Effects of hyperbaric oxygen and platelet derived growth factor on medial collateral ligament fibroblasts.

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Chan Y-S, Chen A. C-Y, Yuan L-j, Lin S-S, Yang C-Y, Lee S-S, Ueng S. W-N. Effects of hyperbaric oxygen and platelet derived growth factor (PDGF-BB) to determine their combined effects on fibroblasts from rabbit medial collateral ligament (MCL). Method: Cells were divided into four groups: (I) Control, (II) HBO2 treatment, (III) PDGF-BB treatment and (IV) HBO2 combined with PDGF-BB treatment. All hyperoxic cells were exposed to 100% O2 at 2.5 atmospheres absolute (ATA) in a hyperbaric chamber for 120 minutes per 48 hours. Measurement of cell growth was based on increase in cell number. Cell cycle modulations were analyzed by fluorescence-activated cell sorter (FACS). Quantity of Type I and Type III collagen was determined by western blotting and image analyzer. Results: Treatment doses of HBO2 alone or PDGF-bb alone dependently increased cell growth. A combination of HBO2 treatment plus PDGF-bb treatment had an additive effect on cell growth in comparison with HBO2 treatment alone or PDGF-bb treatment alone. FACS analysis revealed that HBO2 alone, PDGF-bb alone and PDGF-bb plus HBO2 treatment increase the percentage of cells accumulated in S-phase. Western blotting analysis revealed that Type III collagen content was decreased significantly after HBO2 treatment alone or HBO2 plus PDGF-bb treatment but not in PDGF-bb treatment alone. In contrast, although Type I collagen content was increased after HBO2 treatment, the increase in Type I collagen (increase/original) was not statistically significant. Conclusion: HBO2 or HBO2 plus PDGF-bb treatment decreases the Type III collagen/Type I collagen content, which could result in mechanically stronger collagen fibrils. We propose HBO2 therapy as a potentially effective treatment for MCL healing.

INTRODUCTION

Ligament injury is a common soft tissue athletic injury (1). Athletes must quickly recover from such injuries to resume sporting activities. In the current study, the medial collateral ligament (MCL) was selected as a model for ligament healing which theoretically can be applied to other ligament injuries (2, 3, 4). After MCL injury, the ligament heals in three overlapping phases: inflammation, proliferation and remodeling (5, 6, 7). The inflammatory phase begins soon after injury with the invasion of white blood cells followed by the formation of granulation tissue (5). Fibroblast proliferation and matrix synthesis mark the onset of the proliferation phase. Histological examination illustrates that fibroblasts become the dominant cell 3 weeks after ligament transaction (5, 6, 7). The remodeling phase begins at approximately the sixth week when the fibroblasts decrease in number and size and their nuclei align along the long axis of the ligament (5, 6). The replacement tissue continues to mature for at least 48 weeks, but the mechanical properties of the injured ligament remain different from those of a normal ligament. Laboratory and clinical studies have demonstrated that, even with various treatment methods such as suture repair or early motion, normal ligament...
properties are difficult to restore during MCL healing after an injury (2, 4, 8, 9).

Hyperbaric oxygen (HBO2) and growth factors are known to stimulate fibroblast proliferation and remodeling in healing wounds (10, 11, 12). Since wounds and ligaments heal through similar cellular events, HBO2 is thought to play a synergistic regulatory role with these growth factors in ligament healing.

Ligaments consist of bundles of collagen fibrils. Normal medial collateral ligaments are composed of six genetically distinct types of collagen. Collateral ligaments undergo changes in their complement of collagens during fetal, postnatal development, aging and healing (13-20). Type I collagen is the major fibrillar collagen of the MCL, and types III and V collagen are quantitatively minor components (13, 19, 20). The role of types III and V collagen in ligaments remains unclear. Type III collagen content increases with age or after ligament injury (16). Previous studies have demonstrated that during early phases of ligament healing, type III collagen is highly elevated relative to type I collagen (18), which is believed to produce small collagen fibrils (14). Healed ligaments with smaller collagen fibrils are mechanically weaker than those with normal collagen fibrils (17).

