Control of tissue composition and organization will be a key feature in the development of successful products through tissue engineering. However, the mechanism of collagen fibril formation, growth, and organization is not yet fully understood. In this study we have examined collagen fibril formation in a wound healing model in which the newly formed fibrils were kept distinct from preexisting tissue through use of a porous tubular biomaterial implant. Samples were examined after 4, 6, 14, and 28 days by light microscopy, in situ hybridization, and immunofluorescence microscopy. These showed a normal wound healing response, with significant collagen formation at 14 and 28 days. Individual collagen fibrils were isolated from these samples by gentle extraction in a gentamicin-containing buffer which allowed extraction of a large proportion of intact fibrils. Examination by transmission electron microscopy showed that ~80% of the intact fibrils showed a single polarity reversal, with both ends of each fibril comprising collagen amino-terminal domains; the remaining fibrils had no polarity reversal. All fibrils had similar diameters at both time points. Immunoelectron microscopy showed that all labeled fibrils contained both type I and III collagens. These data indicate that this wound healing model provides a system in which collagen fibril formation can be readily followed.

INTRODUCTION

The rapidly emerging field of tissue engineering provides a unique opportunity to provide homologous tissue for regeneration of diseased or damaged tissues (Langer and Vacanti, 1999). Research has shown that if appropriate cells can be taken from a patient and grown on a designed bioresorbable molecular scaffold in a bioreactor, then replacement tissue for the patient can be produced. It is important that the organization and composition of the collagenous tissue match that of the natural tissue that is being replaced so that the durability and complex mechanical properties of the natural tissue are accurately reproduced. For example, in ligaments the collagen fibrils are highly oriented along the length of the tissue, and their diameters can be correlated with the load requirements for the tissue (Parry and Craig, 1988). These characteristics are quite distinct from the collagen composition and organization in a general wound healing response, where a different collagen organization and composition are found (Bailey et al., 1975). This wound response could emerge during the production of a tissue-engineered replacement. Thus, in order to match the appropriate tissue characteristics it will be necessary to control the organization and composition of the new collagen that is deposited in the tissue-engineered construct.

Although there is extensive information on the biosynthesis of collagen and on the properties of collagenous tissues, the processes by which procollagen forms into functional fibrils in tissues are less well understood. Evidence suggests that the nucleation steps (Silver et al., 1992) that initiate generation of fibrils may occur within cell surface crypts (Birk and Trelstad, 1984), and that nonstaggered SLS aggregates may be involved in this process (Bruns et al., 1979), although it is possible that nucleation may occur in the matrix further from the cell. There have been various in vitro studies (see Veis and Payne, 1988) on fibril formation, many utilizing pepsin or acid-soluble collagen extracted from tissue. Of most interest, however, have been
the in vitro studies that use pN-, pC-, and intact pro-collagen with fibril formation initiated by addition of the specific N- and C-proteases (Miyahara et al., 1984; Kadler et al., 1990). This approach leads to the formation of fibril-like moieties with the characteristic collagen D-periodic banding pattern, but with a distinctive, needle-like morphology that is different from fibrils isolated from native tissue (Kadler et al., 1990; Silver et al., 1992). These fibril structures show a single polarity reversal where the orientation of the collagen organization is reversed (Kadler et al., 1990). Newly formed fibrils with characteristic D periodicity have been isolated from various embryonic tissues (Birk et al., 1995, 1996; de- vente et al., 1997). These fibrils also frequently show a single polarity reversal (Birk et al., 1995; Holmes et al., 1994). The mechanism of how these fibrils would grow into larger fibrils is not clear. Data suggest, however, that formation of longer fibrils may occur by the fusion of small (1–10 μm) segments (Birk et al., 1995), and that lateral association of long or short fibrils may lead to thicker fibrils (Parkinson et al., 1994). Immunogold labeling of type III pN collagen in embryonic tissues showed the presence of this collagen on the newly forming fibrils (Fleischmajer et al., 1983), suggesting that mature type III collagen may be colocalized in type I fibrils and that the N-propeptide has a role in regulating fibril growth. A range of other molecules, including the small proteoglycans decorin and lumican, have also been proposed as being important in the control of fibril growth (Neame et al., 2000), as has hyaluronic acid which is also present in large amounts in developing connective tissues (Scott and Parry, 1992). Fibrils extracted from different tissues suggest that the detailed mechanism may also be dependent on the site and function of the particular tissue. For example, in developing tendon, the fibril length increases rapidly between Day 16 and Day 18, whereas developing fibrils in the cornea and dermis lengthen more gradually (Birk et al., 1996).

