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Normal and healing ligament vascularity: a quantitative histological assessment in the adult rabbit medial collateral ligament

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ABSTRACT

Normal and healing adult rabbit medial collateral ligaments (MCL) have been assessed for microvascular anatomy using a quantitative image analysis methodology. MCL preparation by ink-gelatin perfusion enabled acceptable visualisation of microvascular channels within the tissue. Fifteen adult rabbits were studied; 3 normal rabbits formed an external time-zero control group; 12 animals received a standardised gap injury to the right MCL. The ligament injury was permitted to heal for 3, 6, 17 or 40 wk (3 animals in each healing group). Results confirmed that the normal MCL is hypovascular (about 1.46% vascularity by area) and that microvascular channels are highly organised and oriented longitudinally deep within the tissue. Healing MCL scar becomes twice as vascular as normal ligament early on, but returns to near normal values by 40 wk. Microvascular channels appear less organised in scar than in contralateral controls, but remodel with time. The directional scatter and spatial extent of ligament microvascular channels is quantifiable in normal and healing tissues.

Key words: Image analysis; joints; microvasculature; injury; repair.

INTRODUCTION

Vascularity is thought to be crucial to normal tissue health and function, yet very little is known (in quantitative terms) about the microanatomical distribution and physiological roles of blood vessels in articular ligaments. Compared with other vascular joint tissues such as synovium or bone, ligament appears to be relatively hypovascular (Bray et al. 1993). Despite this limited vascularity, it is widely held that blood supply is important to normal ligament health and function (Marshall et al. 1979; McFarland et al. 1986), and that the vascular response to ligament injury or transplantation is a potentially important determinant of the rates and endpoints of the healing process (Arnoczky et al. 1979; Oegema et al. 1988).

Current concepts about ligament blood supply are largely based on subjective observations that ligaments have a limited but grossly visible blood supply (Bray et al. 1990); that blood supply to tendons and ligaments can change, possibly as a result of ageing (Carr & Norris, 1989) or chronic damage (Clancy, 1990); and that certain ligaments demonstrate a limited vascular response to injury, possibly resulting in inadequate or incomplete healing in some circumstances (Alm & Stromberg, 1974; Arnoczky et al. 1979; Oegema et al. 1988). Recently, it has been shown that tendon cells demonstrate aerobic metabolic pathways implicating an important role for O_2 delivery even in these hypovascular tissues (Birch et al. 1994). In fact, functional demands are known to influence blood flow in the anterior cruciate ligament (ACL) (Dunlap et al. 1989; Simkin et al. 1990), where data suggest that important acute and chronic vascular reactions occur in joint tissues in response to imposed loading conditions.

Experimental studies using methods of vascular perfusion with opaque dyes have helped to elucidate a number of important histological characteristics of ligament blood vessels (Brookes & Harris, 1957;

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Scapinelli, 1968; Arnoczky et al. 1979; Weiss, 1988; Bray et al. 1993). Ligaments contain a sparse but clearly organised microvasculature, and different regions of ligament tissue appear to have distinct microvascular patterns and distributions (Arnoczky et al. 1979; Bray et al. 1990; Chowdhury et al. 1991). The epiligamentous (covering) layer of ligaments appears to contain a relative abundance of blood vessels. Vessels in the epiligament are randomly dispersed in a loose connective tissue matrix; they branch extensively, forming anastomotic networks of interconnected vessels (Arnoczky & Warren, 1988; Chowdhury et al. 1991). In contrast, intraligamentous vessels appear to be more organised, running parallel with the collagen fibre bundles of the densely organised matrix (Arnoczky, 1983, 1985; Arnoczky & Warren, 1988; Bray et al. 1990).

Despite many investigations of ligament vascularity, it is still not known whether different ligaments, or anatomically similar ligaments from different species have comparable blood supplies with similar microvascular functions. Moreover, as a result of limited quantitative information about ligament microvascular densities and distributions, and the fact that studies of healing ligaments are often difficult to compare (because of methodological differences), the processes of ligament revascularisation during healing have only been described in subjective terms.

