Accelerated post traumatic osteoarthritis in a dual injury murine model

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S U M M A R Y

Objective: Joint injury involving destabilisation of the joint and damage to the articual cartilage (e.g., sports-related injury) can result in accelerated post-traumatic osteoarthritis (PTOA). Destabilised medial meniscotibial ligament (DMM) surgery is one of the most commonly used murine models and whilst it recapitulates Osteoarthritis (OA) pathology, it does not necessarily result in multi-tissue injury, as occurs in PTOA. We hypothesised that simultaneous cartilage damage and joint destabilisation would accelerate the onset of OA pathology.

Methods: OA was induced in C57BL/6 mice via (a) DMM, (b) microblade scratches of articular cartilage (CS) or (c) combined DMM and cartilage scratch (DCS). Mice were culled 7, 14 and 28 days post-surgery. Microcomputed tomography (µCT) and histology were used to monitor bone changes and inflammation. Dynamic weight bearing, an indirect measure of pain, was assessed on day 14.

Results: Osteophytogenesis analysis via µCT revealed that osteophytes were present in all groups at days 7 and 14 post-surgery. However, in DCS, osteophytes were visually larger and more numerous when compared with DMM and cartilage scratch (CS). Histological assessment of cartilage at day 14 and 28, revealed significantly greater damage in DCS compared with DMM and CS. Furthermore, a significant increase in synovitis was observed in DCS. Finally, at day 14 osteophyte numbers correlated with changes in dynamic weight bearing.

Conclusion: Joint destabilisation when combined with simultaneous cartilage injury accelerates joint deterioration, as seen in PTOA. Thus, DCS provides a novel and robust model for investigating multiple pathological hallmarks, including osteophytogenesis, cartilage damage, synovitis and OA-related pain.

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Introduction

Osteoarthritis (OA) is globally the most prevalent musculoskeletal disease. OA is characterised by significant structural changes in joint tissues such as articular cartilage degradation, osteophyte formation (bony outgrowths), subchondral osteosclerosis, epiphyseal bone expansion, synovitis and pannus formation. Although OA is often associated with increasing age, an accelerated progression is recognised in individuals suffering previous (often sport-related) joint injury involving multi-tissue damage. Notably, 66% of patients presenting with articular surface damage in their joints progressed towards OA. Furthermore, current data suggests that patients with chronic OA who suffered an earlier articular trauma are, upon diagnosis, >10 years younger than those patients with no history of joint injury. Taken together, post-traumatic OA (PTOA), as characterised by accelerated joint degeneration, pain and dysfunction, accounts for ~12% of all OA cases.

Lapine models used to investigate PTOA resulting from multi-tissue joint injury, usually involve traumatic rupture of the
anterior cruciate ligament (ACL) and damage of surrounding tissues including the meniscus via mechanical impact on the patellofemoral joint. These have yielded valuable insight into PTOA progression, but involve relatively undefined and uncontrolled damage to various joint tissues. In comparison, surgically-induced murine models that develop OA-like pathology include the destabilised medial meniscotibial ligament (DMM) model and anterior cruciate ligament transection (ACLT) murine model. While both recapitulate features of OA pathology, these do not arise directly from an initial multi-tissue injury, as occurs in PTOA. This may be an important limitation, as increasing evidence suggests that joint tissue interactions may play a significant role in driving disease progression. Alternative murine models include articular injection of agents that induce inflammation and/or tissue toxicity/damage, but questions remain as to how well these represent the joint damage seen in OA. To better understand accelerated OA progression, what is needed is a murine model that combines joint destabilisation with defined/controlled joint tissue injury, and is accessible to genetic manipulation, while enabling comprehensive evaluation of disease-related changes in relevant joint tissues (e.g., cartilage, bone, meniscus). The present study is the first to present a dual injury murine model involving simultaneous cartilage damage and joint destabilisation, and was developed to test our focused hypothesis that concomitant cartilage damage and joint instability accelerates the onset of OA pathology as seen in PTOA.

