American Journal of Pathology, Vol. 138, No. 5, May 1991

Quantitative Assessment of the Age of Fibrotic Lesions Using Polarized Light Microscopy and Digital Image Analysis

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Reliable histologic methods for gauging the maturity of fibrotic lesions are limited, making interventions in the healing process difficult to assess. As collagen ages there is enhanced birefringence due to increased molecular and fibrillar organization. The purpose of this study was to develop a microscopal technique to quantify this process and to determine its ability to distinguish scars of varying ages. Fibrosis in the rat gracilis muscle was studied 5 to 63 days after superficial injury. Sections were stained with picrosirius red and illuminated with monochromatic, polarized light. The microscope fields were digitized using a computer-video system yielding an image in which noncollagenous material was dark (gray level 0) and collagen was depicted by grey levels 1 to 255. In the fibrosis model used, the collagen area fraction plateaued at 80% by day 21. The median collagen grey level increased progressively as the scar aged. It is concluded that this histologic, nondestructive technique can reliably quantify age-related optical properties of fibrotic collagen and that this could be used to determine the maturity of fibrotic lesions. (Am J Pathol 1991, 138:1225–1231)

The deposition of collagen is a fundamental feature of repair following muscle cell death and serves to restore the tensile strength of the damaged tissue. Once deposited, fibrotic collagen undergoes a highly regulated maturational process that enhances its mechanical strength and resistance to degradation.[1,2] This process consists primarily of intermolecular cross-linking but also includes progressive loss of ground substance[3] and water[4] and a conversion in the type of collagen synthesized from type III to type I.[2,5–7] The active nature of the tissue during this time is in contrast to its relatively homogeneous and invariant appearance on routine light microscopy. Yet the ability to characterize histologically the maturity of a scar would be of considerable value in dating the onset of tissue injury and in evaluating the efficacy of measures aimed at modulating the rate of fibrogenesis.

Recently we reported the use of a polarization microscopy-video densitometry technique that allowed for rapid quantification of collagen in histologic sections of myocardium.[8] This approach exploited the high birefringence of collagen stained with picrosirius red. Collagen birefringence, however, is not constant but will increase as the tissue ages.[9] Such changes in collagen birefringence may manifest as changes in brightness of the polarization image and the polarization brightness of a fibrotic network may therefore be a valuable and easily quantifiable parameter with which to characterize the maturity of a scar. We report here on the combined use of polarized light microscopy and digital image analysis to characterize fibrotic tissue and to distinguish quantitatively scars of different ages.

Materials and Methods

Experimental Model of Fibrosis

The intensity of brightness of collagen viewed with polarized light depends not only on collagen birefringence (ie, the degree to which it retards linearly polarized light) but also on the orientation of the fiber with respect to the polars. If fibers in a network are analyzed individually, this can be controlled by analyzing all fibers at identical orientations. From a practical standpoint, however, the ability to evaluate a larger region of the scar (eg, a microscope field) would be desirable. In this regard two types of scarring may be considered—those in which progressive alignment of individual collagen fibers is a significant

Supported by a grant from the Heart and Stroke Foundation of Ontario.

Dr. Boughner is a research associate of that foundation and Dr. Pickering was supported from a fellowship from the Heart and Stroke Foundation of Canada.

Accepted for publication January 7, 1991.

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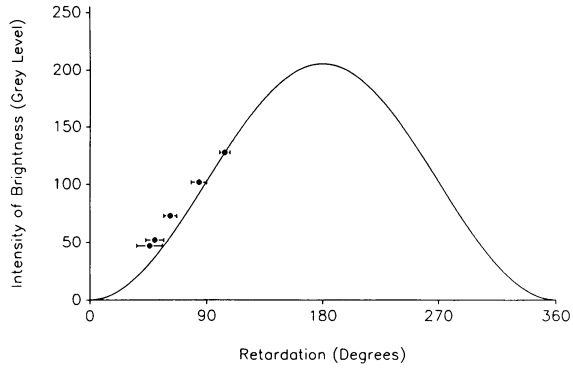


Figure 1. *Relation between intensity of brightness of rat tail tendon, stained with picrosirius red, and retardation. The theoretical relation[13] is shown also (sinusoidal curve). The intensity of brightness of collagen was measured as the median grey level of the collagen-depicting pixels.*

component of the maturation process, and those in which this is not the case. In the former instance an increase in either fiber alignment or fiber birefringence could lead to a time-dependent increase in brightness of the network. While both events may be considered features of maturation, we wished to minimize the influence of fiber alignment and specifically address changes in birefringence because this would only reflect changes in molecular orientation and packing. After preliminary study we found that following a superficial injury to the gracilis muscle of the rat, alignment of the collagen fibers was not a prominent feature and this was therefore chosen as the method of fibrosis induction.

