cultured in collagen gels and seeded on pretensioned sutures were found to align when subjected to a constant load of 5 N[19]. Application of a cyclic load to the BMSCs showed an up-regulation of ligament fibroblast markers[3]. The influence of mechanical stimulation of BMSCs on PLGA scaffolds, which are more likely to be used for TE of the ACL, has not been studied. Given the role of the ECM in downstream signaling of mechanical stimuli, partial differentiation with concomitant matrix deposition may be required for adequate translation of these stimuli. Thus the timing of application of a mechanical load to cell-seeded synthetic biodegradable scaffolds could influence its final effects.

In the current study, cell proliferation and matrix synthesis by undifferentiated BMSCs seeded onto a porous three-dimensional (3D) braided poly(L-lactide/glycolide) acid scaffold were studied under various regimes of static mechanical load.

MATERIALS AND METHODS

Design

Culture-expanded goat BMSCs were dynamically seeded onto a 3D knitted scaffold made of PLGA. The cell-seeded scaffolds were cultured without the application of any load, with static load starting during seeding, immediately after seeding, or 2 days after culture. All samples were analyzed for proliferation and differentiation on days 0 (after seeding), 5, 11, and 23.

Cell isolation and expansion

Goat BMSCs were isolated and expanded as described earlier[153]. In summary, BMSCs were obtained from goat (aged 1 year) iliac wing bone marrow aspirate. The aspirate was cultured until confluence in culture medium consisting of alpha minimum essential medium supplemented with 10% fetal bovine serum (Gibco,GrandIsland,NY), L-glutamine (2 mM, Invitrogen, San Diego, CA), ascorbic acid (0.2 mM, Sigma, St. Louis, MO), and penicillin/streptomycin (each 100U/mL, Gibco). This culture medium was supplemented with basic fibroblast growth factor (bFGF, 1 ng/mL, Instruchemie, Hilversum,The Netherlands). Cells were cryopreserved at the end of passage 1. Within 6 months, the cryopreserved cells were thawed and replated at 10,000 cells/cm2. When confluent, the cells were trypsinized and cultured in the abovementioned culture medium with omission of bFGF at 5000 cells/cm2. The cells were used for seeding at passage 4.

Scaffolds

The degradable scaffolds were made of braided Panacryl (size 2/0, Ethicon, Sommerville, NJ), which is composed of 11µm PLGA bundles consisting of approximately 40 monofilaments. These bundles of 40 monofilaments were used to braid a porous 3D braided structure with a length of 15mm ± 0.5mm (Fig. 1A). Both ends of the braided structure were clamped together using a small piece of a 21-gauge injection needle. The scaffolds were sterilized using repeated cycles of incubation in ethanol 70% (EtOH, 4 times for 15 min) and after air-drying were washed with sterile phosphate buffered saline (PBS; 4 times for 15 min). The night before seeding, the scaffolds were incubated in the above-mentioned culture medium.

Seeding and 3D culture

The BMSCs were used at passage 4. The cells were trypsinized (0.25% trypsin, Biowittaker Europe, Verviers, Belgium), counted in duplicate using a Coulter counter Z2 (Beckman Coulter, Krefeld, Germany), and resuspended in the above-

mentioned culture medium. The cells were seeded as described earlier[72]. Briefly, one million cells per scaffold were seeded in 2mL of culture medium in a 2-mL tube (Eppendorf, Hamburg, Germany). The tubes with the cells and scaffolds were sealed using a hydrophilic filter (Eppendorf). The scaffolds that were loaded from the moment they were seeded were put on an orthodontic spring wire and seeded in 10mL of culture medium in a 10-mL tube using the same concentration of cells as the scaffolds seeded in the 2-mL tubes. As a control for seeding efficiency, unloaded scaffolds were seeded under the same conditions as the scaffolds loaded during seeding in 10-mL tubes. All tubes containing scaffolds were placed on a roller bank (1 rpm, New Brunswick Scientific, Edison, NJ) at 37°C and 5% carbon dioxide (CO_2) for 24 h. After seeding, the scaffold loops were put on orthodontic spring wires, with or without applied load (see below), and cultured in 25-well bacterial culture plates (Sterilin, Stone, UK) on a XYZ shaker at 30 rpm (37°C and 5% CO_2). The scaffolds loaded during seeding were also transferred to 25-well bacterial culture plates and cultured under the same conditions. Medium was changed twice a week.

Load application

The load applied was based upon previous studies investigating the effect of a static load on BMSCs[19]. Load was applied by putting the cell-seeded scaffolds on a sterile orthodontic spring wire (Fig. 1B). These spring wires were made under standard conditions, and the load applied to the scaffolds was measured as 6.7 ± 1.6 N. After seeding, unloaded scaffolds were put on a spring wire that did not apply any axial load to the scaffolds (Fig. 1C). The scaffolds that were loaded during seeding were seeded and cultured on the same loaded spring wire. The cell-seeded scaffolds that were loaded after seeding were put on a loaded spring wire after seeding and the scaffolds loaded after two days of culturing were first put on an unloaded spring wire after seeding and during culturing were transferred to a loaded spring wire at day 2 (Fig. 1B).

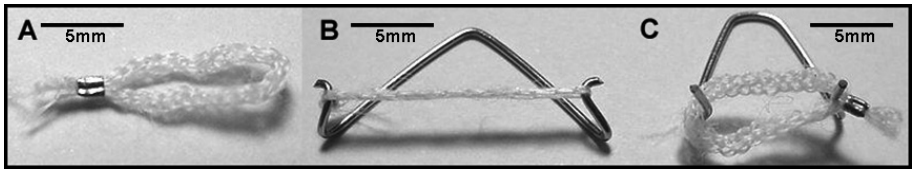

Figure 1. *Three-dimensional braided scaffold (length 15mm ± 0.4 mm). (**A**) Scaffold before culture, (**B**) loaded scaffold on a spring wire, and (**C**) unloaded scaffold on a spring wire.*

Outcome parameters

Cell attachment and matrix structure. Methylene blue block staining and environmental scanning electron microscopy (ESEM; Philips, Eindhoven, the Netherlands) were used to qualitatively evaluate cell attachment and matrix synthesis. The scaffolds were rinsed with PBS and fixed with 1.5% glutaraldehyde in 0.14M cacodylate buffer overnight at 4°C. The scaffolds were stained with methylene blue and analyzed using stereomicroscopy (SM2-10A, Nikon, Tokyo, Japan). For ESEM, the scaffolds were then dehydrated, critical point-dried, and gold sputter coated.

Cell proliferation. Cell proliferation was assessed using fluorometric quantification of DNA using the CyQuant Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR). After rinsing with PBS, all samples were digested in a 1.0-mg/mL proteinase K solution (Sigma) for 16 h at 56°C, after which the samples were treated according to the manufacturer's instructions. The fluorometric analysis was done at 480/520 nm excitation/emission using a fluorometer (PerkinElmer, Buckinghamshire, England).

Collagen quantification. As a parameter for matrix synthesis, we determined the total amount of collagen, using the hydroxyproline assay described by Brown et al[36]. Briefly, aliquots of 100 µL of the proteinase K digest were hydrolyzed overnight with 200 µL of 6M of hydrochloric acid (Merck, Darmstadt, Germany) at 110°C in a heating block (Techne, Cambridge, England). Fluid was removed using nitrogen evaporation (Evaporator, Pierce, Rockford, IL). After resuspension

in demineralized water, the hydrolysates were oxidized using Chloramine T (Sigma) followed by the Ehrlich reaction, by adding p-methylamino benzaldehyde (Sigma) dissolved in a propanol and perchloric acid solution (Merck). Absorbance was measured at 570 nm with a microplate reader (Biokinetics reader, Bio-Tek Instruments, Winooski, VT). The collagen content was calculated by assuming that the content of hydroxyproline in collagen is 14%[213].

Histology. For histology, the scaffolds were rinsed with PBS, fixed with 1.5% glutaraldehyde in 0.14M cacodylate buffer overnight at 4°C, dehydrated in a graded ethanol series, and embedded in glycol methacrylate. Using a microtome, 5 mm sections were sawed and stained with hematoxylin and eosin. Sections were analyzed using light microscopy (Eclipse E600, Nikon).

Statistical analysis

Differences in cell proliferation and collagen synthesis between the different groups (n=6 per group per day) were assessed using analysis of variance variation analysis (including Bonferroni correction). A p-value ≤ 0.05 was considered statistically significant. All data are presented as means ± standard deviations.

RESULTS

Macroscopic appearance

After 11 and 23 days of culture, all scaffolds resembled cord-like bundles (Fig. 2). The surface was white and shiny upon macroscopic inspection. At 23 days, the arms of the loops had grown into one single bundle in the unloaded group (Fig. 2). This was also the case in the loaded scaffolds, although not always completely. At day 23, the filaments of the scaffold were hardly visible on the outer surface of the structure. After releasing the load, the scaffolds cultured with load had a smaller diameter than the scaffolds cultured without load.

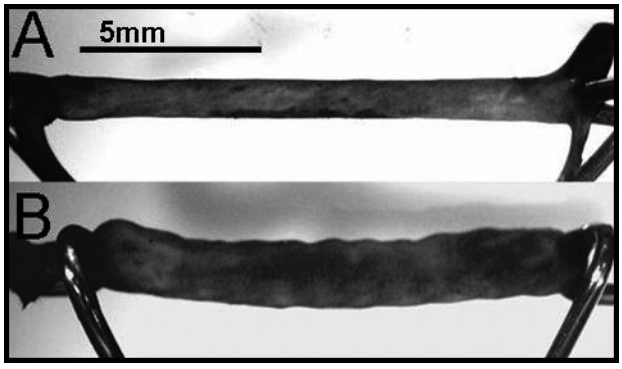

Figure 2. *Gross morphology of (**A**) loaded scaffold and (**B**) unloaded scaffold at day 23.*

Cell attachment and matrix production

The BMSCs readily attached to the scaffolds, as observed using methylene blue block staining and ESEM (Fig. 3A), and flattened along the axis of the monofilaments. Until day 11, an increase in tissue deposition was demonstrated according to ESEM (Fig. 3B–D) for all scaffolds. Between day 11 and day 23, only a small additional increase in volume was seen. At day 23, a difference in tissue production became apparent, with unloaded scaffolds seemingly containing the highest amount of matrix.

As demonstrated using light microscopy of the glycol methacrylate–embedded scaffolds, tissue was present throughout the whole scaffold for all different load regimes (Fig. 4). At day 5, the centre of the scaffolds was filled with cells and tissue. On the outer surfaces of the scaffold, the cells were densely packed in layers. In the unloaded scaffolds, the cells and matrix present inside the scaffold seemed looser than the tissue formed in the loaded scaffolds (Fig. 5A–D). As found with the macroscopic observations, applying load to the scaffolds appeared to have resulted in smaller overall diameters (Fig. 5E-H). However, because the number of scaffolds analyzed using histology was too small, no statistical analysis could be performed to confirm this impression.

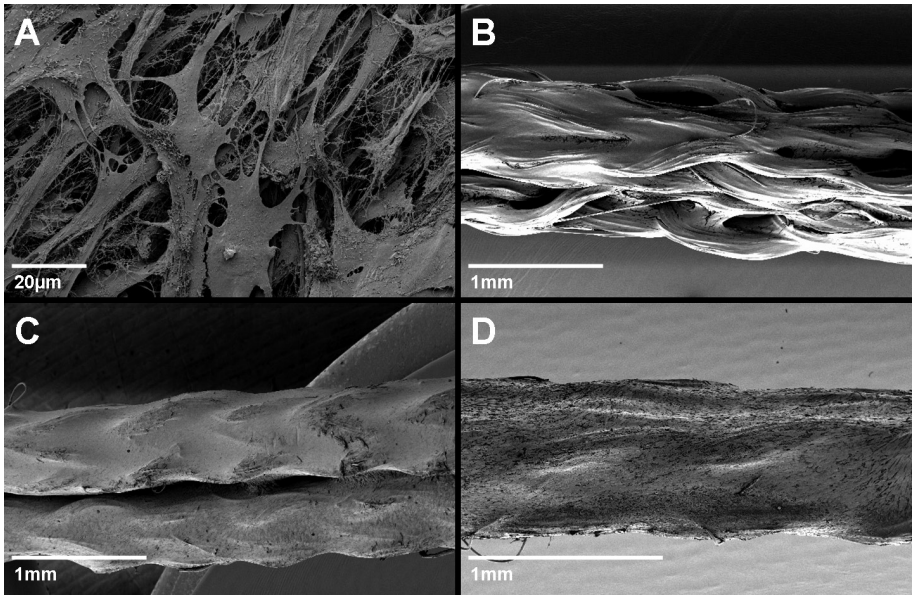

Figure 3. *Environmental scanning electron microscopy demonstrating cell attachment and matrix deposition in an unloaded scaffold. (**A**) At day 0, cells attached to the scaffold. From (**B**) day 5 to (**C**) day 11, an increase in matrix was seen. (**D**) Between day 11 and day 23, only a small increase in matrix was seen, but the loops of the scaffold had grown into one bundle at day 23.*

DNA content

At day 0, no statistical difference in DNA was seen between all scaffolds, and no statistically significant differences were seen in DNA between the scaffolds loaded during seeding and the unloaded scaffolds seeded under the same conditions in the 10-mL tubes (control group for seeding efficiency, data not shown). DNA analysis reflecting cell number showed an increase in DNA content for all different scaffolds at day 11 as compared to day 1 (Fig. 6). The scaffolds loaded immediately after seeding and after 2 days of culture demonstrated a delay in increase of cells from day 0 to day 5, although this was statistically significant only for the scaffolds loaded with a 2-day delay. These scaffolds loaded with a delay showed a lower cell number between day 5 and day 23 than the unloaded scaffolds and the scaffolds loaded during seeding ($p<0.01$).

At day 23, no statistically significant differences were found between the other scaffolds, although there seemed to be more cells on the unloaded scaffolds. For all scaffolds, the amount of total DNA increased significantly in the culture periods between day 5 and day 23 ($p<0.05$).

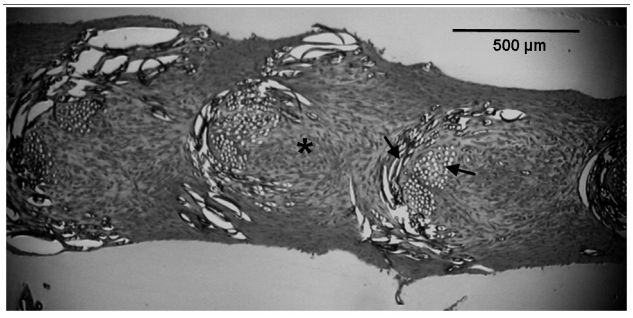

Figure 4. *Histology of unloaded scaffold seeded with bone marrow stem cells and cultured for 11 days (hematoxylin and eosin staining, *center of the scaffold, fibers of the scaffold are indicated with arrows). (See full colour section page 179)*

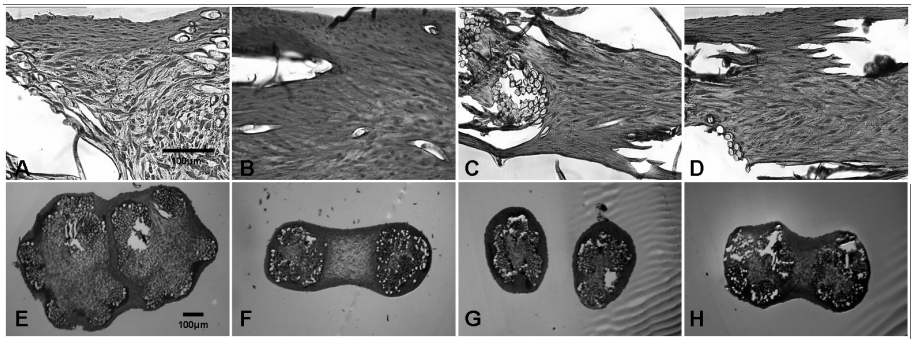

Figure 5. *Histology of scaffolds cultured for (**A–D**) 11 and (**E–H**) 23 days, stained with hematoxylin and eosin. Cells and matrix are densely packed at the outer surfaces. In the (**A**) unloaded group, tissue appears looser toward the center of the scaffold than in the loaded scaffolds (loaded before seeding (**B**), after seeding (**C**), and 2 days after culture (**D**)). (See full colour section page 179)*

Hydroxyproline content

Total hydroxyproline content as a parameter of collagen production increased continuously during the whole culture period for all scaffolds, with the exception

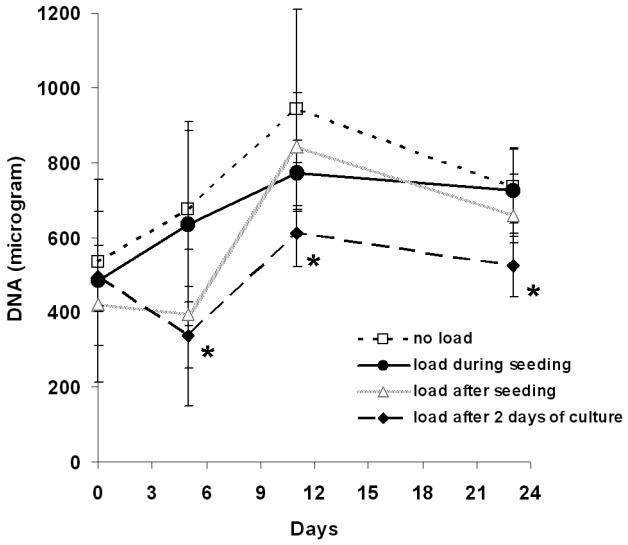

Figure 6. *Amount of total DNA in time (mean ± standard deviation). *p<0.01 for scaffolds loaded 2 days after culture compared with unloaded scaffolds and scaffolds loaded during seeding.*

of the scaffolds loaded after 2 days of seeding showing an initially low collagen content at day 5 as compared to the other scaffolds (Fig. 7; $p<0.05$). After this culture period, the collagen production of these scaffolds had increased to levels comparable with those of the other scaffolds. At day 5, matrix production on the scaffolds loaded during seeding was highest ($p<0.01$). At day 11, no statistically significant differences were found. However, final collagen content at day 23 was highest for the unloaded scaffolds ($p<0.01$). Collagen content on scaffolds loaded immediately after seeding was significantly higher at day 23 than on the scaffolds loaded with a delay of 2 days ($p<0.01$).

