Flexor tendon repair in zone II is complicated by adhesions to the surrounding fibro-osseous sheath. Adhesions between the tendon and tendon sheath impair the gliding mechanism of tendons and result in marked impairment of hand function.

Tendon healing is the product of both cells within the tendon as well as cells of the epitenon and tendon sheath. There has been great debate over whether intrinsic or extrinsic mechanisms of healing predominate; it is now clear that cells of the tendon sheath, epitenon, and endotenon contribute to tendon healing and possibly adhesion formation.1–7 Efforts over the past 2 decades have focused on the development of strategies to decrease adhesion formation experimentally, with the long-term goal of improving clinical outcome after tendon injury and repair. Biochemical agents such as antihistamines, steroids, β-aminopropionitrile, and hyaluronic acid have not led to notable reduction in postoperative adhesions in clinical flexor tendon injury and repair.8–12 The ability to modulate adhesion formation is clearly contingent on a better understanding of the repair process.

Postoperative scarring is the end result of a complex cascade of biochemical reactions. The importance of lactate in stimulating the production of scar has been well described.13,14 Lactate is an early
mediator in the wound healing process. Tissue hypoxia stimulates lactate production from tissue macrophages and lactate, in turn, is a strong stimulant of collagen production. Lactate is also capable of stimulating growth factors, including transforming growth factor (TGF)-β, which are known to play an important role in tendon healing.\textsuperscript{15–17}

We have developed a rabbit flexor tendon wound healing model and previously described the important role of TGF-β in adhesion formation. We have also demonstrated the ability to alter tendon healing and scarring by exogenous supplementation with TGF-β.\textsuperscript{18–20}

Using our model of flexor tendon healing we examined proliferation of tendon sheath fibroblasts, epitenon tenocytes, and endotenon tenocytes; \textit{in vitro} collagen production by 3 different cell lines; and influence of lactate on cell proliferation and collagen production by all 3 cell types.

**Materials and Methods**

**Animal Model**

All rabbit experiments complied with the institutional animal protocols. Adult male New Zealand white rabbits (4.0–4.5 kg) were anesthetized with an intramuscular injection of ketamine (50 mg/kg), xylazine (5 mg/kg), and acepromazine (0.01 mg/kg). The middle digit flexor digitorum profundus equivalent was identified and isolated in each forepaw. The tendon and tendon sheath were transected.

**Cell Culture and Morphology**

Cells from the flexor tendon profundus sheath, epitenon, and tendon were isolated and cultured using a previously described protocol with minor modifications.\textsuperscript{21} Briefly, the intact flexor tendons and tendon sheath were separated by sharp dissection. The tendon sheath was digested with 0.5% collagenase (Sigma, St Louis, MO) in 20 mmol/L HEPES buffer for 10 minutes at room temperature. The tendon sheath fibroblasts were plated and cultured in Ham’s F12 medium supplemented with 10% fetal bovine serum.

The intact tendons were treated with 0.25% trypsin at 37°C for 20 minutes to release the epitenon tenocytes. The epitenon tenocytes were plated and cultured in Ham’s F12 medium. The remaining tendon was treated with 0.5% collagenase to release endotenon tenocytes, which were similarly plated and cultured in Ham’s F12 medium.

Each cell line was grown to confluence at 37°C in a humidified, 5% CO\textsubscript{2} incubator. At confluence cells were passaged by washing with phosphate-buffered saline (PBS) and detached with trypsin/EDTA. The cells were then plated at a density of 1.5 × 10\textsuperscript{4}/well. Cell counts were performed using a hemacytometer at days 1 to 4 to quantify proliferation. Cell lines were cultured in triplicate.

Cell cultures were stained with methylene blue and photographed using phase-contrast microscopy at ×100 magnification.

**Effects of Lactate on Cell Proliferation**

Sheath fibroblasts, epitenon tenocytes, and endotenon tenocytes were detached from their culture dishes and seeded onto 24-well plates at a density of 2 × 10\textsuperscript{4} cells/well. The cells were grown in serum-supplemented medium as described previously for 24 hours. The cells were washed with PBS and changed to serum-free medium and 0.2% lactalbumin (Sigma). Half of the cell cultures for each cell line were supplemented with 25 mmol D-L-lactate (Sigma). The remainder were grown in nonsupplemented culture medium.