The main objective of this study was to define various aspects of the microvasculature of normal and healing medial collateral ligaments quantitatively. We also wanted to determine the effect that an injury has on the quantity (vascularity by area) and quality (vessel angular dispersion) of the microvasculature of a ligament scar. A standardised injury of the rabbit medial collateral ligament (MCL) was studied because rabbit MCL microvascular anatomy has been described (nonquantitatively) in some detail elsewhere (Bray et al. 1990), and an image analysis technique has been developed to quantify the degree of vascularity and blood vessel segment angular distribution in this structure (Eng et al. 1992). We wished to describe certain aspects of normal ligament vascularity and to characterise the vascular response to ligament injury. The hypothesis tested is that healing MCL scar will show measurable microvascular differences compared to normal. Some of this work has already been published in abstract form (Bray et al. 1993).

MATERIALS AND METHODS

Animal groups

A total of 15 adult (12-month-old) female New Zealand White rabbits obtained from a single supplier (Riemans Fur Ranche, St Agatha, Ontario, Canada) were studied. Three animals were used as external controls; external controls were killed and MCL tissues were studied at an interval designated as 'time zero', usually within 2 wk of supply. The remaining 12 animals comprised 4 experimental groups of 3 each; experimental animals all received a standard 4 mm gap injury (Chimich et al. 1990) to the right MCL at 'time-zero', and were allowed to heal over 3 early (3, 6 and 17 wk) intervals and 1 late (40 wk) interval. The healing MCLs are referred to as 'MCL scar' because only the repair tissue filling the original 4 mm gap injury site is assessed in this study. The left knees of the experimental animals were not operated on, providing internal or 'contralateral' MCL controls. All animals were allowed free unrestricted activity in cages of size $380 \times 800 \times 610$ mm (height, width, depth).

MCL preparation and processing

At the time of killing of the animals, both femoral arteries were cannulated and perfused with an India ink and gelatin solution according to a standardised protocol (Bray et al. 1990). The lower limbs were then removed at the hip joint and cooled to -4 °C for 2–4 h. The MCLs were fixed in situ with phosphate-buffered formalin (PBF) at a controlled knee flexion angle of 90°. Ligaments were removed in toto excluding their bony insertion sites, and further fixed for several days in PBF at 4 °C. MCL specimens were then individually frozen, cut into serial 50 μm thick sections, and contact mounted on glass slides maintaining a parasagittal orientation for image analysis (Fig. 1). The normal MCL and MCL scar tissues were retrieved as very specific and reproducible samples.

In the normal MCL, deep ligament tissue can be distinguished from epiligamentous covering tissue by using a polarising light microscope; deeper ligament tissue is characterised by a distinctive crimp pattern of the collagenous matrix, but this pattern is absent in epiligamentous tissue where the matrix is organised differently (Chowdhury et al. 1991). Only normal and control contralateral tissue from deep within the ligament (as opposed to superficial epiligamentous tissues) was analysed in this study (Fig. 2*A*). After creation of the MCL gap injury, the site was marked

