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The effects of reduced oxygen tension on cell proliferation and matrix synthesis in synovium and tendon explants from the rabbit carpal tunnel: an experimental study in vitro

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Abstract

Local ischemia may play an important role in the development of tendon degeneration as well as entrapment neuropathies. In order to investigate the cellular effects of hypoxia on tendon and synovial tissue from the carpal canal, dose response effects of oxygen on cell proliferation and synthesis of matrix components were examined in segments of synovial and flexor digitorum profundus tendon from the carpal tunnel of rabbits during short term culture. Explants were incubated in airtight containers flushed with either 0%, 1%, 3%, 20% O_2 plus 2% CO_2 and N_2 to balance and labeled with either ^{3}H-thymidine or ^{3}H-proline and ^{35}S-sulfate. Cell proliferation was significantly inhibited by hypoxia in synovium but not in tendon ($P = 0.03$). In parallel, the synthesis of non-collagenous proteins was significantly reduced in synovium but not in tendon ($P = 0.006$). In both tissues hypoxia significantly inhibited collagen synthesis. On the other hand, hypoxia had no significant effect on the synthesis of new proteoglycans as determined by ^{35}S-sulfate incorporation. Hypoxia can inhibit cell proliferation and alter synthesis of matrix components in synovial tissue, but may only have minor effects on non-collagen protein synthesis in tendon explants from the carpal canal of rabbit forepaws.

Introduction

Tendons, their surrounding synovium and other adjacent tissues (e.g., nerve) may be exposed to prolonged ischemia due to localized tissue compression. This may occur at sites where the tissues wrap around a bony prominence or in 'confined' spaces such as the carpal tunnel [18]. For example, within the carpal tunnel the hydrostatic pressure varies throughout the day and can rise, in conjunction with some hand postures, to levels above 6 kPa [23,24]. These pressures are above those known to reduce microvascular flow in animal models (e.g., 4 kPa) [19,25,26]. The reduced blood flow may limit delivery of substrates, such as oxygen, or may limit the clearance of metabolites.

The initial cellular effects of compromised blood flow or reduced oxygen tension on synovium and tendon are unknown. The purpose of this study was to investigate, in vitro, the hypothesis that reduced oxygen tension reduces cell proliferation and modifies the synthesis of extracellular matrix macromolecules in synovial and tendon tissue from the rabbit carpal tunnel. The rabbit carpal tunnel is similar to the human in that the carpal bones and the transverse carpal ligament form a stiff passageway at the wrist through which the flexor tendons and the median nerve travel (Fig. 1). As in humans the tendons are surrounded by a firm synovium. Recently, it has been demonstrated that cellular activities, including cell proliferation and the synthesis of matrix components may differ between tendon and synovial sheaths during healing [31].

Methods

Two similar experiments were conducted, each with mixed gender Swedish Loop rabbits of ages 3–4 months, weighing 1.9–2.3 kg. Prior to the use of animals the study was approved by the Lund University Committee on Animal Research.

During the first experiment, the tissue explants from six rabbits were labeled with ^{3}H-thymidine and during the second experiment the explants from six different rabbits were labeled with ^{3}H-proline and

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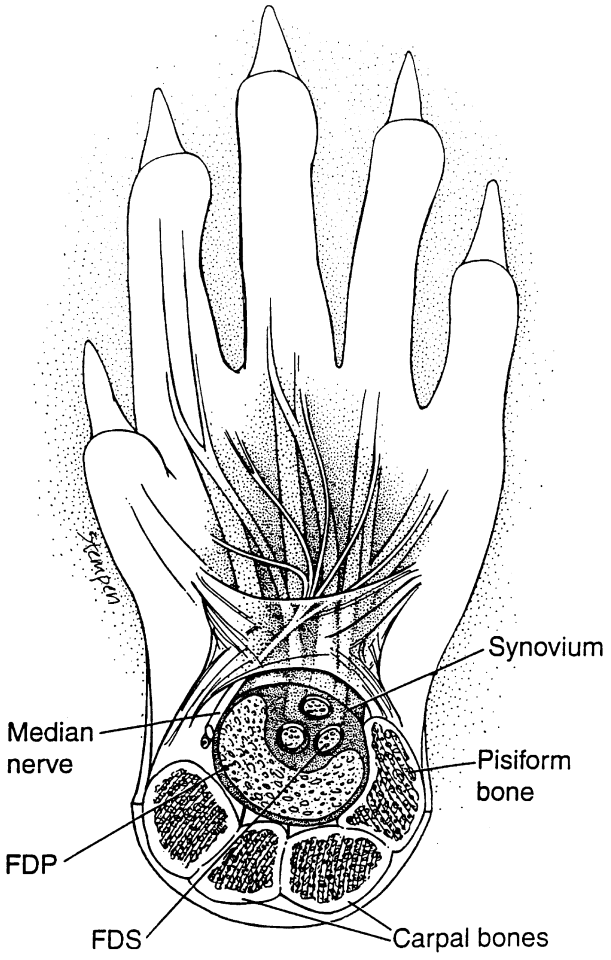

