**EXTENDED REPORT**

Lifelong voluntary joint loading increases osteoarthritis in mice housing a deletion mutation in type II procollagen gene, and slightly also in non-transgenic mice


Objectives: To investigate the effects of voluntary running on the incidence and severity of osteoarthritis (OA) and associated changes in cartilage matrix and subchondral bone in a transgenic Del1 mouse model for OA.

Methods: Del1 mice and their non-transgenic littermate controls were housed from the age of 5–6 weeks to 15 months in individual cages with running wheels. The running activity of each mouse was monitored for the entire 12 month period. Addition Del1 and control mice were housed in individual cages without running wheels. At the end of the experiment the severity of OA was evaluated by light microscopy, and the articular cartilage matrix changes by digital densitometry and quantitative polarised light microscopy.

Results: Lifelong voluntary running increased the incidence and severity of OA significantly in Del1 mice (transgenic runners), and slightly also in non-transgenic runners. Severe OA changes increased from 39% in transgenic non-runners to 90% in transgenic runners (p=0.006) in lateral tibial condyles, and from 24% to 80% (p=0.013) in lateral femoral condyles, respectively. The proteoglycan content of articular cartilage was reduced in transgenic runners in comparison with transgenic non-runners (p=0.0167), but a similar effect was not seen in non-transgenic runners compared with non-transgenic non-runners. No attributable differences were seen in the collagen network of articular cartilage or in the subchondral bone between any of the groups.

Conclusion: The Del1 mutation has earlier been shown to disturb the assembly of the cartilage collagen network and thereby increase the incidence and severity of OA with age. In this study, voluntary running was shown to increase further cartilage damage in the lateral compartments of the knee. This suggests that articular cartilage in Del1 mice is less resistant to physical loading than in control mice. Despite severe OA lesions in the knee joint at the age of 15 months, Del1 mice continued to run voluntarily 2–3 km every night.

Osteoarthritis (OA) is the most common form of arthritis. It is a disorder of the whole synovial joint organ, consisting of articular cartilage, subchondral bone, synovium, and synovial fluid. The most characteristic abnormality in OA is gradual degradation of articular cartilage. However, it has also been suggested that articular cartilage is just an innocent bystander, as OA may result from traumatic injuries to cartilage, ligament, or bone, or excessive loading of cartilage. Increased body weight in humans has been associated with an increased prevalence of OA and, at present, obesity is regarded as a definite risk factor for OA.1 2 The intensity of physical exercise has been shown to correlate with the incidence of OA in humans. Recreational light sports appear not to increase it, but strenuous physical exercise, requiring repetitive kneeling and squatting, or strenuous sports activities with a possibility of joint injuries and/or dynamic joint instability, seem to be definite risk factors.3 6 On the other hand, running training of light intensity and relatively short length has improved the biological properties of young canine articular cartilage.10 11

Gross defects in the articular cartilage involve the breakdown of the matrix backbone, especially of the type II collagen fibril network.13 14 This is preceded by a complex interplay between inflammatory mediators (tumour necrosis factor α, interleukin (IL) 1α (IL1α), IL1β, IL6, IL17, and leukaemia inhibitory factor) of the synovium and cartilage, disturbed synthesis and turnover of the matrix constituents, activation of matrix metalloproteinases (collagenases, stromelysins, gelatinases, and membrane-type matrix metalloproteinases), and altered synthesis of specific tissue inhibitors of metalloproteinases.15 16 However, it is not known whether the increased production of cytokines and proteolytic enzymes precedes or follows the initial cartilage defects.

The relation between OA and aging is well documented. It remains to be shown to what extent the pathogenesis of OA shares common pathways with age associated alterations of cartilage metabolism, or to what extent OA is a time dependent disorder distinct from normal aging with separate causative mechanisms working at the genetic, metabolic, behavioural, or environmental levels.17 18 In some families, mutations have been identified in collagen genes which predispose the affected subjects to early onset OA, sometimes associated with features of chondrodysplasias.19 20

Many investigations on murine OA have been performed in mouse strains carrying a transgene which either disturbs type II procollagen formation,20 21 or causes targeted inactivation of type II,22 23 type IX,24 25 or type XI collagen genes.24 25 Normal laboratory mice have been shown to develop OA spontaneously, the incidence of which is increased by forced running exercise and aging.26 Heterozygous inactivation of Col2a1 gene