Following the general definition of differentiation as the process during which stem cells acquire specialized functions, the production of ECM molecules such as collagen can be considered indicative of differentiation of the BMSCs. Cell differentiation expressed as total collagen per cell (w/w% hydroxyproline/DNA) showed a similar increase in time for all scaffolds (Fig. 8). However, at day 5,

cells on the scaffolds loaded during seeding and immediately after seeding had produced more collagen per cell than the scaffolds loaded with a delay of 2 days ($p<0.01$). At days 11 and 23, no statistically significant differences were seen between any of the load regimes.

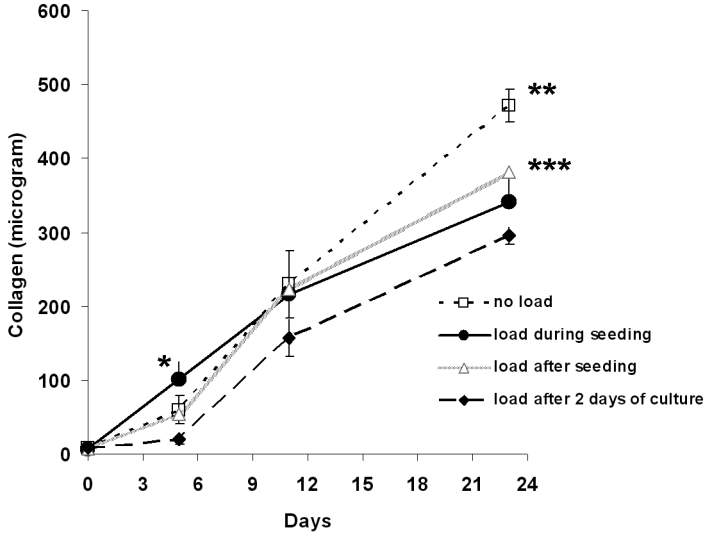

Figure 7. *Total amount of collagen continued to increase throughout the whole culture period (mean ± standard deviation). *p<0.01 for scaffolds during seeding and p<0.05 for scaffolds loaded 2 days after seeding. **p<0.01 for unloaded scaffolds compared with other scaffolds. ***p<0.01 for scaffolds loaded immediately after seeding compared with scaffolds loaded with a delay of 2 days.*

DISCUSSION

The current study is the first to describe the effect of static load on undifferentiated BMSCs seeded and differentiated onto braided PLGA scaffolds. On all scaffolds, the BMSCs were found to have attached, aligned, and proliferated. Cells had migrated throughout the scaffold and produced matrix between the monofilaments. In the unloaded scaffolds, cells and matrix seemed to be distributed more loosely inside the scaffold.

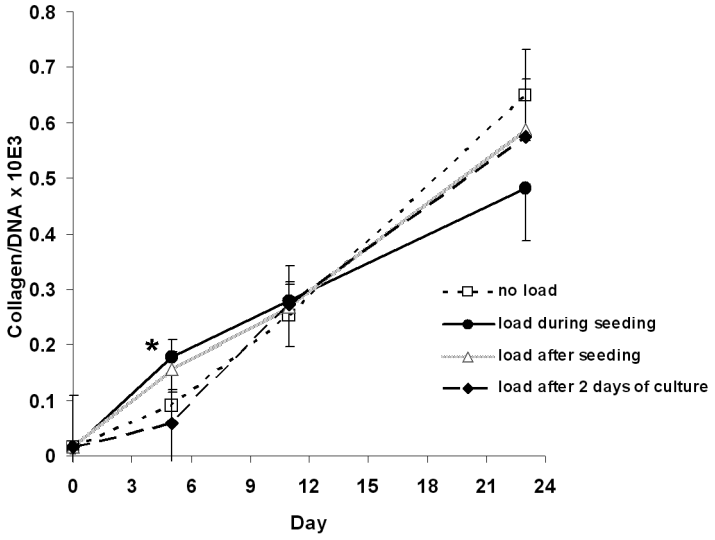

Figure 8. *Total collagen per DNA showed a similar increase over time for all four scaffolds (mean ± standard deviation). At day 23, no statistical significant difference was seen between all scaffolds. *p<0.01 for scaffolds loaded during seeding and immediately after seeding compared with scaffolds loaded after a delay of 2 days.*

Cells proliferated until day 11, after which cell number decreased, resulting in a decrease of final cell number from day 11 to 23 for all loading regimes. Total collagen production and collagen production per cell continued to increase in all conditions, but at day 23, total collagen content was statistically significantly highest for the unloaded scaffolds. This might have been due to a slightly, albeit not significantly, higher cell number on the unloaded scaffolds. Thus, at the end of the culture period, the mechanical stimulation applied had not significantly influenced cell number and collagen production. However, after the first 5 days of culture, the proliferation and matrix production profiles differed significantly depending on the load regime. In particular, the scaffolds loaded with a delay of 2 days contained fewer cells at day 5 than the unloaded scaffolds and the scaffolds loaded during seeding. In addition, collagen content was lower than in unloaded scaffolds or scaffolds loaded at earlier time points. At day 5, collagen content was higher in scaffolds loaded during seeding ($p<0.01$) than in unloaded

scaffolds or scaffolds loaded after 2 days of culture. When collagen content was normalized to cell number as a parameter for differentiation, unloaded scaffolds and scaffolds loaded after 2 days showed a delay in differentiation. Thus, it appears that, during the first 5 days, cells on unloaded scaffolds proliferated but did not differentiate substantially, cells loaded after 2 days of culture onwards showed a delay in both parameters, loading immediately after seeding resulted in a delay of proliferation but a substantial differentiation, and loading during seeding induced proliferation and differentiation.

The lower proliferation on scaffolds loaded after seeding than on unloaded scaffolds may indicate an adaptive response of the cells to the change in mechanical conditions, which may temporarily preclude cell division. On the other hand, load seems to stimulate cell differentiation in these first 5 days of culture, apparently at least partly independent of alterations in the biomechanical environment. After longer periods of culture, however, the differences in cell number and proliferation were minimized.

Because the current study focused on the ability of a mechanical load to affect cell proliferation and matrix production per se as a mere tool in enhancing TE, we have not studied the actual biomechanical properties of the constructs generated. However, it has been reported previously that the application of static load improves the biomechanical properties of constructs consisting of tenocytes cultured on PGA for 5 weeks[46]. In addition, tissue maturation was clearly enhanced, which was suggested in this study as well, with more compacted tissue seen in the loaded scaffolds at early time points. It has been suggested that the effects of application of load are dependent on the presence of ECM. Static compression applied to chondrocytes in agarose discs at different time points between 2 and 41 days of culture[41] inhibited ECM production only if produced matrix was already present in the discs. In addition, medial collateral ligament cells have shown a difference in load dependent inhibition of growth, depending on the surrounding ECM[107]. The current study seems to partly support this observation, because late application of load, after 2 days of

initial matrix deposition, resulted in an initial delay in collagen deposition. This is related to a delay in collagen production per cell, reflecting differentiation, and in proliferation. However, the ECM deposited under load during the first few days of culture (before or immediately after seeding) does not inhibit further ECM production. Possibly it is the organization of this ECM deposited in the absence of load that affects the initial response of cells to the application of load. This discrepancy in effect of the presence of ECM between the results presented here and those of previous studies might be related to the undifferentiated state of the cells used here. Undifferentiated cells may be more sensitive to various stimuli than already differentiated cells like tenocytes or medial collateral ligament cells. It is not clear whether the increase in differentiation found was based on increased collagen production per differentiating cell or whether the proportion of differentiating and hence collagen-producing cells had increased. To what extent BMSCs had actually differentiated into real tendon-like tissue is not clear, because many connective tissue cells synthesize collagen. Previous studies on BMSCs seeded and cultured on PLGA[133,249] or silk[2] have shown that at least collagen type I and III are produced, which are likely to be fibroblast- or tenocyte-derived. Moreover, although matrix synthesis and cell content were similar at the end of the culture period for all loading regimes, it cannot be excluded that the mechanical properties of the scaffolds were different. Various parameters, such as collagen content, fibril diameter and cross-linking, and relative ratio of collagen type I to type III, determine ligament tissue strength[5]. However, the PLGA fibers were likely to display failure strength that is still higher than that of the soft connective tissue formed to detect any tissue-related biomechanical differences in the constructs. This is based on previous biomechanical data on PLGA fibers[243] after spontaneous hydrolysis over 20 days[86] and the strength of anchored fibroblast- containing collagen gels[108].

BMSCs oriented and elongated along the axis of the load applied, as is characteristic for ligaments and tendons, in accordance with previous data on rabbit BMSCs cultured on loaded monofilaments covered with a collagen gel[19]. A

study on the application of cyclic mechanical load to bovine and human BMSCs embedded in collagen gel also reported alignment of cells and matrix[3]. Here also an upregulation of ligament fibroblast markers was seen. A similar response seems to be found in the current study, despite the purely synthetic scaffolds used. Whether the type of mechanical stimulus applied in the current study is optimal is not clear, because the effect of mechanical loads on cells is not completely understood. Determinants such as type of load and frequency, force, and timing of loading after seeding will influence the effect of biomechanical stimuli in TE. Although it may be argued that dynamic loads are more likely to mimic the natural situation, several studies have shown positive effects of static loads. The loss of mechanical strength of in vitro load-deprived rabbit tendon fascicles could be completely prevented using static[263] and cyclic[264] strain using similar (1-2.5 mPa) loads, and likewise the induction of the ECM degrading collagenase using in vitro load deprivation of rat tendons could be prevented using static[13] and cyclic loading[159]. Fibroblasts in collagen gels were even reported to yield stiffer constructs when exposed to static loading than dynamic loads[78]. Stem cells are particularly sensitive to mechanical stimuli. When subjected to hydrostatic forces, they are directed toward the chondrogenic phenotype[259] whereas stem cells that are subjected to shear forces will start to produce bone markers and thus are directed along the osteogenic lineage[51] and load may induce fibrous tissue formation[3]. Altogether, even if constant load is a non-physiological way of loading, it may be helpful in directing undifferentiated cells such as bone marrow stromal cells into the desired pathway. Moreover, the notion that dermal fibroblasts seeded on PLGA scaffolds differentiated toward tendon-like tissue upon implantation in vivo suggests that even more important than in vitro cues is the in vivo environment of the cells[167]. Thus in vitro stimulation of cell proliferation and matrix production and subsequent further differentiation in vivo may be the clue to efficient ligament TE.

In conclusion, static load does not negatively affect BMSCs seeded on braided PLGA scaffolds but induces alignment, proliferation, and differentiation,

although the latter two parameters are in the end not different from unloaded cells. Application of load during seeding seems to be favorable in terms of proliferation and differentiation as opposed to application of load after seeding (immediately or after a delay) when a quick response is required. Further research into the biochemical and biomechanical properties of the tissue and constructs formed is warranted.

7

IN VIVO MATRIX PRODUCTION OF BMSCS SEEDED ON PLGA SCAFFOLDS FOR LIGAMENT TISSUE ENGINEERING

ABSTRACT

Ligament tissue engineering based on cell-seeded biomechanically functional constructs is a commonly studied strategy towards native anterior cruciate ligament replacement. Little is known about the survival and differentiation of the seeded cells after the transplantation. We applied retroviral genetic marking to trace implanted cells and studied their differentiation by species-specific immunolabelling of the extracellular matrix produced.

Goat bone marrow stromal cells were transduced with a MoMuLV-based vector encoding the ΔLNGFR gene. Transduced cells were seeded onto PLGA fibers and implanted subcutaneously into nude mice and left for various periods up to 6 weeks.

Immunohistochemistry for LNGFR expression showed survival of the seeded cells after transplantation for up to 6 weeks. Immunohistochemistry for collagen type I and III showed the production of fibrous tissue inside the scaffolds. Moreover, using a goat-specific anti-collagen type III, donor-derived matrix could be demonstrated. We conclude that bone marrow stromal cells survived in vivo and at least partially differentiated after implantation.

INTRODUCTION

One of the challenges in the field of regenerative medicine is engineering a ligament with the characteristics of a native anterior cruciate ligament (ACL)[2,19,33,46,61,164,172,220]. The ACL is an intraarticular structure that has poor healing capacities and requires surgical replacement in patients with persistent knee instability. Current surgical strategies such as the use of auto- or allografts have several drawbacks, such as donor-site morbidity and risk of disease transmission[63,126,141]. Engineering ligament grafts by combining autologous cells with a biodegradable and biomechanically functional scaffold is an interesting strategy to bypass these drawbacks. Differentiated[60,164] as well as undifferentiated[3,190] cell types have been used in tissue engineering of tendon and ligaments. Promising candidates are bone marrow stromal cells (BMSCs), which have been studied extensively and can be cultured and directed towards several lineages of differentiation including tendon and ligament[118]. Previously it was shown that BMSCs seeded and cultured on PLGA scaffolds proliferate more readily and produce more matrix as compared to more differentiated cell types, including ACL fibroblasts, and therefore may be more suitable for tissue engineering of the ACL[72].

Although many in vitro and in vivo studies have focused on tissue engineering of ligaments, little is known about the fate of the seeded cells after the transplantation. By use of physical separation from donor and host tissue and selective destruction of native cells, it was shown that fibroblasts in a reconstructed patellar tendon autograft for ACL reconstruction in a rabbit model died after reconstruction in the knee joint and the graft was rapidly repopulated by host cells 4 weeks after transplantation[146,147]. Rabbit skin and anterior cruciate ligament fibroblasts labeled with PKH26-GL and seeded on collagen scaffolds were found to be present in the intra-articular environment after 6 weeks[25]. However, as is a common problem with fluorescent membrane labels, label transfer and signal dilution hamper proper data interpretation.

A means to unequivocally study the presence of donor cells in an implanted construct is retroviral transduction with non native marker genes. LacZ (β-galactosidase) labeling has been applied to trace allografted and autografted cells in two rabbit models of ligament repair and autografting[117,178], but until now, no tissue-engineered ligament constructs have been evaluated for the presence of genetically labeled donor cells after implantation.

Another issue in addition to the actual presence of viable donor cells in vivo is their functionality. It is not known whether implanted cells actively take part in tissue formation, let alone whether successful tissue engineering actually requires the implanted cells to produce the desired matrix in vivo. To demonstrate that transplanted cells do not only survive but also produce extracellular matrix, the origin of the deposited tissue should be identified. One option to address this problem is to use species-specific recognition of proteins produced by the donor cells.

