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The chondrogenic potential of periosteum decreases with age

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Abstract

Periosteum contains undifferentiated mesenchymal stem cells that possess the potential for chondrogenesis during cartilage repair and in fracture healing. With aging, the chondrogenic potential of periosteum declines significantly. An organ-culture model was used to investigate the relationship between the chondrogenic potential of periosteum and aging. A total of 736 periosteal explants from the proximal medial tibiae of 82 rabbits, aged 2 weeks to 2 years, were cultured in agarose suspension conditions conductive for chondrogenesis, and analyzed using histomorphometry, collagen typing, wet weight measurement, ^{3}H-thymidine and ^{35}S-sulfate uptake, autoradiography, and PCNA immunostaining. The rabbits were skeletally mature by 6 months and stopped increasing in weight by 12 months. Chondrogenesis declined significantly with age ($P < 0.0001$) and was maximal in the 1.5–2 month-old rabbits. Explants from the 6 month-old rabbits formed 50% less cartilage, and by 12 months chondrogenesis reached a steady state minimal level. In parallel with this decrease in chondrogenic potential similar decreases were measured in ^{3}H-thymidine uptake ($P < 0.0001$), ^{35}S-sulfate uptake ($P = 0.0117$), as well as the thickness ($P < 0.0001$) and the total number of cells in the cambium layer of the periosteum ($P < 0.0001$). Autoradiography with ^{3}H-thymidine and PCNA immunostaining confirmed the measured decrease in proliferative activity in the cambium layer where the chondrocyte precursors reside, although the percentage of proliferating cells did not change significantly with age. The most dramatic change was the marked decrease (87%) in the thickness and total cell number in the cambium layer of the perisoteum between the 2 and 12 month-old rabbits ($P < 0.05$). These data confirm a decline in the chondrogenic potential of periosteum with aging. Thus, one possibility for improving cartilage formation by periosteal transplantation after skeletal maturity would be to stimulate an increase in the total number of cells in the chondrocyte precursor pool early during chondrogenesis.

Introduction

Biological repair of damaged articular cartilage is currently considered a realistic goal towards which clinicians and researchers are striving based on experimental studies showing the potential for cartilage repair through the use of transplanted chondrocytes [7,8,22], mesenchymal stem cells [3,44], perichondrium [33–35, 40,41] and periosteum [25,26,31,36,37]. Periosteum contains undifferentiated mesenchymal stem cells that have the potential for cartilage formation. This is an important factor in the repair of damaged articular surfaces and in fracture healing. Periosteum regenerates both cartilage and bone [9,10,13,25,26,29,31,44]. A limiting factor in chondrogenesis is the age of the subject. Unfortunately, young adults are precisely the target group for whom 'biological resurfacing' of damaged joints is most needed. Thus, we are compelled to understand the influence of age on periosteal chondrogenesis, if it is to be successfully applied to adult patients.

There are indications that the chondrogenic potential of periosteum is qualitatively and quantitatively inferior in mature and older rabbits than in immature ones [25]. This correlates with the observation that the biological activity of periosteum diminishes with age following the completion of skeletal growth [5,18,25]. Nakahara et al., were able to grow cartilage from human rib periosteal cells obtained from patients younger than 20 years old but not those taken from patients older than 22 years of age [21]. Now that periosteum is being used clinically to regenerate cartilage and resurface defects in joint surfaces, it is imperative that we improve our understanding of the effect of aging on the potential for biological regeneration of cartilage using periosteum. Other investigators have found that delayed fracture healing in

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old rats is due to impaired chondrogenesis rather than alterations in the process of endochondral ossification [2].

To investigate the age-dependence of periosteal chondrogenesis, we have proposed a model of cartilage formation by chondrocyte precursors in periosteum [23]. Our model involves three sequential phases: proliferation, differentiation, and matrix formation. The goal of this study was to investigate the influence of age on the chondrogenic potential of the periosteum by determining exactly how age affects the three phases of our model.

Methods

The relationship between the chondrogenic potential of periosteum and the age of the animal from which it is obtained was determined. Periosteal explants were harvested from rabbits aged 2 weeks to 24 months. A screening study was performed initially using 2 rabbits at each of following ages: 2 weeks, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 18, 21, and 24 months. After culturing in a standardized cartilage yield assay, these data were analyzed by histology and histomorphometry as well as by collagen typing (see below). As shown in the results section, the histological and biochemical data showed best results in the rabbits at 1.5–3 months of age, with linear decreases until 12 months of age, after which little or no chondrogenesis was evident. For more detailed studies, the age groups were then narrowed down to 4 groups in all subsequent studies: 2, 6, 12, and 24 months (6 rabbits per group). These ages were chosen because chondrogenesis was maximal at about 2 months and minimal by 12 months. Skeletal maturity in New Zealand white rabbits occurs by 6 months [11]. We also included 24 month-old rabbits that are well past the age (12 months) at which chondrogenesis was no longer evident in the initial studies.