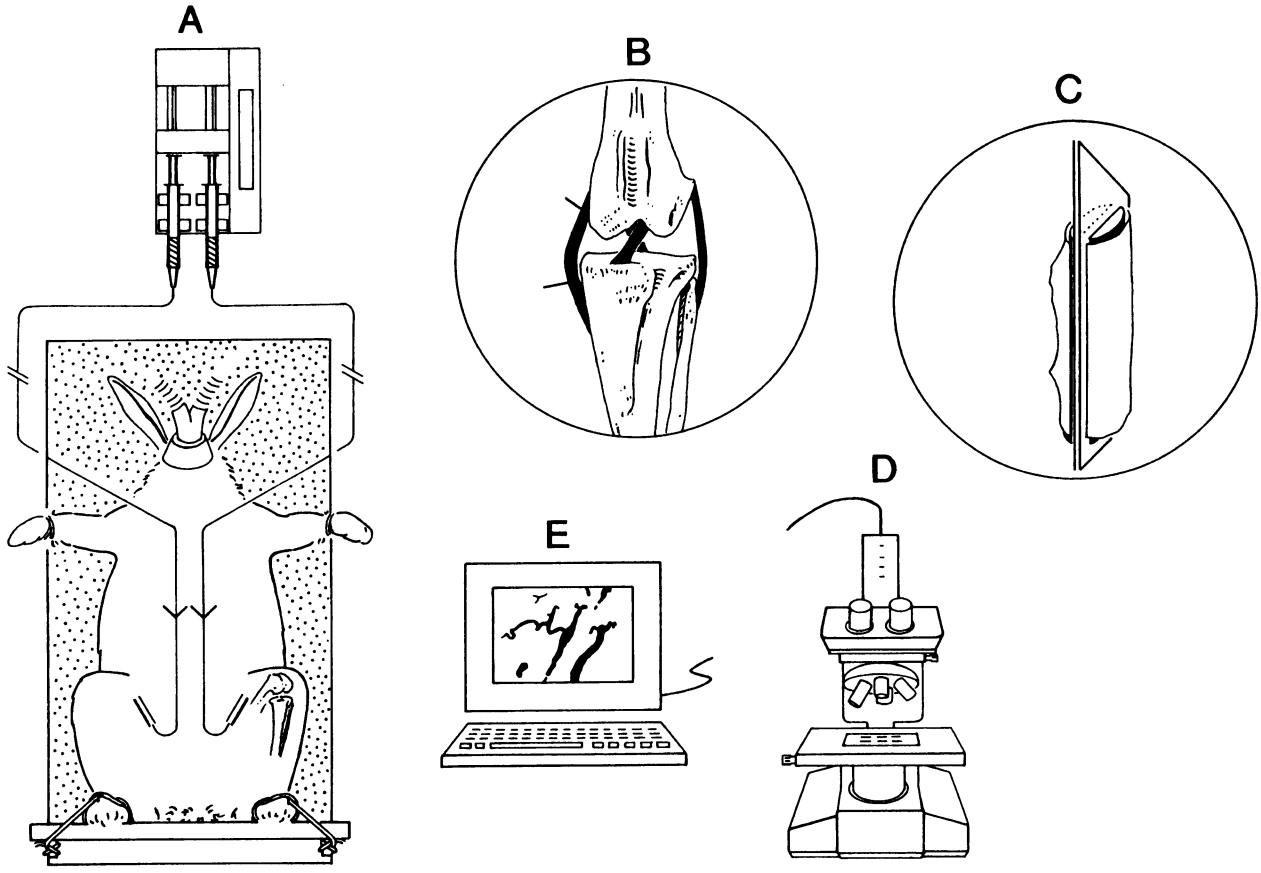

Fig. 1. Composite schematic diagrams of tissue processing of ink perfused tissue from normal and healing MCL samples. (*A*) Ink-perfusion setup, demonstrating the controlled perfusion of gelatinised ink from a syringe pump to both femoral arteries of an anaesthetised rabbit. (*B*) Left knee joint, with associated ligaments, showing the segment of MCL excised for histological assessment by image analysis. (*C*) Representation of the parasagittal plane of ligament serial sectioning. (*D*) Photomicrography of ink-perfused sections of MCL. (*E*) Image analysis of blood vessels.

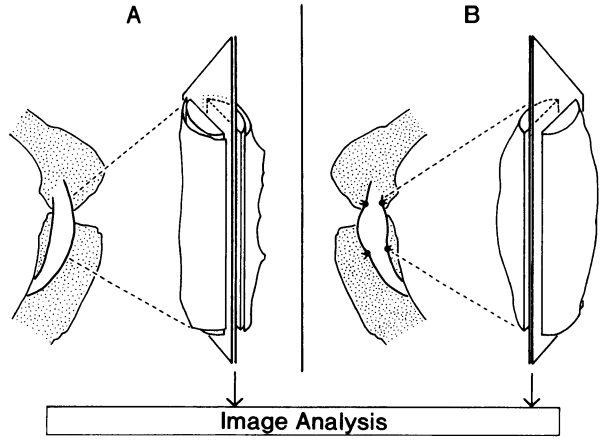

Fig. 2. Schematic diagram depicting typical sample sites (medial view of the knee) from a left contralateral MCL (*A*) and a healing right MCL scar (*B*). Similar anatomical regions of normal or scar MCL were removed for comparative analysis by image processing.

with 4 sutures (Fig. 2*B*) so that when scar tissue was later retrieved, the repair tissue occupying the initial 4 mm gap injury site could be analysed separately from the original ligament ends. Only the tissue that has filled the original 4 mm gap segment is assessed.