Methods

Animals

Experiments were performed on 10 week old adult 22–28 g wild-type C57BL/6 male mice (Envigo, UK). Mice were housed at the University of Glasgow or University of the West of Scotland and maintained under standard animal husbandry conditions in accordance with UK home office regulations. All procedures were performed in accordance with UK Home Office Regulations, and with study approval by Animal Welfare Ethical Review Boards at the University of Glasgow and the University of the West of Scotland.

Induction of OA

OA was induced by one operator (to minimise surgical variability) under aseptic conditions via (a) transection of the medial meniscotibial ligament (DMM), (b) microblade scratches of articular cartilage [cartilage scratch (CS); Supplemental Fig. 1(A)] or (c) combined DMM and cartilage scratch (DCS) [Supplemental Fig. 1(B)]. Three C57BL6 mice were made to the superficial cartilage on the medial side of the tibia under anaesthetic conditions in accordance with UK Home Office regulations. All procedures were performed in accordance with UK Home Office Regulations, and with study approval by Animal Welfare Ethical Review Boards at the University of Glasgow and the University of the West of Scotland.

Mice were individually recorded for 5 min, 1 min of which was validated and subsequently analysed. The parameters examined included initial cartilage lesions inflicted by microblade scratches in both cartilage damage and DCS models in our scoring. Synovitis was assessed by two blinded scorers using a recently developed scoring system which we modified from the original to focus only on pannus formation, synovial membrane thickening and sub-synovial hyperplasia. For both scoring systems, there was good agreement between scorers with intraclass correlation coefficient for cartilage scoring of 0.96 (95% CI 0.92 to 0.98), and synovitis scoring of 0.83; (95% CI 0.65 to 0.92).

dynamic weight bearing

As an indirect measurement of pain, limb weight bearing was assessed in mice 14 days after surgery using the BioSeb Dynamic Weight Bearing (DWB) chamber (BioSeb, Marseilles, France). All animals were individually recorded for 5 min, 1 min of which was validated and subsequently analysed. The parameters examined were load on ipsilateral (injured) hind paw and load on front paws.

Statistical analysis

Prism 6 (Graphpad) was used to perform statistical analysis. For normally distributed data, analysis involved one-way analysis of variance (ANOVA) and multiple comparisons using Bonferroni correction. Student t-tests were also used. Data not normally distributed was analysed by Kruskal–Wallis one-way ANOVA and multiple comparisons using Dunn's corrections. Linear regression curves were generated and Spearman’s correlation coefficient was used to assess strength of relationship. P values less than 0.05 were considered significant.

Results

Accelerated osteophyte development after dual injury

Evaluation of osteophyte formation at an early timepoint (7 days post-surgery) revealed that although osteophytes could be detected in some mice within each of the experimental groups [Fig. 1(A)]...
and (B) there was an increase in detectable osteophytes in the DCS model. Notably, in the DCS group 6 out of 8 mice had observable osteophytes whilst only 3 out of 8 mice in the DMM and CS showed signs of osteophytogenesis [Fig. 1(B)]. Moreover, at day 7 the osteophytes in the DCS group were quantifiably larger in size [Fig. 1(C)] and in some mice more numerous [Fig. 1(B)]. In comparison, the DMM group osteophytes were reduced in both number and volume [Fig. 1(B) and (C)]. Analysis of osteophyte BV/TV at day 7 did not show any detectable increased bone volume across any of the three experimental groups [Fig. 1(D)].

To determine how these osteophytes develop over time in these models, we investigated osteophyte morphology and structural characteristics at day 14. In the DMM group, arboreal-like osteophytes were observable in 6 out of 8 mice, whilst in the CS and DCS mice, 100% of mice exhibited osteophytes [Fig. 2(A),(B) and (C)]. Importantly, at this time point, DCS mice had significantly more osteophytes than the DMM and CS groups [Fig. 2(C)], with a minimum of two observable osteophytes. However, 5 out of 8 DCS mice presented with 3 osteophytes per joint [Fig. 2(C)]. In comparison, both DMM and CS alone animals presented a maximum of two osteophytes, which were only seen in 3 out of 8 of mice in each group [Fig. 2(C)]. Both CS and DCS groups exhibited significantly larger osteophyte volume compared to DMM and sham groups [Fig. 2(C)]. However, the percentage of bone volume within these osteophytes did not differ significantly between experimental model groups [Fig. 2(E)].