The objective of current study was to trace BMSC cells seeded onto braided PLGA scaffolds and to demonstrate their viability and functionality in terms of matrix production in vivo. Therefore, as a first step to demonstrate the applicability of this method, goat BMSCs were transduced with the ΔLNGFR gene, seeded on braided PLGA scaffolds, and implanted in an ectopic nude mice model. The origin of the produced matrix inside the scaffolds was qualitatively studied by species-specific immunostaining of collagen.

METHODS

Cell culture

Goat BMSCs from bone marrow aspirates of a female Dutch milk goat (weight 60 kg, 19-24 months old) were harvested and expanded as described earlier[153]. The cells were cryopreserved at passage 1. Within 6 months, the goat BMSCs (passage 2) were thawed and replated in standard culture medium consisting of

αMEM supplemented with 30% fetal bovine serum (FBS, Gibco, Paisly, Scotland, lot# 3030960S), L-glutamine (2 mM, Invitrogen, San Diego, USA), ascorbic acid (0.2 mM, Sigma, St Louis, USA), and penicillin/streptomycin (each 100 U/ml, Gibco). When confluent, the cells were detached and replated at 5000 cells/cm^2 in culture medium containing 15% FBS.

The Phoenix-Ampho retrovirus packaging cell line was cultured in DMEM (Gibco) supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml) and 2 mM L-glutamine. Cultures were passaged twice a week and selected for gag, pol, env expression every 8 weeks using hygromycin B (300 µg/ml, Roche diagnostics, Mannheim, Germany) and diphtheria toxin A (1 µg/ml, Sigma-Aldrich, Zwijndrecht, The Netherlands). The construction of the retroviral vector pLZRS-TK has been described previously[254]. Twenty µg DNA of a retroviral plasmid construct was transfected into 70% confluent Phoenix-Ampho packaging cells by calcium phosphate precipitation[145]. Twenty-four hours after transfection, medium was replaced with fresh culture medium. The following day, retroviral supernatant was collected, filtered through a 0.45 µm filter and stored at -80 °C. For additional harvest of retroviral supernatant, transfected Phoenix-Ampho cells were cultured for 3 days in the presence of puromycin (1 µg/ml Sigma) followed by 2 days in culture medium without puromycin.

Retroviral transduction of goat BMSCs

Transduction was performed by replacing the standard cultured medium of the BMSCs (P3) by a three-fold dilution of the retroviral supernatant in culture medium supplemented with 6 µg/ml polybrene (Sigma). Culture medium supplemented with polybrene alone was used for mock-transduction (control). The BMSCs were cultured for another 24 hours, after which the medium was renewed. Two days later the cells were harvested and analyzed.

Transduction efficiency was determined by flow cytometry analysis. The BMSCs were labeled with primary mouse anti-LNGFR monoclonal antibody (20.4 culture supernatant, 1:20) for 20 min at 4°C, washed, and then incubated with goat anti-

mouse phycoerythrine-conjugated IgG_1 (Southern Biotechnologies, Birmingham, US). Cells were washed in PBS-1% FBS and resuspended in standard culture medium, immediately before analysis of 10.000 events on a flow cytometer (FACS Calibur, Becton and Dickinson, San Jose, USA)[154,254]. In vitro culture was conducted to investigate long-term label expression. Two separately produced batches of transduced cells were subcultured further in 25 cm^2 flasks for six weeks and analyzed by FACS at each passage. The remainder of the labeled BMSCs were cultured until confluence and subsequently seeded.

Transduction efficiency and long-term stability of cells

In a separate study in vitro culture was conducted to investigate long-term label expression[154]. Two batches of transduced cells were cultured in 25 cm^2 flasks for six weeks and analyzed by FACS at each passage. To assess proliferative capacity, transduced and mock-transduced cells were co cultured in a 50%/50% mixture in a separate study[154].

Scaffolds

The degradable scaffolds were made of Panacryl (size 2/0, Ethicon, Sommerville, NJ), which is composed of poly(L-lactide/glycolide) 11 µm bundles, containing approximately 40 monofilaments[72]. Nine bundles were organized in parallel, and both ends of the structure were sutured together (1 cm in length). The scaffolds were sterilized by repeated cycles of incubation in ethanol 70%, and after air-drying washed with sterile PBS. Before seeding, the scaffolds were pre-incubated overnight in standard culture medium.

Cell seeding

After trypsinization, both transduced and control BMSCs (P4) were resuspended at $2.5x10^6$ cells per mL in standard culture medium. The scaffolds were seeded with 250.000 cells/ scaffold and allowed to attach for two hours, after which the scaffolds were submerged in culture medium consisting of αMEM supplemented

with 15% FBS, L-glutamine (2 mM), ascorbic acid (0.2 mM) and penicillin/streptomycin (each 100 U/ml). All scaffolds were cultured for seven days to allow for proliferation of seeded cells in 25-well bacterial culture plates (Sterilin, Stone, UK) before implantation.

Surgical procedure

All animal experiments were conducted with approval of the ethics committee of the University of Utrecht. Ten female NMRI nude mice (age 4-6 weeks, weight 20-25 grams, Harlan, the Netherlands) were anaestesized with an intraperitoneal ketamine (100µg/ml), xylazine (20µg/ml) and atropine (0.5mg/ml) solution. After desinfection of the back with 70% alcohol, separate subcutaneous pockets were created for implantation of the scaffolds on the back of the mice. Implantations included two mice per evaluaton period and two scaffolds with transduced cells and one scaffold with mock-transduced cells in each mouse (in addition to three tissue engineered bone constructs that were implanted in the course of a separate study). The mice were sacrificed after time periods of 2, 4 and 10 days, and after 4 and 6 weeks. The implants were retrieved and processed for histological evaluation.

Histology

Samples were fixed in 10% buffered formalin. Dehydration was performed through a graded alcohol series prior to paraffin embedding. Four µm thick longitudinal sections of the scaffolds were deparaffinized, rehydrated, stained with haematoxylin and eosin and analyzed with light microscopy (E600 Nikon Eclipse, Nikon, Japan). In addition, parts of the sections were stained for 60 minutes in Picrosirius Red (Direct Red 80, Aldrich, Milwaukee, WI). The presence of a collagenous matrix was visualized using polarization microscopy (E600 Nikon Ecplipse, Japan)[138,139,189]. Collagen type I appears as thick, bright (strongly birefringent) yellow or red filamentous structures shining against a dark background, whereas collagen type III highlights as thin filamentous structures with a weak birefringence of a greenish color[189].

Immunohistochemistry

Paraffin-embedded scaffolds were deparaffinized, rehydrated and endogenous peroxidase activity was blocked with 1.5% H_2O_2 in phosphate-citrate buffer for all samples. The samples were stained for LNGFR-positive cells and the presence of collagen type I and III to asses survival and functionality of the transplanted cells.

For the LNGFR-immunolocalization, the Dako Ark™ kit was applied according to the manufacturers' recommendations (Dako Corporation, Carpinteria, USA) to prevent non-specific staining due to reactivity of the secondary anti-mouse IgG antibodies with the surrounding murine tissue. For the primary antibody a mouse anti-LNGFR (2,56 µg/ml, Dako Corporation) was used.

For collagen immunodetection the sections were first treated with pepsin for 15 minutes at 37°C. Subsequently, the samples were incubated with rabbit anti-human collagen type I (26,7 µg/ml) or collagen type III (1:200, both Biogenesis, Dusseldorf, Germany) for 1 hour at room temperature (RT). As secondary antibody ready-to-use PowerVision (poly-HRP-anti-rabbit IgG, Immunologic, Duiven, the Netherlands) was applied according to the manufacturers' recommendations. Diaminobenzidine (Liquid DAB+ Substrate, Dako Corporation) was used to obtain a signal on the sections. To verify whether the primary antibodies for collagen type I and III could be used for species-specific immunolabelling, control sections containing goat muscle and skin and mouse skin and tendon tissue were labeled with each antibody.

An additional type III immunolocalisation with an antibody known to detect both goat and mouse tissue was performed to demonstrate the presence of collagen type III in mouse tissue. Sections of mouse tendon were blocked with 1.5% H_2O_2 in phosphate-citrate buffer and subsequently treated with pepsin for 15 min at 37°C. Subsequently, the samples were incubated with rabbit anti-rat collagen type III (1:80, Division of Morwell Diagnostics, Zürich, Switzerland) for 1 hour at RT. As secondary antibody a biotinylated goat anti-rabbit (3 µg/ml, Vector laboratories) was applied for 1 hour at RT. Subsequently streptavidin horse radish

peroxidase (SA-HRP, Beckman Coulter, Fullerton, USA) was applied for 1 hour at RT. Finally, diaminobenzidine was used to obtain a signal in matrix.
All samples were counterstained with hematoxylin and analyzed by light microscopy (E600 Nikon Eclipse, Japan).

RESULTS

Transduction efficiency and stability of labeled cells

The genetic labeling procedure of goat BMSCs resulted in a 40-60% transduction efficiency, measured three days after transduction. In vitro long-term culture showed a slight decrease of the percentage of labeled cells in the first week, but it stabilized at 30-40% positive cells for up to six weeks. In the co culture experiment the actual percentage of labeled cells showed to be initially lower during the first week, indicating a relative decreased proliferation compared to the unlabeled cell fraction. However during the subsequent weeks, the percentage transduced cells in the co culture reached the expected percentage, indicating a minimal influence on cell proliferation and viability at the long term[154]. Viability immediately before seeding was in the range for normal BMSC P3 cells. Bone formation of the transduced BMSCs was described in a separate study[154], showing their multipotency.

Histology

All mice survived the implantation periods and all samples were retrieved without signs of infection. As demonstrated by light microscopy of the paraffin-embedded scaffolds, cells and extracellular matrix were present throughout the whole scaffold (Fig. 1). At day 10 the cells and tissue in and around the fibers of the scaffold showed alignment along the fibers. At this time also small blood vessels were seen in the centre of the scaffold. With time, the tissue inside the scaffold appeared much denser as demonstrated by the haematoxilin and eosin staining.

Although no quantitative analysis was done, the cell density in this formed tissue appeared comparable to the earlier time points. Picrosirius Red staining likewise demonstrated a comparable increase in collagen matrix inside the scaffolds in time. Polarization analysis indicated that both collagen type I and III could be seen inside the scaffolds, with collagen type III also seen adjacent to the scaffolds (Fig. 2).

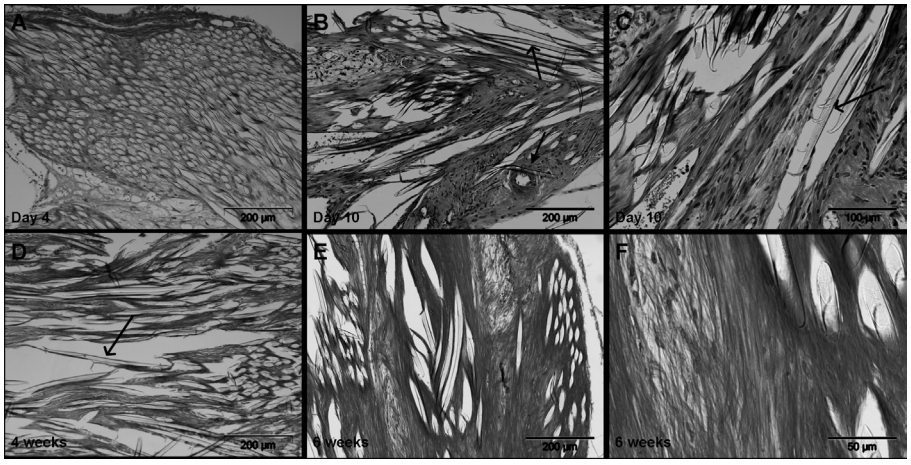

Figure 1 *Histology of scaffolds stained with haematoxilin and eosin (**B-F**) or hematoxylin with Picrosirius Red (**A and D**). Small blood vessels were seen in the centre of the scaffold (closed arrow, **B**). From day 10 the cells and tissue around the fibers showed alignment (**C**). In time, the tissue inside the scaffold appeared much denser as demonstrated by the haematoxilin and eosin staining (B-C, 10 days in contrast with E-F, 6 weeks). A fiber of the scaffold is indicated with an open arrow (**B-D**). The dense tissue around the fibers of the scaffold showed alignment after 6 weeks (**F**). (See full colour section page 180)*

Immunohistochemistry for detection of LNGFR expression

Labeled cells could be traced unequivocally from day two up to six weeks after implantation. The labeled cells were spread homogenously throughout the scaffold, without a specific preference for localisation (Fig. 3). Due to the small sample size, no statements can be made about differences in cell density in time. Control scaffolds with mock-transduced cells and sections incubated with secondary antibody only were always negative (data not shown).

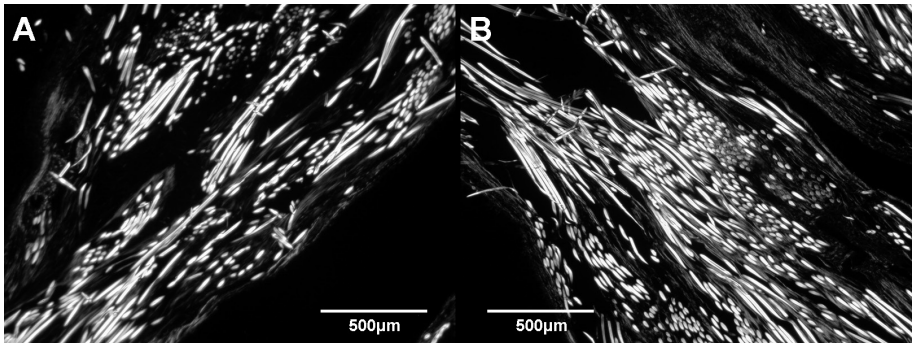

Figure 2 *Polarization microscopy of scaffolds cultured for 10 days (**A**) and 6 weeks (**B**), stained with Picrosirius Red. Red staining denotes collagen type I and greenish staining type III. The scaffolds appear as white fibers. (See full colour section page 180)*

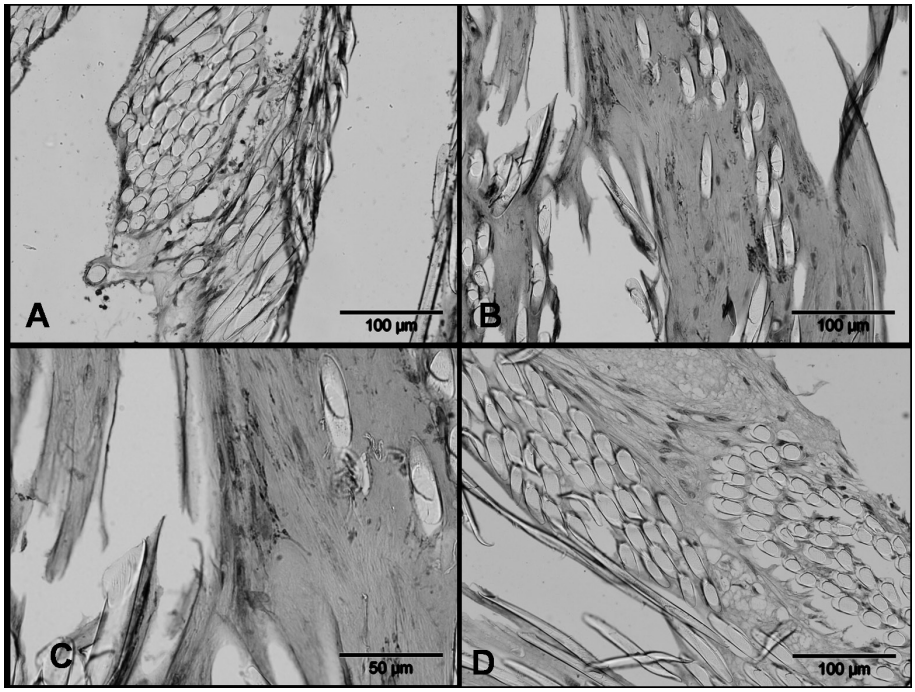

Figure 3 *Immnunohistochemistry for LNGFR-labeled cells at day 2 (**A**) and 6 weeks (**B and C**). Brown staining denotes the presence of LNGFR-positive cells. Controls were always negative (**D**). (See full colour section page 181)*

Collagen I labeling

Both mouse tendon and skin and goat skin tissue showed immunoreactivity for collagen type I. Inside the scaffolds there was a very dark positive staining which increased from day 10 to day 42. Around the scaffolds the tissue stained lightly (Fig. 4).

