Abstract: Tissue engineering for fibrocartilage regeneration using mesenchymal stromal cells (MSC) and biomaterial scaffolds is emerging as a promising strategy, but inhibiting vascularization to prevent endochondral ossification is important to develop stable implants. The objective of this study was to investigate the effect of angiotatin on inhibition of angiogenesis and promotion of chondrogenesis by collagen scaffolds with or without MSC implanted subcutaneously in rats. One scaffold from the following groups was implanted in each animal: Collagen scaffolds only, scaffolds functionalized with angiotatin, scaffolds loaded with MSC and scaffolds functionalized with angiotatin and loaded with MSC. The various scaffolds were harvested after 2 and 8 weeks for histological analysis. Real-time quantitative polymerase chain reaction (RT-qPCR) and immunofluorescence quantification. Results demonstrated significantly decreased expression of inflammatory (interleukin 1 alpha and beta) and angiogenic genes (platelet and endothelial cell adhesion molecule 1) in scaffolds functionalized with angiotatin after 2 weeks in vivo. Histologically, after 8 weeks, the scaffolds with angiotatin had less inflammatory cells and more collagen matrix formation, but no fibrocartilage formation was detected. Thus, although angiotatin suppressed angiogenesis, it did not stimulate ectopic chondrogenesis in tissue engineered constructs in vivo.

Keywords: angiotatin, fibrocartilage, stromal cells, tissue scaffolds

Introduction

Temporomandibular disorders (TMDs) are a significant global health problem [1]. Symptoms of TMD are categorized by regional pain in the facial and preauricular areas, around the temporomandibular joint (TMJ), or by limitation and/or interference in jaw movement [2]. Approximately 5% of cases do not respond to conservative treatment and are thus considered for surgical intervention [3]. In severe cases, degenerative changes are observed and fibrocartilaginous structures such as the TMJ disc and subchondral bone of the TMJ condyle are altered [4]. The avascular nature and low cellularity of fibrocartilage leads to poor healing capacity [1], thus leaving the joint without functional components whenever structures are too degenerated and surgically removed due to its unsalvageable state. Several grafts, e.g. dermis, fat, and muscle–have been applied as interpositional materials to avoid further degenerative changes; however, to date there is no ideal disc replacement material [5].

Tissue engineering often combines scaffold biomaterial(s) and progenitor cells, e.g. mesenchymal stromal cells (MSCs), which may overcome the limitations of current treatment options for severe TMDs and promote regeneration of damaged or lost structures from trauma and/or degeneration. MSCs can easily be isolated from bone marrow, adipose tissue, or synovial fluid [6], and subsequently massively expanded in culture in a undifferentiated state for different therapeutic applications. The chondrogenic differentiation potential of MSCs make them applicable for cartilage regeneration. Additionally, MSCs have anti-inflammatory and immunosuppressive properties that can protect the cartilage from further destruction and also facilitate regeneration [7]. Previous studies have proposed inflammation suppression to be a key factor to tissue regeneration [8] and cartilage repair [9], and the use of growth factors may contribute to overcoming this hurdle.

When selecting biomaterial scaffolding, the unique structure and composition of fibrocartilage needs to be considered [10]. Unlike hyaline cartilage, which contains chondrocytes (CC) that mainly synthesize collagen type II (COL2)–the TMJ disc is fibrocartilaginous, containing a mixed cell population of fibroblasts and CC which almost exclusively synthesize collagen type I (COL1) [10]. Due to its abundance and distribution of in fibrocartilage, biocompatibility, and strong cell adhesion, COL1 may be an ideal candidate biomimetic biomaterial for regeneration of cartilage [11].

Previous work has emphasized the importance of vascularization in tissue regeneration, but inhibition of vascularization in regenerated cartilage is another crucial factor in cartilage repair to prevent ossification [12]. However, there are few studies investigating angiogenesis inhibition in tissue engineering of naturally avascular fibrocartilage [13]. Endostatin has previously been used for hyaline cartilage tissue engineering [12,13].