**Dual injury promotes subchondral osteosclerosis**

Analysis was undertaken to investigate the impact of DMM, CS and DCS on the subchondral bone. At day 7, µCT analysis showed an increase of subchondral osteosclerosis in all 3 OA models when compared with sham controls [Fig. 3(A)]. At day 14 the level of subchondral osteosclerosis in the CS group remained constant, however there was a further increase in subchondral BV/TV in both DMM and DCS models; possibly due to joint instability and

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Fig. 2. Osteophyte development 14 days post-surgery. (A) Three-dimensional μCT imaging of developing osteophytes (arrows) following DMM; cartilage damage; and DCS in C57Bl/6 mice, and (B) corresponding sagittal plane images. Quantitative data showing accelerated osteophytogenesis in the DCS model exhibited by (C) osteophyte number (Kruskal–Wallis with Dunns multiple comparison); (D) total osteophyte volume (Student’s t-test), (E) % of bone present within osteophytes. *P < 0.05; **P < 0.01; ***P < 0.001; n = 8. DMM, DMM; CS; DCS.

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increased biomechanical loading [Fig. 3(B)]. There were no observable differences in metaphyseal trabecular bone in the ipsilateral (injured) leg at day 7 post-surgery [Fig. 3(C)]. However, at day 14, DCS mice exhibited a significant increase in trabecular BV/TV in the injured leg [Fig. 3(D)].

**Dual injury increases cartilage degradation and synovitis**

While cartilage damage and/or synovitis were not normally detectable at day 14 post-induction, we sought to investigate whether these might emerge in the DCS model at this early time-point as an indicator of accelerated PTOA. Histological analysis of the medial compartment of the knee joint at 14 days following DMM, CS or DCS surgery was performed. Over and above the initial scratch-induced cartilage damage, we found significant further cartilage degradation in the DCS model on the tibia when compared with DMM and CS models [Fig. 4(A)–(D)]. Consistent with this finding we also observed increased cartilage degradation on the femur in the DCS model compared with DMM and CS models [Fig. 4(E)]. Furthermore, although there was observable synovitis in the DMM and CS groups, there was a significant increase in synovitis in the DCS model [Fig. 4(F)]. Correlation analysis revealed a strong relationship between cartilage damage and synovitis levels ($\rho = 0.77, P = 0.0004$) [Fig. 4(G)], as well as between cartilage damage and osteophyte number ($\rho = 0.59, P = 0.003$) [Fig. 4(H)]. Synovitis also positively correlated with the number of osteophytes present ($\rho = 0.49, P = 0.02$) [Fig. 4(I)]. In addition, we sought to investigate if accelerated cartilage damage and synovitis in the DCS model persists at 28 days post-surgery [Fig. 5(A)–(C)]. Notably, both increased cartilage damage in the medial tibia and femur [Fig. 5(D) and (E)] and synovitis [Fig. 5(F)] was observed in the DCS group at 28 days post-surgery. Moreover, we also observed remodelling of the cartilage at day 28 in the DCS model [Fig. 5(C)], a feature linked with increasing severity of OA pathology.17 Taken together this indicates that DMM combined with CS promotes an acute inflammatory environment with progressive and accelerated cartilage degradation, remodelling and enhanced osteophytogenesis.

**Osteophyte number and cartilage damage correlate with pain-related behaviour**

Prior work by Huesa et al. has shown that DWB can be used to monitor pain-related behaviour.18 Fourteen days post-surgery, mice in the DCS group demonstrated a significant loss of loading on their ipsilateral (injured) leg when compared with DMM alone [Fig. 6(A)]. No significance was observed in load on the contralateral (non-injured) hind limb between all groups. Consistent with this, mice in the DCS group also demonstrated a significant increase in front paw load compared with the other models [Fig. 6(B)], indicating an increase in OA-related pain. Importantly, there was a strong positive correlation between osteophyte number and front paw load at this time point ($\rho = 0.65, P = 0.0004$) [Fig. 6(C)]. Suggesting that the severity of OA-related pain may be related to the extent of osteophytogenesis. As we observed a positive correlation between osteophyte number and both cartilage damage and synovitis, we further investigated these parameters in relation to OA-pain related behaviour; however, neither cartilage damage nor synovitis exhibited a significant correlation with increase front paw load ($\rho = 0.35, P = 0.09$ and $\rho = 0.28, P = 0.19$ respectively).