In the present study, we have examined tissue obtained from a wound repair model, associated with a biomaterial implant, at specific time points postwounding. Histology, in situ hybridization, and immunofluorescence studies were used to characterize the collagen formation in this model. Gentle disruption of the newly formed tissue enabled isolation of entire, unbroken collagen fibrils. The dimensions of these fibrils were determined by transmission electron microscopy, showing that the average diameter of fibrils remained essentially constant between 14 and 28 days. Immunoelectron microscopy showed that all fibrils were heterotypic, containing both type I and type III collagens.

**MATERIALS AND METHODS**

**Implants.** Lengths of expanded polytetrafluoroethylene (ePTFE) tubing (Goretex; W. L. Gore and Associates, Flagstaff, AZ; 4 mm i.d. \( \times \) 20 mm) were implanted subcutaneously in the backs of 3 Merino wethers (castrated male sheep), each about 3 years of age. These samples were removed for examination after implantation times of 4, 6, 14, and 28 days. Collagen formation in the center of the tube was examined by histology, in situ hybridization, immunofluorescence, and transmission electron microscopy on gentamicin-treated extracts (de-vente et al., 1997).

**Histology.** Tissue from the center of the tube was fixed at each time point in 10% neutral buffered formalin overnight, dehydrated in ascending grades of ethanol, cleared in Histoclear (Sigma, St Louis, MO), and processed through to paraffin wax by standard protocols. Sections 4 μm thick were cut, the paraffin was removed, and the sections were stained using Harris’ hematoxylin and eosin.

In situ hybridization. In situ hybridization for mRNA encoding collagen type I was performed using UTP-32P detection using 4-μm paraffin sections collected onto 3-aminopropyltriethoxysilane-treated slides, following the method of Bisucci et al. (2000). The type I collagen riboprobe was 600 bp in length, derived from rat type I collagen, and was subcloned into pGEM3z (Nakatsuka et al., 1990).

Immunofluorescence microscopy. Sections, 4 μm thick, were cut from frozen samples and examined using purified monodonal antibodies to type I (SD8-G9/Col1) and type III (2G8-B1/Col3) collagens (Werkmeister et al., 1990; Werkmeister and Ramshaw, 1991). After incubation, sections were washed 3 times for 5 min in PBS, and then the secondary antibody, fluorescein isothiocyanate-conjugated, sheep anti-mouse antibody (Silenus Laboratories, Melbourne, Victoria) diluted 1:50 in PBS, was applied. After a further 3 washes each for 5 min in PBS, sections were mounted in glycerol/PBS (9/1, v/v) containing 1 mM 1,4-diazabicyclo(2,2,2)octane (Sigma, St Louis, MO). Sections were examined using an Optiscan Confocal F900E microscope system.

Transmission electron microscopy of individual fibrils. Small tissue pieces (1-2 mm³) were dissected from the center of the tubes and placed in 1 ml of gentamicin solution (de-vente et al., 1997) (40 mg/ml, pH 7.1) for 16 h at room temperature on a reciprocating shaker. The samples were vortexed for 45 min at about 1400 vibrations per minute. The residual tissue pieces were removed from the solution and the gentamicin extract was centrifuged at 4750g for 5 min. The supernatant was removed and samples reconstituted in 20 μl of PBS, containing 0.03% sodium azide. Samples of this preparation (1 μl) were placed onto upturned, freshly glow-discharged carbon-coated 300 mesh gold grids and allowed to settle for 30 s. Grids were then rinsed briefly in 3 μl of PBS and stained in 2% aqueous uranyl acetate for 2 min. Grids were examined using a J EOL J EM-2000FX microscope.