The surgical procedure was approved by the animal ethics committee at the University of Western Ontario, London, Canada. Twelve male 250-g Sprague-Dawley rats were anesthetized with sodium pentobarbitol (40 mg/kg). A 1.5-cm incision to the skin over the medial aspect of the left thigh was made exposing the gracilis muscle. With the use of an operating microscope (American Optical Co., Markham, Ontario, Canada), a partial-thickness elliptical incision of the gracilis muscle was made using iridectomy scissors. Then the skin was oversewn with absorbable sutures. Of the 12 animals studied, two were killed on each of 5, 9, 14, 21, 42, and 63 days after the injury and the healing tissue was recovered. During this procedure, the loose connective tissue immediately beneath the skin was carefully dissected away until the underlying gracilis muscle, with a zone of superficial scarring, could be identified. This portion of the gracilis muscle was freed from the adjacent tissues, excised, and placed in a solution of 10% neutral buffered formalin. Samples were embedded in parafin and sections were cut at 7-μm thickness and stained with picrosirius red according to Constantine and Mowry.[10] The sirius red molecules of this stain bind to collagen in an oriented fashion such that collagen birefringence is enhanced seven times.[11] With polarization microscopy collagen stained in this way appears either bright orange-red or bright green.[12]

Digital Imaging Methods

The imaging system consisted of a Nikon Optiphot polarizing microscope (Nikon, Mississauga, Ontario, Canada) with a solid-state CCD videocamera (Series 4810, Cohu, San Diego, CA) mounted on the eyepiece tube. The video signal underwent eight-bit digitization by a video frame grabber (PCVISION Plus, Imaging Technology, Woburn, MA), in a PC-AT computer, with a resolution of 640 (horizontal) by 480 (vertical) pixels. This yielded an image with 256 possible grey levels that was displayed on a high-resolution monitor (Model C-3479, Mitsubishi, Torrance, CA). A × 10 objective and a × 1 television relay lens were used for all measurements and resulted in a pixel size of 1.82 μm^2. A linear relationship between the intensity of light and the grey level of the digitized image was confirmed using a series of neutral density filters with the respective optical densities confirmed by a densitometer (Model 301, X-rite, Grand Rapids, MI).