Periosteal explant model

Periosteal explants, 1.5 by 2 mm (small), 2 by 3 mm (large), or 3 by 4 mm (strips) were taken from the medial side of the proximal tibia of male New Zealand white rabbits using sharp subperiosteal dissection [30]. These explants were all taken from the same 4 × 6 mm^2 horizontal rectangular area that started 1–2 mm below the growth plate. All periosteal explants were obtained within 30 min of death to control for post-mortem effects on chondrogenic potential [28]. The small explants were used for histomorphometry and collagen typing, the larger explants were used for the isotope uptake studies, and the strips were used to assess day 0 periosteal morphology. The larger explants behave similarly to the smaller one in culture [20]. The culture conditions were as reported in the periosteal agarose explants model by O'Driscoll et al. [29]. Immediately after surgical harvesting, the periosteal explants were placed in Dulbecco's modified eagle media (DMEM), (12100-046 GIBCO BRL, Grand Island, NY, USA), with penicillin/streptomycin (Pen/Step: 50 u/mg and 50 μg/mL)(15145-014, GIBCO BRL, Grand Island, NY, USA) and 1 mM proline (Sigma Chemical, St. Louis, MO, USA) at 4°C for no more than 1.5 h prior to placement into culture wells. 24 well flat bottom culture plates (25820, CORNING, Corning, NY, USA) were prepared using standard techniques [29]. The wells were precoated with high T_m agarose gel (Bio-Rad Laboratories, Richmond, CA, USA). The explants were suspended in 1 mL of a 1:1 mixture of 1.0% low T_m agarose gel (Bio-Rad Laboratories, Richmond, CA, USA) and 2 × normal concentration DMEM. The final suspension medium contained 0.5% low T_m agarose gel, 1X DMEM, 1 mM proline, Pen/Strep (50 U/mL & 50 mg/mL), and 10 ng/mL transforming growth factor-β1 (TGF-β1 from porcine platelets, R& D Systems, Minneapolis, MN, USA). The agarose gel was allowed to fully congeal at 4°C for 5 min. Each 1.0 mL gel layer was covered with 1.0 mL DMEM containing: 10% fetal calf serum (Lot # 37N0840, FCS: GIBCO BRL, Grand Island, NY, USA), 1 mM proline, Pen/Strep (50 u/mL & 50 μ/gmL), 50 μg/mL ascorbic acid, and 10 ng/mL TGF-β1. The medium above the gel layer was replaced every second day. Vitamin C was added daily for a final concentration of 25 μg/mL. The TGF-β that was added to the medium for the first 2 days of culture, was also added to the DMEM used to make the agarose so that the concentration was homogeneously distributed throughout the agarose and liquid culture medium. Cultures were maintained at 37°C and 5% CO_2/ 95% air.

After the preliminary study across multiple age groups, each age group (2, 6, 12, 24 months) consisted of six rabbits. Explants were divided equally amongst the analysis groups. These were removed from culture at the time points at which each respective outcome normally peaks (tritiated thymidine uptake: day 3, sulfate uptake: day 21, type II collagen and cartilage histomorphometry: 6 weeks) [19]. The experiments were performed twice on different days using an additional 24 rabbits (six rabbits per age group). This permitted testing of repeatability of the experiment and reliability of the data. A total of 736 periosteal explants from 82 rabbits were used.

Skeletal maturity

Skeletal maturity was evaluated by weighing the rabbits and examining the distal femoral and proximal tibial growth plates grossly and histologically at the time of periosteal harvesting. Whole joints including the distal femora and proximal tibiae, were decalcified and sectioned for histological examination, except for the 24 month-old rabbits, whose skeletal maturity was certain. This was done using an additional 6 rabbits (2 rabbits per age group). Safranin O and H & E stains were obtained. Thus, a total of 88 rabbits were used in this study.

Histomorphometry

After 6 weeks in culture, specimens were fixed in 10% neutral buffered formalin, embedded in paraffin, and a 3 μm thick section cut from the middle of each specimen and stained with safranin O/fast green. This sampling method has been shown to be reproducible and to represent the percentage of cartilage in an explant [6]. They were analyzed by a blinded observer using computerized histomorphometry to determine the percentage of the tissue that was cartilage [27]. This was performed with a custom-designed software application using the Vidas 2.1 Image Analysis Program from Zeiss (Kontron customized by Carl Zeiss Canada, Don Mills, Ontario, Canada). The analysis of cartilage is based on the uptake of safranin O stain, with the thresholds and combinations of red, green and blue colors distinguished automatically. With the cartilaginous areas staining red with safranin O and the non-cartilaginous areas staining blue-green with fast green, the computer calculates the cartilage yield (i.e., percent area of cartilage) in each explant by measuring the red-stained region of the histological section and by expressing it as a percentage of the whole section's area [27].

Collagen typing

After 6 weeks in culture, quantitative collagen typing was performed using a published technique for measuring the relative amount of type II collagen with respect to type I collagen in tissue samples [32]. This technique has been modified to permit the analysis of very small samples (1–10 μg) without initial purification of the collagen [24]. Samples were weighed for wet weight, and the collagen peptides were cleaved with 0.5 ml 5% cyanogen bromide (CNBr) in de-aerated 88% formic acid. In preparation for electrophoresis, the samples were dissolved in a sample buffer containing 0.063 molar Tris-HCL, pH 8, 3.3% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue, at a concentration of 8 μg (wet weight) of sample per microliter of sample buffer. A 1 microliter volume of sample was loaded onto 20% gels, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out using a Phast System (Pharmacia LKB, Uppsala, Sweden). A standard derived from articular cartilage was run in the last lane in each gel and analyzed to ensure that the banding pattern for 100% type II collagen was accurately represented in each gel. The gels were stained with Coomassie blue and scanned on a laser densitometer (Pharmacia LKB). The percentage of type II collagen with

respect to type I collagen was determined by measuring the ratio of the α1(II)CB10 to the α1(I)CB7,8 and α1(II)CB11 peaks in each lane.

^{35}S-sulfate uptake

^{35}S-sulfate incorporation was measured on day 21 to be used as an indicator of glycosaminoglycan synthesis. To measure ^{35}S-sulfate incorporation, the explants were incubated with 5 μCi/ml of ^{35}S-sulfate for 24 h. The explants were digested overnight in 0.05% proteinase K (Boehringer Mannheim, Indianapolis, IN 6250-0414). The digested explants were then dissolved with 0.5 N NaOH, eluted on PD10 columns (17-0851-01 Pharmacia Biotech, Uppsala, Sweden), and counted on a scintillation counter [12]. Incorporated radioactivity was normalized to explant wet weight.

^{3}H-Thymidine incorporation

Proliferation was evaluated by measuring ^{3}H-thymidine uptake on day 3 [1,15]. Explants (2 × 3 mm^2 in size) were labeled for 24 h with 5 μCi of [methyl-^{3}H] thymidine (Amersham Life Sciences, United Kingdom), then digested in 0.05% proteinase K (Boehringer Mannheim, Mannheim, Germany). DNA was precipitated with 10% TCA using bovine serum albumin as a carrier, dissolved in 0.25 M NaOH, and ^{3}H-thymidine incorporation counted on a scintillation counter [14]. To determine the labeling index explants from 2, 6, 12 and 24 month-old rabbits were cultured for 1–14 days (in daily increments), and labeled with ^{3}H thymidine for 24 h before being taken from culture.