Abbreviations: AIOD, area integrated optical density; AIR, area integrated retardation; DD, digital densitometry; IL, interleukin; OA, osteoarthritis; PG, proteoglycan; PLM, polarised light microscopy
coding for type II procollagen made the articular cartilage softer,\textsuperscript{42} and increased the rate and severity of OA,\textsuperscript{30} but a lifelong voluntary running exercise of the same mice reduced the incidence of OA.\textsuperscript{30} Transgenic Del1 mice harbouring a Col2a1 transgene coding for a mutated pro\(\alpha_1(II)\) chain have earlier been noted to develop OA changes in knee joints already at the age of 3 to 4 months. This is significantly earlier than in the control mice.\textsuperscript{43} Our study aimed at determining the effects of lifelong voluntary running on the incidence and severity of degenerative changes in the knee joints of Del1 mice.

**METHODS**

**Transgenic mice**

Production of transgenic Del1 mice housing six copies of the 39 kb Col2a1 transgene containing an engineered 150 bp deletion including the 45 bp exon 7 has been described earlier.\textsuperscript{28} Mice heterozygous for the Del1 locus (Del1 +/-) were mated with non-transgenic mice sharing the same C57Bl\times DBA background.\textsuperscript{28} Non-transgenic offspring of such matings served as controls. For this study 25 male Del1 mice and 26 of their non-transgenic littermates were used. The experiment was carried out in the National Laboratory Animal Centre of the University of Kuopio, Finland. The Animal Care and Use Committee of the University of Kuopio approved the study protocol.

**Voluntary running wheel training**

The experiment was started when the mice were 5–6 weeks old. The mice were divided into four groups of 10–16 mice. Fifteen Del1 mice (transgenic non-runners) and 16 of their control littermates (non-transgenic non-runners) were placed into individual cages 25\times 50\times 15 cm in size and housed under standard conditions:\textsuperscript{44} temperature 21\pm 2°C, humidity 50\pm 20%, 12/12...
hour light rhythm, R36 mouse food (Lactamin AB, Stockholm, Sweden), and water freely until killed. Ten Del1 mice (transgenic runners) and 10 of their control littermates (non-transgenic runners) were housed under similar conditions in similar cages but equipped with a running wheel (width 8 cm and diameter 23 cm) to which they had free access. The running activity of the mice was measured with infrared sensors connected to a computer. All mice were weighed monthly until the age of 15 months when the experiment was stopped. Average daily running distances and running speeds as well as the total lifetime running distances were calculated.

Specimen preparation

The health of the mice remained good throughout the study. Six mice died during the 12 month observation period: 3/16 (19%) in the non-transgenic non-runner group, 2/15 (13%) in the transgenic non-runner group, and 1/10 (10%) in the non-transgenic runner group. Incomplete decalcification in sample preparation also slightly reduced the samples available for grading of OA changes in the knee joints. The number of mice evaluated for OA/entering the study was as follows: 10/16 (63%) of non-transgenic non-runners, 13/15 (87%) of transgenic non-runners, 9/10 (90%) of non-transgenic runners, and 10/10 (100%) of transgenic runners. At the end of the experiment, the mice were anaesthetised by an intraperitoneal Avertin injection (0.54 mL of 0.04% Avertin in 50% 2% ethanol) and killed by decapitation. The left knee joints were dissected free of surrounding tissues, fixed, and embedded in paraffin, and cut in a frontal direction to produce 3 μm thick sections.

OA grading

For OA severity grading, the sections were stained with safranin O, dehydrated, mounted with DPX (Difco, East Moline, IL, USA), and analysed as described earlier. Articular cartilage lesions were graded as: 0, intact cartilage; 1, superficial fibrillation; 2, deep defects extending to uncalcified cartilage; 3, defects extending to calcified cartilage; and 4, defects extending to the subchondral bone (fig 1). The grading was performed in four compartments of the knee joint, the medial and lateral femoral and tibial condyles. The grading was performed by an investigator who did not know from which mice the sections originated. The sections used for evaluation came from the central area of the condyles limited by the tibial attachments of the anterior and posterior cruciate ligaments in the anteroposterior direction. Three sections, approximately 200 μm apart, were evaluated from every knee joint.

