Chondrocyte cloning in aging and osteoarthritis of the hip cartilage

Morphometric analysis in transgenic mice expressing bovine growth hormone

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ABSTRACT An arthritic disorder resembling human osteoarthritis occurs in transgenic mice expressing the fusion gene coding for bovine growth hormone (bGH). In these animals, we performed a morphometric evaluation of cellular density, clonal density and the relationship between both parameters in articular cartilage. These parameters were determined in the femoral head of bGH (+) mice at 1, 6 and 12 months of age and compared to values in the control mice. The transgenic mouse showed a reduction in cellular density of the superficial and middle zones of the articular cartilage with age. In the uncalcified cartilage at 6 and 12 months of age, cellular density was significantly lower in age-matched transgenic mice than in the control group. In the former, the changes in cellular density were accompanied by a gradual reduction in the clonal density in the superficial and middle zones. The clonal density / cellular density ratio was similar in transgenic mice and the control group at 12 months of age.

These findings indicate that these transgenic mice develop an osteoarthritic process characterized by loss of cellularity and a gradual decline in chondrocyte cloning in the superficial and middle zones.

Osteoarthritis (OA) is a common degenerative chronic disease in the elderly, although its incidence has recently increased in younger people (Gelber et al. 2000). This disease is characterized by focal areas of superficial fibrillation, hypertrophy of articular cartilage and occasional destruction of the articular surface (Mankin 1974). Histological studies of human femoral heads suggest that differences may exist between the changes in aging cartilage, and osteoarthritic cartilage lesions (Vignon et al. 1974). Some authors have found that aggregated chondrocytes or clones increase with the severity of OA (Mitchell et al. 1992, Kouri et al. 1996, 1998), but the relationship between chondrocyte cloning and age has not been determined.

The local characteristics of the cartilage damage can be studied better in experimental models where it is easier to control the parameters that may damage the tissue. Several animal models have been used to study aging and OA, including cynomolgus macaques (Carlson et al. 1996), guinea pigs (Bendele and Hulman 1988, De Bri et al. 1995) and rats (Mohr and Lehmann 1992). Nevertheless, the use of low-cost small animals such as mice, permits the study of the articular cartilage and its possible involvement in aging and OA during a short period.

The transgenic mouse expressing bovine growth hormone (bGH) has been accepted as a reliable model for studying osteoarthritic processes in joint tissues. In bGH transgenic mice more than 6 months of age, histological examination of

the articular cartilage showed marked changes resembling an osteoarthritic process (Ogueta et al. 2000), characterized by localized chondrocyte hypertrophy, disorganization of the superficial zone and presence of cartilage clefts. Our main aim was to compare zonal variations in the cellularity and clonal density from the articular surface to the subchondral bone in transgenic and control mice. We examined the articular cartilage of the femoral head, using a morphometric light-microscopic method, and the changes were correlated with age and depth from the surface.

Animals and methods

Animals

The data were based on the findings in the femoral head of the bGH transgenic mice and control mice without overexpression of the hormone.

Male transgenic mice were generated by M M McGrane. They were kept in their cages where they moved freely. Food (Panlab S.L., Madrid, Spain) and water were available for ad libitum consumption.

The mice were divided into 3 groups, killed at 1, 6 or 12 months—i.e., most of their life span. The increase in body size (Tg+ group) of these animals was compared to that of animals of normal size—i.e., control mice (Tg– group). The latter were killed at the same ages. Each age and group therefore consisted of 10 mice, and a total of 60 male mice were included in the study.

Histological studies

The joint tissues were collected at necropsy, after the animals had been killed with an overdose of CO. Proximal femoral epiphyses were fixed in a neutral buffered 10% formaldehyde solution for 72 hours, then decalcified at room temperature in a 0.07% EDTA solution (pH 7.2) for 5–7 days, which permitted the joints to be cut along the anterior aspect into two approximately equal portions (medial and lateral epiphyses). Next, the samples were immersed in graded alcohols and xylene, and embedded in paraffin. Starting at random, 12–15 sections were made through each joint. Samples were oriented to obtain a sectional plane exactly perpendicular to the articular surface. The paraffin sections (6 μm- thick) were prepared and stained with hematoxylin-eosin and alcian blue-PAS to show the acid glycosaminoglycans. We then studied the light microscopic features of the articular cartilage and, in particular, its clones.