Autoradiography

Autoradiography was performed by Molecular Histology Labs (Gaithersburg, MD, USA), on tissues that had been grown in culture for one through 14 days. They were labeled for 24 h prior to harvest with [methyl-^{3}H] thymidine, washed with PBS and stored in 10% neutral buffered formalin. Each explant was meticulously oriented 'on edge' during the paraffin embedding process such that all of the sections obtained from it provided cross-sections of the cambium and fibrous layers. To determine the percentage of cells undergoing proliferation, a 'labeling index' was measured. The labeling index was defined as the number of labeled cells divided by the total number of cells multiplied by 100. The method of cell counting involved counting the cells in approximately 30% of the cross-sectional area of each histology section [39].

Periosteal morphology

Periosteal morphology was measured on day 0 with the periosteum intact on the bone (Table 1). (We also measured it on the 3 × 4 mm^2 explants, but found that the measurements were more consistent when the periosteum was left intact.) (Table 2). One periosteal explant was taken from each rabbit to study the periosteal morphology. Just as in the autoradiography method above, each explant was meticulously oriented 'on edge' during the paraffin embedding process such that all of the sections obtained from it provided perfect cross-sections of the cambium and fibrous layers. Using techniques previously published, the cambium layer thickness and total cell count were measured at the time of explantation for each of the four main age groups (2, 6, 12, 24

Table 1
Periosteum intact on bone[a]

		Rabbit age (months)				P-value[b]	Significant comparisons[c]
		2	6	12	24		
Cambium layer	Layer thickness (μm)	118 ± 43	26 ± 9.6	15 ± 2.6	8.3 ± 3	<0.0001	2 > 6, 12, 24
	Normalized cell number (cells/mm)	393 ± 106	80 ± 27	50 ± 10	29 ± 5	<0.0001	2 > 6, 12, 24 6 > 12, 24
	Cell density (cells/cm^2)	3.6 ± 0.9	3.4 ± 1.4	3.6 ± 1.1	3.8 ± 1	NS	N/A
Fibrous layer	Layer thickness (μm)	172 ± 42	121 ± 27	106 ± 45	68 ± 21	<0.0001	2 > 6, 12, 24
	Normalized cell number (cells/mm)	200 ± 61	112 ± 28	97 ± 22	61 ± 21	<0.0001	2 > 6, 12 > 24
	Cell density (cells/cm^2)	1.2 ± 0.3	1 ± 0.3	1 ± 0.4	1 ± 0	NS	N/A

[a] Data shown are means ±1 S.D. with $n = 12$.
[b] Global comparison performed using 1 Factor Analysis of Variance (ANOVA). Pairwise differences identified using Duncan's New Multiple Range post-hoc testing.
[c] Using $\alpha = 0.05$.

Table 2
Eplanted periosteum[a]

		Rabbit age (months)				P-value[b]	Significant comparisons[c]
		2	6	12	24		
Cambium layer	Layer thickness (μm)	130 ± 22	37 ± 8.6	21 ± 9	8.1 ± 9	<0.0001	2 > 6, 12, 24
	Normalized cell number (cells/mm)	300 ± 34	72 ± 16	39 ± 11	16 ± 12	<0.0001	2 > 6, 12, 24 6 > 12, 24
	Cell density (cells/cm^2)	2.4 ± 0.7	2 ± 0.7	2 ± 0.7	1.7 ± 1	NS	N/A
Fibrous layer	Layer thickness (μm)	248 ± 36	179 ± 34	162 ± 33	155 ± 35	<0.0001	2 > 6, 12 > 24
	Normalized cell number (cells/mm)	154 ± 12	86 ± 5	67 ± 4	64 ± 4	<0.0001	2 > 6, 12 > 24
	Cell density (cells/cm^2)	0.6 ± 0.2	0.5 ± 0.2	0.4 ± 0.1	0.4 ± 0	NS	N/A

[a] Data are shown ±1 S.D. with $n = 12$.
[b] Global comparison performed using 1 Factor Analysis of Variance (ANOVA). Pairwise differences identified using Duncan's New Multiple Range post-hoc testing.
[c] Using $\alpha = 0.05$.

months). The total cell number in the cambium layer, is simply the mathematical product of the cell density and the total volume of tissue. To represent this, we derived a normalized cell number, or cellularity, which is the product of cell density and cambium layer thickness.

Data Analyses

Sixteen small periosteal explants (eight per leg), or four large periosteal explants (two per leg) could be obtained from each rabbit. To control for rabbit, only one periosteal explant from each rabbbit was assigned to any one group. Therefore, the sample size (n) represents not just the number of periosteal explants but also the number of rabbits. Each experiment was executed in two identical sub-experiments a week apart, by dividing the total number of rabbits into two equal groups (each with sufficient sample sizes to stand on their own as individual experiments) which were then combined into one group after confirming that the results were the same in the two sub experiments. The advantage of this design includes not just opportunity to confirm reproducibility, but also improved management of the randomization and controlling for rabbit with large sample sizes, given that the surgeries have to be performed all in 1 day. Data were then combined and averaged for analysis. Statistical analyses were performed using an ANOVA with Duncan Multiple Range post-hoc testing to determine significance among groups at $P < 0.05$. The number of explants in each group was calculated to give a sample size of $n = 12$. Data are represented as means ±1 standard error unless otherwise stated, as the data were averaged from two or more experiments.

These experiments were approved by the Institutional Animal Care and Use Committee at Mayo Clinic.

Results

Rabbit weights and skeletal maturity

The weights of the rabbits increased steadily until six months of age, at which time they reached a plateau around 4 kg (Fig. 1(A)). Gross and histological examinations at the time of sacrificing the animals for periosteal harvesting, revealed that the growth plates in all of the 2 month-old rabbits were open, while those in the 6 and 12 month-old rabbits were closed (Fig. 1(B)). These observations are consistent with published data, which have shown that skeletal maturation occurs by the age of 6 months [10]. Regarding the subsequent studies comparing 2, 6, 12, and 24 month-old rabbits, the exact ages were as follows. The '2-month-old' rabbits were all 2 months and 13 days old; the '6-month-old' rabbits were all 6 months and 18 days old. Rabbits in the '12-month-old' group were 12 months and 10–18 days, with an average of 12 months and 15 days. In the '24-month-old' group the ranged from 24 months and 21 days to 25 months and 23 days with an average of 25 months and 10 days.