Fig. 1. A palmar view sketch of left rabbit forepaw at the level of the carpal tunnel with radial side to the left. The flexor digitorum profundus (FDP) and flexor digitorum superficialis (FDP) tendons are identified. The synovium and FDP tendon were harvested in a block just distal to the pisiform and beneath the transverse carpal ligament.

^{35}S-sulfate. Cell proliferation was assessed by uptake of ^{3}H-thymidine. Synthesis of new proteoglycans was indirectly assessed by ^{35}S-sulfate uptake. ^{3}H-proline is incorporated into procollagen and then hydroxylated; therefore, ^{3}H-hydroxyproline count provides an estimate of collagen synthesis. The incorporation of ^{3}H-proline was corrected for the known relative frequency of proline in collagen relative to non-collagenous protein. Thus ^{3}H-proline represents the de novo synthesis of non-collagen. The proportion of collagen synthesized out of the total protein synthesized was thereby corrected for the known relative frequency of proline in collagenous and non-collagenous protein [12].

Tissue blocks

The rabbits were anesthetized with an intramuscular injection of fentanyl-fluanisone (Hypnorm®; Janssen Pharmaceutica, Belgium; 0.5 ml/kg body weight) and euthanasia was administered with an intravenous injection of pentobarbital (8 ml; 60 mg/ml). Using a dissecting microscope, a block of synovial tissue and the flexor digitorum profundus (FDP) tendon from the carpal tunnel, just distal to the pisiform bone, were removed from both forepaws using sterile technique, and placed immediately in culture media (Fig. 1). The synovial tissue from each paw was split once longitudinally to produce four explants per animal. Each FDP tendon was cut into two 1-mm thick cross-sectional slices also producing four explants per animal. Each explant, therefore, included similar quantities of central and edge material.

Tissue culture and labeling procedures

Each group of four explants was rinsed in MCDB 105 culture medium (M-6395, Sigma BioSciences, MO; McKeehan 78) then separated to one of four 24 multidish plates, one explant per well (Fisher, Pittsburg, PA) to which MCDB 105 medium supplemented with gentamycin (50 μg/ml), ascorbic acid (50 μg/ml), and bovine serum albumin (1 mg/ml) had been added. To the explants that were later to be labeled with ^{3}H-thymidine, non-radioactive thymidine (50 μg/ml) was also added.

Explants were cultured for three days. During the first day all were incubated at 37°C in a water-saturated atmosphere of 2% CO_2 and 19% O_2. For the second and third days, the four plates were divided to four 2-l airtight containers which were flushed with a custom gas mixture of either 0.0%, 1.0%, 3.0% or 20.0% O_2 plus 2.0% CO_2 and N_2 to balance (Air Liquid Gas AB, Kungsangen, Sweden), then sealed at 101 kPa and incubated at 37°C. The oxygen tensions were selected on an empiric basis. Sterile water (40 ml) had been added to each container to water saturate the interior atmosphere.

On the second day, the culture media was replaced with supplemented media also containing fetal calf serum (10%). On the third day, the media was replaced with media containing either ^{3}H-thymidine (10 μCi/ml) or ^{3}H-proline (10 μCi/ml) and ^{35}S-sulfate (40 μCi/ml; Radiochemical Centre, Amersham, UK) to allow labeling for 24 h. On the fourth day, the explants were rinsed then chase incubated twice for 30 min in supplemented media containing non-radioactive thymidine or proline, then the explants were removed from the culture media and frozen at −20°C.