Digital densitometry (DD)

For DD of matrix proteoglycans (PGs) the 3 μm thick sections were stained with safranin O, which exhibits stoichiometric binding to PGs. Measurements were carried out in three sections of each knee joint at contact sites of the medial femoral and tibial condylar cartilage not covered by the meniscus. Severe OA changes in the lateral condyles made it impossible to perform reliable DD analyses in the lateral knee joint compartments. The area analysed consisted of one third of the total width of the medial condyles. Safranin O absorbances from defined cartilage depth zones were measured by DD by a 12 bit, Peltier cooled, CCD camera (Photometrics Inc, Tucson, AZ, USA) connected to a Leitz BK II microscope equipped with 16 x 0.45 NA objective (Leitz, Wetzlar, Germany) using 492 nm monochromatic light. The cartilage thickness was divided into 12 fractions of equal thickness. The results were pooled to represent the absorbances of the superficial, intermediate, and the deep cartilage zones, as defined by the polarised light microscopic measurements, see below. Zonal area integrated optical density (AIOD) estimators were calculated for absorbances. If superficial or intermediate cartilage zones were eroded, the results were pooled only from the remaining cartilage zones.

Polarised light microscopy (PLM)

Unstained 3 μm thick sections were used for PLM analyses in areas corresponding to those of the DD analyses of the medial tibial condyle. The microscope and the camera system were the same as in DD, except for a crossed polariser analyser pair in the light path. Monochromatic light at 595 nm was used. The thickness of the collagen fibril layers having a preferential tangential (superficial zone), oblique (intermediate zone), and radial (deep zone) course in articular cartilage were first determined with linear polarisation. Actual measurements for collagen-induced birefringence were made using semicircularly polarised light produced by phase plates. Area integrated retardations (AIRs) of polarised light were calculated for the superficial, intermediate, and deep cartilage zones—or in the case of erosion—for the remaining cartilage zones. AIR is an indicator of the degree of parallel orientation of collagen fibrils and the content of collagen in tissue. Also the height of the subchondral bone plate, volume fraction (%) of birefringent subchondral bone, and mean birefringence per standard unit volume of subchondral bone were measured.

Statistical methods

OA grading results, DD and PLM data were analysed with Mann-Whitney’s U statistics.

RESULTS

Monthly monitoring of the body weights of the mice showed that the runners living in cages with running wheels were 10–13% leaner than the corresponding non-runners (fig 2). The weight difference was significant from 3 months onwards (p=0.004**–0.026**) for transgenic mice, and from 6 months
onwards ($p=0.01^{**}-0.028^{**}$) for non-transgenic mice. At the end of the study the non-transgenic non-runners weighed 45 (3) g (mean (SD)), transgenic non-runners 43 (5) g, non-transgenic runners 40 (4) g, and transgenic runners 38 (5) g.

Monitoring of the running behaviour of the mice showed that voluntary running was most active between the 2nd and 4th months of age (fig 3). Non-transgenic runners ran up to 6.5 km and the transgenic runners up to 5 km a night. During subsequent months, the running distance of the non-transgenic runners remained 12–41% higher than that of the transgenic runners. The total lifetime running distance varied between 630 and 2900 km. The running speed reached its peak during the 3rd month, which probably represents the age when mice reach their musculoskeletal maturity. The running speed of non-transgenic runners was 7–12% higher than that of the transgenic runners (fig 4). Owing to large variations in the activity of individual mice within the experimental groups, the differences between the groups in running distance or speed did not reach statistical significance despite the systematic trend.

Figure 5 summarises the severity and prevalence of cartilage degeneration in the knee joints of mice at the age of 15 months using the scoring system from grade 0 to 4 (fig 1). In all four groups, OA changes were more abundant in the lateral compartments. Furthermore, several consistent findings were made between the groups. Firstly, as expected from earlier studies, the prevalence of OA was higher in transgenic non-runners than in non-transgenic non-runners in all four compartments of the knee joint. The difference (20% in non-transgenic non-runners vs 59% in transgenic non-runners) was significant in the medial femoral condyles ($p=0.044^{*}$). Secondly, in transgenic mice, voluntary running clearly increased the prevalence and severity of OA in the lateral tibial condyles (severe changes grade 2 or higher: 39% in non-runners vs 90% in runners, $p=0.006^{**}$) as well as in the lateral femoral condyles (24% in transgenic non-runners vs 80% in runners, $p=0.013^{*}$, respectively). Voluntary running was also seen to increase slightly the prevalence of OA in the lateral tibial condyles of non-transgenic mice. Finally, a strong positive correlation was found between the OA scores of lateral femoral and tibial condyles (all groups, $p=0.000^{***}-0.01^{**}$) as well as those of the medial femoral and tibial condyles (all groups except non-transgenic non-runners, $p=0.000^{***}-0.004^{**}$) representing “the kissing effect”.