Chondrogenic potential

Chondrogenic potential was measured using a standard cartilage assay, which involves culturing the explants in a chondrogenic environment for 6 weeks, then determining the percentage of total tissue that is cartilage using automated histomorphometry as previously published and described in the methods [27]. The horizontal study across age groups from 2 weeks to 2 years showed that the chondrogenic potential of periosteum decreased significantly with age ($P < 0.0001$) in a pattern that was inversely related to the weight of the rabbits (Fig. 2 vs. Fig. 1(A)). Chondrogenesis was maximal in the 1.5 month-old rabbits (cartilage yield =

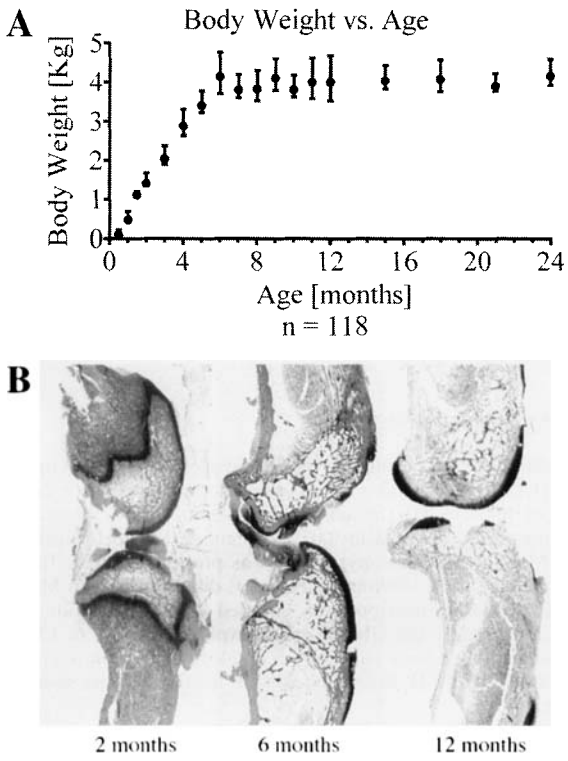

Fig. 1. (A) The weights of the rabbits increased steadily until 6 months of age, at which time they reached a plateau around 4 kg; (B) The growth plates (arrows) in all of the 2 month-old rabbits were open, while those in the 6, 12 and 24 month-old rabbits were closed, confirming that skeletal maturity had occurred by 6 months of age in these rabbits.

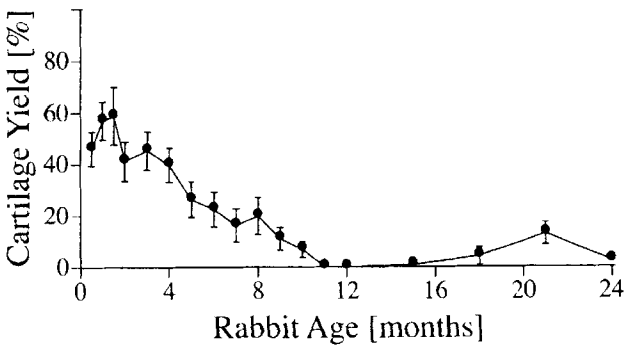

Fig. 2. The chondrogenic potential of periosteal explants decreased significantly with age ($P < 0.0001$) as indicated by the decrease in cartilage yield (i.e. % area of cartilage). At each time point, values represent the means and S.E. of eight explants obtained from each of two rabbits; total n = sixteen per time point).

59 ± 11%), then decreased progressively until 12 months of age. Thereafter, periosteal chondrogenesis was minimal in the 15, 18, 21 and 24 month-old rabbits. Only one rabbit in the 15–24 month age group had explants that formed any significant amount of cartilage. By the time of skeletal maturity at 6 months, periosteal chondrogenesis had declined to less than half (cartilage yield = 23 ± 7%) compared to that in the 2 month-old rabbits.

The experiments involving large numbers of periosteal explants from rabbits aged 2, 6, 12 and 24 months revealed routine production of hyaline cartilage in the explants from the 2 and 6 month-old rabbits, but rarely in those from rabbits aged 12 or 24 months (Fig. 3). Quantitative histomorphometry demonstrated statistically significant differences in chondrogenic potential among these age groups (Fig. 4; $P = 0.0002$). The cartilage yield form the periosteal explants taken from the two month-old rabbits averaged 28 ± 11% cartilage. This was higher (though not statistically different) than that in the periosteal explants from the 6 month-old rabbits (13 ± 8%), the age of skeletal maturity. The quality, and well as the quantity, of cartilage was inferior in the older rabbits (Fig. 3). Chondrogenesis in the periosteal explants from the 12 and 24 month-old rabbits was minimal, with cartilage yields of only 0.006 ± 0.006% and 0.4 ± 0.4% respectively. These were significantly lower than those of the 2 and 6 month-old rabbit explants, but not from each other ($P < 0.05$). The cartilage yields from the periosteal explants from the 2 to 6 month-old rabbits were significantly higher than those from the 12 and 24 month-old rabbits ($P < 0.05$).

Matrix proteins

Indicators of glycosaminoglycan synthesis and type II content correlated with the cartilage yield data, with statistically significant decreases with age of the rabbits from which the periosteal explants were obtained (Fig. 4). The content of type II collagen decreased significantly from 27 ± 8% in the 2 month-old group to 5 ± 2% in the 12 month and 3 ± 2% in the 24 month-old groups ($P < 0.0001$) (Fig. 4(B)).

^{35}S-sulfate incorporation on day 21 of culture, decreased similarly with the age of the donor rabbits (Fig. 4(C)). Uptake was maximal in the periosteal explants from the 2 month-old rabbits, then decreased in a pattern similar to the decline in cartilage yield in the six to 24 month groups (Fig. 4(C); $P < 0.05$).