**Discussion**

Trauma to the joint is known to promote early osteoarthritis, but the mechanisms for accelerated disease are not well understood. This study presents a novel dual injury murine model to address the important question of whether concomitant joint destabilisation and defined cartilage damage leads to early OA characterised by progressive cartilage destruction, osteophytogenesis, synovitis and pain. Critical to model selection and/or development, is the specific research hypothesis being addressed; and how translatable these...
findings are to human OA. There are various well-established and widely used experimental models of OA across a range of species. While none are ideal in modelling human disease, Little and colleagues have identified preferred characteristics, which include recapitulating human joint disease in a manner amenable for measurement and monitoring, and within a reasonable time frame. Model types range from surgical induction and traumatic injury, to chemical induction methods, each with their strengths.

**Fig. 4. Cartilage degradation and synovitis 14 days post-surgery.** Histological appearance of the medial compartment of the knee joint 14 days following (A) DMM (B) Cartilage damage or (C) DCS, showing pannus formation (green arrow), synovial hyperplasia (red arrow), sub-synovial hyperplasia (black arrow) and Subchondral bone/osteophyte formation (blue arrow). Scale bar = 200 μm. Cartilage damage scores for the (D) medial tibia and (E) medial femur, and (F) Synovitis scores in mice following DMM, cartilage damage or DCS surgery at 14 days post-surgery. Correlation curves depicting significant correlations between (G) cartilage damage and synovitis; (H) osteophyte number and cartilage damage; (I) osteophyte number and synovitis. 95% confidence intervals are indicated by dotted lines. *P < 0.05; **P < 0.01; ***P < 0.001; n = 8. DMM; CS; DCS.

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and limitations. For example, blunt trauma results in relatively undefined multi-tissue injuries, surgical approaches risk surgical artefact whilst chemical approaches such as intra-articular injection of monoiodoacetate (MIA) likely evoke joint changes by mechanisms different from those underpinning OA.

Murine models are important tools for research on various human diseases, and for OA, one of the most widely used is the DMM surgical induction model generated by Glasson et al.\textsuperscript{14} This induces joint instability and recapitulates the cartilage damage, osteosclerosis, osteophytogenesis and pain that characterise OA in humans, although it is technically demanding with respect to surgical skill and need for aseptic conditions. Nevertheless, an experienced researcher can achieve good reproducibility with minimal surgical artefact, and thus we consider DMM to be a robust model in our OA research programme. However, DMM does not reproduce the acute trauma-induced damage to surrounding soft tissue (including cartilage) that is observed in human sport-related injuries.\textsuperscript{20,21} Rabbit ACL rupture models involving blunt impact trauma are available to study PTOA.\textsuperscript{7,8,23} Whilst these models can imitate and reproduce multi-tissue damage similar to that of human knee joint impact traumas, they are limited in not being able to target and confine this trauma specifically to defined tissues within the joint in a reproducible fashion. To this end we have developed a novel dual-injury murine model specific for investigation of OA arising from simultaneous cartilage damage and joint destabilisation. Our DCS procedure now enables generation of a controlled multi-tissue injury murine model mimicking significant trauma to a healthy knee. The DCS model is thus highly relevant to human disease, offering significant potential in meeting a currently unmet scientific need for a model that more accurately represents the subcohort of young otherwise healthy individuals suffering accelerated PTOA resulting from sporting injuries. Such injuries involve simultaneous damage to various joint tissues, including ligaments, bone and cartilage. With this model we

Fig. 5. Cartilage degradation and synovitis 28 days post-surgery.\textsuperscript{1}Histological appearance of the medial compartment of the knee joint 28 days following (A) DMM (B) cartilage damage or (C) DCS, showing pannus formation (green arrow), synovial hyperplasia (red arrow), sub-synovial hyperplasia (black arrow) and subchondral bone/osteophyte formation (blue arrow). Scale bar = 200 μm. Cartilage damage scores for the (D) medial tibial and (E) medial femur, and (F) synovitis scores in mice following DMM, cartilage damage or DCS surgery at 14 days post-surgery. *\textit{P} < 0.05; **\textit{P} < 0.01. DMM, DMM; CS, CS; DCS, DCS.