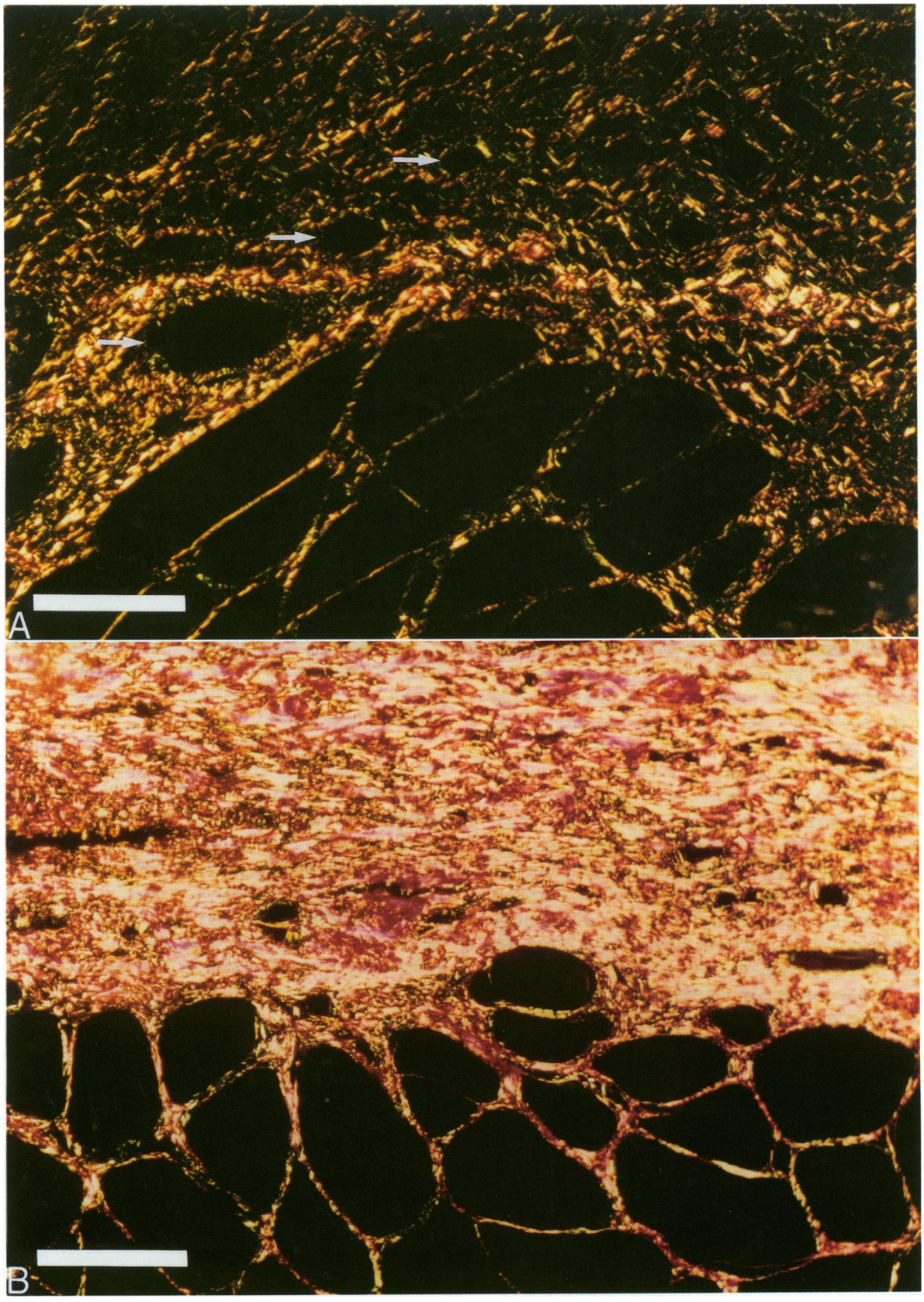
To avoid any variations in camera response to light due to differences in the light wavelength, monochromatic illumination (600 ± 5 nanometers) was used for all collagen measurements (Optikon, Waterloo, Canada). We previously found that when illuminated with this particular wavelength, both orange-red and green fibers were optimally detected by the video system.[8] Collagenous components were thus depicted by grey levels 1–255 and noncollagenous, and therefore nonbirefringent, tissue by grey level zero.

Collagen Measurements

Collagen Content

From each specimen 10 randomly selected regions of the fibrotic zone were digitized. The total collagen content was determined by the area fraction of visible (nonzero) pixels. Artifactual gaps in the section were excluded by a mouse-driven tracing routine (Jandel Video Analysis, Jandel Scientific, Corte Madera, CA). Good correlation between the videodensitometric measurements and hydroxyproline content was established previously.[8]

Figure 2. *Surface of rat gracilis muscle 5 days* (A) *and 63 days* (B) *after partial-thickness injury. Initially there is a loose network of fibrotic collagen appearing either green or orange-red. Blood vessels are abundant (arrows) but appear dark because the luminal contents are nonbirefringent. By 63 days the collagen fibers are packed more densely and generally appear brighter. Bar = 100 μm.*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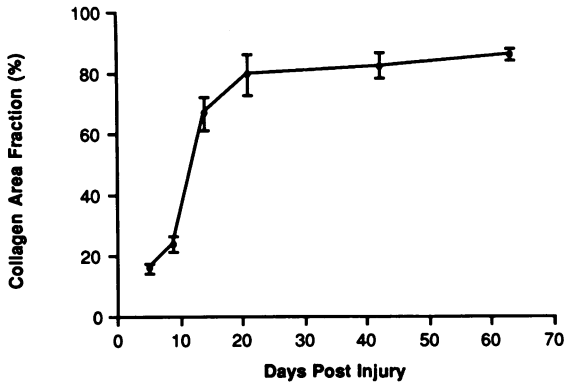