Fibroblast proliferation and collagen synthesis are closely related to oxygen availability and cannot occur without the presence of oxygen (10, 21). Oxygen is required for hydroxylation of proline and lysine, an essential step for synthesis of collagen by fibroblast (22). Under hypoxic conditions, fibroblasts produce, but fail to release, an intracellular peptide collagen precursor (23, 24). Maturation and cross-linking of collagen increases linearly as ambient oxygen concentration increases (25). Collagen synthesized under hypoxic conditions is produced at a slow rate and has less mechanical strength than collagen produced in normoxic conditions (26).

Hyperbaric oxygen enhances oxygen transport in hypoxic tissue and increases oxygen diffusion through tissue fluids. HBO2 treatment affects various cell and tissue activities in diverse systems, including fibroblast proliferation (24); endothelial cell fibrinolysis (27); healing in bone (28, 29), tendon (30), ligament (31, 32), muscle (33) and skin (10, 34); and collagen synthesis of tendon and ligaments (30, 32). In addition, HBO2 treatment of cancer cells can induce cell cycle modulation with an S phase or G2/M phase accumulation (35, 36). Platelet-derived growth factor-BB (PDGF-BB) is released naturally by activated platelets (37), macrophages (38) and fibroblast (39), all of which actively participate in tissue repair. In vitro studies reveal that PDGF has chemotactic effects (40), and regulates collagen synthesis dose dependently (41), and stimulates resting cells to advance from G0 to G1 of the cell cycle (42). In vivo studies of this growth factor in animals and humans have demonstrated enhanced healing properties in wounds and ligament (11, 12, 43). Although the individual effects of HBO2 and PDGF-BB have been reported, the combined effects remain unclear.

Zhao et al. (34) reported that combined PDGF and HBO2 can completely reverse deficient wound healing caused by ischemia in a rabbit dermal ulcer model. Bonomo et al. (44) reported that combined treatment with HBO2 plus PDGF-BB synergistically up regulates mRNA for PDGF β receptor in a rabbit ear wound model. Previous studies have investigated the effects of HBO2 (28, 29, 45) or growth factors (45, 46, 47) on different organ systems. However, the literature includes no animal studies of the effect of HBO2, PDGF-BB and the combination of HBO2 and PDGF-BB on proliferation of fibroblasts from the medial collateral ligament. The present study was initiated and based on the hypothesis that HBO2 alone or PDGF-BB therapy alone dependently increases fibroblastic proliferation and increases collagen formation, and that HBO2 plus PDGF-BB therapy produces only an additive effect on fibroblasts. To determine their effects on MCL fibroblast growth and cell
cycle modulation, HBO\textsubscript{2} and PDGF-BB were tested both individually and in combination. Following each treatment, presence of type III collagen was compared to presence of type I collagen.

**MATERIALS AND METHOD**

**Cell isolation and cell culture**

New Zealand white rabbits weighing 3 kg were sacrificed by intravenous injection of ketamine hydrochloride (Ketalar, ParkeDavis, Taiwan, ROC) and Rompum (Bayer, Leverkusen, Germany). The knee joints were exposed by medial parapatellar incision. Under aseptic conditions, MCL ligaments were harvested and collected with a sterile biological safety hood. A 2-mm portion of the ligaments at the tibial and femoral insertion sites was discarded to avoid possible cartilage and bone tissue contamination. The remaining ligament was cut into pieces approximately 1 mm long and placed in 60 mm culture dishes (Falcon) containing 4 ml of growth medium. Growth medium consisted of HSE 3:1 (DMEM: Ham’s F12) supplemented with 10% (vol/vol) FBS and antibiotics (mixture of 100 units/ml of penicillin and 100 μg/ml of streptomycin; Gibco, Grand Island, NY). Cultures were incubated in a humidified atmosphere of 5% CO\textsubscript{2} / 95% air (non-HBO\textsubscript{2}) throughout the experimental protocol. All hyperoxic cells were exposed to 100% O\textsubscript{2} for 25 minutes then to air for 5 minutes at 2.5 ATA (atmospheres absolute) in a hyperbaric chamber (Sigma II, Perry Baromedical Corporation, Riviera Beach, FL, USA) with a total treatment of 120 minutes per 48 hours. Cells were plated at 10\textsuperscript{5} cells per 60-mm tissue culture dish (Falcon) in 4 ml of medium HSE 3:1 containing 2% FBS for 24 hours until cells were quiescent. At 48, 96, and 144 hour intervals, cell number was determined from triplicate dishes by harvesting cells by trypsin treatment and then counting cells in a Coulter Multisizer II (Beckman Coulter, Inc., Taiwan Branch). Measurement of cell growth was based on increase in cell number after hyperbaric or normobaric treatment.