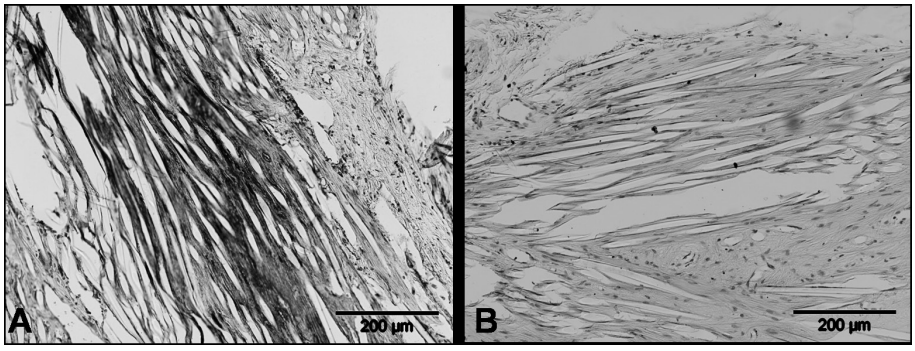

Figure 4 *Immnunohistochemistry for collagen type I inside the scaffolds at 6 weeks (**A**). Brown staining denotes the presence of collagen type I. Controls were always negative (**B**). (See full colour section page 181)*

Collagen III labeling

Immunohistochemistry for collagen type III using the rabbit anti-human antibody demonstrated a goat tissue-specific staining in goat muscle and skin sections (Fig. 5A), with mouse skin and tendon tissue sections consistently negative (Fig. 5B). Using the rabbit anti-rat antibody, collagen type III could be demonstrated in both the abovementioned mouse tissues, showing collagen type III is indeed present in the mouse skin and tendon (Fig. 5C). Staining with the goat-specific rabbit anti-human antibody collagen type III could be specifically identified inside the scaffold (Fig. 5D-G). Collagen type III could be identified from day 10 up to six weeks after implantation. The control tissue (PBS, mouse tissue) was always negative.

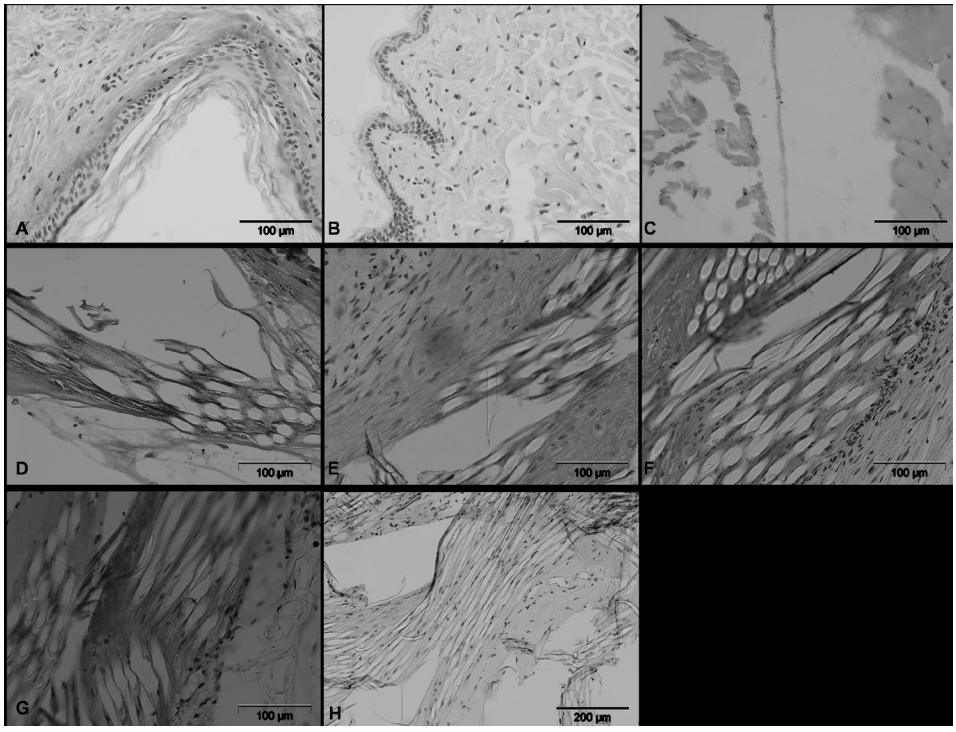

Figure 5 *Immnunohistochemistry for collagen type III for goat skin (**A**) and mouse skin (**B**) using the rabbit anti-human antibody. Mouse tissue showed negative staining (**B**), indicating that this antibody was specific for collagen type III formed by goat cells. Immunohistochemistry for collagen type III for mouse ligament using the rabbit anti-rat antibody (**C**) indicating that collagen type III was indeed present in these mouse tissues. Immunohistochemistry using the species specific rabbit anti-human antibody showing collagen type III inside the scaffolds at 4 days (D, 1:100), 10 days (E, 1:200), 4 weeks (F, 1:200) and 6 weeks (G, 1:200) indicating that transplanted goat cells produced collagen type III at all these time points. Controls were always negative (**H**). (See full colour section page 182)*

DISCUSSION

In the current study we demonstrate the capacity of BMSCs to survive in vivo in a tissue-engineered construct for the replacement of ligamentous structures. Moreover, to our knowledge, this is the first study showing that this contribution

represents more than just stimulating invading host cells by the release of factors, known to be produced by BMSCs, but that the cells actually also produce matrix. After seeding on PLGA scaffolds, goat BMSCs survived for up to 6 weeks when transplanted subcutaneously in nude mice. At least part of the extracellular matrix was indeed produced by the transplanted goat BMSCs as shown by species-specific labeling of type III collagen, thus indicating some form of differentiation. Conventional and Picrosirius Red histological staining suggested an increase in tissue density within the scaffold in time.

The first aim of current study was to follow the fate of seeded ΔLNGFR-transduced goat BMSCs implanted in an ectopic nude mice model. LNGFR has been applied successfully for tracing many cell types, by both flow cytometry and immunohistochemistry[14,31,152,154,254]. Transplantation of genetically modified autologous cells may be more challenging in more clinically relevant models of immunocompetent, larger animal models such as in a goat. However, several in vivo studies have already been conducted introducing foreign genes, such as luciferase and GFP in vivo without any serious immunological reaction being reported[180,183]. Previous research aiming at tracing transplanted and transduced cells showed variable survival periods for such cells. The density of GFP-transduced allogeneic chondrocytes embedded in alginate and transplanted into osteochondral defects in rabbits decreased to 15% within 4 weeks[183]. EGFP-labeled allogeneic chondrocytes transplanted into a similar rabbit model could not be detected after six weeks anymore[184]. It was unclear whether a decrease in density of labeled cells was due to cell death, through rejection by the host cells, or due to loss of expression. Also, the process of transduction itself could have had an effect on the behaviour of transplanted cells. Both studies showed a down-regulation of cartilage gene expression of the transfected cells in vitro, but neither study investigated to what extent this down-regulation changed the actual functionality of the cells after transplantation. In the current study, the effect of transduction on in vitro cell viability and proliferative capacity in current study was minimal, as shown previously with co-culture experiments on

proliferation of labeled and unlabeled cells[154]. Still it cannot be excluded that transduction had some effects on in vivo differentiation. The method used to isolate the BMSCs has been shown to generate BMSCs capable of differentiation into the chondrogenic, osteogenic and adipogenic cell line in vitro[32,153,206]. Also, the multipotency of the same transduced population used in this study has been shown by their induction of bone formation in vivo [154].

Despite unequivocal demonstration of donor cells in a given in vivo situation, nothing can be concluded concerning the actual functionality of the implanted cells. The presence of labeled cells inside osteocyte lacunae may be regarded as proof of the bone-forming capacity of these cells[65,154], however, in other tissues such as ligament, it is more difficult to pinpoint the origin of the produced matrix. The abovementioned study on the fate of transgenic transplanted chondrocytes into articular defects did not show integration of these cells into the repair tissue and therefore the authors concluded that the cells did not form the repair tissue[183]. In the current study, in addition to showing the presence of the transplanted cells, the second aim was to prove their differentiation by species-specific immunostaining of collagen type III produced after xenotransplantation. This is an important step towards demonstrating the functionality and thus the need for implanted cells in orthotopic locations. As to the application of the biomaterials used, PLGA-based constructs have been evaluated extensively before. They were found to allow for efficient attachment and proliferation of and matrix production by seeded goat BMSCs in vitro[72,133]. Maximum loads for braided constructs were shown to be around 60 (PLLA+PLGA)[91] to 100 N (PLGA or PLLA)[173], with ultimate tensile strength decreasing to 50% after 16 weeks in vitro. However, the maximal tensile load of PLA/PLGA constructs after 20 weeks in vivo had decreased to about 10% of native ligaments, indicating biomechanical functionality at later time-points is only partial and stressing the need for efficient stimulation of matrix deposition after implantation[90].

In addition to collagen type III, also production of collagen type I inside the scaffolds was clearly demonstrated, although we were not able to show this

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was formed by the transplanted cells. Collagen type I and III are found in bone and in soft connective tissues like skin and are major extracellular matrix components of tendon and ligaments[166]. Wound healing in general is associated with relatively high contents of type III collagen, which has also been shown for regenerating tendon tissue[6], and therefore the tissue formed by the labeled cells may partly reflect scar tissue. As the major goal of this study was to investigate the in vivo behaviour of BMSCs implanted on biomaterial scaffolds for ligament reconstruction, rather than the production of ligament tissue, no efforts were undertaken to further specify the nature of the tissue. Putative markers further identifying the tissue as ligament-like would have been fibronectin, tenascin-C, scleraxis and tenomodulin, but in the end none of these are truly specific, as they are also found in other tissues and cells such as cartilage, dermis, testis and sclerae[109,155,191,229,242,267,269]. For future in vivo studies on the functionality of transplanted cells, a combination of the markers mentioned above, as well as the ratio of collagen type I versus III may prove some insight into the nature of the tissue formed. Also the unique orientation of the collagen fibers in ligaments and tendons resulting in its high mechanical properties are important determinants to investigate. However, as has been shown extensively that under unstrained conditions extracellular matrix is deposited without any particular orientation[3,46] , the latter parameter is only meaningful when analyzed on constructs that actually have been biomechanically loaded in vivo, in contrast to the current study.

Still the lack of a subset of ligament markers in any given construct at an ectopic location may not preclude functionality at orthotopic locations. Exposure to the proper biomechanical cues in vivo may be sufficient to direct differentiation into the tenogenic/ligament lineage, as has been shown for fibroblasts differentiating into functional tendon tissue in vivo[137,167].

In conclusion, we demonstrate that BMSCs survive in vivo transplantation on PLGA scaffolds and are able to produce collagenous matrix inside these scaffolds designed for tissue engineering of the ACL. Future studies are required to show whether this also occurs in seeded constructs implanted at orthotopic locations

8

SUMMARY AND CONCLUSIONS

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SUMMARY

Rupture of the anterior cruciate ligament (ACL) is a traumatic event, often as a consequence of twisting a weight bearing limb. The ACL has no intrinsic healing capacity, and rupture can lead to instability of the knee. Primary repair and augmentation of the torn ACL were shown to be unsuccessful. The ideal ligament replacement should be readily available with sufficient length and diameter; it should have mechanical properties similar to the ligament it replaces; it should not disturb normal structures and it should retain or develop a vascular supply. Long-term studies evaluating the subjective and objective outcomes of both autograft and allograft replacements, which are the most commonly used grafts, have greater than 90% success rates. However reports with a follow-up of more than ten years are rare and donor-site morbidity with the use of autografts, and risk of disease transmission and immunologic reaction with the use of allografts are still serious drawbacks. Also the commonly used autografts still have long-term mechanical properties that are far from those of a normal non-injured ACL.

The limitations in currently used techniques in ACL reconstruction surgery have prompted ongoing research aimed at developing a tissue-engineered ligament graft that mimics the biologic and mechanical properties of the native ACL. Regenerative medicine, also called tissue engineering, is a field of multidisciplinary interactions aimed at incorporating the principles of biochemistry, engineering, clinical knowhow and materials science to develop substitutes for replacing injured or diseased tissues. The concept of regenerating a functional ligament requires a system that combines appropriate differentiated cells that have the capability to proliferate and produce relevant matrix. Furthermore it needs a biocompatible carrier or scaffold with high mechanical properties that guides these cells and provides initial strength.

Literature on tissue engineering of ligaments shows promising results to overcome the drawbacks with currently used grafts. However still a lot of determinants have to be explored.

The aims of this thesis were formulated as follows:

1. To study the long-term outcome following arthroscopic assisted anterior cruciate ligament reconstruction using allografts and to investigate the clinical relevance of immunologic reactions against the donor;
2. To investigate the optimal cell source for ligament tissue engineering;
3. To study the effect of different growth factors on proliferation and differentiation of bone marrow stromal cells seeded onto poly(L-lactide/glycolide) scaffolds;
4. To study the effect of static load on proliferation and differentiation of bone marrow stromal cells seeded onto poly(L-lactide/glycolide) scaffolds;
5. To develop a method to study the survival and function of bone marrow stromal cells in vivo.

The results with regard to the different aims are each described in the following paragraphs.

1. The long-term outcome following arthroscopic assisted anterior cruciate ligament reconstruction using allografts and the clinical relevance of immunologic reactions against the donor

The use of allografts in ACL reconstruction surgery has risen tremendously the past decade. Its use has several advantages above autografts, but also important disadvantages are associated with the use of the allografts. These are the risk of disease transmission and the risk of an immune response by the recipient. The clinical importance of such an immune response is currently unknown, but it may affect graft incorporation, revascularization, and graft remodeling. The use of allografts has been shown variable stability rates and functional results in studies with a follow-up between two and five years. The outcome of both autografts

as well as allografts in the long term is still unpredictable, because reports on ACL reconstruction surgery with a follow-up of more than ten years are rare. Furthermore the clinical importance of a possible immunologic reaction with the use of allografts is still a subject of debate.

The purpose of the study presented in **chapter 3** was to evaluate the long-term outcome following arthroscopic assisted ACL reconstruction using deep frozen bone-patellar tendon-bone allografts; furthermore we studied the clinical relevance of the induction of donor specific human leucocyte antigens (HLA) antibodies measured in the blood until six years after the reconstruction. Although the techniques in ACL reconstruction surgery have been improved since the start of this study, we expected the clinical outcome to be comparable to what is described in literature for allografts with a follow-up of five to ten years. Furthermore we aimed to demonstrate that an immunological reaction after reconstruction of a bone-patellar tendon-bone allograft did not influence the clinical outcome.

Between 1991 and 1992, 38 patients underwent an allograft ACL reconstruction by a single surgeon. Of these patients, 35 were available for full examination 15 years after the reconstruction, which included a questionnaire (KOOS), functional scores (IKDC), KT-1000 arthrometer testing and X-rays. Furthermore blood samples from the patients were collected pre-operative for HLA-typing. Three months, 3-4 years and 6-7 years after the reconstruction blood samples were taken to assess HLA antibody reactivity against the donor. Clinical failure (IKDC group C or D, KT-1000 left-right difference of > 5mm or rupture of the allograft) occurred in 21 of the 35 patients (60%) and failure of the allograft occurred in 6 patients (17%). Three of these 6 patients had a traumatic rupture more than ten years after the reconstruction. The IKDC score deteriorated between the 4 and 15 year follow-up period (p= 0.002). Donor specific HLA antibodies were detected in 8 patients, but no significant association was found between specific antibody induction and clinical failure of the graft.

The clinical failure rate of 60% described in the current study is relatively high, but this can partly be explained by the fact that our patient population also included patients who already had a history of knee surgery of the index knee, including cartilage repair procedures, lateral reconstruction, and even ACL reconstruction. Also 22% of the patients were classified as having articular cartilage injury grade 3 or 4 at the time of the reconstruction and a large number of patients had moderate or severe impingement of the tibial tunnel as assessed by the Taylor score on radiographs. This impingement is correlated with instability. The results of this study indicate that clinical outcome of allografts for ACL reconstruction may deteriorate at the long-term (15 years) follow-up. Unfortunately this study is only a small patient series and randomized controlled studies with a long-term follow-up would be needed to determine the success of allograft versus current replacement strategies. We did found immunoreactivity in 25% of the patients, however no significant association between these reactions and clinical outcome could be found. The clinical failure of 60% and allograft failure of 17% of this study were disappointing and supported us, to search for novel strategies in ACL reconstruction surgery.