Figure 3. *Time course of the collagen content of the scar forming on the surface of injured rat gracilis muscle. Sections were viewed with the picrosirius-polarization technique and collagen content was determined by the area fraction of visible pixels.*

Collagen Maturity

The relationship between brightness and birefringence is sinusoidal.[13] Therefore an increase in birefringence will manifest as an increase in brightness only if values fall within the ascending limb of the sinusoidal curve. Because we wished to use brightness as a surrogate for birefringence, it was necessary to establish if this was the case for collagen stained with picrosirius red. A portion of rat tail tendon, fixed in 10% neutral-buffered formalin, was embedded in paraffin, sectioned at 2, 4, 6, 8, and 10 μm thickness, and stained with picrosirius red. For each section 10 measurements of retardation were made according to the method of de Sénarmont.[14] The microscope images of each specimen then were digitized and the overall intensity of brightness was determined based on the median grey level of the nonzero pixels. All brightness measurements were made under identical illumination conditions and without knowledge of the corresponding retardation measurement. The range of the measured retardation was 40° to 105°C. This range falls within the linear portion of the theoretical relation with brightness and the brightness measurements closely approximated the theoretical values (Figure 1).

Collagen brightness in the healing muscle wound was assessed in a similar manner to that for the tendon. A histogram of collagen grey levels (number of pixels *versus* grey level) was generated for each specimen. The median grey level served as the index of collagen brightness. To avoid variation in collagen brightness due to different staining conditions, the fascial covering of the muscle opposite the scarred region served as control tissue. This also served to normalize changes due to aging of the rat as opposed to aging of the wound. Thus

$$\text{relative brightness of fibrotic collagen} = \frac{\text{median grey level of fibrotic collagen}}{\text{medial grey level of fascial collagen}} \times 100$$

All collagen measurements were made without knowledge of the age of the injury.

Statistics

Results were expressed as mean ± standard deviation. Comparisons between groups were done using one-way analysis of variance and Scheffé's multiple-range test.

Results

On day 5, the injured regions consisted of granulation tissue with abundant blood vessels, thrombi, and a loosely packed network of thin collagen fibers. With polarization microscopy, the fibers appeared green (Figure 2a). With time, collagen became more predominant, with thicker fibers (up to 14 μm) that were densely packed and appeared orange-red under crossed polars (Figure 2b). This process appeared to start in the region immediately adjacent to the muscle.