Cell counts were obtained 3 days after addition of lactate to the culture medium. Cultures were grown in triplicate; cell numbers were compared between lactate- and nonlactate-supplemented cell lines using the Student’s \textit{t}-test.

**Immunocytochemical Staining of Collagen I, II, and III Production**

The same 3 cell lines, sheath, epitenon, and tendon, were grown on glass coverslips in Ham’s F12 medium. On day 4 the cells were fixed in 2% formalin. The cells were rinsed in PBS and the endogenous peroxidase was quenched with 0.3% hydrogen peroxide and permeabilized with 0.1% saponin. Cells were stained separately with antibodies to collagen I (Sigma), II (Neomarkers; Union City, CA), and III (Chemicon, Temecula, CA) and incubated for 1 hour at room temperature. Cells were washed with PBS and biotinylated secondary antibodies. Vectastain (Vector Laboratories, Burlingame, CA) reagent was added; the cells were stained with diaminobenzidine, mounted with Permount (Fisher, Pittsburgh, PA), and photographed.
Quantification of Collagen Production by Enzyme-Linked Immunoabsorbent Assay

Cells were seeded at a density of $2 \times 10^4$ cells/well and cultured in 48-well plates overnight in Ham’s F-12 medium supplemented with 10% fetal bovine serum. The medium was changed to either lactate-supplemented or nonsupplemented serum-free Ham’s F-12 medium for 3 days. The cells were washed with PBS and fixed with 2% formalin. Endogenous peroxidase was quenched with 0.3% hydrogen peroxide and permeabilized with 0.1% saponin. Antibodies to collagen I, II, and III were added and the cells were incubated at room temperature for 1 hour. Secondary antibodies and peroxidase substrate were added for 30 minutes. The reaction was stopped with 0.5-N sulfuric acid. Next 100 μL of each sample was transferred to a 96-well plate and collagen production was measured spectrophotometrically at an optical density of 450 nm (OD_{450}). Collagen production for each cell line by supple-

![Figure 1](image1.png)

**Figure 1.** Cell growth on tendon sheath, epitenon, and endotenon cells at days 1 to 4. Data are given as mean number of cells ± SEM.

![Figure 2](image2.png)

**Figure 2.** Cell growth with (■) and without (□, control) lactate supplementation of culture media. S, sheath cells; E, epitenon cells; T, endotenon cells. Data are given as mean number of cells ± SEM.
mented and nonsupplemented media was measured in triplicate and compared using a 2-tailed Student’s t-test.

Dose-Response Effects of Lactate Concentration on Collagen I Production

To examine if there was a dose-response effect of lactate collagen I production was measured at varying concentrations of lactate (0, 25, 50, 100, and 200 mmol/L) in the sheath, epitenon, and endotenon cultures. The pH of each solution was maintained at 7.4 using HEPES buffer. Each concentration was studied in triplicate. Collagen I production was quantified using an enzyme-linked immunosorbent assay (ELISA) as described earlier. Collagen production at each concentration was compared with that of control culture medium (no lactate supplementation) using a 2-tailed Student’s t-test.

Results

Isolation and Culture of Sheath Fibroblasts, Epitenon Tenocytes, and Endotenon Tenocytes

Three different cell lines were isolated from each tendon specimen: sheath fibroblasts, epitenon teno-
cytes, and endotenon tenocytes. Growth curves for each cell line are shown in Figure 1. Initially each cell line proliferated at a similar rate. Over days 2 to 4, however, the sheath cells proliferated at a significantly higher rate than the epitenon and tendon cells (p < .05). Fibroblast cell morphology was confirmed by methylene blue staining of each cell line.

**Effects of Lactate on Cell Proliferation**

Cell counts of each cell line were measured on day 3 with and without lactate supplementation of the culture medium (Fig. 2). There was no statistically significant difference in cell proliferation at each time point within each cell line. Therefore, lactate did not affect cell proliferation in any of the 3 cell lines.

**Effects of Lactate on Collagen Production**

Collagen I, II, and III production was detected by immunohistochemical staining of tendon sheath cells (Fig. 3). Immunohistochemical staining of the epitenon and endotenon cells also showed the presence of collagen I, II, and III production. Collagen production was quantified using ELISA assay (Fig. 4). All cell lines produced collagen I in the greatest amount. Collagen II and III were produced in lesser amounts.

