Synovial pathology in an ovine model of osteoarthritis: effect of intraarticular hyaluronan (Hyalgan[®])

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Abstract Objectives

Published scoring methods for quantifying synovitis focus on acute inflammatory parameters, and are unsuitable as outcome measures in experimental surgical models of osteoarthritis (OA). The aim of the present study was to define a modified histopathological scoring system for ovine synovium more suited to the chronic pathology induced by ovine meniscectomy, and to apply it to detect any therapeutic effects following intraarticular injection of hyaluronan (HA) (Hyalgan®).

Methods

OA was induced in 12 sheep by bilateral lateral meniscectomy, before weekly intraarticular injections of HA or saline vehicle from 16-20 weeks post-operatively, prior to sacrifice at 26 weeks. Six matched sheep were used as controls. Synovial sections were qualitatively scored for hyperplasia, inflammatory infiltrate, fibrosis, and hypervascularity; cell number, depth of fibrosis, and vessel number were also quantified using a graticule.

Results

OA synovia had significantly elevated scores for inflammatory cell infiltration, subintimal fibrosis, vascularity, and aggregate score relative to controls. HA-treated sheep had significantly lower vascularity score (p=0.015), aggregate score (p=0.007), depth of fibrosis (p=0.003) and vessel number (p=0.048) compared to saline-injected sheep.

Conclusion

This study confirms the presence of a chronic synovitis in this OA model, characterised by subintimal fibrosis and hypervascularity (but only modest infiltrate and minimal intimal hyperplasia), which is partially ameliorated by intraarticular hyaluronate therapy.

Key words

Synovitis, osteoarthritis, animal model, hyaluronan.

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The cavity of a synovial joint is enclosed within a strong fibrous capsule, lined by a thin synovial membrane that produces and regulates the synovial fluid essential for lubrication and nutrition of articular cartilage. Normal synovium is nominally divided into two layers: a thin (1-3 cells deep) intima containing primarily those cells designated 'synoviocytes', and a mixed subintima of fibroblasts, macrophages, immune cells, nerves, and vascular elements in a loose connective tissue matrix (1). However the histologic appearance of synovium is highly variable, even between different regions of a single joint (2). This is particularly evident in the subintima, which generally conforms to one of three tissue types: areolar, fibrous or adipose-like tissue. The subintimal tissue type, which reflects the functional geography of the synovia, often alters the intimal lining, which is generally sparse and tightly adherent over dense fibrous tissue, but in areolar synovium is often folded and convoluted into vascularised villi, a phenomenon which becomes exaggerated in many joint diseases including osteoarthritis (OA). Changes in synovial membrane composition also occur with age, with more numerous villi and increasing irregularity of blood vessel and cell distribution (3).

Although OA is historically not considered an inflammatory disease, and OA synovia are often used as control samples in studies of rheumatoid arthritis (4), some degree of synovitis is often present and is likely to be important in the disease pathogenesis. For example, synovial thickening detected by magnetic resonance (MR) is uniquely associated with the presence and severity of knee pain in OA (5). It is becoming recognised that OA synovium can demonstrate the same inflamed and activated phenotype as rheumatoid tissue, and that the difference between the diseases is more quantitative than qualitative (6, 7). Smith et al. (1997) found that in synovial biopsies from patients with early OA, a significant increase in thickness, vascularity, inflammatory cell infiltration, and cytokine production was consistently detected in early stages of the disease (8). Altered synovial vascularity (dilation of existing vessels and angiogenesis), vessel permeability and subsequent leakage of serum proteins promotes synovial oedema, increased synovial fluid volume, and the accumulation of surface fibrin (9). Infiltration of inflammatory cells (especially T and B lymphocytes) is variable and generally focal in OA synovia, but can be considerable (10, 11) and in a subset of OA patients, can include prominent lymphoid aggregates (12).