Image analysis

Approximately 10% of ligament or ligament scar serial sections were photographed according to a standardised protocol (Eng et al. 1992) using a polarising light microscope (Leitz Orthoplan, with rotating polariser and analyser polaroids; Leitz Wetzlar, Germany) at × 175 magnification. A total of 3063 black-and-white photomicrographs of ink-injected specimens were analysed for percent vascularity (by area) and directional scatter of vascular segments using image analysis. The method is briefly described below.

Each photomicrograph was digitised using a Fairchild CCD 3000 camera (Fairchild Camera and Instrument Corp., Palo Alto, CA) and an Imaging Technology series 100 digitising image frame buffer (Imaging Technology Inc., Bedford, MA) housed in a

SUN 3 computer (Sun Microsystems Inc., Mountain View, CA). The resolution of digitisation was measured to be 2.32 μm per pixel. Each image was binarised, and the area covered by the ink-perfused vessels was computed. The image was then skeletonised so that each vessel was represented by a 1 pixel thick line signifying its central axis. The skeletal line patterns were then analysed for directionality by a least squares linear regression procedure. This procedure assigns an angle to each pixel in the skeletal lines as given by the best-fitting straight line at the pixel to the skeletal pattern on the basis of a neighbourhood of 11 pixels. The angle values were grouped into 30 bands of 6° each, covering the full range of 0–180°. The angular distribution computed included the net area of all ink-perfused vessel segments having an orientation within the corresponding angle band. The distributions were plotted as rose diagrams for display and visual analysis. The data in the distributions were also subjected to statistical analysis using the standard deviations (S.D.) of the distributions.

For the purposes of grouping the ligament data for comparative analysis, the following procedure was employed. Angular distribution data from all images from any given ligament were grouped into a single normalised distribution, and its S.D. was computed. In order to obtain an averaged statistical measure over a group of rabbits at a certain stage of healing, each variance (square of the S.D.) value was then weighted by the number of images taken from the corresponding ligament, and the weighted sum of the variances was divided by the total number of images of all the ligaments in the group. The square root of this weighted-averaged variance was taken as the final (weighted) S.D. of the angular distribution (in degrees) for the group.

Data on the area covered by the ink-perfused vessels from the various images of each ligament was pooled and converted to a percentage measure of area covered, computed with reference to the total area of each image in pixels. When combining such percent vascularisation measures from various rabbits within a group, the individual values were weighted by the number of images analysed of each ligament, and the weighted sum was normalised by the total number of images in the entire group.

Statistical analyses

The dispersion of angular distributions was compared using Bartlett's test of homoscedasticity (Bartlett's Test; Statistical Package for the Social Sciences; SPSS User's Guide, 1990). Vascularisation measures were compared initially with a 'global' statistical procedure (MANOVA, multivariate analysis of variance; Statistical Package for the Social Sciences; SPSS User's Guide, 1990), and individual differences were tested using the Student-Neuman-Keuls test for multiple comparisons. An alpha level of $P < 0.05$ was used for all procedures. Statistical results were reported as 1-sided or 2-sided depending on whether a 1-tail or 2-tail procedure was used.

RESULTS

Normal MCL vascularity

Blood vessels were not commonly observed in deep MCL tissue sections. Many high-power fields were devoid of ink-injected vessels. Normally, blood vessel segments were found in parallel arrangements (Fig. 3). Vessels from deep ligament matrix often showed ladder-like connections between parallel channels (usually one thicker than the other), in the form of short intervening perpendicular vessel segments (Fig. 3*A*). Confirmation that vessels were located deep within normal MCL matrix was accomplished by using a polarising filter to confirm the typical background crimp pattern (Fig. 3*B*).

From a total of 491 photomicrographs representing about 10% of available ligament matrix, determined