**Response of MCL fibroblast cultures to PDGF-bb**

Cells were plated at 10\textsuperscript{5} cells per 60-mm tissue culture dish (Falcon) in 4 ml of medium HSE 3:1 containing 2% FBS for 24 hours to make the cells quiescent. The PDGF-bb treated cells were exposed to medium with various concentrations of PDGF-bb (0.1 ng/ml, 1 ng/ml, 10 ng/ml and 25 ng/ml). After 48 hours, cell number was determined from triplicate dishes by harvesting cells by trypsin treatment and then counting cells in a Coulter Multisizer II (Beckman Coulter, Inc., Taiwan Branch). Measurement of cell growth was based on increase in cell number after PDGF-bb treatments.

**Response of MCL fibroblast cultures to PDGF-bb plus HBO\textsubscript{2} treatment**

Cells were plated at 10\textsuperscript{5} cells per 60-mm tissue culture dish (Falcon) in 4 ml of medium HSE 3:1 containing 2% FBS for 24 hours to make the cells quiescent. The hyperoxic cells were exposed to 100% O\textsubscript{2} for 25 minutes then to air for 5 minutes at 2.5 ATA in a hyperbaric chamber with a total treatment time of 120 minutes. The PDGF-bb treatment cells were
exposed to 1 ng/ml of PDGF-bb. The combined treatment cells were exposed to 1 ng/ml of PDGF-bb plus HBO2 treatment. After 48 hours, cell number was determined from triplicate dishes by harvesting cells by trypsin treatment and then counting cells in a Coulter Multisizer II (Beckman Coulter, Inc., Taiwan Branch). Measurement of cell growth was based on an increase in cell number after treatments.

**Cell cycle detection by flow cytometry**

Forty-eight hours after treatment, cells were trypsinized, pelleted (1200 revolutions per minute for 5 minutes) and washed twice with cold phosphate buffered saline (PBS)-1% bovine serum albumin (BSA) (Sigma, St. Louis, MO) and fixed in ice cold 70% ethanol. Cells were kept for at least 1 hour at –20°C, washed once at 4°C with PBS, and finally resuspended in 1 ml of 0.5% Triton X-100, 0.05% RNase (Sigma, St. Louis, MO) PBS solution and incubated for 1 hour at 37°C in a temperature controlled bath. Following incubation, 1 ml of 50μg/ml Propidium Iodide solution (in PBS) was added, and the solution was incubated in darkness for 30 minutes at 37°C Samples were analyzed in a FACS (Beckman Coulter, Inc., Taiwan Branch).

**SDS-polyacrylamide gel electrophoresis and immunoblotting for Type I collagen, Type III collagen.**

Forty-eight hours after treatment, cell culture conditioned medium was collected and the cells washed with ice-cold PBS three times. Cells were lysed by freeze-thawing on dry ice and supernatant fraction collected by centrifugation at 3,000 g for 10 min. The collected conditioned medium was lyophilized and combined with the cell fraction. Aliquots of samples were first treated with 100 μg/ml pepsin (Sigma) dissolved in 1 M acetic acid at 4°C for 12 hours, dialyzed against 0.05 M ammonium bicarbonate for 48 hours and lyophilized. Protein content was estimated using a commercially available protein assay kit (Sigma). Samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 10% gel according to Laemmli under reducing conditions. Gels were stained with Coomassie blue-R250, destained and photographs taken. Prestained proteins (Bio-Rad) were employed as molecular weight markers.