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were also present in the CS model at day 7 [Fig. 1(A) and (B)]. However, the DCS model presented with quantifiably larger, and in most mice, more numerous osteophyte formation at day 7 [Fig. 1(A) and (B)]. These data would suggest dual trauma consisting of cartilage damage and joint destabilisation can accelerate the formation of osteophytes as early as 7 days post-injury. Previous studies have reported that secondary osteophyte growth is initiated by cartilage damage,26–28 however the factors influencing the formation and growth of osteophytes are poorly defined. Cartilage lesions have been associated with the formation of larger osteophytes,29 supporting our day 14 findings of both cartilage damage and DCS models presenting with larger, more numerous osteophytes compared with the DMM model [Fig. 2(C) and (D)], where no initial cartilage damage was induced. This provides evidence that osteophytogenesis is accelerated by cartilage damage when combined with simultaneous joint loading imbalance. The specific site of cartilage damage may itself alter the type of pathologic outcome. Our study focussed on cartilage damage within the medial compartment however, there could be value in future studies focussing on the impact of cartilage damage on alternative sites (i.e., non-weight-bearing areas of cartilage) to determine how this affects pathologic outcome. Whilst the DCS model was developed to test a specific hypothesis, it has considerable potential to extend beyond OA, to investigation of osteophytogenesis in various pathological scenarios. Examples include ankylosing spondylitis and degenerative disk disease. Additionally, the study of osteophytogenesis could provide a controlled tool to investigate the initiation of endochondral ossification.

Another prominent feature of OA is the development of osteosclerosis in subchondral bone. When the cartilage becomes damaged there is an increased transfer in load to the subchondral bone,30 as well as the release of inflammatory and osteoclast stimulation factors, which can lead to an increase in bone remodelling.31,32 Osteosclerosis was measured as an increase in bone volume (% BV/TV) in the medial subchondral region of the tibia. At both 7 and 14 days post-surgery, all disease model groups exhibited evident osteosclerosis;33,34 however the factors influencing the formation of osteophytes as early as 7 days post-injury. Previous studies have reported that secondary osteophyte growth is initiated by cartilage damage,26–28 however the factors influencing the formation and growth of osteophytes are poorly defined. Cartilage lesions have been associated with the formation of larger osteophytes,29 supporting our day 14 findings of both cartilage damage and DCS models presenting with larger, more numerous osteophytes compared with the DMM model [Fig. 2(C) and (D)], where no initial cartilage damage was induced. This provides evidence that osteophytogenesis is accelerated by cartilage damage when combined with simultaneous joint loading imbalance. The specific site of cartilage damage may itself alter the type of pathologic outcome. Our study focussed on cartilage damage within the medial compartment however, there could be value in future studies focussing on the impact of cartilage damage on alternative sites (i.e., non-weight-bearing areas of cartilage) to determine how this affects pathologic outcome. Whilst the DCS model was developed to test a specific hypothesis, it has considerable potential to extend beyond OA, to investigation of osteophytogenesis in various pathological scenarios. Examples include ankylosing spondylitis and degenerative disk disease. Additionally, the study of osteophytogenesis could provide a controlled tool to investigate the initiation of endochondral ossification.

The observation that cartilage degradation is more extensive and progressive in the DCS model than either of the other models, suggests that interactions arising from the dual injury exacerbates cartilage breakdown. Trauma to the articular cartilage results in the activation of genes encoding for morphogens and the secretion of soluble inflammatory mediators into the joint cavity.36–37 The formation of microcracks and increased vascularisation associated with abnormal bone remodelling in joints during OA facilitate molecular transport between cartilage and bone3, therefore soluble inflammatory mediators present in the joint cavity as a result of cartilage damage and joint destabilisation may potentiate further cartilage destruction/bone remodelling, thus accelerating joint pathology in PTOA. Interestingly, we observed a significant correlation between cartilage damage and osteophyte number [Fig. 4(H)], raising the intriguing possibility that soluble factors secreted by the damaged cartilage may contribute to accelerated osteophytogenesis. Cartilage damage also exhibited a positive correlation with synovitis [Fig. 4(C)] suggesting a link with inflammatory factors.