Figure 5 Osteoarthritic changes in the knee joints of mice at the age of 15 months. Mann-Whitney’s U test.

![Femur, medial condyles](image)

![Femur, lateral condyles](image)

![Tibia, medial condyles](image)

![Tibia, lateral condyles](image)

Figure 6 Articular cartilage thickness [mean (SEM)] in medial tibial and femoral condyles at the age of 15 months. NS, non significant, Mann-Whitney’s U test.
No correlation was found between the OA scores and total lifetime running. Neither did reduction in the running activity during the past month, past three months, or past six months correlate with the OA score in either of the runner groups. A significant positive correlation was seen between the increasing OA score in the medial tibial condyles and body weight at 12 months (R=0.691, p=0.027*) and at the time of killing (R=0.66, p=0.038*) in the non-transgenic non-runner group. No other correlations were found.

Analysis of the qualitative changes in articular cartilage at the age of 15 months did not disclose abnormalities in the normal looking articular cartilage or subchondral bone. However, the thickness of articular cartilage in the medial tibial condyle was reduced in transgenic mice in comparison with non-transgenic mice (p=0.018–0.04*) (fig 6). A similar trend was seen in the femoral condyles. In all zones of the tibia of transgenic runners the PG content was reduced (p=0.001***–0.04*) in comparison with transgenic non-runners (fig 7A). A similar reduction in PG content was seen in the superficial zone of the medial femoral condyle in transgenic runners (p=0.03*) (fig 7B). In the deeper zones of the femur the pattern was the same, but not statistically significant. Cumulatively, the PG content of all zones was greater in the tibia than in the femur. Voluntary running caused a clear reduction in PG content (p=0.0167*) in transgenic mice due to running. A similar effect was not seen in the non-transgenic mice.

PLM analyses of the normal looking areas of the medial tibial condyles showed, at the age of 15 months, no significant differences between the groups in the birefringence of collagen network (AIR) in any of the zones (table 1). Running training produced no detectable effect on the collagen network of the healthy looking medial tibial condyles in either non-transgenic or transgenic mice (table 1). Differences were seen in the thickness of the cartilage zones between the groups, but not in the relative thickness.

Analysis of the height of the subchondral bone plate under the medial tibial condyle showed no significant differences between the groups. Neither were there any significant differences between the groups in the volume fraction of the subchondral bone, or in the mean birefringence (AIR) per standard volume unit of subchondral bone (table 1). When the relative variation in magnitude of the bone parameters (table 1) in all of the mouse groups was compared, the percentage of SEM of the mean values was the highest for the volume fraction of the subchondral bone plate (14–18%). For the subchondral bone plate thickness and AIR estimates the relative variation was about 6–10% and 3–9%, respectively. The transgenic genotype or running neither increased nor decreased the magnitude of variation between individual mice.

DISCUSSION

The most important finding of this study was the observation that lifelong voluntary running activity causes a statistically significant increase in the incidence and severity of degenerative changes in the articular cartilage of the knee joints of transgenic mice. This effect is not observed in non-transgenic mice.
transgenic Del1 mice. We believe this results from inferior structural properties of all cartilages in these mice. When expressed at high level, the transgenes containing the Del1 mutation produce proα1(II) collagen chains which have been shown to severely disturb the assembly of type II collagen fibrils and result in structurally inferior cartilage.

Although structural weakening of cartilage has not been demonstrated directly in the heterozygous Del1 mice used in this study, their predisposition to early onset OA degeneration of articular cartilage has been well documented. In an earlier study, reduced birefringence of articular cartilage was seen in the lateral compartment of Del1 knee joints at the age of 6 and 9 months, suggesting that a decreased or disorganised collagen fibril network precedes the degenerative lesions.

Although the size, weight development, and running activity were slightly reduced in the transgenic mice in comparison with the non-transgenic littermate controls, none of these changes reached statistical significance. Running training was effective in reducing the weight of the runners in both groups. There were no statistical differences in the running activity between the two mouse lines, so the physical loading was about equal for both runner groups. In non-transgenic non-runners, the increased body weight appeared to increase the severity of OA in the medial tibial condyles. Surprisingly, running activity did not predict the development of OA, and vice versa, the severity of OA did not affect the running activity of transgenic mice.