Cell proliferation

Cell proliferation, as indicated by the uptake of ^{3}H-thymidine into the periosteal explants decreased significantly with age of the donor rabbits from which the periosteal explants had been obtained ($P < 0.05$) (Fig. 4(D)). The trend was similar to the decline seen in chondrogenesis, collagen type II content, and ^{35}S-sulfate uptake (Fig. 4(A–C)). Fig. 4(D) shows that ^{3}H-thymidine in the sixth month group was reduced to 43% of

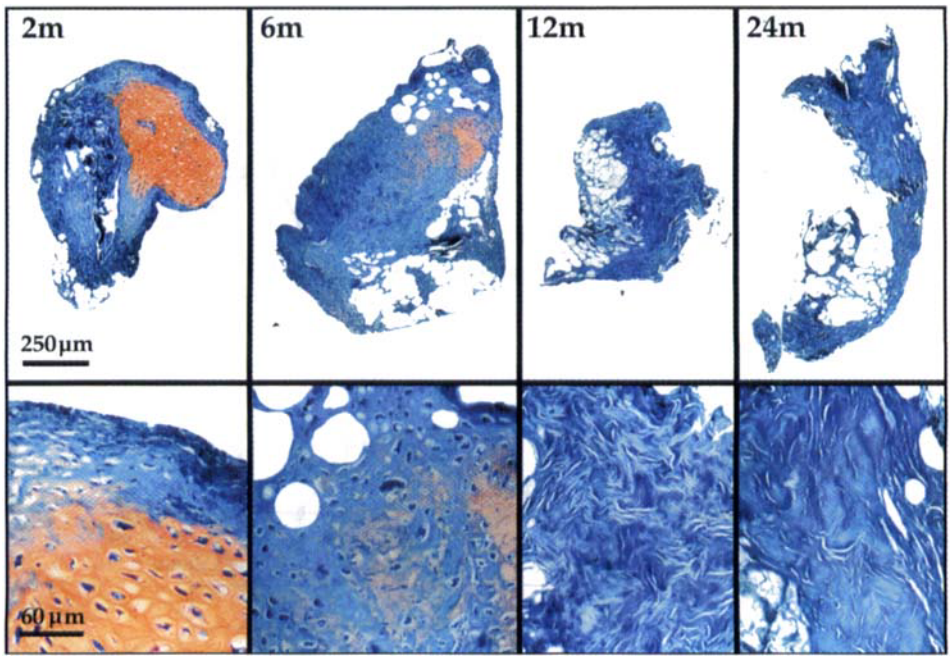

Fig. 3. Photomicrographs representative of the mean cartilage yield assay results (i.e., % area of cartilage) from the 2, 6, 12 and 24 month-old rabbit explants. Hyaline cartilage, which was routinely seen in the explants from the 2 and 6 month-old rabbits, was rarely seen in the 12 or 24 month-old groups.

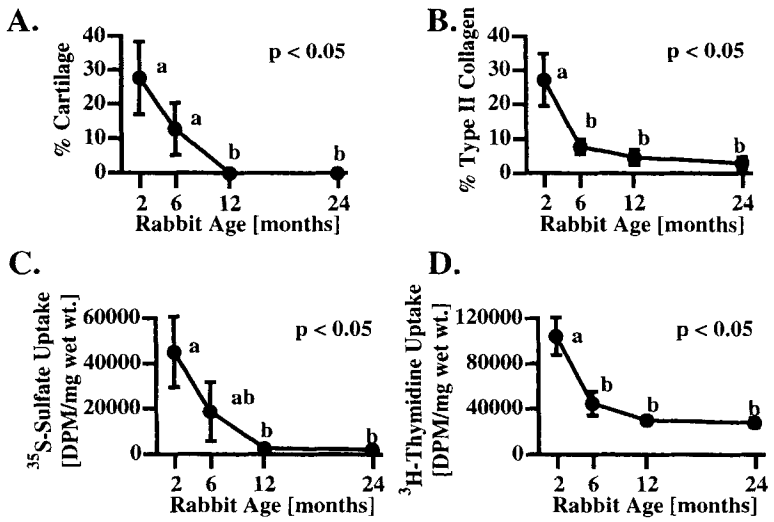

Fig. 4. Comparative data from analyzing periosteal explants from four different age groups – 2, 6, 12 and 24 months. Cartilage yield (i.e. % area of cartilage) (A) and type II collagen (with respect to type I collagen) (B) were assayed after 6 weeks in culture. ^{35}S-sulfate incorporation (C) was measured on day 21 and ^{3}H-thymidine (D) on day 3. The present cartilage, type II collagen and sulfate contents all decreased in similar patters with age, to minimal levels after completion of growth. The correlation between these parameters of chondrogenesis and cell proliferation, as indicated by ^{3}H-thymidine uptake in D was strong. Letters *a*, *b*, and *c* indicate the result of Duncan's New Multiple Range post-hoc testing. Groups with a letter in common are not statistically different from one another.

that seen in the 2 month group, and further reduced in the 12 and 24 month to just 29% of that in the 2 month rabbits ($P < 0.05$).

To determine whether the decline in proliferative activity with age reflected a reduction in the number of cells in the periosteum or a decrease in the percentage of cells undergoing proliferation (or both), explants from all age groups were cultured for 1–14 days, and labeled with ^{3}H thymidine for 24 h before being taken form culture. The autoradiographic sections were obtained and the 'labeling index' was measured. That is, the number of labeled cells were divided by the total number of cells multiplied by 100. Fig. 5 shows that the peak intensities of proliferation at days 4–6 in the cambium layer demonstrated no consistent pattern of change with age.