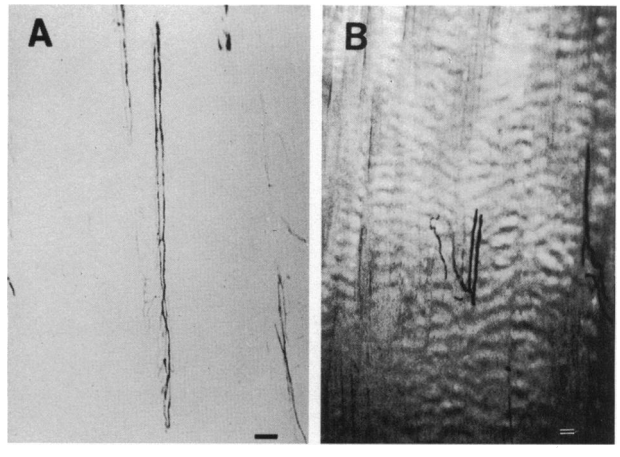

Fig. 3. Time-zero (normal), ink injected, unstained, MCL sections showing the typical longitudinal ladder-like vascular plexuses deep in the MCL. The long axis of each MCL image runs from top to bottom. Image shown in (*A*) without polarisation. Different image shown in (*B*) using polarising filter and illustrating background crimp pattern (confirming location in deep MCL tissue). Bar, 50 μm.

Table *Weighted mean values of percent vascularisation and weighted* S.D. *values of angular distribution of vessel segments from ink-perfused rabbit medial collateral ligaments and scars for various healing intervals*

Healing interval	Number of rabbits	Specimens tested	Number of MCLs	Number of images	Percent vascularity (weighted mean ± S.D.)	Angular distribution (weighted S.D.)
Time zero	3	Left and right	6	491	1.46 ± 0.30	38.13
3 wk	3	Contralateral	3	257	0.99 ± 0.42	35.81
		scar	3	263	2.34 ± 0.81	43.03
6 wk	3	Contralateral	3	352	1.95 ± 0.14	44.35
		scar	3	734	2.78 ± 0.47	48.61
17 wk	3	Contralateral	3	130	1.44 ± 0.61	33.74
		scar	3	256	2.74 ± 1.07	42.43
40 wk	3	Contralateral	3	237	0.58 ± 0.14	27.72
		scar	3	343	0.96 ± 0.48	38.20
Totals	15		30	3063		

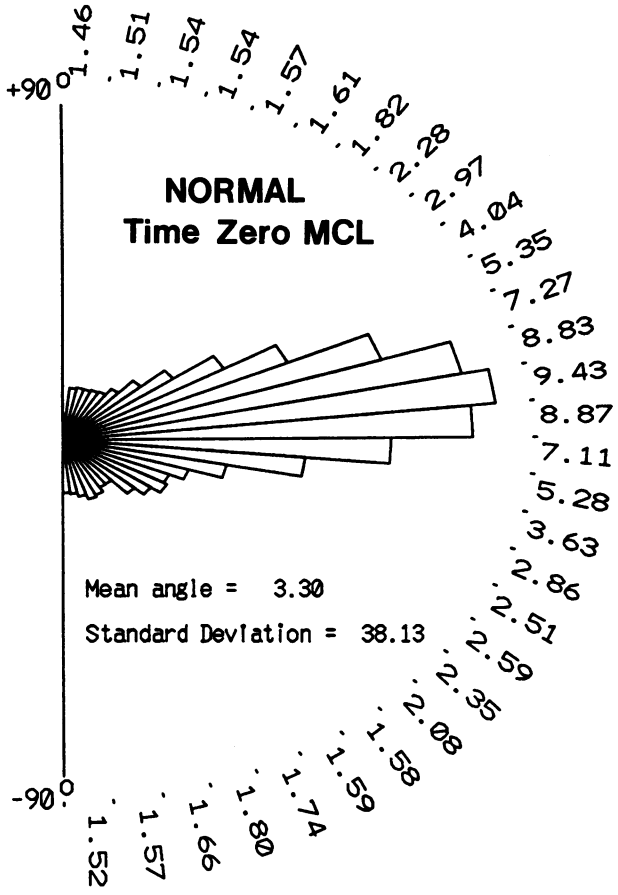

Fig. 4. Composite rose diagram plot of 491 photomicrographs of normal MCL.

that 1.46% of normal (deep) MCL was occupied by ink-perfused blood vessels (see Table). Blood vessel segment alignment showed a consistently tight distribution (Fig. 4), reflecting the highly organised, longitudinal alignment of ink-perfused vessel segments in the normal MCL.

MCL scar vascularity

Ink-perfused sections of MCL scar showed more densely and chaotically distributed vessels. In the earlier stages of healing (3, 6 and 17 wk) vessel segments were very disorganised and appeared thicker than normal. After 40 wk of healing, however, MCL scars became less vascular (Fig. 5).