For immunoblotting, proteins were separated by SDS-PAGE on 10% gel and transferred onto nitrocellulose membranes electrophoretically using a protein transfer unit (Hoeffer, CA, U.S.A.). After blocking with 5% non-fat milk, membranes were incubated for 1.5 h at room temperature with 300-fold diluted mouse monoclonal antibodies to Type I collagen (Oncogene, MA, U.S.A.) or Type III collagen (Oncogene, MA, U.S.A.). After washing, membranes were further incubated for 2 hours with 1,000-fold diluted goat anti-mouse IgG conjugated to horseradish peroxidase. Membranes were washed and rinsed with ECL detection reagents (Amersham Pharmacia Biotech,UK). Band images were photographed using ECL Hyperfilm (Amersham Pharmacia Biotech,UK). Each image was captured by a digital camera (DCS 460; Eastman KODAK, Rochester, NY), and density of each band image was quantified by KODAK 1D image analysis software (Eastman KODAK company, Rochester, NY).

**Statistical analysis**

Differences between two groups were compared by two-tailed Student’s t test. Values were expressed as the mean ± SD; p < 0.05 was considered statistically significant.

**RESULT**

**Dose-dependent effect of HBO2 on MCL cell growth**

Treatment dose of HBO2 dependently increased MCL cell number in comparison
Effects of HBO2 and PDGF on MCL fibroblasts

Dose dependent HBO2 treatment increased MCL cell number in comparison with control cells cultured under the same medium. Data are expressed as mean ±SD of findings in six rabbits in each group; p < 0.05 was considered statistically significant.

with control cells cultured under the same medium (Fig. 1, Control v. HBO2 treatment one time: 2.3 X 10^5 ± 1.4 X 10^4 v.s.2.8 X 10^5 ± 1.5 X 10^4, p<0.05; Control v. HBO2 treatment two times: 3.6 X 10^5 ± 3.2 X 10^4 v.s.5.2 X 10^5 ± 2.4 X 10^4, p<0.05; Control v. HBO2 treatment three times: 5.2 X 10^5 ± 3.1 X 10^4 v.s.6.6 X 10^5 ± 2.9 X 10^4, p<0.05; n = 6).

Dose-dependent effect of PDGF-bb on MCL cell growth

Treatment dose of PDGF-bb dependently increased MCL cell number in comparison to control cells (Fig. 2, Control: 2.3 X 10^5 ± 2.1 X 10^4; 0.1 ng/ml: 2.6 X 10^5 ± 1.3 X 10^4, p>0.05; 1 ng/ml: 3.7 X 10^5 ± 1.1 X 10^4, p<0.05; 10 ng/ml: 4.5 X 10^5 ± 3.6 X 10^4, p<0.05; 25 ng/ml: 4.0 X 10^5 ± 3.8 X 10^4, p<0.05; n = 6).

Additive Effect of PDGF-bb plus HBO2 treatment on MCL cell growth

Treatment with HBO2 treatment and 1 ng/ml of PDGF-bb significantly affected MCL cell growth. Combined treatment of HBO2 plus 1 ng/ml of PDGF-bb produced an additive effect on MCL cell growth in comparison to HBO2 treatment alone (p<0.05) or PDGF-bb treatment alone (p<0.05), respectively (Fig. 3, Control: 1.6 X 10^5 ± 7.8 X 10^3, HBO2: 1.8 X 10^5 ± 5.5 X 10^3, PDGF-bb: 2.1 X 10^5 ± 9.8 X 10^3, HBO2 plus PDGF-bb: 2.5 X 10^5 ± 8.6 X 10^3; n = 6).

Cell cycle detection

Using PI staining and FACS analysis, the results for control group, HBO2 group, PDGF group and HBO2 plus 1 ng/ml of PDGF-bb treatment on cell cycle alteration shown in Table 1. Treatment with HBO2 or PDGF-bb
significantly increased the percentage of cells in the S-phase and decreased percentage of cells in the G$_0$/G$_1$ phase but did not produce a significant difference in the proportion of cells in G$_2$/M phase. Accumulation in S-phase had a more significant effect on cell cycle alteration after HBO$_2$ plus PDGF-bb treatment than after separate HBO$_2$ or PDGF-bb treatment.