In the present study, the potential of angiostatin for regeneration of fibrocartilage was investigated. Angiostatin is a fragment of plasminogen and is an angiogenesis inhibitor, thus preventing endothelial cell proliferation [14]. It is further reported to be a novel anti-inflammatory factor by inhibiting leukocyte recruitment [15]. The objective of the study was to investigate the effectiveness of angiotatin in inhibiting angiogenesis and promoting chondrogenesis by MSC-collagen constructs in a subcutaneous rat model.

Materials and Methods

Isolation and culture of MSC

The study protocol was independently reviewed and approved by the Ethical Board of the Norwegian Animal Research Authority (according to the European Convention for the Protection of Vertebrates Used for Scientific Purposes, local approval number FOTS ID: 5787). This study was conducted in a facility approved by The Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). MSCs were isolated from the femur bone marrow of six female Lewis rats, 4 to 6 weeks old, and expanded using previously established protocols [16]. The rats were kept under uniform conditions for one week and then euthanized by carbon dioxide overdose. The femurs were carefully scrubbed to remove soft tissues and washed for 5 min in phosphate buffered saline (PBS, Invitrogen, Carlsbad, CA, USA), supplemented with 3% penicillin–streptomycin (PS, HyClone, Chicago, IL, USA). The metaphyseal ends of the femurs were excised and the marrow cavity was flushed with complete alpha minimum essential medium (αMEM, Invitrogen) which contained 1% PS and 15% fetal bovine serum (FBS, HyClone) into a sterile Falcon tube. The cells were then resuspended in fresh, complete αMEM medium and plated in culture flasks (NUNC A/S, Roskilde, Denmark). On the next day, the medium was changed with fresh, complete αMEM medium containing 1%
PS (FBS, HyClone) and 10% FBS (FBS, HyClone). Using phase-contrast microscopy (Nikon Eclipse TS100, Nikon, Tokyo, Japan), spindle-shaped cell morphology was observed on day 3 and cells reached 70–90% confluence within 3 days after, when they were passaged.

**Functionalization of the scaffolds with angiostatin**

Sterile COL1 scaffolds (diameter 5 mm, height 1.5 mm, Optimaix 3D, Matricel, Herzogenrath, Germany) were used for this experiment. Scaffolds were functionalized with recombinant human angiostatin (Merck Millipore, Burlington, MA, USA) by using aliquots of 5 μg angiostatin, diluted in 50 μL distilled water and manually dropped onto each scaffold. The scaffolds were then placed on an orbital shaker for 30 min at 300 rotations per min (rpm) to obtain a homogenous protein distribution in the porous scaffold.

**Cell seeding of scaffolds**

Each scaffold in the two groups with cells was manually seeded with 5 × 10^3 MSC in a 48-well plate. Scaffolds were placed on an orbital shaker for 5 min at 500 rpm to facilitate homogenous cell distribution. All scaffolds were incubated for 3 h (37°C, 5% CO2) for cell attachment, prior to implantation.

**Animal experiment and surgical procedures**

All animals received care at the animal core facility at the University of Bergen, approved by AAALAC International. Female Lewis rats (n = 24), 12 weeks old, weighing approximately 200 g (NOVA-SCB AS, Nittedal, Norway) were used for the experiment. Animals were acclimatized for 2 weeks in polystyrene cages prior to treatment, with three to five rats per cage. The animals were kept in sterile housing conditions with 12 h light/dark cycles and fed with standard rodent chow and water ad libitum. Animals were marked with permanent marker on their tails to identify which experimental group they belonged to.

After induction of general anesthesia (isoflurane, USP, Abbott Laboratories, Chicago, IL, USA), the animals were weighed, and the operating field was shaved and disinfected with ethanol. Two incisions of 10 mm were made, each on the upper and lower on the dorsum of each rat, and two subcutaneous pockets of each incision were created by blunt dissection on each side of the spine. Each animal received four scaffolds that were randomly implanted in the surgically created subcutaneous pockets (Fig. 1). The groups were as follows: control with scaffold only (−/-), scaffold functionalized with angiostatin (−/-A), scaffold loaded with MSC (M−/−) and scaffold loaded with MSC and functionalized with angiostatin (M/A).