Immunoelectron microscopy. Grids containing collagen fibrils were prepared as described above, except that they were not stained with uranyl acetate. For immunostaining, grids with collagen were pretreated with 0.001% pepsin (Worthington Biochemical Corporation, Freehold, NJ) in 10 μM acetic acid for 1 min at 37°C, rinsed in PBS, and then placed in 1% BSA in PBS for 30 min. Grids were then incubated on a 20-μl drop of either purified anti-collagen type I monoclonal antibody (SD8-G9/Col1) (Werkmeister et al., 1990) used at a final dilution of 0.1 mg/ml, or purified anti-collagen type III monoclonal antibody (2G8-B1/Col3) (Werkmeister and Ramshaw, 1991) used at a final concentration of 0.2 mg/ml. Grids were incubated for 90 min at room temperature, washed 6 times in PBS each for 4 minutes, and placed for 60 min into a 20-μl drop of 6 nm gold-labeled goat-anti-mouse IgG (H&L) (Proscitech, Thuringowa, Queensland) diluted 1/30 in PBS/1% BSA. Grids were washed in 1.8 M
NaCl/1% BSA and then a further 5 times in PBS/1% BSA. Grids that were labeled with one antibody were then rinsed in water, fixed in 2% glutaraldehyde for 1 min, rinsed in water, and stained in 2% aqueous uranyl acetate for 2 min. Grids that were dual-labeled were then incubated in the other diluted antibody solution for 90 min at room temperature, washed 6 times in PBS each for 4 min, and placed for 60 min into a 20-μl drop of 10 nm gold-labeled goat-anti-mouse IgG (H&L) (Proscitech, Thuringowa, Queensland) diluted 1/30 in PBS/1% BSA. The procedure was finished as in the single labeling. All combinations of antibody and large or small gold were included and examined. In addition, some grids had the primary antibody omitted and were labeled with the secondary gold probes only, to ensure that the gold label was not attaching nonspecifically.

RESULTS

Hematoxylin and Eosin Staining

After 4 days, the center of the tube of the explanted sample was empty, with no tissue formation, while at 6 days, this central tubular region was filled with a loose red blood clot, but no obvious collagenous tissue. After 14 days the center of the tube had been filled with extensive collagenous tissue, and histology showed that a large number of fibroblast-like cells, often in distinctive swirling groups, were present (Fig. 1A). These cells had become even more abundant by 28 days. At both 14 and 28 days there was no evidence of multinucleated giant cells.

In Situ Hybridization

The samples examined by H&E histology were also examined by in situ hybridization, using a riboprobe toward type I collagen. In the 4- and 6-day explants there was no evidence of collagen synthesis within the central tubular region of the implant, although strong staining was present in the wound response tissue that surrounded the implanted material. By 14 days, an extensive cell and collagenous network was present in the center of the tubular implant and in situ hybridization showed that most, if not all, cells were synthesizing type I collagen. Similar results were also obtained from 28 day samples (Fig. 1B).

Immunofluorescence Histology

Examination of the tissue formed in the center of the tubular implant at both 14 and 28 days showed that there was immunostaining for both type I and type III collagens, and that both these collagens were uniformly distributed within the newly formed tissue (Fig. 1C).

Transmission Electron Microscopy of Isolated Fibrils

At 4 and 6 days, histology did not show any new collagen within the center of the explant sample, and no individual collagen fibers were identified from this region by transmission electron microscopy of tissue or tissue extracts. After 14 days, this showed the presence of many small collagen fibrils
in the center of the explant. Examination of 47 isolated fibrils showed that they had an average diameter of 48 ± 11 nm (Fig. 2), and a smaller subgroup of these that were identified across grid bars as intact fibrils had an average length of 9.2 μm. On rare occasions short fibrils, about 1 μm long, were seen. Since there was no evidence of tissue in the center zone of the implant at 6 days, these new fibrils would be up to 8 days old.