**Effects of HBO$_2$ or PDGF-bb on Type I collagen and Type III collagen synthesis.**

After quantification by immunoblotting and image analyzer, no significant changes were apparent in Type I collagen content after each treatment (Fig. 4, HBO$_2$/Control ratio: 111.2% ± 8.2%, PDGF-bb/Control ratio: 97.2% ± 13.7%, HBO$_2$ plus PDGF-bb/Control ratio: 111.8% ± 12.1%; n = 2). Type III collagen content was significantly decreased after HBO$_2$ treatment (p<0.05) and after HBO$_2$ combined with PDGF-bb treatment (p<0.05) but no significant changes were noted after PDGF-bb treatment alone (Fig. 5, HBO$_2$/Control ratio: 59.5% ± 7.6%, PDGF-bb/Control ratio: 107.5% ± 9.4%, HBO$_2$ plus PDGF-bb/Control ratio: 63.5% ± 8.9%; n = 2). Table 2 lists total data for six samples. Treatment with HBO$_2$ or HBO$_2$ plus PDGF-bb significantly decreased Type III collagen/Type I collagen content ratio.

| Table 1. Effects of treatments on cell cycle phases (n = 6) |
|--------------------|----------------|----------------|----------------|
|                   | Control         | HBO$_2$         | PDGF-bb        | HBO$_2$ + PDGF-bb |
| G$_0$/G$_1$        | 74.1% ± 2.6%    | 68.5% ± 3.4%    | 66.1% ± 2.3%   | 64.7% ± 3.7%      |
| S                  | 4.4% ± 0.6%     | 8.0% ± 1.2%     | 7.1% ± 1.0%    | 8.9% ± 1.1%       |
| G$_2$/M            | 12.6% ± 2.6%    | 14.3% ± 2.5%    | 12.9% ± 2.1%   | 13.5% ± 1.9%      |

**DISCUSSION**

Collagen is the most abundant molecule in ligament (48), and the mechanical properties of ligaments depend primarily on collagen.
fibers. More than 90% of collagen in normal ligaments is Type I collagen (48). Ligament injuries heal by scar formation rather than by actual ligament regeneration (12). The functional recovery of ligaments is most apparent in an examination of their tensile properties. Ultimate load and stiffness are the most common measures of the tensile properties of healing ligament (2, 31, 32, 43, 48). Horn et al. (31) demonstrated that administration of HBO₂ increases the ultimate load in lacerated MCL despite no significant difference in the stiffness. However, no previous studies have related the effects or mechanism of HBO₂ to fibroblasts from MCL. The rationale for HBO₂ therapy is that oxygen is required for ligament healing. This therapy increases tissue oxygen tension and improves collagen synthesis and angiogenesis. Furthermore, due to complex cell-to-cell interaction and the activity of soluble factors controlling ligament healing in vivo, determining the direct effects of oxygen on cells themselves and predicting optimal oxygenation conditions for the function of these cells is difficult.

Tompach et al. investigated the effects of HBO₂ on cells involved in wound healing (24). Cultured endothelial cells and fibroblasts were exposed to HBO₂. To measure the effect of HBO₂ on the P₀₂ of the culture medium, a small-bore catheter was threaded through a port in the HBO₂ chamber with the tip placed in the culture medium. After cultures were exposed to HBO₂ at 2.4 ATA for 90 minutes, the partial pressure of oxygen in the medium of culture cells was over 1011 mmHg. The current study followed the same in vitro model and cell culture medium as Tompach et al. The rapid increase in P₀₂ of the culture medium with HBO₂ treatment could be achieved. Tolerance to HBO₂ can be increased by intermittent exposure when HBO₂ treatments are required for humans. This study applied an HBO₂ therapy protocol almost identical to the protocol used by many centers clinically. Treatment with HBO₂ every 48 hours may reduce free radical damage to cells. Cells secreted cytokines, growth factors or extracellular matrix to the cell culture media. The cells and media were pooled for collagen measurements. Cells were seeded in T-75 tissue culture flasks until cell confluence was achieved then plated at 10⁵ cells per 60-mm tissue culture dish for control group or HBO₂ group. Although extracellular collagen deposits might have remained in the T-75 flasks, the purpose of the procedure was to study the effect of HBO₂ on cells cultured in the 60 mm tissue culture dish.