Transgenic non-runners had more OA in all four compartments of the knee joint at the age of 15 months. At 15 months, most mice did not have all of the cartilage zones available for DD and PLM measurements. Therefore, when interpreting the quantitative DD and PLM data from articular cartilage it is important to note that the quantitative estimators are slightly biased because the results are derived from normal looking tissue only.

More OA was observed in the lateral femoral and tibial condyles in all experimental groups. Changes induced by running were also more prominent in the lateral condyles. In
non-transgenic mice, running training increased the incidence and severity of OA in the tibia. This agrees with the results of an earlier study of the non-transgenic C57BL/6 mice. In transgenic mice, the running training also enhanced significantly the prevalence and severity of OA in the lateral femoral and tibial condyles. Interestingly, in the medial condyles, running training did not have such an effect. In fact, the rate of severe changes appeared to be reduced by running training. This may be an indicator of uneven distribution of loading in the joint and ultimately of a varus deformity as shown in fig 8. In non-transgenic mice, this kind of differential reactivity within the joint was not seen. In an earlier study with C57BL/6 mice with a haplo insufficiency of the Col2a1 mutations that the production of mutant procollagen chains has more deleterious consequences than reduced production of normal procollagen chains because of haplo insufficiency. The effects of the two types of mutations in mice (Del1 and heterozygous Col2a1 knockout mice, respectively) are therefore in line with these observations. This also explains the different types of outcomes in these mice in relation to physical exercise.

In the Del1 mice, age related, progressive soft tissue calcification and osteophyte formation in the knee joint start at the early age of 3 months. Progression of the degenerative changes seems to resemble human OA. At 15 months of age, the prevalence and severity of the degenerative changes were high in all the condyles of the knee joint. Both mild and more severe lesions appeared constantly on the joint contact areas uncovered by the menisci. This suggests the importance of mechanical forces in the initiation and progression of degeneration.

Interestingly, at 15 months of age, in the PLM analysis of collagen in the medial condyle of the tibia, we could not find any significant differences between the non-transgenic and transgenic mice or any effects attributable to the running training. However, in this same transgenic mouse line, at the age of 6 months, we have found a reduced birefringence of collagen network compared with non-transgenic mice. The difference was no longer present at the age of 9 months, indicating a slower development and maturation of the collagen network in the Del1 mice (Hyttinen et al, unpublished data).

This developmental delay may increase susceptibility of the mice to loading-induced collagen degradation and is possibly the reason for the increased prevalence of OA later in life. Earlier evidence implies that certain areas of the murine tibiofemoral joint are more susceptible to degradation of collagen fibrils than those in the patellofemoral joint.

In the subchondral bone under the medial condyles of the transgenic Del1 mice, the bone birefringence per standard unit volume of the tissue decreased gradually until 9 months, but was not changed thereafter (Hyttinen et al, unpublished data). Similarly, the volume fraction of the subchondral bone was increased between 6 and 9 months. Between 9 and 15 months, there was no further increase. Osteophyte formation and soft tissue calcification was seen, especially in the lateral joint compartments of Del1 mice. These data suggest that there was no uniform, progressive subchondral sclerosis under the loadbearing articular cartilage at 15 months. However, at the age of 15 months, subchondral bone sclerosis and flattening of the medial tibial condyle could be detected in x-ray images (fig 8). Qualitatively, the most commonly seen subchondral sclerosis was in the vicinity of osteophytes in the lateral margin of the condyles.

The subchondral bone plate data (table 1) were obtained from the central region of the medial femoral condyle covering the medial third of the condyle. Interestingly, subchondral bone volume fraction estimates did not differ between the genotypes or after running. Comparisons between the relative variations suggest a remarkably larger biological variation for the bone volume fraction phenomena than for the subchondral bone plate thickness or AIR of bone. We made PLM of subchondral bone from decalcified specimens. The AIR analysis consisted also of examination of the highly organised non-mineralised matrix of bone. The results imply that there were no major differences in the microstructure of the organic matrix of subchondral bone. We suggest that sclerosis of the subchondral bone seen especially in the medial femoral condyles of transgenic mice is located cortically close to the osteophytes.

The Del1 mutation appears to delay the maturation of the collagen network of cartilage. This seems to allow more wear and destruction of the collagen network. This in turn makes it possible for PGs to leak from damaged cartilage. Our findings from this and earlier work strongly suggest that the Del1 mutation in transgenic mice leads to a mechanically incompetent collagen network which is more sensitive to trauma resulting from loading of the joint.

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Exercise and type II collagen mutation


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