Morphometry

The morphometric evaluation was done with a projection light microscope. A video camera mounted on the light photomicroscope was employed to capture ×400 images. A transparent paper, used to count points, was superposed on the monitor screen. The test system for estimating morphometric values from histological sections consists of a set of regularly-spaced points and test lines joining a few crosses. Three histological sections of each specimen were examined morphometrically. The chondrocyte nuclei were counted and the cellular density determined with Gundersen's method (Gundersen et al. 1988).

We counted the clones with the stereoscopic microscope in the square of the transparent model projection imaging; and determined the number of clones per field ×400. A second parameter—i.e., the ratio of clonal density to cellular density was multiplied by 10^6.

The following parameters were calculated: cellular density, clonal density and clonal density/cellular density ratio. We evaluated the 4 zones of articular cartilage lining the femoral head in all sections separately. From the surface towards the bone, these zones were the superficial, middle, deep and calcified cartilage. The calcified zone was delimited from the uncalcified cartilage by the tidemark.

On each sample, we performed quantitative analysis on 3 sections cut in different planes throughout the tissue, and calculated mean values from at least 10 individual counts for each femoral head. All counts were made by two blinded observers (MFM and ED).

Statistics

The findings were expressed as the mean, the mean and range. We compared the overall values of the morphometric parameters in the Tg+ and Tg– groups (age-matched animals) with the Mann Whitney U test. The statistical significance of the differences between the groups and its rela-

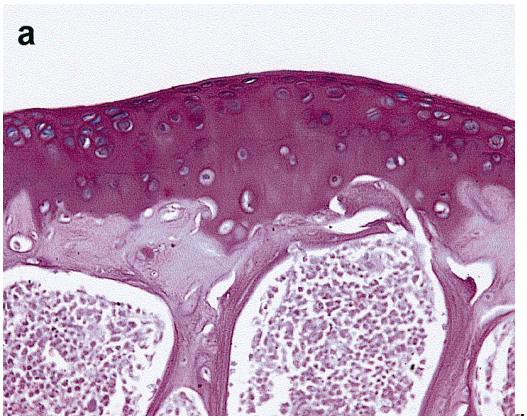
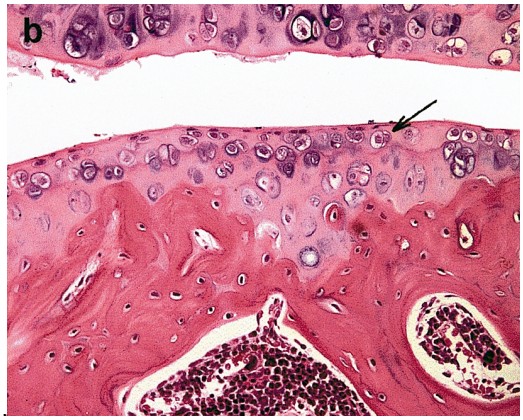


Figure 1. A. Histological section of the hip joint from a 12-month-old control mouse with loss of staining in the calcified cartilage zone. Alcian blue-PAS stain. Original magnification ×400.



B. Photomicrograph obtained from a 6-month-old control mouse with clonal groups mainly in the middle zone (arrow). Hematoxylin-eosin stain. Original magnification ×400.

tion to age was determined, with a general linear model (GLM) and "post hoc" analyses with the Bonferroni/Dunn test. The statistical significance was defined as a probability value less than 0.05. We used the SPSS for Windows version 10.0.7 software package (SPSS Inc, Chicago, IL).

Results

Qualitative morphological features

Control mice (Tg– group). At 1 month of age, the articular cartilage had a normal appearance and no chondrocytic cloning. The subchondral bone plate could not be evaluated at this age.

At 12 months the changes were characterized by a decrease in alcian blue staining in all zones of the articular cartilage; this decrease was greater in the calcified cartilage zone (Figure 1A). In animals aged 6 and 12 months, the middle and deep zones had abundant aggregates of chondrocytes or clones (Figure 1B).

Transgenic mice (Tg+ group). At 1 month of age, we found no cloning of chondrocytes. The articular surface was occasionally irregular with short splits in 6-month-old animals. In some samples, many multicellular clones of chondrocytes were organized in the middle and deep zones (Figure 2A).