Addition of lactate to cell culture medium increased collagen production in all 3 cell lines (Fig. 4). Collagen type I production increased more than 70% by tendon sheath cells compared with baseline levels. Collagen I production by epitenon cells increased 15% and by endotenon tenocytes by 12%; both increases were statistically significant (p < .05). Collagen II and III production was also increased in each cell population.

**Discussion**

Adhesion formation after tendon injury and repair can compromise tendon gliding mechanisms and overall hand function. In this study we isolated 3 different cell lines from zone II rabbit flexor tendons: tendon sheath fibroblasts, epitenon tenocytes, and endotenon tenocytes. All 3 cell lines produced collagen in vitro. Therefore, tendon adhesion formation in vivo likely results from disordered collagen production by 3 separate cell populations, including cells within the substance of the tendon and those within the epitenon and tendon sheath.
Figure 4. Collagen (A) I, (B) II, and (C) III production measured spectrophotometrically with (■) and without (□, control) lactate supplementation of culture medium by sheath, epitenon, and tendon shells. Data are given as mean OD$_{450}$ ± SEM. *p < .05.
The classification of adhesion formation as simply intrinsic or extrinsic may be expanded to reflect the production of collagen from 3 distinct regions: the substance of the tendon (endotenon), the epitenon, and the tendon sheath. Effective strategies to minimize adhesion formation, both surgically and medically, could target all 3 areas.

In culture tendon sheath fibroblasts showed the greatest proliferative capacity and produced the greatest amount of collagen. As expected most of the collagen produced by each cell line was collagen type I. Collagen types II and III were also detected by immunohistochemical analysis, however. Because we used different antibodies, with potentially different affinities, to collagen I, II, and III, the quantities of collagen production may not be directly comparable.

Addition of lactate to cell culture media significantly increased collagen production by all cell lines. The greatest increases were again observed in the tendon sheath cell line. Interestingly, there was an increase in collagen production in all cell types but the absolute number of cells did not increase significantly. Therefore, lactate increased collagen production not by increasing cell proliferation but by upregulating collagen production by each cell.

The effects of lactate on collagen production are concentration dependent. We initially observed an increase in collagen production that peaked at 50 mmol. At higher concentrations collagen production decreased to below baseline levels, possibly because of cellular toxicity at higher lactate levels. The pH of each culture media was buffered to eliminate any effects of H⁺ on cell proliferation and collagen production. Changes in collagen production were therefore directly attributable to lactate.

The importance of lactate in the inflammatory response after cellular injury is well established. Lactate production is a direct result of tissue hypoxia and is therefore an early mediator in the wound healing cascade. Hunt et al showed that the addition of lactate to extracellular fluid closely correlates with the stimulation of collagen synthesis by fibroblasts. This stimulatory effect is caused by upregulation of prolyl hydroxylase, an essential enzyme in collagen synthesis.

The significant increase in collagen production in all 3 cell lines after addition of lactate in vitro suggests that lactate may be a key modulator of adhesion formation after injury in vivo. In addition to directly stimulating collagen production, lactate is capable of upregulating TGF-β production, another known stimulant of adhesion formation.

These observations raise several important issues. Lactate is a direct product of cellular metabolism, capable of stimulating a chain of reactions that increase local cytokine and growth factor levels. Therefore, the ability to manipulate lactate levels or mitigate the stimulation of collagen production through competitive inhibition of prolyl hydroxylase, for example, could alter the cascade of events that result in adhesion formation. The influence of lactate...
on growth factors such as TGF-β, whose critical role in adhesion formation is well described, also merits investigation.

The results of this study confirm that 3 distinct cell lines, tendon sheath fibroblasts, epitenon tenocytes, and endotenon tenocytes, can be isolated from rabbit flexor tendons and each has different potentials for proliferation and production of extracellular matrix. The traditional paradigm of adhesion formation as either extrinsic or intrinsic should be expanded to reflect the collagen production by 3 distinct regions: sheath, epitenon, and endotenon. Lactate significantly increases collagen production by all 3 cell lines, providing another potential target in the development of strategies to mitigate adhesion formation.

References

Erratum
In the article by Klein et al entitled “Flexor Tendon Wound Healing In Vitro: The Effect of Lactate on Tendon Cell Proliferation and Collagen Production,” which appeared in the September 2001 issue (Vol 26A, No 5, pp 847-854), an error in calculation was made. On page 851, the sentence beginning on the last line of the first column should read: “Collagen type I production increased more than 37% by tendon sheath cells compared with baseline levels.” The data in Figure 4 is correct as it appears.