2. The optimal cell source for ligament tissue engineering

Little is known about the optimal cell source for tissue engineering of ligaments. The appropriate cell type for tissue-engineered ligaments must show enhanced proliferation and production of an appropriate extracellular matrix. The tissue formed must be able to survive in an intra-articular environment in the patient's knee. ACL fibroblasts are cells specialized in producing all ligament constituents and maintaining the ligament tissue in the appropriate conformation. However, it is well known that the ACL has poor healing capacities. BMSCs are cells that have been shown to be capable of differentiating toward cells specific for various types of connective tissue, such as bone and cartilage and also ligament fibroblasts. Bone marrow stromal cells (BMSCs) are easily harvested and are also

rapidly expanded in culture media. Skin fibroblasts are also easily harvested and have been shown to survive in an intraarticular environment for up to 4 weeks. In **chapter 4** we describe a study in which we seeded BMSCs, ACL, and skin fibroblasts onto a resorbable suture material (poly(L-lactide/glycolide) (PLGA)) at five different seeding densities, and cultured for up to 12 days. All cell types tested attached to the suture material, proliferated, and synthesized extracellular matrix rich in collagen type I. On day 12, the scaffolds seeded with BMSCs showed the highest DNA content ($p < 0.01$) and the highest collagen production ($p < 0.05$ for the two highest seeding densities). Scaffolds seeded with ACL fibroblasts showed the lowest DNA content and collagen production. Accordingly, BMSCs appeared to be the most suitable cells for further study and development of a tissue-engineered ligament. The higher proliferation of the BMSCs could be expected since these cells are thought to be less differentiated as compared to ACL and skin fibroblasts. Apparently the BMSCs were not only capable of showing a high proliferation rate, but in the end also produced more collagen as compared to the more differentiated fibroblasts. This and the fact that these cells are easily harvested in a clinical setting makes these cells highly attractive to use for our purpose. Seeding of the PLGA scaffolds with al different cell types resulted in attachment and alignment along the longitudinal axis of the scaffolds after which the cells started to proliferate and produce collagen. From this study we learned that the scaffold material we used is biocompatible with the selected cell population, making it attractive to use in further studies.

3. The effect of different growth factors on proliferation and differentiation of bone marrow stromal cells

A promising way of enhancing tissue regeneration is the application of bioactive proteins and growth factors, both in vitro and in vivo. Various growth factors have been used to enhance connective tissue generation. In particular, transforming growth factor (TGF)-β1 is a potent inductor of extra cellular matrix (ECM)

production and promotes the generation of bone, cartilage, and soft connective tissues like ligaments. Another, more specific, growth factor suitable for enhancing soft connective tissue differentiation might be growth differentiation factor (GDF)-5. Ectopic application of GDF-5 has been shown to induce differentiation along the ligament pathway, and this factor is involved in healing of ligamentous tissues. Moreover, addition of GDF-5 improves the mechanical properties of healing tendon lesions. In **chapter 5** we compared the effects of TGF-β1 and GDF-5 on the in vitro generation of ligament like soft connective tissue. As learned from literature, we expected that both growth factors would also improve the behavior of our cells on our PLGA scaffolds. Proliferation and ECM production were determined for BMSCs seeded and cultured on knitted, bioabsorbable, 3-dimensional (3D) PLGA scaffolds in the presence and absence of different concentrations of TGF-β1 and GDF-5. BMSCs attached to the suture material, proliferated, and synthesized extracellular matrix rich in collagen type I and III. No differentiation was demonstrated toward cartilage or bone tissue. The addition of TGF-β1 and GDF-5 increased cell content ($p < 0.05$), but only TGF-β1 also increased total collagen production ($p < 0.05$) and collagen production per cell. With this study we learned that stimulation with TGF-β1, and to a lesser extent with GDF-5, can modulate human BMSCs toward collagenous soft tissue when applied to a 3D hybrid construct. Although not one specific marker for ligaments is available, a limitation of the study was that we could not prove that the tissue formed was really ligament-like. Preferably we should use a combination of markers, such as fibronectin, tenascin-C and collagen type III/I ratio to make a statement about the nature of the tissue that was produced by the cells.

Further studies combining growth factors or growth factors with biomechanical stimuli and subsequent implantation in vivo, analyzed with a combination of the above mentioned markers, may enhance our understanding of the connective tissue that is formed inside the scaffolds.

4. The effect of static load on proliferation and differentiation of bone marrow stromal cells

Among several options to direct differentiation of BMSCs, like the addition of biochemical factors to culture medium as described in chapter 5, or in vivo gene transfer, application of mechanical stimulation may represent an efficient cue. As yet, little is known about the effect of mechanical stimulation of undifferentiated cells such as BMSCs in ACL tissue engineering. Given the role of the extracellular matrix in downstream signalling of mechanical stimuli, partial differentiation with concomitant matrix deposition may be required for adequate translation of these stimuli. Thus the timing of application of a mechanical load to cell-seeded synthetic biodegradable scaffolds could influence its final effects. In **chapter 6**, cell proliferation and matrix synthesis by undifferentiated BMSCs seeded onto a porous 3D-braided poly(L-lactide/glycolide) scaffold is studied under various regimes of static mechanical load. Scaffolds were unloaded, loaded during seeding, immediately after seeding, or two days after seeding. During the first 5 days, proliferation seemed to be inhibited by changing the mechanical environment. During this period differentiation increased with the duration of the load applied. After day 5, differences in cell content and collagen production leveled off. After day 11, cell number decreased, while collagen production continued to increase. Cell number and differentiation at day 23 were independent of the timing of the mechanical stimulation applied. This study demonstrated that static load induces alignment, proliferation and differentiation of BMSCs seeded on braided PLGA scaffolds, although the latter two parameters were in the end not different from unloaded cells. Application of load during seeding seemed to be favourable in terms of proliferation and differentiation as opposed to application of load after seeding, when a quick response is required. Dynamic mechanical load more closely resembles the loading of ligaments in vivo. It would therefore be more attractive to study the effect of dynamic loading on the cell seeded constructs. However with dynamic loading even more variables, such as frequency and rate,

have to be set. Therefore a logic first step towards loading was static loading. The next step would be to study the effect of dynamic loading in an in vitro setting.

5. A method to study the survival and function of bone marrow stromal cells in vivo

With regard to the clinical application of tissue engineering strategies, little is known about the fate of the seeded cells after transplantation. Several studies have found that cells from autografts die after reconstruction into the knee joint and that the grafts are repopulated by host cells. Another issue in addition to the actual presence of viable donor cells in vivo is their functionality. For a tissue-engineered ligament to be successful it is essential that the cells produce the desired matrix in vivo. We searched for a method to be able to show functionality of our cells in vivo. In **chapter 7** we used species-specific recognition of proteins to demonstrate that genetically labeled cells do not only survive after transplantation but also produce extracellular matrix. Cells, transduced with a MoMuLV-based vector encoding the ΔLNGFR-gene, were seeded onto PLGA fibers and implanted subcutaneously into nude mice and left for various periods up to 6 weeks.

Immunohistochemistry for LNGFR expression showed survival of the seeded cells after transplantation for up to 6 weeks. Immunohistochemistry for collagen type I and III showed the production of fibrous tissue inside the scaffolds. Moreover, using a goat-specific anti-collagen type III, donor-derived matrix could be demonstrated. With this study we demonstrated that BMSCs survive in vivo transplantation on PLGA scaffolds and are able to produce a collageneous matrix inside these scaffolds, designed for tissue engineering of the ACL. In this study we did not attempt to prove that the tissue formed by the cells was really ligament-like. Future studies are required to show whether the tissue formed is ligament-like and if the functionality of the cells also occurs in seeded constructs implanted at orthotopic locations. Moreover we will need to address the final mechanical functionality of these constructs.

CONCLUSIONS

Current techniques in ACL reconstruction surgery are satisfactory. However the goal of improving this procedure and eliminating its associated drawbacks is a relevant topic for clinical care and basic science. With the increasing wish for people to continue their participation in sports related activities, the prevalence of ACL injuries will likely continue to rise. Follow-up studies of more than ten years of currently used reconstruction techniques are rare and the techniques in this type of surgery change quickly. The presented study in this thesis indicated that results can deteriorate after fifteen years, as compared to results described in literature obtained the first ten years after surgery. For this reason it is essential to follow these patients for a longer time period in a randomized controlled setting, although this can be difficult with the relatively young and mobile patients. One can also question if we are really interested in the long-term success of these grafts. The only indication for reconstruction of the ACL is instability, and most of those complaints will be when a person is still young and active. Activity decreases when people get older and as a consequence the demand for a highly stable knee will be less. Furthermore there is proof that damage to the cartilage and as a consequence the commonly seen arthritis in these patients is initiated at the time of the trauma, and not, as was earlier believed, caused by instability due to an ACL-deficient knee. A stable knee after at least fifteen years however could be a minimum criterion for a successful reconstruction.

A suitable tissue-engineered ACL replacement may obviate all shortcomings of currently used autograft and allograft techniques. The research in this thesis has found that braided PLGA scaffolds can sustain cell adhesion, growth, and matrix deposition under appropriate conditions, and that growth factors and mechanical stimuli have a role in modulating cellular response. BMSCs seem favourable cells in terms of cell proliferation and differentiation, as compared to the more differentiated ACL fibroblasts. Regarding the clinical application of this method, BMSCs also are more easily harvested; BMSCs can be harvested with a

simple bone marrow aspirate, whereas ACL cells have to be harvested from the ACL needing an arthroscopy of the knee.

Although all advancements made are promising, many obstacles persist. To date no engineered construct has the appropriate biologic architecture or mechanical strength for in vivo implantation. The precise local conditions that promote successful ligament development and repair have yet to be defined. Furthermore as studied in this thesis, moving towards in vivo research it is essential to closely monitor the condition of the seeded cells; do they survive? And if they do, what are they producing?

As research continues, it is expected that ligament engineering techniques will lead to the production of a new generation of compatible scaffolds that will mimic the mechanics of the native ligament and will result in durable ligament replacement. However as the concept of tissue engineering was first described in the late 80s, and still no ready to use tissue-engineered organ has been developed, it is uncertain if a tissue-engineered ligament will be used by the orthopaedic surgeon of today.

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NEDERLANDSE SAMENVATTING EN CONCLUSIES

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SAMENVATTING

Het scheuren van de voorste kruisband (VKB) is een traumatische gebeurtenis, vaak als gevolg van draaien van de knie van het belaste been. De VKB heeft geen intrinsieke genezingscapaciteit en het scheuren kan dus tot instabiliteit van de knie leiden. Het is eerder aangetoond dat primair hechten of augmentatie van de gescheurde kruisband geen genezing van de kruisband bewerkstelligd. Indien de VKB scheurt en instabiliteitklachten geeft, zou deze dus vervangen moeten worden door een substituut. De ideale VKB vervanging zou direct beschikbaar moeten zijn met dezelfde lengte en diameter als de oorspronkelijke. Deze vervangende VKB zou idealiter exact dezelfde mechanische eigenschappen moeten hebben als de oorspronkelijke en zou dezelfde vascularisatie moeten behouden of ontwikkelen als voorheen. De lange termijn studies die subjectieve en objectieve resultaten van de meest gebruikte auto- (lichaamseigen) en allotransplantaten (donor) voor VKB reconstructie analyseren, beschrijven succes percentages van meer dan 90%. Follow-up studies van meer dan tien jaar zijn echter zeldzaam en "donor-site morbidity" (klachten als gevolg van oogsten van lichaamseigen pezen) bij het gebruik van autotransplantaten, het risico van ziekte overdracht en het optreden van immunologische reacties bij het gebruik van allotransplantaten zijn nog steeds relevante nadelen bij klinische toepassing ervan. Afgezien hiervan hebben de meest gebruikte autotransplantaten op de lange termijn veel minder goede mechanische eigenschappen dan die van een normale niet geruptureerde VKB. De beperkingen van de huidige gebruikte technieken bij VKB reconstructie chirurgie, zijn een belangrijke reden voor verder onderzoek gericht op de ontwikkeling van een "tissue engineered" ligament, dat de biologische en mechanische eigenschappen van de oorspronkelijke kruisband zo goed mogelijk nabootst. De regeneratieve geneeskunde, ook wel tissue engineering genoemd, combineert de principes van biochemie, engineering, klinische expertise en materiaalkunde. Het concept van de regeneratie van een functioneel ligament is gebaseerd op geschikte gespecialiseerde cellen die de capaciteit hebben om te prolifereren

en de juiste extra cellulaire matrix (ECM) te maken. Verder is het afhankelijk van een biocompatibele matrix of scaffold met hoge mechanische eigenschappen, die de beoogde initiële sterkte geeft en waarin de cellen kunnen overleven en functioneren.
De huidige strategieën die regeneratie van een VKB nastreven zijn in volle ontwikkeling. Sommige hiervan laten veel belovende resultaten zien. Er is op dit moment echter nog weinig bekend over de precieze locale condities die succesvolle ligament ontwikkeling bevorderen.

De doelstellingen van dit proefschrift zijn:

1. De bestudering van de lange termijn resultaten van arthroscopische voorste kruisband reconstructie met allotransplantaten en de klinische relevantie van het optreden van een immunologische reactie tegen het allotransplantaat.
2. Bepaling van het optimale celtype voor tissue engineering van ligamenten.
3. Bepaling van het effect van verschillende groeifactoren op proliferatie en differentiatie van mesenchymale stamcellen, die gezaaid zijn op poly(L-lactide/glycolide) scaffolds.
4. Onderzoek van het effect van statisch spannen van met mesenchymale stamcellen gezaaide poly(L-lactide/glycolide) scaffolds op proliferatie en differentiatie van deze cellen.
5. Ontwikkeling van een methode om de overleving en functionaliteit van mesenchymale stamcellen in vivo te bepalen.

De resultaten met betrekking tot de verschillende doelstellingen worden elk in de volgende paragrafen beschreven.

1. De lange termijn resultaten van arthroscopische voorste kruisband reconstructie met allotransplantaten

Het gebruik van allotransplantaten in VKB reconstructies is het laatste decennium enorm toegenomen. Het gebruik heeft een aantal voordelen boven autotransplantaten zoals een snellere operatietijd en geen "donor-site morbidity". Er zijn echter ook belangrijke nadelen die geassocieerd zijn met het gebruik van allotransplantaten. Nadelen zijn het risico van ziekte overdracht en het optreden van een immuunrespons bij de patiënt. De klinische relevantie van zo'n immuunrespons is nog onbekend, maar het zou kunnen dat dit de ingroei van het transplantaat, de vascularisatie en het remodelleren van het transplantaat beïnvloedt.

Er worden wisselende resultaten in stabiliteit en functionaliteit van allotransplantaten beschreven in de literatuur met een follow-up tussen de twee en zes jaar. De resultaten van zowel autotransplantaten als allotransplantaten op de lange termijn zijn nog steeds onvoorspelbaar, vanwege het feit dat hier nog nauwelijks onderzoek naar gedaan is. Daarbij is de klinische relevantie van een immuunreactie bij het gebruik van allotransplantaten nog steeds een onderwerp van discussie.

In **hoofdstuk 3** worden de lange termijn resultaten (15 jaar) van arthroscopische VKB reconstructie met diep gevroren bot-patellapees-bot allotransplantaten besproken. Daarbij onderzochten we ook de klinische relevantie van de aanwezigheid van human leucocyte antigens (HLA) antilichamen gericht tegen de donor, gemeten in het bloed van de patiënten tot zes jaar na reconstructie. Hoewel de technieken in de VKB reconstructie sterk verbeterd zijn na de start van deze studie in 1991, verwachtten we dat de klinische resultaten vergelijkbaar zijn met wat is beschreven in studies met een follow-up tussen de vijf en tien jaar. Verder hoopten we te kunnen aantonen dat een immuunreactie na reconstructie van de VKB met een allotransplantaat niet van invloed zou zijn op het klinische resultaat.