After 28 days, the collagenous network was more extensive. Examination of 101 of these isolated fibrils showed that they had diameters very similar to those at 14 days, also having an average diameter of 48 ± 8 nm (Fig. 2). Examination of a smaller subgroup of intact fibrils suggested that they were now longer, with an average length of 28.1 μm. Again, on rare occasions short fibrils, about 1 μm long, were seen. The fibrils in this sample would be up to 22 days old. Although there is an indication that the fibril length may be longer at 28 days, this average value may be an underestimate since the longest fibrils traverse across grid bars and cannot be identified with certainty from other fibrils in that grid bar. In addition, shorter fibrils may be preferentially extracted. At both time points, very few fibrils with broken ends were isolated, perhaps reflecting the mild extraction procedure. At 28 days, transmission electron microscopy studies of conventionally processed, resin-embedded tissue showed the presence of numerous newly formed collagen fibrils, with a mean diameter of 45 nm (n = 243) (data not shown), comparable to the mean diameter observed for isolated fibrils.

In all cases, the fibrils examined appeared to be single fibrils, and did not appear to be made from thinner fibrils that had laterally coalesced. In a very few cases the fibrils thinned at a point along their length before rethickening to a mature diameter. There was no evidence of this being associated with lateral aggregation. Also, there was no polarity reversal to indicate axial aggregation, although these thinner regions could have arisen from an in-phase axial aggregation without any polarity reversal, either as part of natural fibril growth or due to chance during grid preparation (Fig. 3). For both 14- and 28-day samples, when the staining showing the fibril D periodicity was clear, about 80% of fibrils showed a single polarity reversal, suggesting that axial aggregation had occurred (Fig. 4A). The region of the polarity reversal appeared to be less densely packed and slightly broader than the surrounding fibril, suggesting that this region may be where two fibrils had come together axially. When a polarity reversal was present, both tips were formed from the N-terminal ends of the collagen molecules and the C-terminal ends formed the overlap region (Figs. 4B and C). Where no polarity reversals were present, the orientation (polarity) of the asymmetric banding pattern was uniform along the full length of each fibril. In the present study no fibrils with two polarity reversals were observed.

The ends of intact fibrils all showed well-defined tapered tips (Figs. 4B and C). The tapers in the present study generally do not show the clear differences between the two ends of the fibrils. This contrasts with reports for other systems, including in vitro (Kadler et al., 1990) and naturally occurring fibrils (Birk et al., 1996), where the fibrils were observed to have a gradually tapered (α) end and a blunt (β) end. In the present study, the range of values for the distance from the position of half-fibril width (50% of the constant diameter, middle region of the fibril) to the visible end of the tip was 0.18 to 0.44 μm. This range corresponds to the blunt (β), short tapered end of fibrils described previously (Birk et al., 1996). No fibrils with long tapered (α) end characteristics were observed.

FIG. 2. Histogram of fibrils diameters for isolated fibrils at 14 days (n = 47, □) and at 28 days (n = 101, □).
all fibrils contained both type I and type III collagens. Although the type I antibody labeled weakly, labeling was generally visible over the entire central region of fibrils (Fig. 5A). Similarly, type III collagen antibodies labeled fibrils over the entire central region (Fig. 5B). The type III labeling was much stronger than for type I collagen, and a labeling periodicity, seen as an approximate 5D repeat of the label external to the fibril, was evident in many images (Fig. 5C). The difference in staining levels reflects the different affinity of the antibodies rather than giving any indication on collagen quantities. The type I collagen antibody detects an epitope at the C-terminal of pepsin-treated collagen (Werkmeister et al., 1990). Only a brief, mild pepsin treatment was used in the present study to produce epitopes for detection, since more extensive pepsin treatment degrades the fibril structure, potentially limiting the number of epitopes available. Not all fibrils were labeled, with labeling for both type III and type I collagens being absent for small diameter fibrils, typically less than 40 nm in diameter (Fig. 5B). Also, the labeling was not uniform and was absent in the smaller diameter zone of the tapered ends of the fibrils when the diameter was also less than 40 nm. This reflects the smaller diameter of these fibrils or regions of fibrils as adjacent fibrils of larger diameter in the same fields were well labeled (Fig. 5B).