Oxygen levels

The pO_2 and pCO_2 levels (kPa) within each container were measured with a blood-gas analyzer (model ABL505, Radiometer, Copenhagen) at the end of the second and third days. A 40-ml syringe was used to draw off a sample through a vacuum valve on the air-tight container in order to prevent contamination by outside air. For the experiment using labeled thymidine, the respective pO_2 levels at the end of both days, for the air mixtures of 0%, 1%, 3%, 20% O_2, were 0.6, 0.6, 1.2, 1.2, 3.2, 3.3, 19.1, and 18.9 kPa. The pO_2 levels for the experiment using labeled sulfate and proline were 0.5, 3.1, 1.7, 1.6, 3.3, 3.2, 18.7, and 18.3 kPa. Because the pO_2 level in the second experiment, second day, low pO_2 group (underlined value) was elevated, indicating some contamination with outside air, the results for the low pO_2 group are not included in the analysis. Based on pilot studies, the pO_2 levels after 24 h were between 0.2 and 0.9 kPa higher than air samples taken immediately after flushing. All pCO_2 levels were 1.9–2.0 kPa.

Determination of incorporation rates

The frozen explants were lyophilized and weighed. Mean synovial explant dry weight was 3.7 ± 0.9 mg and tendon dry weight was 4.8 ± 0.7 mg. Weights did not differ by oxygen level.

Samples labeled with ^{3}H-thymidine were dissolved in 1 M potassium hydroxide at 37°C for 24 h, extracted with trichloracetic acid and centrifugation twice, dissolved in sodium hydroxide, neutralized, mixed with liquid scintillant and counted in a scintillation counter. Samples labeled with ^{35}S-sulfate and ^{3}H-proline were hydrolyzed in 6 M hydrochloric acid at 100°C for 24 h, evaporated, redissolved in 0.01 M hydrochloric acid, then separated on a column of Aminex A6 (BioRad) eluted with citrate buffer, pH 3.75 at 60°C. Radioactivity in peaks corresponding to ^{35}S-sulfate, ^{3}H-hydroxyproline, and ^{3}H-proline were measured with a radioactivity flow detector (Radiomatic Instruments and Chemical). For a full description, see [3].

The release of labeled macromolecules into the media may lead to an underestimation of protein synthesis. Previous, unpublished studies from this lab found that ^{35}S-sulfate labeled macromolecules in the accumulated media were 13.5% and for ^{3}H-hydroxyproline 7.3%. Thus, approximately 90% of labeled matrix components remain in the tissue segment during these short-term experiments.

Statistical analysis

Incorporation rates are expressed as mean dpm/mg sample dry weight ±SEM ($n = 6$). Mean measured oxygen, tissue type (synovium or tendon) and their interaction term (oxygen × tissue) were used as the independent variables in a repeated measures analysis of variance conducted separately for each incorporation rate (e.g., ^{3}H-thymidine, ^{3}H-hydroxyproline, etc.) and the proportion of collagen synthesized (JMP, SAS Institute, Cary, NC). If the interaction term was significant, then the pattern of the response to oxygen, across all oxygen levels, between synovium and tendon was statistically different. Differences between absolute counts between synovium and tendon are given at the end of each paragraph. Significant findings were followed up with Tukey's test at a procedure-wise error rate of 0.05. A value of $P < 0.05$ was considered statistically significant.

Results

Cell proliferation in the synovium and tendon differed in their response to oxygen level (interaction term: $P = 0.03$) (Fig. 2(a)). For synovium the uptake of ^{3}H-thymidine was $25.3 \pm 4.5 \times 10^3$ dpm/mg in 20% O_2 and was significantly reduced at the lower O_2 levels. For tendon, the uptake was $6.8 \pm 1.6 \times 10^3$ dpm/mg in 20% O_2 and was not significantly different at the lower oxygen levels. Uptake was significantly greater in synovium than tendon.

On the other hand, oxygen tension had no effect on the synthesis of new proteoglycans (Fig. 2(b)). The synovium and tendon response to oxygen did not differ (interaction term: $P = 0.29$). The uptake of ^{35}S-sulfate was not significantly effected by oxygen level in either tissue. For synovium the uptake was $1.07 \pm 0.08 \times 10^3$ dpm/mg in 20% O_2 and for tendon the equivalent uptake was $0.32 \pm 0.06 \times 10^3$ dpm/mg. However, there was statistically greater uptake by synovium than tendon.

Synthesis of non-collagenous proteins differed in synovium and tendon in response to oxygen level (interaction term: $P = 0.006$) (Fig. 2(c)). For synovium the uptake of ^{3}H-proline was $27.6 \pm 2.4 \times 10^3$ dpm/mg in 20% O_2 and was significantly reduced at the lower O_2 levels. For tendon, the uptake was $6.3 \pm 0.7 \times 10^3$ dpm/mg in 20% O_2 and was not significantly different from uptake at the lower O_2 levels. There was greater uptake by synovium than tendon.