Intraarticular hyaluronan (HA) preparations are widely used to treat OA, and many double-blind, placebo-controlled, clinical trials have determined this treatment to be effective, providing symptomatic relief lasting for many months post injection (13, 14). Although initially based on the premise of 'visco-supplementation', preclinical investigations, plus the prolonged relief achieved despite a much shorter intraarticular half-life (15), suggest not only that the primary mode of action of HA is via direct pharmacological action within the joint, but that hyaluronans may have disease-modifying activity in OA (16). We have previously used the well-established meniscectomy model of OA in sheep to examine the efficacy and mechanisms of action of many potential therapies [see (17) for review]. Published schema for the histopathological grading of synovial sections (4, 18) are typically designed for evaluation of rheumatoid arthritis and have proven unsuitable for use in this model, as they tend to focus on acute inflammatory parameters (e.g., leukocytic infiltration) which are often absent at the usual endpoint of six months after OA induction. The aim of the present study was to trial a modified scoring system for ovine synovium which would reliably distinguish chronic OA sections from normal controls, and therefore be more sensitive in the detection of therapeutic effects, in this case following intraarticular injection of hyaluronan (Hyalgan[®]).

Materials and methods

Twelve of 18 aged (6-7 years) Merino ewes were subjected to bilateral lateral meniscectomy to induce OA as

Competing interests: none declared.

published previously (19). Careful haemostasis was applied during surgery using electrocoagulation. After a brief recovery period, all sheep were maintained for the experimental period in small paddocks containing irrigated pasture. From 16 weeks after surgery, meniscectomised ewes were bilaterally injected intraarticularly with 2.0ml of either of sterile 0.9% saline, or 10mg/ml sodium hyaluronate (Hyalgan[®], Fidia SpA, Abano Terme Italy; MW=0.5-0.73x10⁶ Da), weekly for 5 weeks. Approximately 1ml of synovial fluid was drawn from each knee immediately prior to each injection. Five weeks after the last injection (*i.e.*, 6 months post-operatively) all 12 OA and 6 non-operated controls (NOC) were killed. A sample of synovium from the suprapatellar fold was removed from each stifle (knee) joint and placed in 10% (v/v) neutral buffered formalin. After processing to paraffin by standard methods, $4\mu m$ sections were cut and stained with haematoxylin and eosin (H&E) or Masson's trichrome. Sections were coded and scored by three blinded observers (M.S., M.C., and A.Y.) using a trial scoring system. The scale used was similar to that described by Smith et al. (1997) (8), modified to provide better data spread for the low-grade synovitis typically seen in this OA model. Synovium scored was of the loose connective or areolar type; no areas of normal fibrous synovium present in these sections were scored (Fig. 1). Samples were scored from 0 to 3 for each of four tissue criteria, as outlined in Table I and Figure 2. Several other potential criteria were considered and rejected from pilot studies (data not shown), including villous hyperplasia (highly variable and statistically unrelated to OA), and synoviocyte activation (parallels intimal hyperplasia).

Three quantitative parameters (cellularity, depth of fibrosis, vessel number) were also measured using a 10x10mm high-precision eyepiece graticule which, when viewed using a 40x objective, projected a 250x250µm grid onto the slide. Five randomly selected areas were counted per section; areas of synovium without sufficient depth,



Fig. 1. Ovine stifle joint synovial sections stained with Masson's trichrome and viewed under normal (a, c) or differential interference contrast (Nomarski) (b, d) microscopy (x200), comparing normal fibrous synovium (a, b) with synovium showing fibrosis secondary to synovitis (c, d).