While traditionally considered a ‘wear and tear’ disease, inflammation is increasingly recognised to have a role in...
degenerative changes occurring during OA pathogenesis. Histological features consistent with synovitis have been observed in synovium from OA patients as well as expression of pro-inflammatory cytokines and more recently we have reported considerable heterogeneity of various inflammatory indices in OA synovia. Indeed knee arthroscopic studies of OA patients found synovial inflammation to be associated with OA structural severity, whilst another study reported that serum C-reactive protein levels in early osteoarthritists predicted likely progressive disease; c-reactive protein (CRP) also correlates significantly with disease joint count and radiographic score in OA. Whilst the DMM model does recapitulate many of the characteristic features of OA, it is generally considered a non-inflammatory model. Our study, however, supported by previous findings, detected histologically the presence of synovitis at 14 days post-surgery. Nevertheless, given the low-level nature of this synovitis, DMM is not the model of choice for investigation of inflammation in OA joints. While the MIA model evokes joint inflammation, this inflammatory response is not initiated in a manner resembling that of OA. Our DCS model yielded a significant and consistently higher level of synovitis than seen in either the DMM or CS models and, similarly to cartilage degradation, synovitis was positively correlated with increased osteophyte number. Thus DCS presents value in enabling mechanistic interrogation of the putative contribution of synovitis to OA pathogenesis.

Pain is one of the most common and physically limiting symptoms of OA, however the origin of this pain is still poorly understood. DWB presents a behavioural non-invasive means of assessing pain, although other factors (e.g., mechanical impediment) may influence the observed changes. Ipsilateral (injured) hind paw and front paw loading were analysed. There was a significant decrease in injured hind paw load with a corresponding increase in load on the front paws in the DCS model compared with all other groups, suggesting the combination of cartilage damage and DMM enhances pain-associated pathology. There have been conflicting studies regarding the relationship between pain and osteophytes in OA. One previous study presented a significant correlation between knee joint pain in OA patients with osteophytes present in the medial tibial condyle. However, more recently, Sengupta et al. challenged this finding by reporting that the presence of high-signal osteophytes detected on magnetic resonance imaging (MRI) were not associated with pain or pain severity. Our data demonstrates a significant and strong positive correlation between osteophyte number and front paw load at 14 days post-surgery, indicating that synovitis is known to lead to heightened pain sensitivity in knee OA and we therefore elected to further explore this finding in relation to pain-associated behaviour in the DCS model. We did not observe a significant correlation between front paw load and synovitis or an increase in cartilage degradation. The association of osteophytogenesis, cartilage damage, synovitis and pain implicates a causal relationship, and this merits future research to define the nature of any such relationships; this may help identify possible molecular mechanisms driving osteophyte formation, as well as joint pain.

We conclude that the concomitant joint instability and cartilage injury that typify knee joint traumatic injury predispose to accelerated OA progression. To establish this we developed and validated a murine model of acute trauma involving simultaneous controlled damage to multiple joint tissues. This robustly mimicked the accelerated OA pathogenesis observed in otherwise normal individuals with PTOA, as characterised by osteoarthritis and compared with DMM) enhanced cartilage degradation, synovial inflammation, osteophytogenesis and pain. Importantly, this model also provides a valuable research tool for investigating osteophytogenesis in various contexts, as the DCS model generates an accelerated and more extensive formation of osteophytes at an early time-point. The reproducible synovitis associated with this model also presents means to elucidate the role of synovitis in OA pathology. Similarly, the enhanced pain-related behaviour in DCS provides a robust model for in vivo investigation of joint pain.

Author contributions
Conceived and designed the experiments: KM, CH, JCL, CSG. Performed the experiments: KM, CH, LD. Analysed the data: KM, CH, LD, GL, RJvTH, JCL, CSG.; wrote manuscript: KM, CH, JCL, CSG.

Competing interests
The authors declare no competing financial interests.

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Supplementary data
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