Platelet-derived growth factor is comprised of two different polypeptide chains, A and B, which are present in tissue as three isoforms, AA, BB and AB. The most common form is the heterodimer PDGF-AB; however, the most biologically active isoform is the homodimer, PDGF-BB. The effect of PDGF is exerted by binding to membrane-bound receptors. The two receptors to which it binds are designated receptor alpha and receptor beta. The binding of PDGF to its receptors initiates a cascade of intracellular biochemical events which culminate in effects on cellular proliferation, chemotaxis and matrix production (37). The data in the present study indicated that dose-dependent HBO₂ or PDGF-bb treatment increases cell growth. Treatment with HBO₂ plus PDGF-bb had an additive effect on cell growth in comparison to HBO₂ or PDGF-bb treatment, respectively. Treatment with HBO₂ plus PDGF-BB, but not PDGF-bb treatment alone, is reportedly synergistic in upregulating mRNA for PDGF-β receptor in a rabbit ear wound model (26). This effect on receptor modulation may partially explain the observed interaction of HBO₂ and PDGF-bb in the present study.

The differences in regulation of cell cycle kinetics by PDGF-bb and HBO₂ may be another mechanism than receptor modulation. Fibroblasts undergo cell division only after exposure to both competence and progression growth factors (5, 43, 49). Competence growth factors such as PDGF-bb stimulate resting
cells to advance from G₀ to G₁ of the cell cycle and are unable to promote further progress through G₁ (49). Progression growth factors such as epidermal growth factor (EGF) are believed to advance fibroblasts from G₁ to the S phase of the cell cycle and thus stimulate cell proliferation (5, 11, 49). In the S phase, the cell prepares itself for division by replicating DNA. The radiolabeled nucleotide [³H]-thymidine is incorporated into DNA during the S phase of the cell cycle and thus can measure cell proliferation. The current study revealed that PDGF-bb treatment increases the percentage of cells accumulated in S-phase. The required additional components may be present in serum, which contains both competence and progression activity (18, 31). However, prolonged (>12 hours) stimulation of quiescent fibroblasts with this factor reportedly induces entry into S phase (21). In addition, HBO₂ treatment stimulates fibroblast proliferation (50) and induces cancer cell cycle modulation with an S phase or G₂/M phase accumulation (38, 51). Combined HBO₂ and PDGF-bb treatment may provide more competence and progression growth factors to increase the percentage of cells accumulated in S-phase thus producing an additive effect on cell growth.

Collateral ligaments undergo changes in their complement of collagens during fetal development, postnatal development, ageing and healing (13-16, 27, 30, 32, 33). Treatment with HBO₂ increases total collagen synthesis of tendon and ligaments (17, 45), but modulation of collagen fiber-types has not been reported. Exogenous TGF-β1 increases Type I collagen secretion and thus decreases Type III collagen /Type I collagen in healing ligament (35). In vitro studies have shown that PDGF regulates collagen synthesis dose dependently. Increased cell proliferation may contribute to decreased collagen production per cell. Only high concentrations of PDGF tend to decrease Type I and III procollagen mRNA levels and collagen accumulation (20). In this study, we used monoclonal antibodies for Type I collagen and Type III collagen, which did not cross-react with other classes. The data indicated that Type III collagen content significantly decreased after HBO₂ or HBO₂ plus PDGF-bb treatment but not after PDGF-bb treatment alone. Treatment with HBO₂ increased cell growth and collagen synthesis. Because the proportion of Type I collagen was much higher than that of Type III collagen, Type I collagen content was increased after HBO₂ treatment. However, the increased Type I collagen content (increased/original) was not statistically significant. Treatment with HBO₂ produces a stronger regulatory effect on collagen complement modulation than PDGF-bb and results in the suppression of Type III collagen secretion. Treatment with HBO₂ decreases the ratio of Type III collagen/Type I collagen, which may result in mechanically stronger collagen fibrils.

Histological investigations have demonstrated that fibroblast proliferation is an essential step in the process of ligament repair (16). However, complex cell-to-cell interactions and the activity of soluble factors controlling wound healing in vivo make it difficult to determine the direct effects of oxygen on cells themselves and to predict optimal oxygenation conditions for the function of these cells. However, oxygen is required for healing wounds and this is a rationale for HBO₂ therapy. Therefore, in vitro experiments designed to investigate the effects of HBO₂ and growth factors on cellular physiology are important to establish a scientific basis for the optimal use of this therapy. Our future studies will focus on the development of a growth factor delivery system (alginate-PDGF-bb composite release system) and test the combined effects of HBO₂ and growth factors in vivo.

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