Criteria	Score	Observation
Intimal hyperplasia		
	0	normal (intima 1-2 cells deep only)
	1	mild, focal hyperplasia (3 to 4 cells)
	2	mild diffuse (3-4 cells) or moderate focal hyperplasia (5+ cells
	3	moderate-marked, diffuse hyperplasia (5+ cells)
Inflammatory (lymphoe	cytic/ plasmo	cytic) infiltrate
	0	normal (occasional cell)
	1	slight or mild focal infiltrate
	2	mild diffuse or moderate focal infiltrate
	3	moderate-marked diffuse infiltrate or defined lymphoid foci
Subintimal fibrosis		
-	0	none (minimal subintimal collagen)
	1	slight or mild focal fibrosis (average 25-50µm depth)
	2	mild diffuse or moderate focal fibrosis (average 50-100µm depth)
	3	moderate-marked diffuse fibrosis (average >100µm depth)
Vascularity		
	0	normal (0-2 vascular elements per x100 field)
	1	slight increase (3-4 vascular elements)
	2	moderate increase (5-8 vascular elements)
	3	marked increase (8+ vascular elements)
Aggregate score		
0	0 -12	Sum of the 4 criteria above

without a reasonably flattened surface profile, or of normal fibrous synovium were excluded. Cellularity (cells/mm) was determined as the number of defined nuclei along a strip of intima 250x50µm, multiplied by four to yield cells per mm. Average depth of fibrosis was determined in each area, using the graticule grid, to the nearest multiple of 22.5μ m (maximum of 250μ m). Vessel number was quantified as the number of blood vessels above capillary size

Table I. Qualitative criteria for scoring ovine synovial histopathology.



Fig. 2. Representative images of ovine synovium (H&E, x200) yielding scores of 0 (\mathbf{a} , \mathbf{b}), 1 (\mathbf{c} , \mathbf{d}), 2 (\mathbf{e} , \mathbf{f}) and 3 (\mathbf{g} , \mathbf{h}) for intimal hyperplasia (\mathbf{a} , \mathbf{c} , \mathbf{e} , \mathbf{g}) and subintimal fibrosis (\mathbf{b} , \mathbf{d} , \mathbf{f} , \mathbf{h}).

(*i.e.*, approx >10 μ m diameter) within the 250x250 μ m square abutting the intimal surface.

For ordinal histological score data, differences between treatment group means were tested for significance using Kruskal-Wallis ANOVA and the Mann-Whitney U-test, applying Bonferroni's correction for multiple comparisons. Continuous variables, including total histological score, were analysed by two-way ANOVA (treatment group x scorer) and Fisher's protected least significant difference (PLSD) *post hoc* tests. For intimal fibrosis depth, statistical analysis was performed on log-transformed data to approximate normal distribution. Interobserver agreement was calculated for each ordinal variable using Fleiss's adaptation of Cohen's kappa coefficient (κ) for multiple observer agreement, as determined by the MacKappa software module (20).

Results

The described synovial scoring system detected highly significant differences between knee joint synovia from normal (NOC) animals and meniscectomised (OA) sheep for inflammatory cell infiltration, subintimal fibrosis, vascularity, and aggregate score (Table II). Intimal hyperplasia score was slightly but significantly increased, in the saline-treated OA group only (p=0.034). Intraarticular treatment of OA sheep with Hyalgan[®] significantly decreased the vascularity score (p=0.007), compared to saline-treated sheep.

Quantitative indices including the number of intimal cells per mm, depth of intimal fibrosis and number of blood vessels/ $250x250\mu$ m field were significantly greater in the synovium of OA sheep compared to controls, regardless of intraarticular treatment (Fig. 3). Hyalgan[®] treatment significantly reduced both the depth of fibrosis (*p*=0.003) and vessel number (*p*=0.048) compared to saline-injected joints.

Interobserver agreement for hyperplasia, infiltration, fibrosis, and vascularity score was 39, 32, 54, and 39% respectively. Fleiss's multiple observer kappa coefficient for these respective variables was -0.001, +0.044, +0.36, and +0.10. Thus subintimal fibrosis was the only scoring characteristic with fair and statistically significant interobserver agreement, especially for fibrosis scores 0 and 3, which showed kappa statistics of 0.64 and 0.41 respectively.