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Tussen 1991 en 1992 ondergingen 38 patiënten een allotransplantaat VKB reconstructie door één orthopaedisch chirurg. Van deze 38 patiënten waren er 35 na 15 jaar beschikbaar voor volledige evaluatie. Deze evaluatie bestond uit een vragenlijst (KOOS), bepaling van functionele scores (IKDC), KT-1000 arthrometer testen en de uitslagen van röntgenfoto's. Daarbij werd er pre-operatief een HLA-typering gedaan van zowel patiënt als van de donor. Drie maanden, 3-4 jaar en 6-7 jaar na de reconstructie werd er bij de patiënten bloed afgenomen om een test op HLA reactiviteit tegen de donor van het allotransplantaat uit te voeren. Klinisch falen (een IKDC score C of D, een KT-1000 rechts-links verschil van > 5mm of ruptuur van het allotransplantaat) trad op bij 21 van de 35 patiënten (60%) en failure van het allotransplantaat zelf trad op bij 6 patiënten (17%). Drie van deze zes patiënten had een traumatische ruptuur meer dan tien jaar na de reconstructie. De IKDC score verslechterde tussen de 4 en 15 jaar follow-up periode (p=0.002). Donor specifieke HLA anti-lichamen werden gedetecteerd bij 8 patiënten, maar er kon geen significante associatie aangetoond worden tussen de aanwezigheid van specifieke antilichamen en klinisch falen van het allotransplantaat. Het percentage van 60% van klinisch falen van het allotransplantaat is relatief hoog, maar dit kan gedeeltelijk toegeschreven worden aan eerdere operaties aan dezelfde knie voor de reconstructie met het allotransplantaat, waaronder kraakbeen reparatie procedures, laterale reconstructies en zelfs eerdere VKB reconstructies bij deze patiënten. Ook had 22% van de patiënten een chondropathie graad 3 of 4 ten tijde van de allotransplantaat reconstructie en een groot aantal patiënten had matige of ernstige impingment van de tibia tunnel, gemeten met de Taylor score op de röntgenfoto's. Impingment is gecorreleerd met instabiliteit. De resultaten van onze studie laten zien dat de klinische uitkomsten van een allotransplantaat voor VKB reconstructies op de langere termijn wel eens zouden kunnen verslechteren (≥ 15 jaar). Helaas beschikten wij voor onze studie slechts over een relatief kleine patiënten serie en gerandomiseerde gecontroleerde studies zullen nodig zijn om het succes van allotransplantaten ten opzichte van andere gebruikte reconstructie

technieken op deze lange termijn te bepalen. We vonden een immuunreactie bij 25% van de patiënten, maar konden geen significante associatie tussen de klinische uitkomst en deze reactie aantonen. Het klinisch falen bij 60% en het falen van het allotransplantaat bij 17% van de patiënten was teleurstellend en ondersteunde ons in het zoeken naar nieuwe strategieën voor VKB reconstructie.

2. Het optimale celtype voor tissue engineering van ligamenten

Er is maar weinig bekend over de beste celtypering voor tissue engineering van ligamenten. Het optimale celtype moet goede proliferatie tonen en moet de juiste ECM maken. Het weefsel dat vervolgens door de cellen gevormd wordt, moet overleven in de intra-articulaire omgeving van de knie van de patiënt. VKB cellen zijn gespecialiseerd in het produceren van alle componenten van een ligament en het behouden van de structuur van een ligament. Het is echter bekend dat de VKB een slechte helingscapaciteit heeft na beschadiging. Van mesenchymale stamcellen is aangetoond dat zij kunnen differentiëren in voor verschillende soorten weefsels specifieke cellen, zoals bot-, kraakbeen- en ook ligamentcellen. Mesenchymale stamcellen zijn eenvoudig te verkrijgen bij de patiënt en zij kunnen zich snel vermenigvuldigen in een kweekmedium. Huidcellen zijn ook eenvoudig te verkrijgen bij de patiënt. Bovendien is aangetoond dat deze minimaal 4 weken intra-articulair kunnen overleven in de knie.

In **hoofdstuk 4** beschrijven we een studie waarin we mesenchymale stamcellen, VKB cellen en huidcellen zaaien op een resorbeerbaar hechtmateriaal (poly(L-lactide/glycolide) (PLGA)), de scaffold, met vijf verschillende zaaidichtheden en voor 12 dagen kweken. Alle verschillende typen cellen hechtten aan het hechtmateriaal, prolifereerden en produceerden extracellulaire matrix, rijk aan collageen type I. Na 12 dagen toonden de scaffolds gezaaid met mesenchymale stamcellen het hoogste DNA gehalte ($p < 0.01$) en de hoogste collageen productie ($p < 0.05$) voor de twee hoogste zaaidichtheden. Scaffolds die gezaaid waren met VKB cellen, toonden de laagste hoeveelheid DNA en collageen productie.

De mesenchymale stamcellen blijken dus het meest geschikt te zijn voor verder onderzoek naar en ontwikkeling van een "tissue engineered" VKB. De hogere proliferatie van mesenchymale stamcellen was voorspelbaar omdat van deze cellen gedacht wordt dat ze het minst gedifferentieerd zijn in vergelijking met de VKB- en huidcellen. Maar blijkbaar waren de mesenchymale stamcellen niet alleen in staat tot de hoogste proliferatie, maar produceerden ze uiteindelijk ook meer collageen in vergelijking met de andere meer gedifferentieerde cellen. Deze bevinding en het feit dat deze cellen eenvoudig te oogsten zijn in een klinische setting, maakt dat de cellen uiterst geschikt zijn voor ons doel.
Daarnaast bleken de verschillende celtypen zich uitstekend te hechten aan de PLGA scaffolds en bleken deze hun cellichaam uit te rekken in de richting van de lengteas van de fibers. Uit deze studie leerden we dat ons scaffold materiaal biocompatibel is met de geselecteerde celpopulaties en dus geschikt is voor onze vervolg studies.

3. Effect van groeifactoren op proliferatie en differentiatie van mesenchymale stamcellen

Een veelbelovende manier om weefsel te stimuleren is het toedienen van bioactieve eiwitten en groeifactoren, zowel in vitro als in vivo. Verschillende typen groeifactoren zijn al toegepast ter stimulering van weefselgroei. In het bijzonder is de transforming growth factor (TGF)-β1 een potentiële stimulator van extracellulaire matrix productie die de productie van bot, kraakbeen en zachte bindweefsels zoals ligamenten stimuleert. Een andere, meer specifieke, groeifactor die geschikt is voor differentiatie in zachte bindweefsels, is mogelijk de groei differentiatie factor (GDF)-5. Ectopische applicatie van GDF-5 heeft al aangetoond dat deze groeifactor differentiatie in de richting van ligament induceert en ook betrokken is bij het helingsproces van ligamenten. Verder verbetert GDF-5 ook de mechanische eigenschappen van genezende peesletsels.
In **hoofdstuk 5** werden de effecten van TGF-β1 en GDF-5 op in vitro generatie

van ligamentachtig weefsel bestudeerd. Op basis van de literatuur was in onze studie te verwachten dat beide groeifactoren ook het gedrag van onze cellen, gezaaid op de PLGA scaffolds, verbeterden. Proliferatie en ECM productie van mesenchymale stamcellen, gezaaid op gevlochten 3-dimensionale (3D) PLGA scaffolds, werden gemeten in aan- of afwezigheid van verschillende concentraties TGF-β1 en GDF-5. Mesenchymale stamcellen hechtten aan de scaffolds, prolifereerden en maakten ECM rijk aan collageen type I en III. Er kon geen differentiatie in de richting van kraakbeen of botweefsel aangetoond worden. Het toevoegen van zowel TGF-β1 als GDF-5 verhoogde het aantal cellen ($p<0.05$), maar alleen TGF-β1 verhoogde ook de totale collageen productie ($p<0.05$) en de collageen productie per cel. Wij vonden dat stimulatie met TGF-β1, en in mindere mate GDF-5, mesenchymale stamcellen in een 3d hybride scaffold kan stimuleren in de richting van collageenrijk bindweefsel. Helaas konden wij niet aantonen dat het weefsel gevormd door de cellen echt ligamentachtig was, ook al omdat er op dit moment niet één ligament specifieke marker is. Het liefst zouden we een combinatie van markers gebruiken, zoals fibronectine, tenascin-C en de ratio collageen type III/I, om een statement te kunnen maken omtrent de aard van het weefsel dat door de cellen geproduceerd werd. Toekomstige studies met een combinatie van verschillende groeifactoren, toepassing van biomechanische stimuli, in vivo implantatie van de constructen en analyse hiervan met de hierboven genoemde markers, zullen het inzicht in de aard van het gevormde weefsel in de scaffolds verbeteren.

4. Het statisch spannen van met mesenchymale stamcellen gezaaide poly(L-lactide/glycolide) scaffolds en het effect hiervan op proliferatie en differentiatie

Naast het toedienen van biochemische factoren aan het groeimedium (zie hoofdstuk 5) of in vivo genetische transfer, kan mechanische stimulatie een efficiënte methode zijn om mesenchymale stamcellen te laten differentiëren.

Tot op heden is er echter weinig bekend over het effect van mechanische stimulatie van ongedifferentieerde cellen zoals mesenchymale stamcellen in VKB tissue engineering. Vanwege de rol van ECM in het afremmen van het effect van mechanische stimulatie, kan gedeeltelijke differentiatie met bijkomende matrix afzetting nodig zijn voor adequate vertaling van deze stimuli. Met andere worden de timing van de toediening van een mechanische stimulans aan met cellen bezaaide scaffolds, kan het uiteindelijke effect beïnvloeden. In **hoofdstuk 6** hebben we celproliferatie en matrix synthese bestudeerd van ongedifferentieerde mesenchymale stamcellen die gezaaid waren op poreuze gevlochten PLGA scaffolds en op verschillende tijdstippen aan statische spanning werden blootgesteld. Er waren 4 verschillende opties: de scaffolds werden niet gespannen, gespannen tijdens zaaien van de cellen, meteen na het zaaien of 2 dagen na het zaaien. Tijdens de eerste 5 dagen leek de proliferatie van de cellen geremd te worden door het toedienen van de mechanische spanning. In deze periode was de differentiatie groter naarmate de duur van de mechanische spanning toenam. Na dag 5 vlakten de verschillen tussen celaantal en collageen productie af. Na dag 11 nam het totaal aantal cellen af, terwijl de collageen productie verder toenam. Celaantal en differentiatie waren op dag 23 voor alle opties vergelijkbaar en dus onafhankelijk van het tijdstip van toediening van de mechanische spanning. Deze studie toonde aan dat statische spanning lengte oriëntatie, proliferatie en differentiatie van mesenchymale stamcellen gezaaid op PLGA scaffolds stimuleert, hoewel er tussen wel en niet gespannen cellen ten aanzien van proliferatie en differentiatie na 11 dagen geen verschillen meer waren. De applicatie van spanning tijdens zaaien leek de voorkeur te hebben boven spannen na het zaaien, met name indien er een snelle respons gewenst is. Dynamisch spannen in vergelijking met statisch spannen benadert meer het mechanisch stimuleren van ligamenten in vivo. Daarom is het misschien logischer om het dynamisch spannen van met cellen bezaaide constructen te bestuderen. Bij het dynamisch spannen zijn er echter nog meer variabelen van belang, zoals onder andere frequentie en snelheid. Daarom was het een logische stap om

eerst het effect van statisch spannen te onderzoeken. Vervolgens kan dan het dynamisch laden van de constructen in vitro worden onderzocht.

6. De ontwikkeling van een methode om de overleving en functionaliteit van mesenchymale stamcellen in vivo te bepalen

Er is weinig bekend over het lot van de cellen na transplantatie bij de in vivo toepassing ervan. Meerdere studies hebben aangetoond dat cellen in autotransplantaten dood gaan na reconstructie in het kniegewricht en dat de transplantaten vervolgens bevolkt worden door andere cellen van de patiënt. Behalve het al dan niet overleven van cellen in vivo, is ook de functionaliteit van deze cellen in vivo van belang. Het succes van een "tissue engineered" ligament is onder meer afhankelijk van de productie van de gewenste matrix door de cellen in vivo. We zochten een methode om functionaliteit van onze cellen in vivo te kunnen aantonen. In **hoofdstuk 7** hebben we species specifieke herkenning van eiwitten gebruikt om aan te tonen dat genetisch gelabelde cellen niet alleen overleven na transplantatie, maar ook ECM produceren. Cellen, getransduceerd met het MoMuLV-based vector die het delta-LNGFR-gen encodeert, werden gezaaid op PLGA fibers, en subcutaan geïmplanteerd bij naakte muizen tot een periode van 6 weken.
Middels immunohistochemie voor het aantonen van LNGFR expressie bleken de gezaaide cellen de transplantatie tot de periode van 6 weken te overleven. Immunohistochemie voor het aantonen van collageen type I en III toonde de productie hiervan aan in de scaffolds. Daarbij konden we matrix, geproduceerd door de donor cellen zelf, aantonen met geitenspecifiek anti-collageen type III. Met deze studie toonden we aan dat mesenchymale stamcellen gezaaid op PLGA scaffolds, in vivo transplantatie overleven en dat ze vervolgens in staat zijn om collageen bevattende matrix te produceren. Deze studie had niet tot doel te bewijzen dat de geproduceerde matrix typisch was voor ligamenten. Vervolg onderzoek is nodig om echt ligamentachtig weefsel te kunnen aantonen

en om te onderzoeken of de functionaliteit van de cellen vervolgens ook na transplantatie in meer orthotopische locaties aanwezig is. Bovendien dient dan ook de uiteindelijke mechanische functionaliteit van de constructen bestudeerd te worden.

CONCLUSIES

De huidige technieken voor voorste kruisband chirurgie zijn bevredigend. Echter het streven naar verbetering van deze ingreep en maximale reductie van de geassocieerde nadelen is een uitdaging voor de basale wetenschap en van groot belang voor optimalisering van de patiëntenzorg. Doordat mensen vandaag de dag tot op latere leeftijd willen blijven deelnemen aan sport gerelateerde activiteiten, zal de prevalentie van voorste kruisband letsels waarschijnlijk ook toenemen. Studies met een follow-up van meer dan tien jaar met de huidig gebruikte technieken zijn zeldzaam, ook al omdat de toegepaste technieken voortdurend worden verbeterd. De uitkomsten van onze longitudinale studie met een follow-up van 15 jaar lieten zien dat de resultaten van reconstructie na 15 jaar mogelijk minder gunstig zijn dan in de literatuur beschreven resultaten tot tien jaar. Om deze reden is het essentieel om de patiënten langer dan tien jaar na de reconstructie te vervolgen in een gerandomiseerde gecontroleerde setting. Dit kan echter problemen opleveren omdat jonge, doorgaans mobiele, patiënten vaak uit het oog verloren worden. De enige indicatie voor reconstructie van de voorste kruisband is instabiliteit, en de meeste klachten van instabiliteit zullen optreden als een patiënt nog jong en actief is. Bij het ouder worden neemt de behoefte aan beweging af en als gevolg daarvan ook de behoefte aan een hoog stabiele knie. Verder is er bewijs dat kraakbeen schade, met als gevolg de vaak geziene arthrose in deze patiëntengroep, optreedt ten tijde van het trauma en niet, zoals eerder wel werd gedacht, veroorzaakt wordt door de instabiliteit als gevolg van de gescheurde kruisband. Een stabiele knie gedurende ten minste 15 jaar zou echter een minimaal criterium moeten zijn voor een geslaagde reconstructie.

Een "tissue engineered" VKB reconstructie zou aan alle beperkingen van de huidige technieken met auto- en allotransplantaten tegemoet kunnen komen. Het onderzoek in dit proefschrift toonde aan dat gevlochten PLGA scaffolds geschikt zijn voor cel adhesie, waarna de cellen prolifereren en matrix

produceren onder de juiste condities. Verder toonden we aan dat groeifactoren en mechanisch spannen een rol spelen bij het beïnvloeden van het gedrag van de cellen. Mesenchymale stamcellen lijken de optimale cellen te zijn met betrekking tot proliferatie en differentiatie, in vergelijking met de meer gedifferentieerde VKB cellen. In de klinische applicatie van deze cellen zijn de mesenchymale stamcellen ook eenvoudig te oogsten met een beenmerg biopt, terwijl de VKB cellen met behulp van arthroscopie geoogst moeten worden.

Hoewel er vooruitgang geboekt is, zijn er nog vele obstakels die overwonnen moeten worden. Zo is er tot op heden nog geen "tissue engineered" ligament met de geschikte biologische architectuur of de mechanische sterkte voor in vivo implantatie in de knie. De precieze locale condities die succesvolle ligament ontwikkeling bevorderen, moeten nog gedefinieerd worden. Verder is het, zoals in dit proefschrift aannemelijk gemaakt, van groot belang om de conditie van de cellen na in vivo transplantatie nauwgezet te volgen. Belangrijke vragen daarbij zijn of ze overleven en zo ja, wat ze dan produceren?