The chondroepiphysis had a fibrillated surface and no superficial zone was seen in some regions of the cartilage in 12-month-old animals (Figure 2B). In these animals, all layers of the matrix were poorly stained with alcian blue (Figure 2C). The necrotic chondrocytes, which had eosinophilic remnants of cellular nuclei, were mainly observed in the middle zone. The chondrocytes in the deep zone were disorganized and usually grouped in clones.

Quantitative data

The control mice at all ages, showed a decrease in cellular density from the superficial zone to the calcified cartilage. In the calcified cartilage at 6 months, this density was about 10% of that in the superficial zone. The density in the middle, deep and calcified cartilage zones of the control mice increased with age (6–12-month-old animals). In the zones of uncalcified cartilage in 12-month-old transgenic mice, this parameter was significantly lower than in the control mice. However, the cellular density of calcified cartilage in transgenic mice was similar to that in the control mice (Table 1).

With aging in both groups, we found a reduction in clonal density in the superficial and middle zones. Unlike in the control mice, the 6-month-old transgenic animals showed statistically significant changes in the clonal density of superficial and calcified cartilage zones (Table 2).

At 6 and 12 months of age, the clonal density to cellular density ratio in the control and transgenic mice gradually increased from the superficial to the deep zone. In the calcified cartilage of 6-month-old animals, this parameter differed significantly in transgenic and control mice (Table 3).

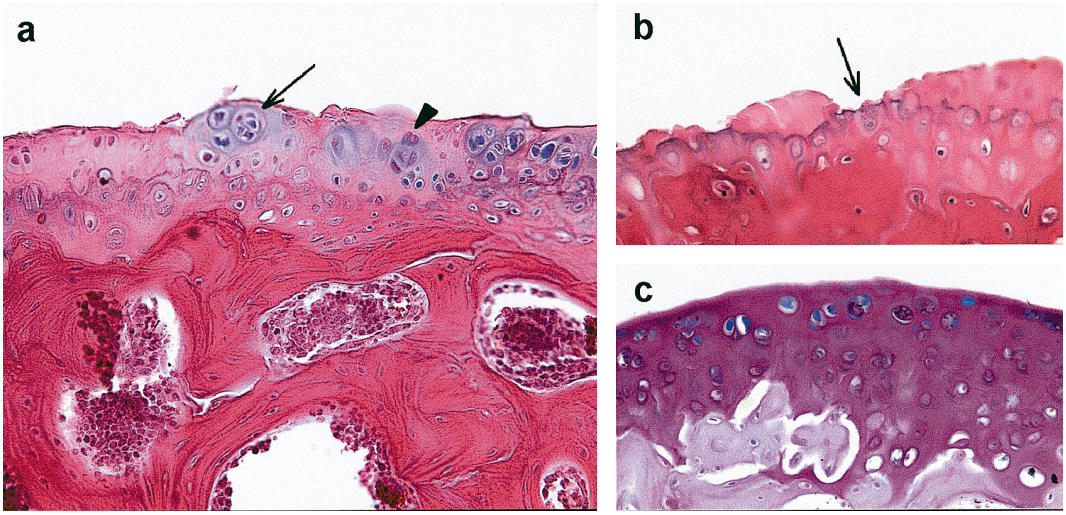


Figure 2. A. Section of articular cartilage from a 6-month-old transgenic mouse with multicellular clones in the un-calcified cartilage (arrow). Note the group of eosinophilic cells (arrowhead) in the middle zone. Hematoxylin-eosin stain. Original magnification ×400.
B. Histological section from a 12-month-old transgenic mouse showing hypocellularity and loss of the superficial zone (arrow). Hematoxylin-eosin stain. Original magnification ×400.
C. Photomicrograph of the articular cartilage from a 12-month-old transgenic mouse. In the calcified cartilage, matrix staining is reduced, while the middle and deep zones have PAS (+) cells. Alcian blue-PAS stain. Original magnification ×400.