Discussion

This study has confirmed that synovial membrane pathology is a significant feature of the joint disturbances induced in this ovine surgical model of OA. Osteoarthritis induced by meniscectomy caused qualitative and quantitative increases in cellularity, fibrosis and vascularity of the synovial intima sampled at six months post-surgery. Whilst significant changes in inflammatory cell infiltration and intimal cellularity were detected, the most prominent changes were a distinct fibrosis, and to a lesser extent hypervascularity of the synovial subintima. The mean depth of fibrosis beneath the intimal

Table II. Synovial scoring results for each treatment group (mean \pm standard error; n=12 joints per group)*.

	NOC	OA+saline	OA+Hyalgan®	
Intimal hyperplasia	1.1 ± 0.1	$1.5 \pm 0.1^{*}$	1.4 ± 0.1	
Inflammatory infiltrate	0.4 ± 0.1	$1.4 \pm 0.2^{***}$	$1.2 \pm 0.2^{**}$	
Fibrosis	0.5 ± 0.1	$2.1 \pm 0.1^{***}$	$1.8 \pm 0.1^{***}$	
Vascularity	1.4 ± 0.1	$2.6 \pm 0.1^{***}$	$2.2 \pm 0.1^{*** \dagger a}$	
Aggregate score	3.3 ± 0.2	$7.7 \pm 0.3^{***}$	6.6 ± 0.3*** ^{†b}	

*p < 0.05, **: p < 0.005, ***: p < 0.0005 vs. NOC; [†]a: p = 0.015, [†]b: p = 0.007 vs. OA+saline.



Fig. 3. The effect of meniscectomy and intraarticular hyaluronate (Hyalgan[®]) treatment on synovial lining cell number (cells/mm), depth of fibrosis (μ m) and number of blood vessels per 250x250 μ m field (mean \pm standard error; *n*=12 joints per group). **p*<0.05, ****p*<0.0005 *vs.* NOC; *a*: *p*=0.003, *b*: *p*=0.048 *vs.* OA+saline.

surface increased more than four-fold in the OA sheep, an event which was also easily and reliably detected using the qualitative scoring system. In support of this, fibrosis showed the greatest interobserver agreement of all the variables assessed. Prominent perivascular fibrosis was also observed around subintimal vessels in many OA sheep synovia, a change that paralleled the increase in both number and prominence of subintimal blood vessels that ranked as the second-most prominent disturbance in this model. Similar neovascularization and perivascular fibrosis has been noted as a frequent change in human OA (4).

By contrast, cellular changes were more subtle. In particular, this study demonstrates that synovial intimal hyperplasia is not a feature of the osteoarthritis-like pathology generated in this ovine model. Whilst a small but statistically significant increase in hyperplasia score was detected, the very low kappa statistic for this variable demonstrates the difficulty in reliably assessing the subtle changes observed. Typically, OA synovia showed focal regions of increased intimal thickness (i.e., 3-6 cells deep) but this was regionally variable and rarely diffuse. Synovia from normal sheep rarely conformed to the classic description of an intimal monolayer, thus contributing to the lack of discrimination. While quantitative estimation of cellularity (cell nuclei per mm, in the topmost 50µm) did show a small increase in cell number, this was as likely the result of cell infiltration into the topmost subintima rather than lining cell hyperplasia. However whilst an increase in inflammatory cell infiltration was clearly evident in OA sheep, it was typically modest and lymphoid aggregates were observed in only a few samples. Changes in this sheep model therefore more resemble the 'fibrotic' subtype of human synovial pathology identified by Oehler et al. (2002), though this was thought to be a late stage variant following earlier hyperplastic or inflammatory synovitis, which was apparently not the case in this model (21).