Toekomstig onderzoek zal ertoe leiden dat ligament regeneratie de productie van een met cellen bezaaide compatibele scaffold bewerkstelligd met de mechanische eigenschappen van de natieve VKB. De verwachting is dat dit zal resulteren in een duurzame reconstructie van de VKB. Het concept van tissue engineering is echter pas voor het eerst in de tachtiger jaren beschreven. Desondanks is er nog steeds geen "tissue engineered" orgaan ontwikkeld dat direct klinisch toepasbaar is. Het is daarom onzeker of er een "tissue engineered" ligament gebruikt zal worden door de orthopaedisch chirurg van vandaag.

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DANKWOORD EN CURRICULUM VITAE

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DANKWOORD

Met de begeleiders die ik heb is het schrijven van een proefschrift makkelijker dan het lijkt. Je moet onderzoek doen leuk vinden en je moet er tijd in willen steken. Maar het belangrijkste nog is, en dat klinkt cliché, dat je mensen om je heen hebt die je stimuleren en bereid zijn je te willen helpen. Mijn geluk was dat ik er daar velen van had. Daarbij komt nog dat ik al die tijd mijn eigen tijdschema kon aanhouden zonder door mijn directe begeleiders met deadlines geconfronteerd te worden. Mede daardoor heb ik dit proefschrift tot het einde met veel plezier kunnen schrijven. Veel dank hiervoor!

Dr. Willems, beste Jaap, jij bent degene die er voor gezorgd heeft dat ík dit onderzoek in de eerste plaats kon opstarten. Tot op heden kan ik geen voorspelling of belofte van je bedenken die niet uitgekomen of nagekomen is. Ik wil je bedanken voor je altijd prettige samenwerking en je geuite vertrouwen in mij. Je hebt me als opleider altijd gestimuleerd op zowel klinisch als wetenschappelijk gebied. Ik ben er trots op jouw promovendus te zijn.

Dr. Saris, beste Daan, jouw hoge werktempo en directe manier van communiceren hebben mij bij de start van mijn onderzoek in Utrecht soms een beetje overrompeld, maar deze eigenschappen werden meteen gewaardeerd. Ik realiseer me dat het juist daarom is dat die twee jaar in Utrecht zo ontzettend productief zijn geweest. Ondanks je eigen drukke werk was je altijd, waar je ook was, beschikbaar voor vragen. Ik heb je altijd gewaardeerd als persoon en wetenschapper, en later ook als opleider. Dank voor al je inhoudelijke bijdragen en steun!

Dr. Creemers, beste Laura, we hebben elkaar eigenlijk via de email leren kennen toen ik met mijn vooropleiding begonnen was. Jij bent van groot belang geweest voor het publiceren van de experimentele studies in het, voor tissue engineering, beste tijdschrift. Je weet niet half hoe dankbaar ik je hiervoor ben. Gelukkig zijn al de uren die je in mijn papers stak allemaal succesvol gebleken!

Prof. Dhert, beste Wouter, jou wil ik bedanken voor je kritische blik, en visie op ontwikkelingen in het orthopaedisch onderzoek waar ik veel profijt van heb gehad. Zie hier het eindresultaat van het onderzoek dat jij en Jaap Willems voor ogen hadden voor mij. Je prettige omgang en je streven naar onderzoek doen op het hoogste niveau hebben mij enorm gestimuleerd.

Prof. Verbout, veel dank voor het in mij gestelde vertrouwen en uw adviezen bij het opzetten van het onderzoek.

Alle mensen van het orthopaedie lab in het UMCU veel dank voor de hulp. Mattie van Rijen veel dank voor de tijd die je stak in de immuno's en de hulp bij het maken van de foto's.
Alle mensen van Isotis, dr. Jens Riesle en de andere mensen van de kraakbeen groep, veel dank voor het begeleiden, trainen en helpen van deze clinicus in de wereld van cellen, kweekmedia, microtomen en gevaarlijke stofjes.

De twee jaar onderzoek in Utrecht zijn alweer een tijdje geleden, maar ik kijk er met veel plezier op terug. Moyo, Steven, Clayton, Jorrit-jan en Marieke bedankt voor de gezellige tijd. Moyo en Natalja veel dank voor het LNGFR onderzoek wat ik met jullie mocht doen. Natalja bedankt voor alle analyses die je gedaan hebt. Jacco bedankt dat jij het onderzoek na mij hebt voortgezet.

Voor het allograft onderzoek moet ik allereerst Arthur Kleipool bedanken, die dit onderzoek mede heeft opgezet en die alle data uit 1991 en 1992 zo netjes heeft verzameld en bewaard. Zonder deze data was het allograft onderzoek niet mogelijk geweest. Mary Boekhorst bedankt voor het traceren van de patienten en het plannen van de polibezoeken.

In het MCA is ook direct iedereen heel behulpzaam geweest om mij te voorzien van oude statussen, de KT-1000 en een polikamer. Bart Burger, Carla Visser, Stan Vos en de anderen veel dank hiervoor.

Mw Witvliet veel dank voor het in 1 uur verhelderen van de handgeschreven HLA aantekeningen uit 1995 die ik al meer dan een jaar probeerde te ontrafelen!
Lea Dijksman en Saskia Rijkenberg bedankt voor de hulp bij de statistiek voor het allograft onderzoek.

Fleur en San, mijn paranimfen uit Amstelveen, twee hele bijzondere vriendinnen. Fleur, we gaan inmiddels richting onze zilveren vriendschap, die begon op het hockeyveld. Met jou begon ook mijn wetenschappelijke carrière; we begonnen samen met cervixscreeningsonderzoek in Canada en uiteindelijk vond jij (niet) poepende kindjes en ik ligamenten op mijn weg! Ik weet zeker dat wij samen ook goud zullen halen!
Sannie, lieve trouwe jaargenoot. De medische wetenschap heeft jou altijd zeer geboeid en je vraagt me altijd het hemd van het lijf over mijn werk. Nu jij daar zoveel van weet, hoop ik dat jij het van mij overneemt op 10 juli als ik een black-out krijg! Tijdens mijn onderzoekstijd in Utrecht spraken we elkaar de hele week als we beiden terug naar huis reden. Zo was ik thuis voordat ik het wist. De heerlijke gesprekken en discussies met jou zijn onvervangbaar!

Lieve familie, vrienden en vriendinnen, dank voor het zo mooi en gezellig maken van mijn leven!

Mijn ouders, Jacques en Cato. Alles wat ik bereikt heb in dit leven, dank ik aan de onbezorgde jeugd die jullie me gaven en het vertrouwen dat jullie altijd in jullie kinderen stelden en nog steeds stellen. Jullie mening en steun zijn tot op de dag van vandaag erg belangrijk voor mij.

En dan mijn mannen; Lieve, lieve Rogier, Sal en Mick. Het afronden van dit boekje is leuk, maar het staat volledig in het niets met het geluk dat ik voel, omdat ik jullie heb. Wat een voorrecht dat we nog een baby'tje krijgen!

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CURRICULUM VITAE

Floor van Eijk werd geboren op 26 mei 1973 in Nijmegen. Samen met haar ouders, broer Luuk en zusje Jakolien bracht zij daar een onbezorgde en sportieve jeugd door. Na haar eindexamen VWO in 1991 (Elshof College te Nijmegen) verhuisde zij naar Amsterdam voor de studie geneeskunde aan de Vrije Universiteit. In 1999 deed zij haar artsexamen. Hierna was zij voor vier maanden poortarts in Almere om vervolgens een half jaar te werken als AGNIO orthopaedie in het Onze Lieve Vrouwe Gasthuis in Amsterdam. Vervolgens deed zij, onder directe begeleiding van prof.dr.W.J.A. Dhert, dr.D.B.F. Saris (beiden UMCU), dr.W.J. Willems (OLVG) en dr. J.Riesle (Isotis bv), onderzoek naar tissue eningeering van ligamenten (oktober 2000 tot oktober 2002). Het grootste gedeelte van dit proefschrift is hierop gebaseerd. Hierna volgde zij vanaf oktober 2002 de vooropleiding chirurgie in het OLVG (opleider dr. N.J. Out), om na 2 jaar met de opleiding orthopaedie in het OLVG te starten (opleider dr.W.J. Willems). In juni 2009 zal zij de opleiding orthopaedie afronden. Floor woont samen met Rogier van Gelder en samen hebben zij twee zoons, Sal en Mick. Een derde held of heldin is op komst.

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Colour Figures

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CHAPTER 2

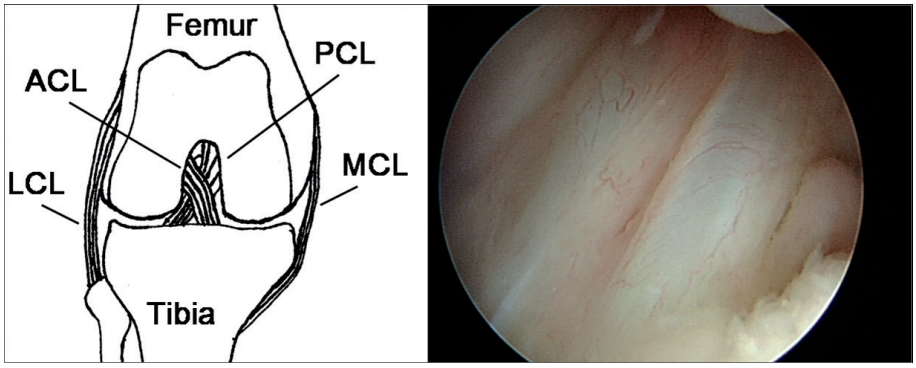

Figure 1. *Left: anatomy of the ligaments in the knee. Right: arthroscopic view of the anteromedial and posterolateral bundle of the ACL.*

CHAPTER 4

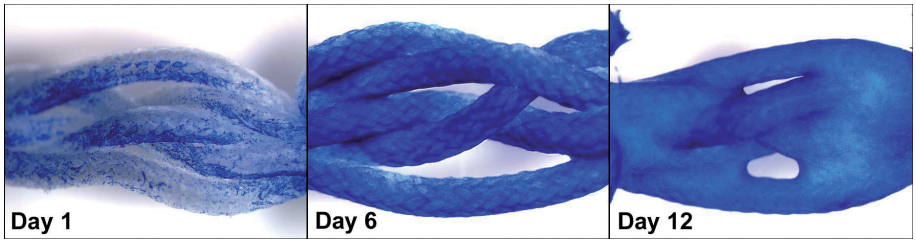

Figure 1. *Effect of culture period on cell content and matrix deposition. Methylene blue staining of scaffolds seeded with 100,000 BMSCs (original magnification, x5). Increased accumulation of cells and matrix is observed with time. Similar effects of culture period were observed for scaffolds seeded with skin and ACL fibroblasts. Arrow: tissue bridging the multifilaments.*

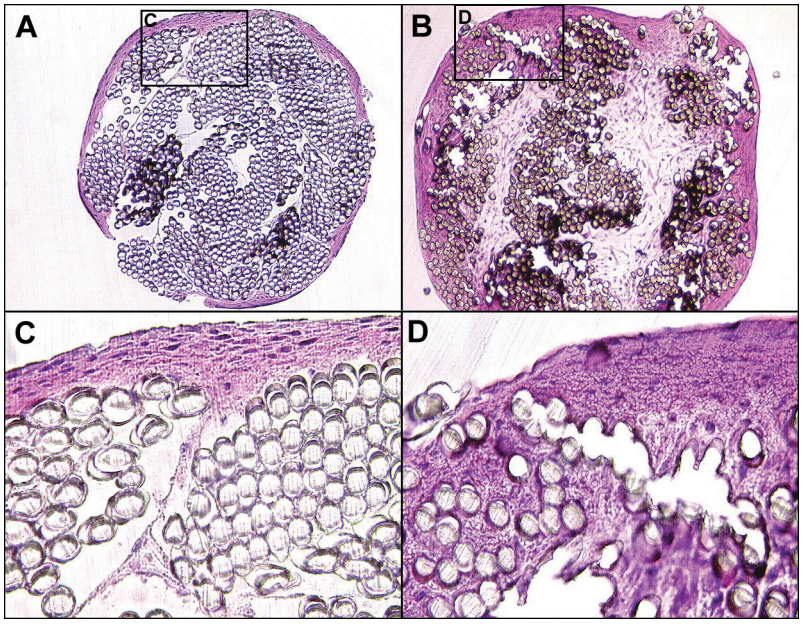

Figure 3.
*Histology of scaffolds seeded with 300,000 BMSCs on day 12. H&E staining of cross sections showing (**A** and **C**) cells and tissue visible only on the outside of the scaffold and (**B** and **D**) cells and tissue present both on the outside and at the center of the scaffold in areas with a more open structure between the monofilaments.*

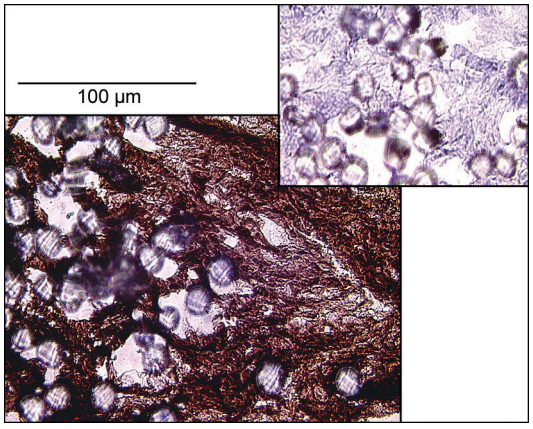

Figure 4. *Immunohistochemistry for collagen type I. Collagen type I staining was demonstrated for all cell-seeded scaffolds independent of the cell type seeded (shown: scaffold seeded with 100,000 skin fibroblasts on day 12.* Inset: *Negative control).*

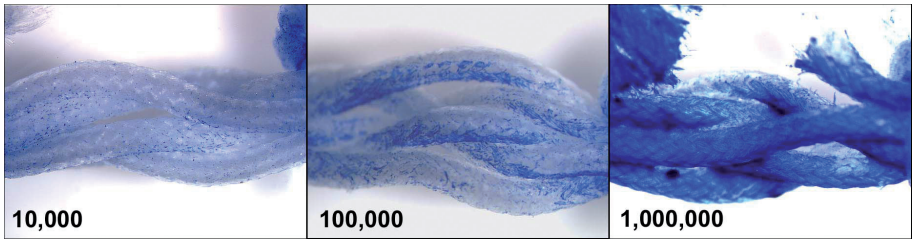

Figure 8. *Effect of seeding density on cell content on day 1. Methylene blue staining of scaffolds seeded with BMSCs (original magnification, x5). Increased accumulation of cells is observed with increasing seeding densities. Similar effects of seeding density were observed for scaffolds seeded with skin and ACL fibroblasts.*

CHAPTER 5

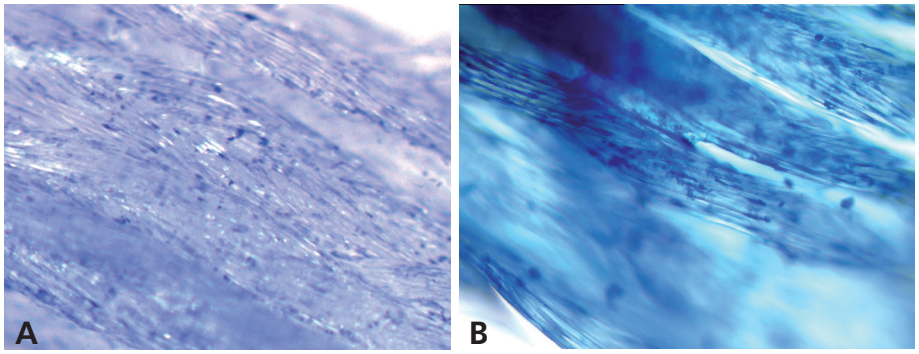

Figure 1. *(**A**) Cell attachment throughout the 3-dimensional (3D) scaffold with deposition of matrix (day 1, transforming growth factor beta 1 (TGF-β1) at 5 ng/mL). Methylene blue staining of 3D scaffolds, imaged with stereomicroscopy (magnification, 125x). (**B**) Cell attachment throughout the 3D scaffold with deposition of matrix (day 3, TGF-β1 at 5 ng/mL). Methylene blue staining of 3D scaffolds imaged with stereomicroscopy (magnification, 100x).*