Table 1. Relation of cellular density in the articular cartilage to zone, group, and age. The findings are expressed as mean (range) x10^6 cells/mm^3

	1 month	P-value [a]	6 months	P-value [a]	12 months	P-value [a]
Superficial						
Tg–	1.77 (0.56–3.22)	0.03	2.5 (1.19–4.03)	<0.001	1.97 (0–6.71)	<0.001
Tg+	2.59 (1.22–4.25)		0.61 (0–1.75)		0.28 (0–1.41)	
Middle						
Tg–	1.45 (1.06–2.41)	0.5	1.12 (0.54–2.19)	0.002	1.34 (0.53–1.84)	<0.001
Tg+	1.39 (0.69–2.37)		0.83 (0.45–1.51)		0.7 (0.2–1.46)	
Deep						
Tg–	0.94 (0.53–1.45)	0.2	0.82 (0.38–1.33)	0.04	1.10 (0.63–1.55)	0.002
Tg+	0.80 (0.4–1.22)		0.70 (0.33–1.18)		0.8 (0.3–1.83)	
Calcified cartilage						
Tg–	0.70 (0.35–0.94)	0.07	0.29 (0.08–0.62)	0.9	0.44 (0.16–0.76)	0.06
Tg+	0.56 (0.26–0.84)		0.27 (0.15–0.43)		0.37 (0.16–0.77)	

Tg– Control mouse; Tg+ Transgenic mouse
[a] Comparison of Tg+ and Tg– age-matched animals, using the Mann Whitney U-test:

Multivariate analysis

The GLM showed a statistically significant reduction in the cellular density of the superficial zone with age ($p < 0.001$), while it increased in the middle ($p < 0.001$), deep ($p = 0.003$) and calcified cartilage ($p < 0.001$) zones. These changes occurred from 6 to 12 months. As regards the development of OA (inter-group comparison of control and transgenic mice), we found a significant reduction in the cellular density of the calcified cartilage ($p = 0.009$).

Clonal density declined significantly with age in the superficial zone ($p < 0.001$), and increased in the deep zone ($p < 0.001$). It also declined in rela-

Table 2. Relation of clonal density in the articular cartilage to a zone, group and age (clones/unit area), expressed as mean (range)

	6 months	P-value	12 months	P-value
Superficial				
Tg–	0.53 (0–2)	<0.001	0	1
Tg+	0.02 (0–1)		0	
Middle				
Tg–	1.81 (0–5)	0.6	1.78 (0–5)	0.05
Tg+	1.74 (0–5)		0.95 (0–3)	
Deep				
Tg–	2.60 (0–7)	0.3	4.98 (0–10)	0.04
Tg+	2.07 (0–5)		3.52 (0–11)	
Calcified cartilage				
Tg–	0.37 (0–2)	0.03	0.20 (0–1)	0.21
Tg+	0.14 (0–2)		0.62 (0–3)	

Tg– Control mice; Tg+ Transgenic mice
At 1 month of age, no clones were seen.
Comparison of Tg+ and Tg- groups (age-matched animals), using the Mann Whitney U-test.

Table 3. Clonal density to cellular density ratio shown as a function of zone, group and age, expressed as mean (range)

	6 months	P-value	12 months	P-value
Superficial				
Tg–	0.24 (0–1.29)	<0.001	0	1
Tg+	0		0	
Middle				
Tg–	1.65 (0 –3.43)	0.7	1.23 (0–3.45)	0.6
Tg+	2.20 (0–6.34)		1.20 (0–4.61)	
Deep				
Tg–	3.24 (0.48–7.93)	0.7	4.67 (0–12.6)	0.4
Tg+	3.00 (0–7.62)		4.15 (0–10.8)	
Calcified cartilage				
Tg–	1.20 (0 –6.25)	0.03	0.44 (0–3.05)	0.2
Tg+	0.47 (0–6.8)		1.37 (0–6.24)	

Tg– Control mice; Tg+ Transgenic mice
Comparison of Tg+ and Tg– groups (age-matched animals), using the Mann Whitney U-test.

tion to the development of OA in the superficial zone ($p = 0.04$).

The ratio of clonal density to cellular density showed a significant decrease with age in the middle zone ($p = 0.03$), but a significant increase in the deep zone over 6 months of age ($p = 0.006$). No relation was noted between this parameter and the development of OA.