**Real-time quantitative polymerase chain reaction (RT-qPCR)**

Two weeks after implantation, samples were harvested for RNA extraction following established protocols [17]. Briefly, RNA was extracted (Maxwell, Promega, Fitchburg, WI, USA) and measured with a spectrophotometer (NanoDrop 1000, Thermo Fischer Scientific, Waltham, MA, USA). cDNA was synthesized from 400 ng of total RNA following the protocol from Applied Biosystems. RT-qPCR was performed according to the manufacturer’s protocol with TagMan Fast Universal PCR Master Mix (Applied Biosystems) with amplification in a 96-well thermal cycle platform, and software (StepOne, Thermo Fischer Scientific) for gene expression detection and analysis. The housekeeping gene glutationaldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. All gene primers were ordered from Life Technologies (Thermo Fischer Scientific) (Table 1).

**Histology**

After 2 and 8 weeks of implantation, samples were embedded in Optimal Cutting Temperature compound (O.C.T., Tissue-Tek, Sakura Finetek, Tokyo, Japan), snap frozen in liquid nitrogen, and cut in 5 μm thick sections (Leica CM 3050S, Leica Microsystems, Wetzlar, Germany). Sections were mounted on slides (Superfrost Plus, Thermo Fisher Scientific) and stained with hematoxylin and eosin (H&E) and Masson’s Trichrome (MTC). The histological sections from all groups were blindly evaluated and described by a pathologist with respect to vessel density, inflammatory cell infiltration, capsule thickness, collagen, and cartilage formation.

**Immunofluorescence staining for CD31**

Cryosections taken from the 2-weeks group were fixed with ice cold acetone in −20°C for 10 min, blocked with 10% normal goat serum (Sigma-Aldrich) for 2 h in room temperature in the dark. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) (1:3,000) for 10 min in room temperature before imaging with a fluorescent microscope (Nikon Eclipse 80i, Nikon). Exposure time and fluorescence intensities were normalized to appropriate control images. Quantification of CD31 positive area divided by total area was calculated using NIS-Elements Advanced Research Software (Nikon).

**Statistical analysis**

After consultation with a professional statistician, normality was tested by applying the Shapiro-Wilk test on the residual from the univariate one-way analysis of variance. These analyses showed that COL1 was non-normally distributed. For this variable, an additional non-parametric test was performed (Mann-Whitney U-test). For the remaining variables, a post-hoc Tukey’s comparison of the mean was used to determine statistically significant differences between groups using SPSS software (IBM, Armonk, IL, USA).

**Table 1 Gene primers used in the study**

| Gene                          | Assay ID          | Amplification length |
|-------------------------------|------------------|----------------------|
| GAPDH                         | Rn99999916_s1    | 87                   |
| ACAN                          | Rn00573424_m1    | 74                   |
| SOX9                          | Rn01735069_m1    | 182                  |
| COL1A1                        | Rn01458448_m1    | 115                  |
| COL2A1                        | Rn01437087_m1    | 97                   |
| COL3A1                        | Rn01437651_m1    | 71                   |
| IL1A                          | Rn00566700_m1    | 73                   |
| IL1B                          | Rn00580432_m1    | 74                   |
| VEGFA                         | Rn01511602_m1    | 95                   |
| PECAM1                        | Rn01467262_m1    | 112                  |

GAPDH, glutaraldehyde 3-phosphate dehydrogenase; ACAN, aggregcan; SOX9, SRY (sex determining region Y)-box 9; COL, collagen; A, alpha; IL, interleukin; VEGF, vascular endothelial growth factor; PECAM, platelet endothelial cell adhesion molecule.
The blinded histological evaluation revealed a thin, fibrous capsule surrounding the scaffold biomaterial in all groups and both timepoints. After 2 weeks (Fig. 3), the group with scaffold only showed empty pores with incipient ingrowth of surrounding connective tissue from the periphery. Mononuclear inflammatory cells were limited to the periphery in the group with scaffold only, while in the other groups, cells were more evenly distributed inside the whole construct, most clearly observed in the groups with angiostatin. In all groups, multinucleated giant cells (MNGC) were evident inside the scaffold, but more distinctly in the groups with angiostatin. Furthermore, the groups with angiostatin had more formation of fibrous tissue, with horizontally oriented fibers. The group with angiostatin and MSC were the most cell rich, with more newly synthesized collagen observed.