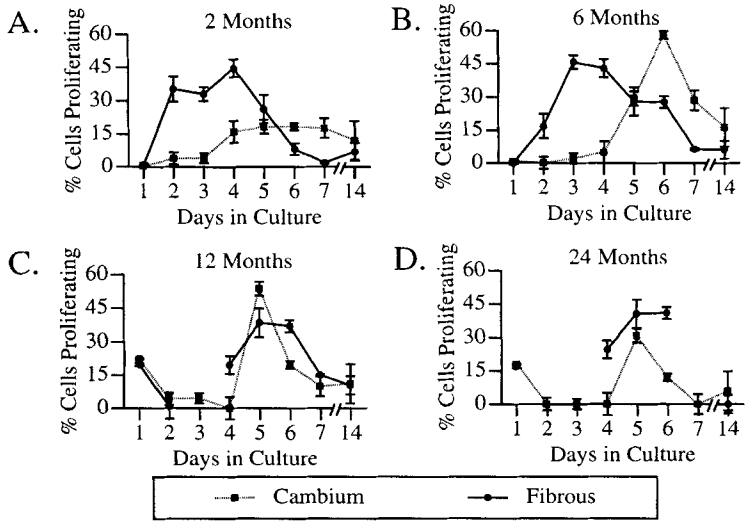

Fig. 5. Labeling indices (Solid line = fibrous layer; dashed line = cambium layer). The labeling index was defined as: (labeled cells/total cells) × 100, as counted on autoradiographs obtained after culturing periosteal explants in the presence of ^{3}H-thymidine. The trends indicate that proliferation in the fibrous layer preceded that in the cambium layer, and that while the labeling indices change over time, there were no obvious age-related differences. In particular, there did not appear to be a reduction in the percentage of proliferating cells in the cambium layer with age.

Periosteal morphology

Periosteal morphology changed significantly with age, as detailed in Tables 1 and 2 and shown in Figs. 6 and 7. Variability was higher in the explanted periosteal samples, which showed some evidence of sporadic areas of cambium cell layer losses. Because the measurements were more consistent when the periosteum was left intact on the bone, we believe the data for periosteal morphology to be more reliable in the intact specimens (Table 1) than in the explants (Table 2). Most notably, the cambium layer become thinner with age, although changes were seen in the fibrous layer as well. At 2 months the thickness of the cambium layer of the periosteum, while still intact on the bone, was 118 ± 43 μm. This diminshed significantly ($P < 0.0001$) in the 6 month rabbits to 26 ± 9.6 μm and 15 ± 2.6 μm at 12 months and 8.3 ± 3 μm at 24 months respectively. Cell density was three times as high in the cambium layer as in the fibrous layer, but did not change significantly with age (Fig. 7(C); $P > 0.8$). The normalized total cell number, or cellularity, in the cambium layer, was calculated as the product of cell density and cambium layer thickness. The normalized total cell number in the cambium layer decreased from 393 ± 106 at 2 months to 80 ± 27 at 6 months, 50 ± 10 at 12 months 29 ± 5 cells per mm of length of periosteum ($P < 0.0001$) (Fig. 7). The 2 and 6 month groups were significantly different from each other and from the 12 and 24 month groups. This indicates that the total number of available chondrocyte precursors decreases with age, as these cells are located in the cambium layer [23].

Changes in the fibrous layer are also evident with age, but less dramatically than the cambium layer (Fig. 7). The fibrous layer diminished significantly in thickness and total cell number ($P < 0.0001$). The thickness declined from 172 ± 42 μm at 2 months to 68 ± 21 μm at 24 months. As the cell density did not change significantly with age, the decline in total cell number reflected the decline in fibrous layer thickness.

Discussion

The present study has demonstrated that the potential of periosteum for chondrogenesis declines with age.

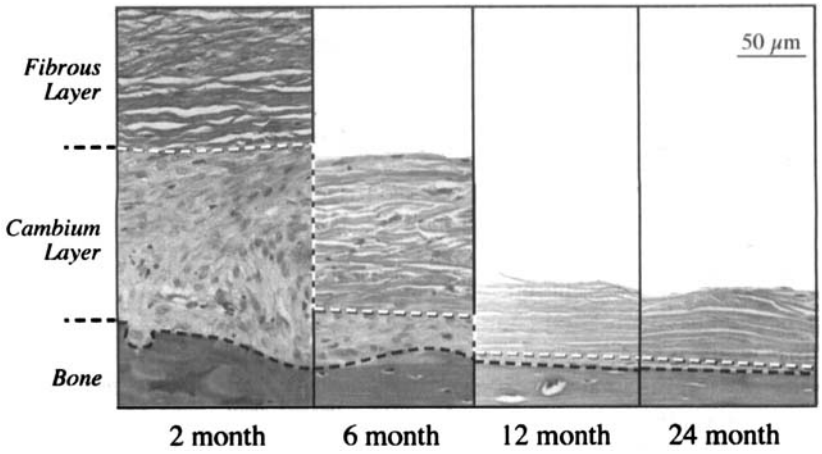

Fig. 6. Representative photomicrographs of intact periosteum still on the underlying tibial bone, from the same four age groups, showing how both cambium and fibrous layers become thinner with age. Most notable is the marked reduction in total cell number in the cambium layer, which contains the chondrocyte precursors.

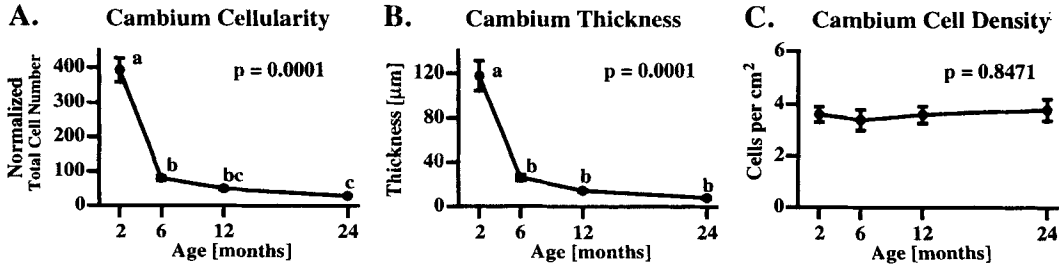

Fig. 7. Normalized total cell number, layer thickness and cell density of the periosteal cambium layer versus age. In the cambium layer, there is a marked decrease in cell number and layer thickness with increased age. The total cell number is normalized to a standardized length of periosteum (1 mm). Letters *a*, *b*, and *c* indicate the result of Duncan's New Multiple Range post-hoc testing. Groups with a letter in common are not statistically different from one another.