Collagen synthesis in synovium and tendon did not differ in response to oxygen (interaction term: $P = 0.77$) (Fig. 2(d)). The ^{3}H-hydroxyproline count in synovium explants was $0.57 \pm 0.04 \times 10^3$ dpm/mg in 20% O_2 and was significantly reduced at the lower O_2 levels. The count in tendon was $0.76 \pm 0.18 \times 10^3$ dpm/mg in 20% O_2 and was also significantly reduced at the lower O_2 levels. The count was not significantly different between synovium and tendon.

The effect of oxygen tension on the synthesis of collagen as a percentage of all protein synthesized (percent collagen) was not significantly different between synovium and tendon (interaction term: $P = 0.90$), nor did oxygen level have a significant effect on percent collagen ($P = 0.64$). However, a greater proportion of collagen was produced by tendon than synovium ($P = 0.0001$). For synovium, the proportion of collagen synthesized was $0.83 \pm 0.13\%$ in 20% O_2 while for tendon the proportion of collagen synthesized was $4.6 \pm 0.8\%$.

Discussion

Synovial tissue from the rabbit carpal tunnel demonstrated a reduction in cell proliferation and an alteration in the synthesis of matrix proteins with exposure to low oxygen tensions during short-term culture in vitro. Cell proliferation was reduced by two-thirds and the synthesis of collagenous and non-collagenous proteins were reduced by one-half and one-third, respectively, when the oxygen level decreased from 20% to 3.4%. On the other hand, the synthesis of proteoglycans and the proportion of collagenous proteins synthesized (percent collagen) were not altered by oxygen tension. In contrast, the tendon explants were either much less sensitive or not sensitive at all to the effects of hypoxia. Cell proliferation and synthesis of collagen and proteoglycans were not significantly effected by hypoxia, but non-collagenous protein synthesis was reduced by one-fourth.

The effects of hypoxia on synovium may be mediated through a short-term injury mechanism and followed by a long-term 'healing' response. Hypoxia rapidly inhibits cell proliferation and the synthesis of matrix. Based on previous studies of healing synovial tissue in vivo this injury may be followed by a cellular reactivation and proliferation and the synthesis of proteoglycan, then non-collagenous proteins and eventually collagen [31]. This may be accompanied by fibrosis, scar formation and an alteration in the physiologic properties of the synovium.

The lack of an effect of hypoxia on ^{35}S-sulfate incorporation in combination with a decline in cell proliferation and synthesis of collagenous and non-collagenous proteins implies an increase in proteoglycan synthesis in relation to collagen synthesis at the low oxygen tensions. A rise in ^{35}S-sulfate incorporation is the earliest response of matrix components in connective tissue to environmental stresses or tissue repair [2,14]. However, these short-term results in vitro do not allow us to judge whether healing is beginning or sulfate incorporation is increasing during hypoxia.

The observed differences in the response of tendon and synovium to hypoxia may be due to differences in vascularity, cellular survival, cellular activity, or repair response. The synovium is well vascularized in comparison to tendon [17], therefore tendon cells with their poor vascularization may be better adapted to repeated hypoxia. Tendon sheaths have a higher cellular activity