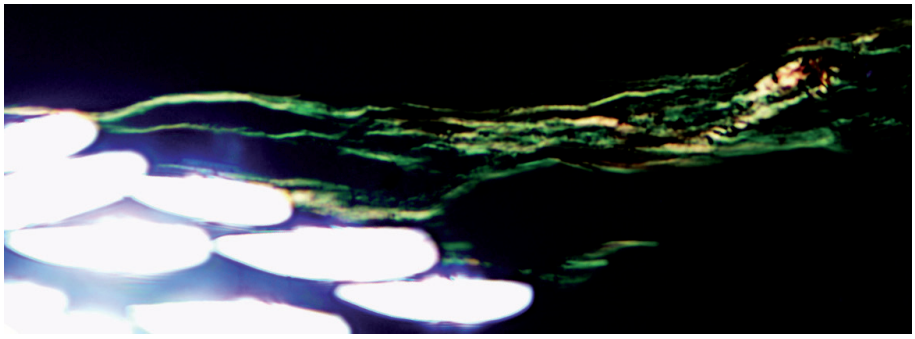

Figure 2. *Picrosirius Red staining of collagen (day 12, transforming growth factor beta 1 at 1 ng/mL) observed using polarization microscopy. Red and green birefringence is seen, demonstrating the presence of collagen type I and type III, respectively (magnification, 400x).*

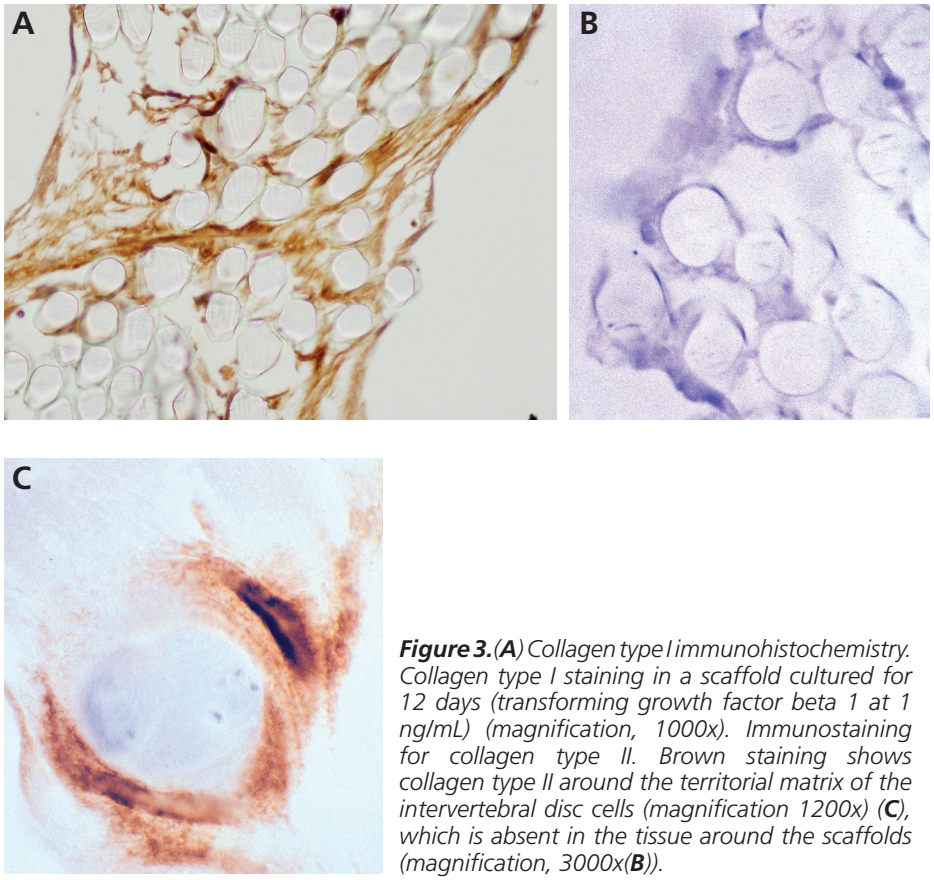

Figure 3. *(**A**) Collagen type I immunohistochemistry. Collagen type I staining in a scaffold cultured for 12 days (transforming growth factor beta 1 at 1 ng/mL) (magnification, 1000x). Immunostaining for collagen type II. Brown staining shows collagen type II around the territorial matrix of the intervertebral disc cells (magnification 1200x) (**C**), which is absent in the tissue around the scaffolds (magnification, 3000x(**B**)).*

CHAPTER 6

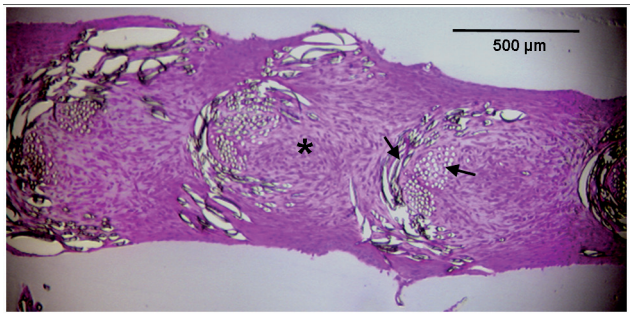

Figure 4. *Histology of unloaded scaffold seeded with bone marrow stem cells and cultured for 11 days (hematoxylin and eosin staining, *center of the scaffold, fibers of the scaffold are indicated with arrows).*

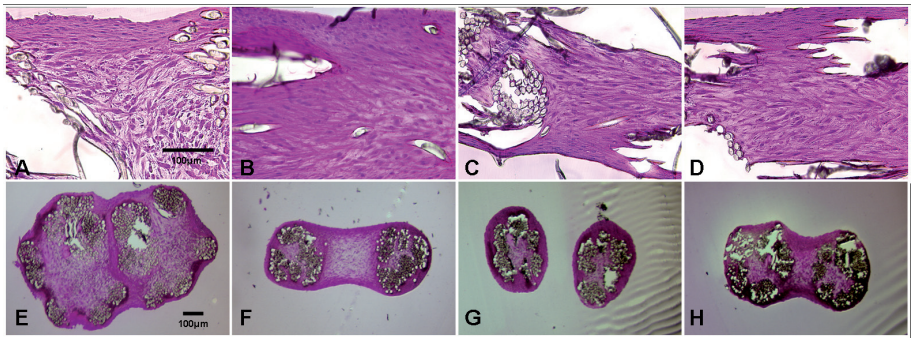

Figure 5. *Histology of scaffolds cultured for (**A–D**) 11 and (**E–H**) 23 days, stained with hematoxylin and eosin. Cells and matrix are densely packed at the outer surfaces. In the (**A**) unloaded group, tissue appears looser toward the center of the scaffold than in the loaded scaffolds (loaded before seeding (**B**), after seeding (**C**), and 2 days after culture (**D**)).*

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CHAPTER 7

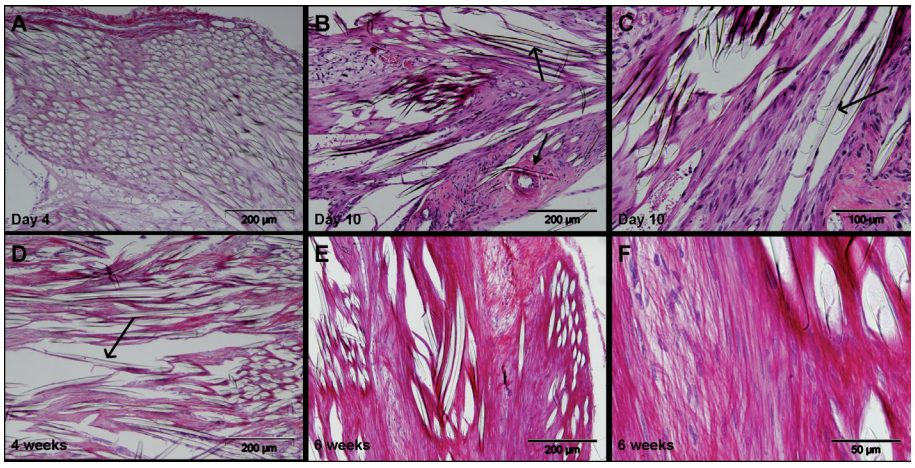

Figure 1 *Histology of scaffolds stained with haematoxilin and eosin (**B-F**) or hematoxylin with Picrosirius Red (**A and D**). Small blood vessels were seen in the centre of the scaffold (closed arrow, **B**). From day 10 the cells and tissue around the fibers showed alignment (**C**). In time, the tissue inside the scaffold appeared much denser as demonstrated by the haematoxilin and eosin staining (B-C, 10 days in contrast with E-F, 6 weeks). A fiber of the scaffold is indicated with an open arrow (**B-D**). The dense tissue around the fibers of the scaffold showed alignment after 6 weeks (**F**).*

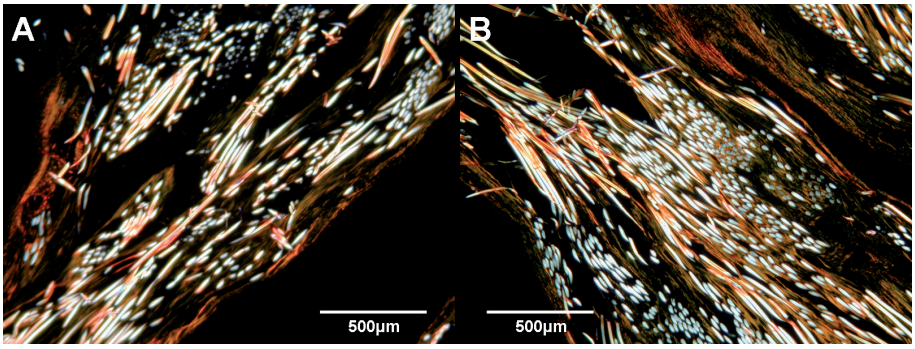

Figure 2 *Polarization microscopy of scaffolds cultured for 10 days (**A**) and 6 weeks (**B**), stained with Picrosirius Red. Red staining denotes collagen type I and greenish staining type III. The scaffolds appear as white fibers.*

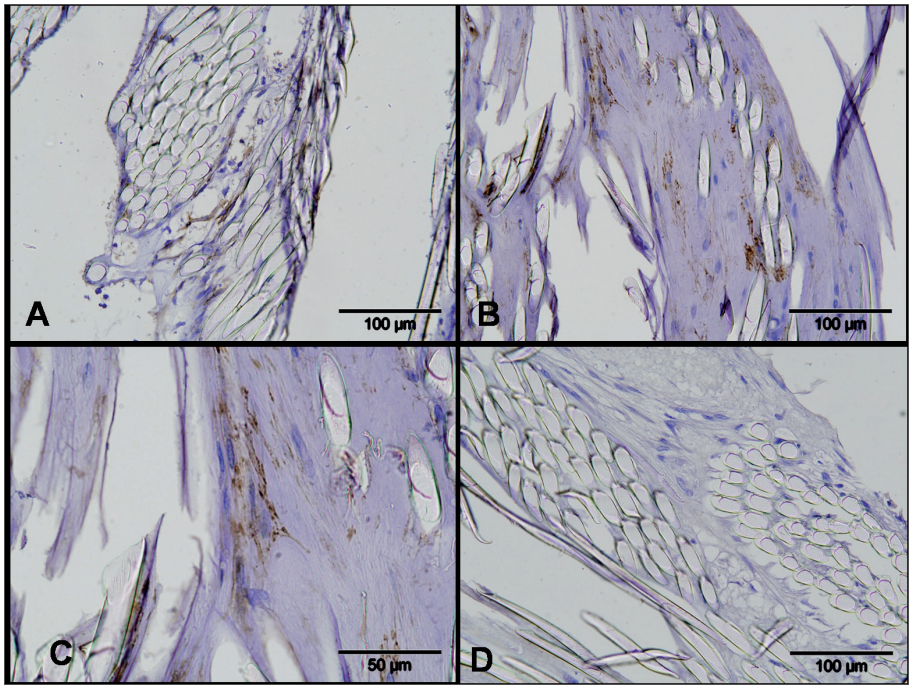

Figure 3 *Immnunohistochemistry for LNGFR-labeled cells at day 2 (**A**) and 6 weeks (**B and C**). Brown staining denotes the presence of LNGFR-positive cells. Controls were always negative (**D**).*

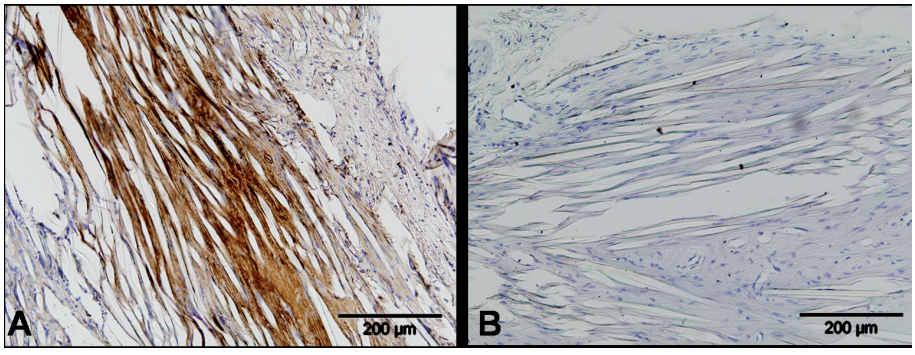

Figure 4 *Immnunohistochemistry for collagen type I inside the scaffolds at 6 weeks (**A**). Brown staining denotes the presence of collagen type I. Controls were always negative (**B**).*

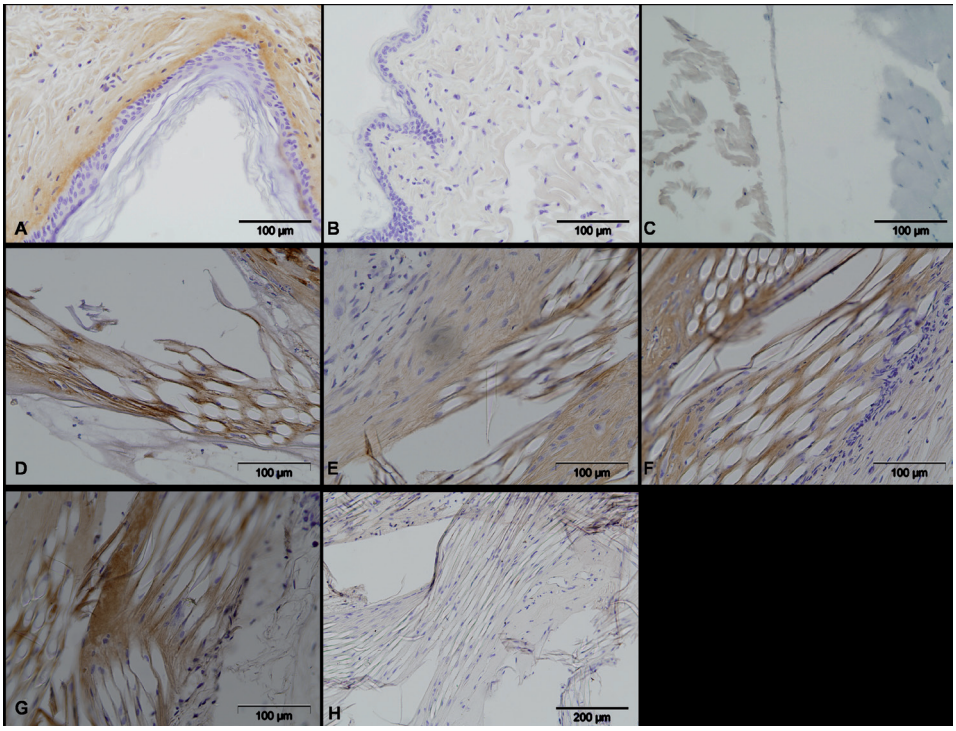

Figure 5 *Immnunohistochemistry for collagen type III for goat skin (**A**) and mouse skin (**B**) using the rabbit anti-human antibody. Mouse tissue showed negative staining (**B**), indicating that this antibody was specific for collagen type III formed by goat cells. Immunohistochemistry for collagen type III for mouse ligament using the rabbit anti-rat antibody (**C**) indicating that collagen type III was indeed present in these mouse tissues. Immunohistochemistry using the species specific rabbit anti-human antibody showing collagen type III inside the scaffolds at 4 days (D, 1:100), 10 days (E, 1:200), 4 weeks (F, 1:200) and 6 weeks (G, 1:200) indicating that transplanted goat cells produced collagen type III at all these time points. Controls were always negative (**H**).*