Percent vascularity of MCL scars was relatively high in the early healing intervals, and by 6 wk was elevated compared with (time-zero) normal MCL ($P = 0.007$, 2-sided); see Fig. 6. At 40 wk, MCL scars returned to percent vascularity values not significantly different from those for time-zero control MCL tissue. When comparing the percent vascularity between experimental and contralateral limbs, however, there was significantly higher vascularity in MCL scar at all healing intervals, except for 40 wk. In the 40 wk case, a measurable difference between MCL scar and contralateral-control values was not evident. Compared with a time-zero undisturbed control, however, the vascularity in the 40 wk contralateral ligament was shown to be lower ($P = 0.023$, 2-sided).

With respect to blood vessel segment alignment, MCL scars from every healing interval showed elevated S.D. values compared with contralateral controls (Table). Elevated S.D. values reflect a greater degree of vessel disorganisation (dispersion of vessel segments relative to the longitudinal axis of the MCL). The angular dispersion of blood vessels is represented in composite rose diagram plots comparing 3, 17 and 40 wk healing MCL scars (Fig. 5). Compared with earlier healing intervals, 40 wk scars showed less vessel segment angular dispersion, but even 'mature' MCL scars continued to show relatively high S.D. values when compared with contralateral controls (Table).

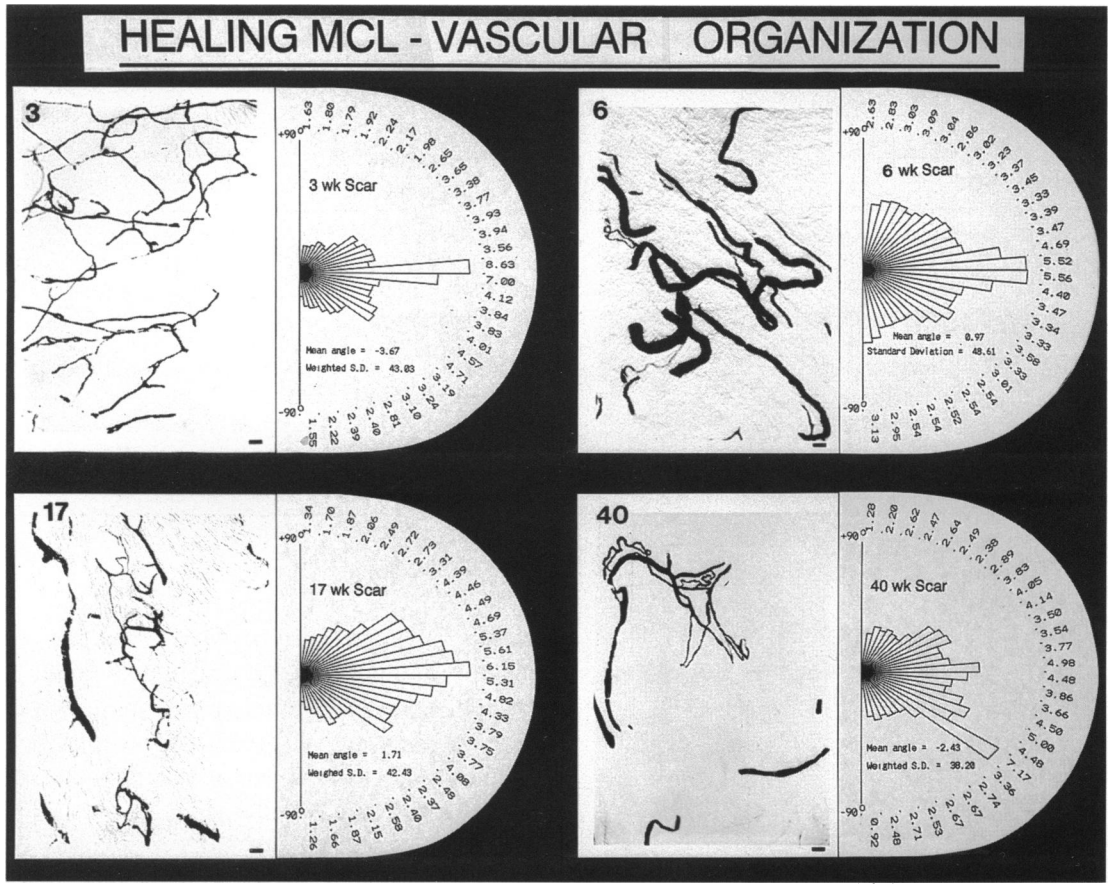

Fig. 5. Photomicrographs of ink-perfused MCL scar sections and composite rose diagram plots for angular distribution obtained from samples at the 3, 17 and 40 wk healing intervals. For the photomicrographs, the proximal-distal axis of each MCL image runs from top to bottom (similar to orientation shown in Fig. 3). Bar, 50 μm. The angular distribution plots represent grouped data from all the images for any given healing interval. It can be seen that the orientation of vascular segments, as represented in S.D. values, show gradual return toward normal time-zero values.

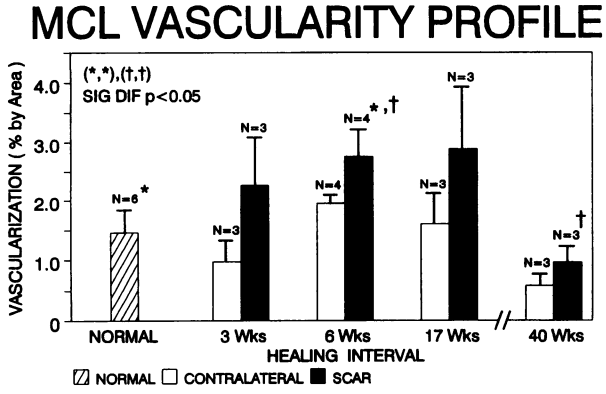

Fig. 6. Vascularity profile for the weighted mean percent vascularity values of MCL scar, contralateral and time-zero control MCL samples. Error bars represent the S.D. of the weighted vascularity values. Values for 6 wk MCL scars were significantly higher than time-zero controls and 40 wk MCL scars and their respective contralateral controls. N, number of ligaments for each sample set of images.

Contralateral MCL vascularity

Contralateral control MCLs showed considerable variability in percent vascularity (Fig. 6) and vascular segment angular distribution over the intervals studied (Table, S.D. values). As noted above, the 40 wk contralateral MCLs were only about one-third as vascular as time-zero controls and the S.D. values for angular distributions of these contralateral ligaments were the lowest of any of the contralateral MCL tissues studied (reflecting substantially less dispersion of vessel segments).