After 8 weeks, the group with scaffold only had more inflammatory cells and MNGC, compared to the 2 week timepoint (Fig. 4). The scaffolds of this group were clearly degraded with empty pores at 8 weeks, but cell infiltration was no longer limited to the periphery. The groups with angiostatin had fewer inflammatory cells after 8 weeks and further increased collagen matrix formation, especially in the group with MSC and angiostatin. The group with MSC only demonstrated increased fibroblast activity with more collagen formation and organized fiber orientation, but inferiorly to the group with MSC and angiostatin. However, cartilage formation was not evident in any group.

With regard to fibrocartilage-related genes, there was lower expression of SOX9 (Fig. 2E) and ACAN (Fig. 2F) in the groups with angiostatin, although this was not statistically significant. For COL1 (Fig. 2G), COL2 (Fig. 2H), and COL3 (Fig. 2I), there were lower levels in the groups with angiostatin compared to control (Fig. 2C), while VEGF was decreased in the group with angiostatin and cells (Fig. 2D). Increased levels of these cytokines have been reported in patients with TMJ disc displacement and osteoarthritis (OA) [20]. Furthermore, IL1B has been reported to be strongly associated with TMJ pain [21]. A TMJ disc implant which could decrease levels of pro-inflammatory cytokines could help re-establish joint homeostasis, inhibit cartilage breakdown, and initiate regeneration.

Discussion

The present study aimed to demonstrate the effect of angiostatin in reducing vascularization and inflammation to induce fibrocartilage formation in subcutaneously implanted collagen scaffolds with or without MSC in immunocompetent rats. The results confirmed the downregulation of inflammatory and angiogenic genes. However, it did not induce subcutaneous fibrocartilage formation in the presence of MSC in rats. Despite vascularization being crucial for bone regeneration, in a naturally avascular tissue such as fibrocartilage of the TMJ, vascularization may interfere with cartilage maturation and subsequently induce undesired ossification. Angiostatin and endostatin have previously been investigated extensively for anti-cancer therapy due to their anti-angiogenic and tumor suppressing effects [14,18]. Recently, several studies investigated endostatin for the regulation of angiogenesis in order to improve hyaline cartilage regeneration [12,13]. However, there is little to no information about the use of angiostatin for cartilage regeneration.

In the present study, angiostatin decreased the gene levels of pro-inflammatory cytokines IL1A and IL1B. These cytokines are secreted by monocytes/macrophages and play a crucial role in cartilage destruction by inducing the production and activity of metalloproteinases (MMPs) [19]. Increased levels of these cytokines have been reported in patients with TMDs such as TMJ disc displacement and osteoarthritis (OA) [20]. Furthermore, IL1B has been reported to be strongly associated with TMJ pain [21]. A TMJ disc implant which could decrease levels of pro-inflammatory cytokines could help re-establish joint homeostasis, inhibit cartilage breakdown, and initiate regeneration.

At the vascularization level, angiostatin also downregulated the angiogenic gene markers PECAM1 and VEGF, which indicates the potential of angiostatin-functionalized collagen scaffold for cartilage regeneration. Previous work shows that VEGF plays a central role in CC hypertrophy during embryonic development, skeletal growth, and endochondral ossification [22]. Moreover, increased VEGF levels are also associated with severity and levels of pain in OA [23]. Suppression of VEGF has been suggested as a strategy for inhibition of OA progression [23] and has thus been proposed as an important target molecule for chemotherapy of TMJ chronic closed lock patients [24].

Previous research has shown that local delivery of the antiangiogenic factor bevacizumab, which is a humanized recombinant monoclonal anti-
VEGF antibody, is successful for \textit{in vivo} hyaline cartilage formation with the use of CC cultured on hyaluronic acid scaffolds \cite{25}. These function-
alized scaffolds were found to be less degraded, with five times lower CD31 positive cells compared to controls. In the present study, similar observations were found in the groups with angiostatin wherein decreased vascularization was confirmed via immunofluorescence staining after 2 weeks of implantation.