In rabbits, this chondrogenic potential of peirosteum begins declining shortly after birth to less than 50% by the time of skeletal maturity at six months. From 6 to 12 months, i.e., an age corresponding to approximately twice that at skeletal maturity, this chondrogenic potential continues to decline, until it reaches a steady state minimal level. Although chondrogenesis was seen in much older rabbits, it was much less frequently.

These data are important for four reasons. First, they establish a baseline for interpretation and planning of future studies related to experimental cartilage formation of repair using periosteum. Second, they offer an opportunity to study and elucidate the mechanisms responsible for the age-dependent decline in chondrogenic potential, not just of periosteum, but in general. Third, if the mechanisms responsible for this age-dependent decline in chondrogenesis can be elucidated and overcome, it may be possible to apply similar technologies to improve the quality of cartilage repair in younger individuals as well.

The periosteal explants were most chondrogenic prior to skeletal maturity, but continued to demonstrate some chondrogenic potential when taken from rabbits approximately twice the age of skeletal maturity. Nakahara et al., found that isolated human periosteal cells displayed chondrogenic potential if the donors were less than 19 years of age, but not if they were 22 years or older [21]. It would be reasonable to anticipate a reduction in satisfactory outcomes with increasing age in adults. Most patients with damaged cartilage are past skeletal maturity, and the prevalence of this problem rises with increasing age. Experimental studies in lab animals show that aging is one of the main obstacles to successful cartilage healing [7,16,17,25,38]. Therefore, it will be necessary to understand the age-related changes in the potential for healing or regenerating cartilage.

The most important observation made in the present study was that the chondrogenic potential was related closely to the total number of cells in the cambium layer of the periosteum, as previously documented for variations in chondrogenic potential with different donor sites [6]. This is the layer in which the chondrocyte precursor cells reside [42]. Tonna Cronkite [42] speculated that the progressive thinning of periosteum with age may have been due to stretching with radial bone growth, incorporation of periosteal cells into the cortical bone layer, and/or a decrease in proliferative capacity with age.

The observation that periosteal chondrogenic potential is determined by the total number of cells in the cambium layer is important, as it stimulates proliferation of the chondrocyte precursors or developing chondrocytes as a possible means for enhancing cartilage repair. As stated by Critchlow et al. [4] cell proliferation is the crucial first step in the development of periosteal cartilage formation. In a recent report we showed periosteum responds to mechanical stimulation in the form of dynamic fluid pressure (DFP) by increasing cell proliferation prior to cartilaginous differentiation, which is also increased [39]. This is consistent with reports by Veldhuijzen et al. [43] in which mechanical factors have been shown to influence chondrocyte proliferation.

In summary, these experiments confirm that chondrogenic potential of periosteum decreases significantly with age. This decrease in cartilage formation is associated principally with a decline in the size of the chondrocyte precursor pool. Thus, one possibility for improving cartilage formation by periosteal transplantation in patients beyond skeletal maturity would be to stimulate cell proliferation early during chondrogenesis and/or to increase the final number of chondrocytes by incorporating chondrocyte precursors, mesenchymal stem cells, or chondrocytes in the treatment.

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References

[1] Benya PD, Padilla SR. Dihydrocytochalasin B enhances transforming growth factor-beta-induced reexpression of the differentiated chondrocyte phenotype without stimulation of collagen synthesis. Exp Cell Res 1993;204:268–77.

[2] Bronk JT, Urabe K, Liang T, Bolander ME. The effect of age on fracture repair in a rat femur model. Trans Orthop Res Soc 1996;21:200.

[3] Bruder SP, Fink DJ, Caplan AI. Mesenchymal stem cells in bone development, bone repair, and skeletal regeneration therapy. (Review). J Cell Biochem 1994;56:283–94.

[4] Critchlow MA, Bland YS, Ashhurst DE. The effects of age on the response of rabbit periosteal osteoprogenitor cells to exogenous transforming growth factor beta 2. J Cell Sci 1994;107:499–516.

[5] Engdahl E. Bone regeneration in maxillary defects. An experimental investigation on the significance of the periosteum and various media (blood, surgical, bone marrow and bone grafts) on bone formation and maxillary growth. Scand J Plast Reconstr SurgSuppl 8 1972:1–79.

[6] Gallay SH, Miura Y, Commisso CN, Fitzsimmons JS, O'Driscoll SW. Relationship of donor site to chondrogenic potential of periosteum in vitro. J Orthop Res 1994;12(4):515–25.

[7] Helbing G. Transplantation of isolated chondrocytes in articular cartilage defects. Regeneration of adult hyaline cartilage with fetal chondrocytes. Fortschr Med 1982;100:83–7.

[8] Hendrickson DA, Nixon AJ, Grande DA, Todhunter RJ, Minor RM, Erb H, Lust G. Chondrocytes-fibrin matrix transplants for resurfacing extensive articular cartilage defects. J Orthop Res 1994;12(4):485–97.

[9] Hoikka VE, Jaroma HJ, Ritsilä VA. Reconstruction of the patellar articulation with periosteal grafts. 4-year follow-up of 13 cases. Acta Orthop Scand 1990;61:36–9.