Effects of time on normal and healing ligament vascularity

The vascular response to injury in this model is biphasic; early on, the MCL scar is hypervascular and the alignment distribution of blood vessel segments is quite dispersed and disorganised; in later healing stages, the degree of vascularity decreases and more uniform alignment of vessels becomes apparent. The contralateral MCL also undergoes alterations of vascularity and vessel organisation, ultimately becoming less vascular but more organised at 40 wk.

DISCUSSION

The objectives of this study were to define a quantitative baseline for normal MCL vascularity in the rabbit, and to evaluate the process of revascularisation of a healing ligament scar in this model. These objectives have been met, but several important limitations of the study need emphasis before discussing the results in detail.

One significant limitation of all perfusion preparation studies is the possibility of incomplete filling of available blood vessels, potentially leading to a fictitiously low estimate of actual vascularity (Linstrom, 1963; Liew & Carson-Dick, 1981; Bray et al. 1990). At least three previous studies have specifically addressed the issue of artifact related to incomplete vascular perfusion. The sense of these studies is that under optimal perfusion conditions, up to 85% of available vessels typically fill with perfusate (Eriksdotter-Nilsson et al. 1986; Proia et al. 1988; Weiss, 1988). Other artifacts of tissue preparation include abnormal distension or possibly vessel shrinkage during perfusion and fixation, potentially causing error in the estimates of percent vascularity. One-way vascular perfusion (as performed in this and most other studies) does not allow distinction between arterial, venous, and capillary vessels. However, reports detailing the size ranges of MCL vessels and the diameters of vessels in the synovium layer covering the patellar ligament, suggest that capillaries comprise the largest proportional fraction of vessels in these tissues (Knight & Levick, 1983; Bray et al. 1990).

The foregoing limitations might affect the accuracy of percent vascularity values, but would be less likely to influence the estimates made on the degree of vascular organisation (unless certain vascular configurations are either preferentially perfused, or alternatively, preferentially underperfused, which could lead to systematic errors in the interpretation of the results). We have not specifically addressed this question, and therefore the magnitude of these latter errors is unknown.