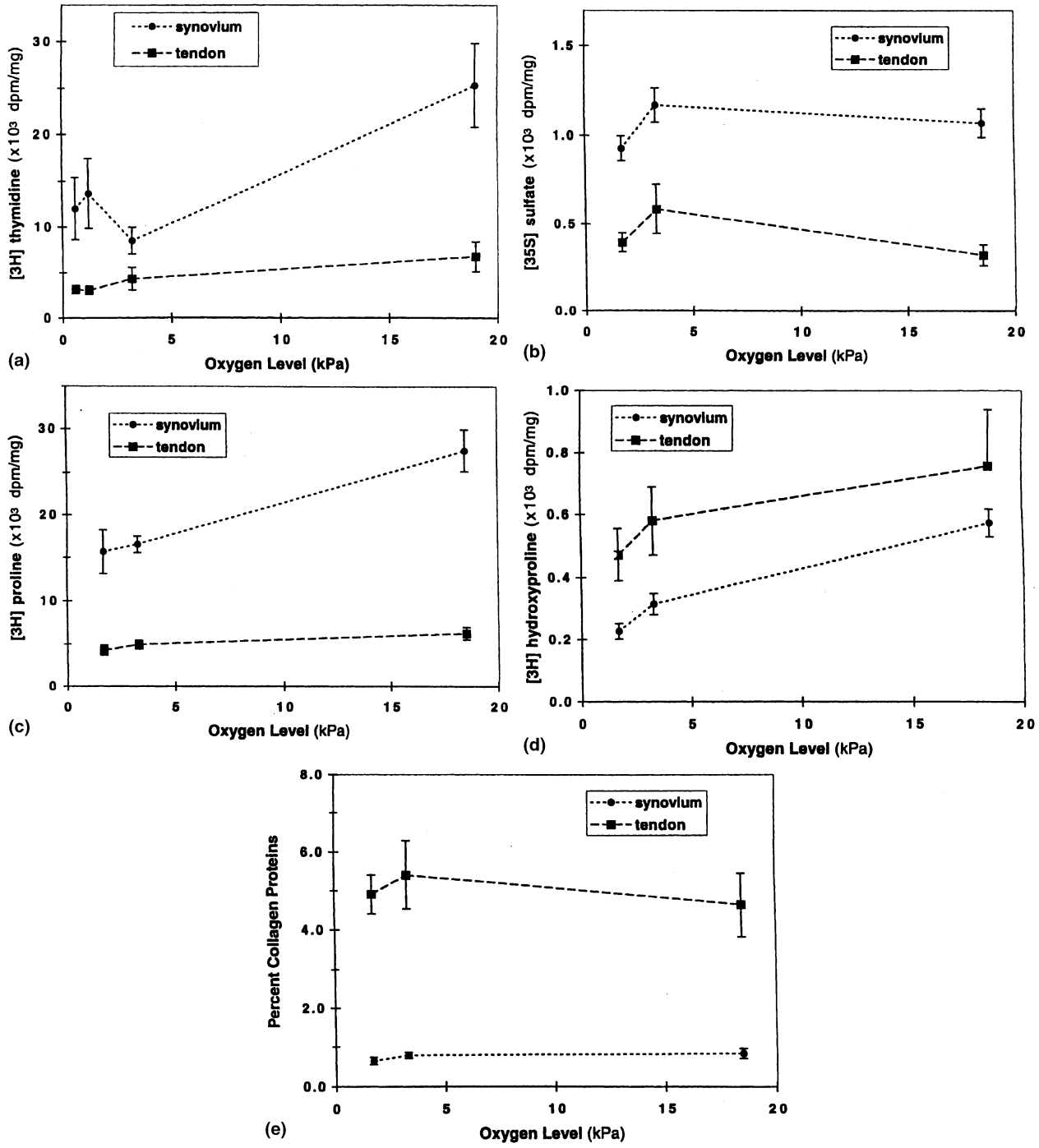

Fig. 2. The effects of different oxygen levels on: (a) cell proliferation, (b) proteoglycan synthesis, (c) non-collagenous protein synthesis, (d) collagen synthesis, and (e) percent collagen of all proteins synthesized, in synovium and tendon explants. Values are presented as mean dpm ($\times 10^3$)/mg dry weight SEM ($n = 6$).

than tendon and the time frame for the repair response of the sheath and tendon differ [31]. During the first five days of repair, cell proliferation in the sheath dropped while in the tendon cell proliferation increased. Collagen and proteoglycan synthesis dropped immediately in both tissues, but then rose to a peak at five days in the sheath and 10 days in the tendon. The response of synovium from the carpal tunnel to two days of hypoxia in this study was similar to the initial response of tendon sheath to injury described by Wigg and collaborators [31]. If the mechanisms are similar one might expect that hypoxia of more than two days duration would lead to a continued decline of cell proliferation but an increased collagen and proteoglycan synthesis.