**Immunoelectron Microscopy of Isolated Fibrils**

Immunoelectron microscopy of 14-day samples using anti-collagen monoclonal antibodies showed that all fibrils contained both type I and type III collagens. Although the type I antibody labeled weakly, labeling was generally visible over the entire central region of fibrils (Fig. 5A). Similarly, type III collagen antibodies labeled fibrils over the entire central region (Fig. 5B). The type III labeling was much stronger than for type I collagen, and a labeling periodicity, seen as an approximate 5D repeat of the label external to the fibril, was evident in many images (Fig. 5C). The difference in staining levels reflects the different affinity of the antibodies rather than giving any indication on collagen quantities. The type I collagen antibody detects an epitope at the C-terminal of pepsin-treated collagen (Werkmeister et al., 1990). Only a brief, mild pepsin treatment was used in the present study to produce epitopes for detection, since more extensive pepsin treatment degrades the fibril structure, potentially limiting the number of epitopes available. Not all fibrils were labeled, with labeling for both type III and type I collagens being absent for small diameter fibrils, typically less than 40 nm in diameter (Fig. 5B). Also, the labeling was not uniform and was absent in the smaller diameter zone of the tapered ends of the fibrils when the diameter was also less than 40 nm. This reflects the smaller diameter of these fibrils or regions of fibrils as adjacent fibrils of larger diameter in the same fields were well labeled (Fig. 5B).
The labeling of fibrils by both the individual antibodies suggested that all fibrils contained both type I and type III collagens. This was confirmed by dual labeling, which showed that each fibril was labeled for both these collagen types (Fig. 5D).

**DISCUSSION**

The present study has demonstrated that newly formed collagen fibrils can be extracted from a wound healing model. Partitioning of the newly formed collagen fibrils through use of the biomaterial implant ensured that these fibrils were newly synthesized and not contaminated by preexisting mature fibrils. The H&E histology data indicated that there was no chronic or unwanted response in the wound healing process in this model that contains a biomaterial implant. This was shown, for example, by the absence of multinucleated cells (Muller-Mai and Gross, 1991). Also, bothabsence of multinucleated cells (Muller-Mai and Gross, 1991). Also, both

The method used in this study to isolate fibrils was gentle and did not include homogenization or ultrasonic treatment, as has been used in certain other studies (Birk et al., 1996; Graham et al., 2000). Instead, the sample was shaken gently in the presence of buffered gentamicin (devente et al., 1997). This was found to lead to significantly less mechanical breakage of fibrils than homogenization methods (data not shown). If gentamicin was omitted, and the fibrils extracted in PBS, the amount of fibril breakage was greatly increased and few intact fibrils were obtained (data not shown). The reason for the effectiveness of gentamicin was not clear.

The present data showed that fibril formation observed in the wound healing model was comparable to that previously observed in various embryonic tissues (Holmes et al., 1994; Birk et al., 1996). For example, the diameters of the shorter fibers were similar to the longer fibers, consistent with previous proposals that they represent a population of fibrils that later grow into the longer functional fibrils of mature tissues (Birk et al., 1997). Tendon fibrils are uniformly thin (~25 nm) during development until a stimulus causes a rapid increase in diameter and a corresponding marked decrease in tissue glycosaminoglycan (Scott and Parry, 1992). The diameter of each fibril was constant along its length. Very few had irregular profiles in cross section, unlike 17-day embryonic tendon fibrils where lateral fusion may be occurring (Birk et al., 1995).

Preparation of fibrils by an in vitro approach (Kadler et al., 1990, 1996) and development of methods for isolation of intact collagen fibrils has enabled the molecular polarity during collagen fibril growth to be examined. Fibrils formed in vitro from acid-soluble collagen are unipolar (Kadler et al., 1996). Those formed by cleavage of pC-collagen by C-propeptidase were exclusively bipolar (Kadler et al., 1996), showing a single polarity reversal in the banding pattern, and having collagen molecules oriented with amino-termini at both ends of the fibrils. Fibrils from echinoderms have a single reversal in polarity, with the amino-termini of the collagen again forming the ends of each fibril (Thurmond and Trotter, 1994). Chicken collagen fibrils formed in vivo also exhibit a polarity reversal, although not all fibrils show this (Holmes et al., 1994). Those fibrils that do exhibit a polarity reversal have amino-termini at the ends of the fibrils (Birk et al., 1996; Trotter et al., 1998). In the present study a reversal in polarity was found in about 80% of intact fibrils, and these also had the collagen with the amino-termini at the ends of fibrils. The regions of polarity reversal appeared to be locations where two fibril tips had come together axially. If many small fibrils were able to axially associate, larger fibrils with multiple polarity reversals along the length would be observed. These were not seen in this wound repair system where only single polarity reversals were observed. The data on newly formed, immature fibrils contrast with those from mature tissues where polarity reversals are not observed.