Other limitations relate to the image processing methods. These have been described in some detail in a previous report outlining development of the image analysis methods (Eng et al. 1992). Briefly, pixel sampling errors become important for very thin vessel segments, and very thick vessels may become subject to a small loss of area during image processing. One 'theoretical' limitation relates to the fact that vascular organisation or the S.D. of the angular distributions of vessel segments is computed relative to the plane of section—i.e. longitudinal parasagittal—and hence the directional analysis is based on a 2-dimensional representation of ligament blood vessels; branching of vessels out of the plane of section (above or below) is not accounted for.

Despite these important limitations, results indicate that normal MCL tissue appears, as predicted, to be hypovascular. We estimate in normal rabbit MCL tissue that about 1.5% (by area) of the total available matrix is occupied by the ligament microvasculature. This figure compares closely with a previous estimate of 1% by area (Eng et al. 1992), based on a sample set of about one-fifth of the present sample size. We are aware of no similar information pertaining to other normal ligaments in the same or different species. Quantitative studies of the vascularity of synovium have been performed in the rabbit (Knight & Levick, 1983), but because of methodological differences, a rigorous comparison of results is difficult. Nonetheless, in that study, areolar or adipose synovium was found to be highly vascular with a capillary density reported in the range of 67000–83000 capillaries/cm^2 of tissue section, compared with the synovial covering layer of the patellar ligament which showed a density of 2000 capillaries/cm^3—the latter being roughly one fortieth as vascular as the former (Knight & Levick, 1983). This information leads to the conclusion that the synovium covering ligaments was 'very low' in vascularity. In a previous report, we studied the analogous synovial tissue covering the rabbit MCL (epiligamentous tissue) and found it to be just slightly more vascular than the deeper MCL tissue described here, which as stated, is very hypovascular (Eng et al. 1992).

Following catastrophic injury, simulated here by removing a large gap segment of MCL mid-substance, a rapid and relatively exuberant revascularisation of the gap healing segment occurs. Only the mid-substance gap healing region was analysed because it was desirable to study pure 'repair tissue' in what would otherwise be considered an extreme injury to an extra-articular ligament. This model of MCL injury appears to be a very potent stimulus for a vascular response. Indeed, by 6 wk the percent vascularity of a healing MCL scar is roughly twice contralateral control values. The orientational alignment of early healing blood vessels, however, is severely altered compared with uninjured MCL. These differences may not seem striking, considering past reports of extensive revascularisation observed in other healing ligament models (Alm & Stromberg, 1974; Arnoczky et al. 1982), but this study attempts to quantify the extent and course of the vascular response of an injured extra-articular ligament.

Another important finding is the biphasic nature of the scar revascularisation process. It appears that an intense neovascular proliferation is followed by vascular regression, ultimately leading to remodelling of MCL scar microvascular patterns. While a similar finding has been shown to occur after about 5 months in models of canine anterior cruciate ligament healing (Arnoczky et al. 1982), it appears to take longer for MCL vascular remodelling to become apparent. Such differences reflect the diverse nature of individual ligament responses to injury. Importantly, therefore, results from the present study should not be generalised in the light of the almost certain model and species-specific aspects of healing ligament revascularisation.

One somewhat surprising result was the observation that contralateral control MCL tissue appears to undergo alterations in the degree of vascularity and vascular organisation (relative to time-zero external normal control MCLs) over the time frame of this study. Some of this may be explained by the relatively small sample size or possibly just expected variance in the data. However, at least two alternative explanations might also explain this curious finding. Such a 'contralateral effect' could be due to natural ageing-related processes in rabbit ligaments, or alternatively to some local or systemic cause attributable to the opposite knee injury (Frank et al. 1994). Neither can be ascertained by this study, but the question of age-related alterations of normal ligament vascularity is the subject of ongoing investigation in our laboratory.

Many questions remain about MCL scar vascularity. This study represents a step towards the question of how vascularity relates to tissue function in healing ligaments. With the quantitative methods described here, we hope to continue work aimed at determining the relationships between MCL vascularity and biomechanical function. Future studies will be directed toward correlations of healing tissue vascularity and mechanical properties to elucidate important relationships between vascularity and other clinically relevant variables such as age, immobilisation and exercise.

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