Discussion

Our study focused on the presence of aggregated chondrocytes or clones in relation to aging and OA. According to previous studies on human articular cartilage (Mitrovic et al. 1983, Quintero et al. 1984), the incidence of these aggregates increases significantly with age, a typical sign of the development of OA. They have also been reported in various experimental models (Bendele and Hulman 1988, Jiménez et al. 1997). We found that chondrocyte aggregation occurs not only in animal OA models (such as this transgenic mouse), but also in age-matched normal controls. A moderate-to-high incidence of clones was evident in control animals between the ages of 6 and 12 months.

The histopathology of OA has been studied by several authors who described several degenerative stages and cellular features in various zones of the articular cartilage (Mitrovic et al. 1983, Quintero et al. 1984, Kouri et al. 1998). We found that all zones in the hip articular cartilage of mice undergo age-related morphological and morphometric changes. However, the changes due to aging and osteoarthritis in cartilage differ. There seems to be a parallel relationship between cellular density in articular cartilage and clonal density. Indeed, we observed that cellular aggregations are related to aging in this animal model. The present study shows that, in the older transgenic mouse bGH (+), chondrocyte cloning in the middle zone does not increase, unlike in other animal models (Colombo et al. 1983, Bendele and Hulman 1988), but it tends to increase in the deep and calcified cartilage zones.

It is generally accepted that the early manifestations of OA occur in the superficial zone of the articular cartilage (Vignon et al. 1974, Mitchell et al. 1992). In the transgenic mouse at 12 months of age, the significant reduction in cellular density and clonal density of the superficial zone may be due to cell necrosis. These changes accord with those of others who hypothesized that the superficial zone is at greater risk because of its greater distance from basal active chondrocytes (Bertram and Lierse 1991).

The cause of this grouping phenomenon is not understood. Biological and biomechanical zonal

characteristics may be involved, since the highest values for the clonal density are found in the deep zone. These aggregates seem to be related to an increase in the frequency of cell division near superficial fissures and clefts in the cartilage (Armstrong and Gardner 1977). The inability of chondrocytes to regenerate means a high risk for the integrity of the articular cartilage. As the animal ages, the total number of mitotic figures diminishes and DNA synthetic activity appears to stop. However, under certain circumstances, such as OA, the articular chondrocyte may again start synthesis and cell division (Mankin 1974).

The pathogenesis of OA has been thought to be related to the presence of clonal cells (Kouri et al. 1996). Our findings show that the absolute number of chondrocyte "clones" and the clonal density/cellular density ratio decline with aging of these animals in the superficial and middle zones. The reduction in cellular density with age of the transgenic animals showed no parallel increase in clonal density, which would suggest that, in the superior zones, the chondrocytes undergo moderate grouping during the development of OA. From these data and others (Mohr and Lehmann 1992), it can be assumed that necrosis of chondrocytes is the first change in the osteoarthritic process and it is followed by the loss of proteoglycans by the loss of proteoglycans from cartilage. The surrounding cells then proliferate to form aggregates, giving the lesion a hypocellular/hypercellular appearance. Certain chondrocytes with abundant filopodia and cilia in a lacuna near the aggregates, as suggested elsewhere (Weiss and Mirow 1972, Kouri et al. 1998), may migrate to form clones by active movement. The aggregates seem to be unable to maintain or modify the matrix, perhaps, because of abnormal synthesis of proteoglycan. The final result is a hypocellular articular cartilage with severe changes in the matrix.

In human aging, chondrocyte death has been thought to be a physiological phenomenon due to "exhaustion" of these cells (Mitrovic et al. 1983), while it has been regarded as a local phenomenon in some models of OA (Mohr and Lehmann 1992). In the human hip, the cartilage thinning in OA has been related to a reduction in matrix synthesis (via the release of incompletely synthesized chondroitin sulfate chains) with only a mild reaction of the viable remaining cells (Vignon et al. 1974). In both groups, we found a decrease in the alcian blue stain in older animals, but the transgenic animals had significantly more changes in staining than the control group.

In summary, the present data are compatible with the view that the changes in the cellular density of the articular cartilage, which tend to decrease in the older transgenic mice expressing bGH, may be related to a parallel reduction in clonal density. Our findings provide further evidence that this animal can be regarded as a reliable model in which to improve our knowledge of OA in human beings. However, more studies are needed to understand the mechanisms underlying this process.

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