At the molecular level, angiostatin-functionalized scaffolds downregu-
lated the expression of fibrocartilage specific genes: SOX9, ACAN, and COL1. Of these, SOX9 is the main chondrogenic transcription factor \cite{26} reported to control the expression of ACAN \cite{27} and COL2 \cite{28}; thus, the downregulation of SOX9 may explain the downregulation of the other two genes. It was previously reported that IL1 decreases the levels of SOX9 in CC \cite{29} and also that VEGF decreases ACAN and COL2 synthesis \cite{30}. Furthermore, IL1 has been reported to inhibit synthesis of COL1 and COL3 in dermal fibroblasts \cite{31}. These data contradict the current study, in that the gene levels found in MSC where the control (scaffold only) group had the highest levels of IL1, also had the highest gene levels of COL1 and COL3. This gene expression corresponds to histological findings at 8 weeks, where more newly formed collagen was evident in the groups with angiostatin and accompanied by lower levels of IL1.

The thin fibrous capsule surrounding the scaffolds and the infil-
tration of chronic inflammatory cells are classical signs after biomaterial implantation \cite{32}. The considerable amount of multinucleated giant cells (MNGC) observed reveals active degradation of biomaterial. It is known that \textit{in vivo} implantation of polymeric materials activates the foreign body

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{Histology at 2 weeks. A thin, fibrous capsule (red arrows) surround the implanted scaffold (blue arrows) in all groups. Multinucleated giant cells (green arrows) were evident in all groups after 2 weeks, with greater prominence in groups with angiostatin. Mononuclear inflammatory cells (yellow star) infiltrated the scaffolds, and were more prominent in the groups with angiostatin. H&E, hematoxylin and eosin; MTC, Masson’s Trichrome; -/-, scaffold only; -/A, scaffolds functionalized with angiostatin; M/-, scaffolds loaded with MSC; M/A, scaffolds functionalized with angiostatin and loaded with MSC.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{Histology at 8 weeks. In the group with scaffold only, some multinucleated giant cells (green arrows) were still evident. Newly formed collagen (black arrows) was observed in all groups, but more pronounced in groups with implanted cells. H&E, hematoxylin and eosin; MTC, Masson’s Trichrome; -/-, scaffold only; -/A, scaffolds functionalized with angiostatin; M/-, scaffolds loaded with MSC; M/A, scaffolds functionalized with angiostatin and loaded with MSC.}
\end{figure}
reaction, thus leading to engulfment of degraded materials [32]. The reaction is more explicit in porous biomaterials with high surface-to-volume ratios compared to implants with a smooth-surface [32]. In the current study, macrophages fused into MNGC was observed histologically in all groups, especially after 2 weeks. The groups with angiostatin had more pronounced MNGC infiltrate after 2 weeks, which was likely due to the use of a recombinant human type of angiostatin. Interestingly, the group with scaffolds only, which demonstrated the lowest degree of MNGC infiltrate after 2 weeks, was by far the most degraded after 8 weeks. Despite the lack of cartilage formation, the group with MSC and angiostatin histologically had the highest collagen formation after 8 weeks.

The experimental method of subcutaneous implantation of scaffold/MSC lacks endogenous chondrogenic factors and mechanical signals necessary to promote ectopic cartilage formation [33]. Nevertheless, they are considered suitable for a proof-of-principle study [33]. The lack of studies to investigate the effect of angiostatin on MSC makes the argument for an essential to promote ectopic cartilage formation [33]. Nevertheless, they are considered suitable for a proof-of-principle study [33]. The lack of studies to investigate the effect of angiostatin on MSC makes the argument for an essential to promote ectopic cartilage formation [33]. Nevertheless, they are considered suitable for a proof-of-principle study [33].

Conflicts of interest

The authors declare no conflict of interest.

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Gene therapy may provide a more controlled release of antiangiogenic factors. The effect of this strategy has been demonstrated with endostatin, with regard to non-viral plasmid transfection of MSC [12] and plasmid-supplemented collagen scaffolds [13]. Despite these promising results for hyaline cartilage regeneration, there are challenges to implementation, such as the transient persistence of gene expressions and also concerns regarding safety [25].

The present study confirmed that angiostatin decreased vascularization and inflammation in vivo, although this alone was not sufficient to induce cartilage formation by MSC-collagen constructs. Future studies should optimize the methods with respect to cell density and chondrogenic induction. Furthermore, an orthotopic model should be implemented for improved clinical translation with regards to mechanical stimulation and the challenge of fixation and integration of the implant into surrounding tissues.
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