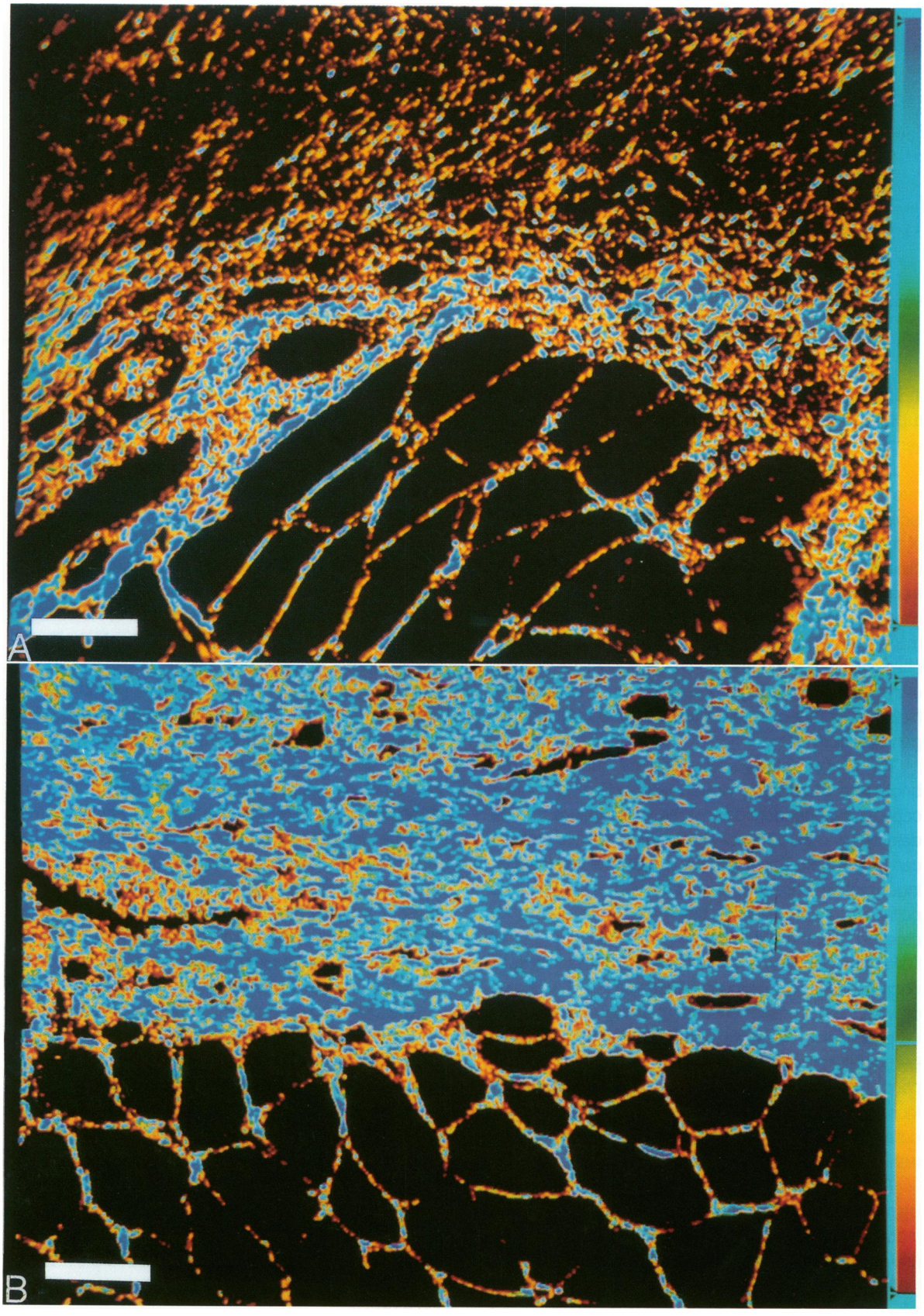
The collagen content at 5 days, determined from the video images, was 16% ± 2%. This increased in a sigmoidal fashion such that after 21 days a plateau was evident at approximately 80% (Figure 3).

Digitized color-encoded images of the sections shown in Figure 2 are depicted in Figure 4a and b. The relative amount of collagen pixels encoded by blue, corresponding to higher grey levels, is much greater in the 63-day specimen. At 5 days the median brightness value for fibrotic collagen, expressed in terms of the median grey level of fascial collagen, was 14.9 ± 2.1 This increased progressively through the entire study period with no clear evidence of a plateau (Figure 5). The average median brightness level of the 63-day scars was 6.6 times that of the scars assessed at 5 days.

Discussion

The presence of scar tissue following tissue damage generally implies irreversible cell injury. The fibrotic process itself, however, may still be modifiable and such modifications may have important clinical impact. For example, enhancement of healing rates after myocardial

Figure 4. *Digitized, color-encoded images of the same sections of healing rat muscle shown in Figure 2 at 5 days* **(A)** *and 63 days* **(B)**. *The proportion of brighter pixels, depicted in blue, increases with time. Bar = 100 μm.*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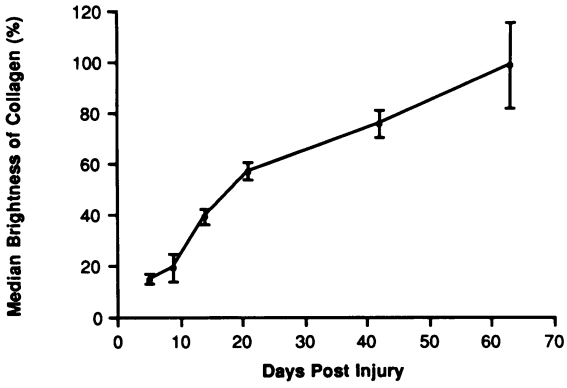