Synovial pathology has been previously documented in other animal models of OA. Moderate to marked synovitis is observed in around 50% of biopsies from OA dogs (22), and develops rapidly following experimental anterior cruciate ligament transection (ACLT) (23). Hyperplasia and inflammatory changes (subsynovial lymphocyte and plasma cell accumulation) are prominent by 8 weeks post-operatively, and fibrosis develops within 13 weeks (24). However, careful haemostasis during open surgery considerably reduces the incidence of synovitis when compared to a blind 'stab' technique (23, 25). In rabbit models of OA, synovial responses are rapid and severe, often preceding observable cartilage changes (26). Synovial hyperplasia is a prominent event, observed rapidly following the Hulth procedure (ACLT and hemimeniscectomy) (27), joint immobilisation (28), 10% NaCl injection (29), or repetitive impulse loading (30). By contrast, in the sheep model used in this study (by comparison a more chronic OA model, in which haemostasis during inductive surgery was carefully controlled) hyperplasia is essentially not a feature, and cellular infiltration, while present, is not particularly prominent.

Intraarticular treatment with Hyalgan[®] significantly lowered the overall aggregate synovial pathology score when compared with saline treatment, and reduced the extent of post-surgical synovial fibrosis and hypervascularity. This finding is important for two reasons: firstly, it demonstrates that HA ameliorates several of the pathological changes associated with chronic synovitis, even when administered some time after the induction of OA (in this case, only 10 weeks before a 26-week endpoint); secondly, it links therapeutic targets and likely sources of pain, if we accept that these histological signs of chronic synovitis are likely to be correlated to MR-imaged synovial thickening, which has been shown to be uniquely associated with the presence and severity of knee pain in human OA patients (5). The observed effect on fibrosis is in agreement with a canine study in which sodium hyaluronate injections inhibited peridural fibrosis after unilateral lumbar hemilaminotomy (31), as well as the in vitro observation that exogenous hyaluronan

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reduces collagen synthesis by human skin fibroblasts (32). However studies with other tissues such as meniscus have suggested that HA may instead promote collagen remodelling (33).

In this study, HA was found to have a significant effect on both quantitative and semi-quantitative indices of subintimal blood vessel density. This demonstrates that the increased synovial vascularity associated with synovitis in this model is the result of angiogenesis (again a chronic, structural change) as well as any acute inflammatory changes such as hyperaemia or vessel dilation, and provides possible evidence of an anti-angiogenic effect from HA therapy. A reduction in the number and size of blood vessels, as well as numbers of lining synoviocytes, has also been shown in a small human trial of HA therapy (34). Hyaluronate has been shown in the rabbit ACLT model to reduce synovial production of nitric oxide (NO) (35), a mediator likely to be involved in synovial hyperaemia and angiogenesis (36). In this rabbit model intraarticular hyaluronate was effective in reducing thickness of the synovial lining layer (37), though it is not clear if this was a persistent effect (38). The observation that HA therapy reduces MMP-3 and IL-1ß mRNA in synovium but not cartilage in the same rabbit model (39, 40), suggests again that synovium may be an important target tissue for HA therapy, a conclusion supported by the findings of the current study. However, another rabbit study found that intraarticular HA had no therapeutic effect on synovial histology in experimental arthritis (41).

This study demonstrates the potential for a tailored histopathological scoring system in assessing synovial changes in a chronic, surgical OA model, despite the apparent variation in control (normal) specimens. The protocol presented here differs in several ways from other published methods (4, 8, 18): (i) in using defined criteria for regions to be scored, particularly exclusion of 'normal fibrous synovium' (which was readily distinguished from pathological fibrosis by its very ordered collagen structure); (ii) in reducing the criteria for higher scores, such that what are (by rheumatoid standards) comparatively modest changes yielded a useful spread of scores; (iii) in giving equal weighting to more chronic, structural changes such as fibrosis and hyperaemia rather than inflammatory and hyperplastic changes, which are shown here to be minor and more difficult to assess in this model. This protocol was used to confirm the presence of a chronic synovitis in this OA model, predominantly manifesting as subintimal fibrosis and hypervascularity, which is partially ameliorated by intraarticular hyaluronate therapy.

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