It is not likely that the synovium and flexor tendons from the carpal tunnel are exposed to static oxygen

levels as used in this study, but are exposed to fluctuating levels depending on local tissue hydrostatic pressures, which are influenced by hand postures [24]. It is possible that the swings in oxygen tension in the synovium and tendon lead to an ischemia/reperfusion injury response [16]. Unfortunately, there are no studies of blood flow rates and oxygen tensions within the tendon and synovium from the carpal tunnel of healthy subjects. Therefore, application of these findings to the pathogenesis of carpal tunnel syndrome may be limited. However, the relevance of testing the low oxygen levels was demonstrated by Takemiya and Maeda [28]. Using an oxygen electrode, in vivo recordings from the rabbit Achilles tendon revealed pO_2 levels of 4.1 ± 2.0 kPa. In addition, there may be wide variations in oxygen levels within micro-regions of the connective tissue. Cater [11] measured pO_2 levels directly over a capillary of 12 kPa, but 10–15 μm from the capillary the pO_2 was zero. As a reference point, arterial pO_2 in humans and rabbits is 9–12 kPa and venous pO_2 is approximately 5 kPa [27]. It is likely that the physiologic oxygen tensions in synovium in the carpal tunnel fall between the oxygen tensions (3.4–20%) that were associated with the greatest changes in cell metabolism.

Compared to other studies of rabbit tendon explants, rates of cell proliferation and macromolecule synthesis of the FDP tendon from the carpal tunnel fall between rates from tension loaded and compression/tension loaded tendon regions of the FDP [3]. Due to the diverging arrangement of the tendons the FDP tendon may be loaded during wrist extension or flexion and some compression of the FDP against the carpal bones may occur. Therefore, the FDP at the carpal tunnel is sheathed by synovium and has biologic and morphologic characteristics of an intrasynovial tensional and partially compressive tendon segment.

In relation to intrasynovial tendons, the rates of cellular activities within synovial explants of the carpal canal during short-term culture correlate with those of digital tendon sheaths and extrasynovial tendons [1,31]. The synovial tissue is the parietal layer of the tendon sheath that surrounds intrasynovial flexor tendons at the levels of the digits and the carpal canal and that along with the visceral epitenon layer of intrasynovial tendons proximally forms the well vascularized, loose connective tissue, paratenon, surrounding extrasynovial tendons. Metabolic and morphologic qualities differ between synovial and internal tendon cells, but undifferentiated mesenchymal cells may be recognized along vessels, frequently found within highly vascularized synovium and extrasynovial tendons [6,13]. Thus, cellular composition and biological activities of synovium, tendon sheaths and extrasynovial tendons correlate but differ from that of intrasynovial tendons. The number of cells, however, expressed as DNA content show only minor variations between different regions and types of flexor tendons and synovium [4,5,10,15,20,22].

The effects of reduced oxygen tension on articular cartilage explants and bone and tendon cells differ from the findings of this study. After seven days of culture of rabbit cartilage explants in different oxygen tensions, cell proliferation was minimally increased at 1% and 5% oxygen tension compared to 21%, while labeled sulfate incorporation was reduced at the lower oxygen tensions [8]. Variations in cell proliferation at low oxygen levels may be due to differences in tissue phenotype or culture duration. In a similar study of bone cell cultures by the same group, cell proliferation was increased at the lower oxygen tensions, but the differences between oxygen tensions were only evident at three days of culture and later [9]. Six week cultures of periosteal explants demonstrated inhibition of type-II collagen formation at lower oxygen tensions [21], findings that parallel our findings in synovium. Fetal tendon cell cultures also respond to hypoxia with a decline in collagen and non-collagen protein synthesis; however, they are more resistant to the effects of hypoxia on cell proliferation [30].

The effects of hypoxia may be mediated by limiting energy available for collagen synthesis and secretion since tendon cells appear to rely on oxidative energy metabolism to maintain cellular ATP [7]. Besides affecting synthesis of matrix components, hypoxia may increase the rates of tissue degradation. Hypoxia can inhibit the hydroxylation of proline and lysine residues and lead to structural alterations of collagen. For example, when embryonic tendon cells were cultured for 90 min in an anoxic environment then exposed to oxygen, the collagen formed was under-hydroxylated (e.g., hydroxyproline residues are replaced with proline and hydroxylysine residues are replaced with lysine) and the stability of the triple helix was reduced [29].

Our results demonstrate that hypoxia can inhibit synovial cell proliferation and alter the mix of matrix macromolecules synthesized during short-term culture. The same effects occur to a lesser degree in tendons. These results suggest that cell damage may occur at low oxygen tensions resulting in a decrease in cell proliferation and synthesis of some matrix components. It may be that hypoxia increases the relative synthesis of proteoglycans or reduces the stability of the collagen structure, thereby increasing the availability of hydrophilic regions within the matrix which may, in turn, lead to the initial edema. The mechanism by which such an injury leads to an altered tissue repair response and scar formation is unknown.

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