[10] Jaroma HJ, Ritsila VA. Reconstruction of patellar cartilage defects with free periosteal grafts. An experimental study. Scand J Plast Reconstr Surg Hand Surg 1987;21:175–81.
[11] Kaweblum M, Aguilar MC, Blancas E, Kaweblum J, Lehman WB, Grant AD, Strongwater AM. Histological and radiographic determination of the age of physeal closure of the distal femur, proximal tibia, and proximal fibula of the New Zealand white rabbit. J Orthop Res 1994;12(5):747–9.
[12] Kimura JH, Caputo CB, Hascall VC. The effect of cycloheximide on synthesis of proteoglycans by cultured chondrocytes from the Swarm rat chondrosarcoma. J Biol Chem 1981;256:4368–76.
[13] Korkala OL. Periosteal primary resurfacing of joint surface defects of the petella due to injury. Injury 1988;19:216–8.
[14] Labarca C, Paigen K. A simple, rapid, and sensitive DNA assay procedure. Anal Biochem 1980;102:344–52.
[15] Livne E, Weiss A. In vitro effect of harmones and growth factors on the incorporation of [^{3}H] leucine, [^{35}S] sulphate and [^{3}H] proline by chondrocytes of aging mice. Mech Ageing Dev 1993;72:213–29.
[16] Mankin HJ. Localization of fritiated thymidine in articular cartilage. II with I. Repair in immature rabbits. J Bone Joint Surg 1962;44-A:688–98.
[17] Mankin HJ. Localization of tritiated cytidine in articular cartilage of immature and adult rabbits after intra-atricular injection. Lab Invest 1963;12:543–8.
[18] Melcher AH, Accursi GE. Osteogenic capacity of periosteal and osteoperiosteal flaps elevated from the parietal bone of the rat. Arch Oral Biol 1971;16:573–80.
[19] Miura Y, O'Driscoll SW. General time course of periosteal chondrogenesis in vitro with TGF-β1. Orthop Trans 1994;18:686.
[20] Miura Y, O'Driscoll SW. Culturing periosteum in vitro: the influence of different sizes of explants. Cell Transplant 1998;7: 453–7.
[21] Nakahara H, Goldberg VM, Caplan AI. Culture-expanded human periosteal-derived cells exhibit osteochondral potential in vivo. J Orthop Res 1991;9(4):465–76.
[22] Nixon AJ, Hendrickson DA, Lust G, Grande DA. Chondrocyte laden fibrin polymers effectively resurface large articular cartilage defects in horses. Orthop Trans 1992. p. 431.
[23] O'Driscoll SW. Articular cartilage regeneration using periosteum. Clin Orthop 1999;367(Suppl S):186–203.
[24] O'Driscoll SW, Commisso CN, Fitzsimmons JS. Type II collagen quantification in experimental chondrogenesis. Osteoarthritis Cartilage 1995;3:197–203.
[25] O'Driscoll SW, Keeley FW, Salter RB. The chondrogenic potential of free autogenous periosteal grafts for biological resurfacing of major full-thickness defects in joint surfaces under the influence of continuous passive motion. An experimental investigation in the rabbit. J Bone Joint Surg 1986;68A:1017–35.
[26] O'Driscoll SW, Keeley FW, Salter RB. Durability of regenerated articular cartilage produced by free autogenous periosteal grafts in major full-thickness defects in joint surfaces under the influence of continuous passive motion. A follow-up report at one year. J Bone Joint Surg 1998;70A:595–606.
[27] O'Driscoll SW, Marx RG, Fitzsimmons JS, Beaton DE. Method for automated cartilage histomorphometry. Tissus Eng 1999;5:13–23.
[28] O'Driscoll SW, Meisami B, Miura Y, Fitzsimmons JS. Viability of periosteal tissue obtained postmortem. Cell Transplant 1999;8: 611–6.
[29] O'Driscoll SW, Recklies AD, Poole AR. Chondrogenesis in periosteal explants. An organ culture model for in vitro study. J Bone Joint Surg 1994;76A:1042–51.
[30] O'Driscoll SW, Salter RB. The induction of neochondrogenesis in free intra-articular periosteal autografts under the influence of continuous passive motion. An experimental investigation in the rabbit. J Bone Joint Surg 1984;66A:1248–57.
[31] O'Driscoll SW, Salter RB. The repair of major osteochondral defects in joint surfaces by neochondrogenesis with autogenous osteoperiosteal grafts stimulated by continuous passive motion. An experimental investigation in the rabbit. Clin Orthop 1986;208:131–40.
[32] O'Driscoll SW, Salter RB, Keeley FW. A method for quantitive analysis of ratios of types I and II collagen in small samples of articular cartilage. Anal Biochem 1985;145:277–85.
[33] Ohlsen L. Cartilage regeneration form periochondrium. Experimental studies in rabbits and dogs. Acta Univ Upsal (Abstract) Upps Fac Med. Ph.D. Thesis. 1976. p. 252.
[34] Ohlsen L. Cartilage regeneration from periochondrium. Experimental studies and clinical applications. Plast Reconstr Surg 1978;62:507–13.
[35] Ritsila V, Alhopuro S. Regeneration of articular cartilage defects with free perichondrial grafts. IRCS Medical Science: Connective Tissue, Skin and Bone; Surg Transplantation 1975;3:49–50.
[36] Rubak JM, Poussa M, Ritsilä V. Chondrogenesis in repair of articular cartilage defects by free periosteal grafts in rabbits. Acta Orthop Scand 1982;53:181–6.
[37] Rubak JM, Poussa M, Ritsilä V. Effects of joint motion on the repair of articular cartilage with free periosteal grafts. Acta Orthop Scand 1982;53:187–91.
[38] Salter RB, Simmonds DF, Malcolm BW, Rumble EJ, MacMichael D, Clements ND. The biological effects of continuous passive motion on the healing of full-thickness defects in articular cartilage. An experimental investigation in the rabbit. J Bone Joint Surg 1980;62A:1232–51.
[39] Saris D, Sanyal A, An KN, Fitzsimmons JS, O'Driscoll SW. Periosteum responds to dynamic fluid pressure by proliferating in vitro. J Orthop Res 1999;17(5):668–77.
[40] Skoog T, Ohlsén L, Sohn SA. Perichondrial potential for cartilagenous regeneration. Scand J Plast Reconstr Surg 1972;6: 123–5.
[41] Skoog T, Ohlsén L, Sohn SA. The chondrogenic potential of periochondrium. Chir Plastica (Berl) 1975;3:91–103.
[42] Tonna EA, Cronkite EP. An autoradiographic study of periosteal cell proliferation with tritiated thymidine. Lab Invest 1962;11: 455–62.
[43] Veldhuijzen JP, Bourret LA, Rodan GA. In vitro studies of the effect of intermittent compressive forces on cartilage cell proliferation. J Cell Physiol 1979;98:299–306.
[44] Wakitani S, Goto T, Pineda SJ, Young RG, Mansour JM, Caplan AI, Goldberg VM. Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. J Bons Joint Surg 1994;76A:579–92.