Figure 5. *Time course of brightness profile of fibrotic collagen. Values represent the median grey level of fibrotic collagen expressed as a percentage of normal fascial collagen.*

infarction may decrease the likelihood of free-wall rupture[15] and limit topographic alterations of the ventricle.[16] Conversely, by inhibiting collagen maturation in primary fibrotic conditions such as scleroderma, the rate at which collagen is degraded *in vivo* may be increased.[17,18] While several methods of altering the rate of fibrogenic repair exist,[17–22] none have gained wide clinical use. Because modulation of the fibrotic process is most likely to be effective in the early, active stages,[1] it would be important to have a means of establishing the maturity of a scar. This may be accomplished with biochemical studies (eg, measurement of post-translational enzymes[23] or procollagen levels[24,25]) but a quantitative, histologic approach would offer both practicality and organ specificity.

The present study shows that by quantifying the brightness of collagen viewed with polarizing microscopy, time-dependent differences in scar tissue can be detected and thus the activity of a given fibrotic process can be gauged. This approach exploits the fact that collagen birefringence increases as the fiber ages. In fibrotic collagen this increase in birefringence is due to increased alignment and packing of molecules and of fibrils through intra- and intermolecular cross-linking,[1,2] decreased interstitial ground substance,[4] and exclusion of water[9] with consequent strengthening of existing hydrophobic and electrostatic bonds.[3] Furthermore collagen deposited in the early stages of fibrosis is predominantly type III,[5–7] which forms thinner fibrils in a relatively loose network[26] with no intramolecular bonding along the coiled part of the molecule[27] and a greater association with interstitial carbohydrate.[4]

The intensity of birefringence generally is determined by measuring the retardation of linearly polarized light by individual structures. This requires both technical expertise and a working knowledge of polarization theory, and perhaps for these reasons is not easily applied to the clinical setting. We found that over a wide range of retardation values for collagen stained with picrosirius red, changes in intensity of brightness closely track changes in retardation. Therefore in this setting the brightness of collagen may be a valuable indicator of molecular and fibrillar order. With video techniques and digital image analysis, both the amount[8] and relative maturity of fibrotic collagen may be ascertained readily.

The particular model of fibrosis used in this study was chosen for two reasons. First muscle was considered to be a suitable substrate for the injury, as opposed to skin or tendon, because degenerating, pre-existing collagen would not be present in the scar. Such degraded collagen has reduced birefringence, which could decrease the overall intensity of brightness independent of fibrogenesis. Second the alignment of the fibers within the scar did not change significantly with time. Therefore any observed changes in brightness would only reflect changes in birefringence, ie, molecular and fibrillar structure. This does not imply that fibrotic maturity could not be assessed in instances in which progressive alignment of fiber bundles is prominent. Indeed greater changes in brightness with time may be expected because both molecular organization and fiber alignment will increase. For this study, however, we restricted the definition of maturity to include only the former type of changes.

In the fibrosis model used we found that the collagen content plateaued by 21 days. This is consistent with previously reported findings of wound healing in rat skin.[28,29] In contrast, throughout the 63-day study period, the median intensity of brightness continued to increase. This suggests ongoing organizational changes at a time when the rate of deposition of new collagen was apparently low. Structural reorganization after the initial phase of collagen deposition may be inferred from studies of mechanical function of healing wounds. Levenson has shown that the tensile strength of rat skin wounds does not reach maximum until approximately 90 days after injury.[30] The described methodology is a means to detect quantitatively histologic changes associated with, and probably contributing to, this sustained augmentation of mechanical properties.

The changing birefringent properties of fibrotic collagen can be exploited to assess quantitatively collagen deposition and maturation after tissue injury. In this way, a quantitative assessment of the activity of fibrosis can be made on histologic sections, a feature that could facilitate the evaluation of interventions used to modify the healing response.

Acknowledgments

The authors thank J. Dixon for the preparation of the histologic specimens and Dr. P. Whittaker for helpful comments regarding this research.

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