Although the polarity reversals provide evidence for axial aggregation, there was no evidence for lateral aggregation in the fibrils examined. Collagen found in mature tissues is arranged into structures that form macroscopically visible fiber bundles. These fiber bundles would require a mechanism whereby individual fibrils can associate and broaden to attain a functional mature tissue form. Our study indicates that the isolated collagen fibrils from a wound model do not associate laterally into stable structures during their early development (<22 days).

In previous studies (Birk et al., 1995, 1996), particularly in vitro studies (Kadler et al., 1990), newly formed fibrils were shown to have distinct tapers at each ends, with initial growth occurring preferentially at the sharp (α) end compared to the blunt (β) end. In contrast to these studies, in the present model the fibrils had symmetrical tips, and these were of the short, blunt taper (β) class. Since the shape of the ends of a fibril is dependent on the concentration of pC-collagen at the onset of fibril assembly (Kadler et al., 1996) the taper at the ends of the newly formed fibrils in this wound repair model could also be determined by the specific enzyme and substrate concentrations.

There are little data on the length of mature fibrils. Long fibrils (up to 1 mm) have been isolated...
unbroken only from echinoderms (Trotter et al., 1998). Studies on mature rat-tail tendon, where over 1000 fibrils were followed over 1.4 μm by serial sectioning, showed no ends (Craig et al., 1989), leading to an estimate for fibril length of up to 10 mm in 6-month-old tendon (Craig et al., 1989). Serial sectioning to measure the length of fibrils in 14-day chicken embryo tendon showed only one complete fibril, but the proportion of ends seen suggested that the fibrils were shorter than in mature tendon (Birk et al., 1989, 1990; Birk and Zycband, 1994). In the present study, potential variations in extraction and fracture of small versus larger fibrils do not allow length data to be accurately assessed.

The present study has examined fibrils from a wound healing system, where type III collagen is found at increased levels (Barnes et al., 1976). Of particular interest was that these fibrils, which later form an extensible tissue containing both type I and type III collagens, all contained both these collagen types at this early stage in fibril formation. Previous studies have indicated that type III is present on the surface of newly formed dermal collagen type I fibrils (Fleischmajer et al., 1990). The dermal fibrils with a diameter greater than 60 nm did not label with type I antibody (Fleischmajer et al., 1983, 1990). In the present study the fibrils had an average diameter of 48 nm. However, as there were very few fibrils that exceeded 60 nm in diameter the loss of labeling for larger diameter fibrils could not be confirmed. Fibril labeling by both antibodies was observed on fibrils greater than 40 nm in diameter, but was very limited or absent for fibrils with a diameter of 40 nm or less. This was also the case for the tapered tips of the larger fibrils where the diameter had fallen to less than 40 nm. The present study has not examined whether there are propeptides or other proteins on the surface of the smaller diameter fibril segments masking the collagen staining. A range of molecules that bind along the collagen fibril, including decorin, lumican, and fibromodulin, may control lateral aggregation of the molecules and regulate axial growth (Neame et al., 2000).

The present study of fibril formation in a wound healing model incorporating a biomaterial allows the newly formed fibrils to remain physically isolated from the nearby mature tissue. This allows them to be extracted, uncontaminated by mature tissue. The model is suitable for use with knockout mice where specific molecules that may have a role in fibril development have been deleted. For example, the role of decorin could be further studied using the present system. Decorin is known to decrease significantly after 18 days in chicken embryo tendons, suggesting that a decrease in fibril-associated decorin is necessary to allow further growth and fusion of fibrils (Birk et al., 1995). Also, the porous nature of the biomaterial will allow biochemical modulation of fibril formation, for example, by release of biologically active molecules into the newly forming tissue. Thus, this model will allow potential biochemical approaches for controlling tissue formation in tissue-engineering constructs to be examined and refined, leading to the more accurate production of natural tissue by a tissue-engineering approach for therapeutic applications.

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