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EXPERIMENTAL STUDY

Effects of hyperbaric oxygen on gene expressions of procollagen, matrix metalloproteinase and tissue inhibitor of metalloproteinase in injured medial collateral ligament and anterior cruciate ligament

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Abstract Animal experiments were performed to investigate whether and how the administration of hyperbaric oxygen (HBO) affects gene expressions of procollagens, matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in injured medial collateral ligament (MCL) and anterior cruciate ligament (ACL). In 64 Sprague-Dawley rats, the MCL of the left knee was lacerated at the mid-substance, and the ACL of the left knee was lacerated adjacent to the tibial insertion in another 64 rats. Of these, 32 rats with lacerated MCL and 32 rats with lacerated ACL were housed in individual cages at normal atmospheric pressure (Groups MC and AC, respectively), while the remaining 64 rats were exposed to 100% oxygen at 2.5 atmospheres absolute for 2 h for 5 days a week (Groups MH and AH, respectively). Rats were sacrificed at 3, 7, 14 and 28 days postoperatively. After macroscopic examination, bilateral MCLs were harvested from Groups MC and MH, and bilateral ACLs from Groups AC and AH. Total RNA was extracted from each specimen and gene expressions of type I and type III procollagens, MMP-2, -9 and -3, and TIMP-1 and -2 were estimated using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Macroscopically, lacerated MCL healed by scar tissue formation, the amount of which appeared to be greater in Group MH than in Group MC. In contrast, no lacerated ACLs united, and little, if any, differences were apparent in macroscopic findings between Groups AH and AC. Gene expression of type I procollagen was significantly greater in Group MH than in Group MC at 7 days postoperatively and was also significantly greater in Group AH than in Group AC at 28 days ($P < 0.05$). No significant differences in type III procollagen gene expression were noted between Groups MH and MC or between Groups AH and AC. In addition, no significant differences in gene expressions of MMPs were seen in either ligament, except that gene expression of MMP-13 was significantly lower at 7 days in Group MH than in Group MC ($P < 0.05$). Gene expressions of TIMPs did not differ significantly between Groups MH and MC in each time interval, whereas gene expressions of TIMPs were significantly greater in Group AH than in Group AC at 7, 14 and 28 days for TIMP-1 and at 3, 7 and 14 days for TIMP-2 ($P < 0.05$). RT-PCR results suggested that HBO enhances structural protein synthesis and inhibits degradative processes by enhancing TIMP activities in the lacerated ACL. However, none of the lacerated ACLs united macroscopically despite administration of HBO, indicating that the effect of HBO is insufficient for healing of the injured ACL. If HBO therapy is used as an adjunctive therapy after primary repair of the injured ACL, the success rate of surgery seems likely to be increased.

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Introduction

The knee joint is the most frequently injured joint in sports activities, and medial collateral ligament (MCL) and anterior cruciate ligament (ACL) injuries are the most common ligament injuries in the knee joint [6]. These ligaments display quite different healing processes after injury. Injured MCL readily heals with distinct scar formation [12], whereas injured ACL consistently fails to heal [1, 24, 31, 43]. Reasons for failure of the ACL to heal have yet to be fully elucidated.

Wound healing is a complex process that requires deposition and accumulation of newly synthesized structural proteins as well as degradation of old or damaged structures composed mainly of extracellular matrix [8, 10]. Therefore, such failure to heal could be influenced by a lower potential for structural protein synthesis leading to a lower amount of scar tissue formation or rapid degradation of ligament substance following injury, or both [2]. Wiig et al. [45] reported that procollagen gene expression was less in fibroblasts of the ACL than in the MCL. Amiel et al. [1] showed a rapidly degenerative process in injured ACL with increased collagenase activity and decreased total collagen, suggesting autodegradation by the ligament.

Collagen is the most abundant substance in ligaments, and more than 90% of collagen is type I, while less than 10% is type III [13]. Structural protein synthesis can thus be accurately assessed by estimating the production of these collagen types. The extracellular matrix in ligaments is degraded predominantly by the activities of matrix metalloproteinases (MMPs) [1, 8, 10]. MMPs are Zn^{2+}-dependent endoproteinases and their hydrolytic activities are blocked by tissue inhibitors of metalloproteinases (TIMPs) [7, 8, 23]. MMP-13 (collagenase 3) degrades type I and III collagens, although it hydrolyzes type II collagen more preferentially [23]. MMP-13 also hydrolyzes other extracellular matrix molecules such as gelatin [23], and it has been suggested that MMP-13 may play an important role in healing, matrix remodeling and degeneration after ligament injury [17]. MMP-2 (gelatinase A) and MMP-9 (gelatinase B) degrade gelatin, as well as type I and III collagens [3, 5]. All TIMPs, including TIMP-1 and -2, are able to inhibit all MMPs, although different TIMP species show preferential inhibition of particular MMPs [7]. The degenerative process of the ligament can thus be assessed by estimating activities of these proteins.

Hyperbaric oxygen (HBO) therapy has been used as a primary treatment for air embolism, decompression sickness, and carbon monoxide poisoning [16, 28], and is also used adjunctively for several other conditions, including burns, crush injuries and compartment syndromes [4, 37, 39, 40]. HBO therapy has also been used by some professional sports teams, in the hope that this approach could help injuries to heal more rapidly [22, 32]. Some studies have shown that HBO promotes healing in the injured MCL [18, 26]. However, no reports have described the effects of HBO on injured ACL. If HBO promotes structural protein synthesis and/or inhibits the degenerative process in the injured ACL, injured ACL may heal with the administration of HBO.

Production of proteins, including collagens, MMPs and TIMPs is initiated with expression of the gene for each protein. Our previous study showed that administration of HBO increased the expression of type I procollagen gene in injured MCL [26]. However, no studies have reported the effects of HBO on gene expression of these proteins in injured ACL. The current study investigated whether and how the administration of HBO affects gene expressions of procollagens, MMPs and TIMPs in injured ACL in comparison with injured MCL. Taking into account the favorable effects of HBO on injured MCL shown in our previous study [26], our hypothesis in this study was that in both injured MCL and ACL, administration of HBO increases the gene expressions of procollagens and TIMPs, and/or decreases gene expressions of MMPs.

Materials and methods

Animals

This experiment was performed under the control of the Animal Care and Use Committee, and in accordance with Guidelines for the Care and Use of Laboratory Animals of our institution. A total of 128 male Sprague-Dawley rats (11 weeks old; weight 356 ± 14 g) were used. Surgery was performed under general anesthesia induced using intraperitoneal injection of pentobarbital (50 mg/kg). With sterile technique, the MCL of the left knee was lacerated at the midsubstance in 64 rats according to the method of Hart and Darners [15]. Ligament ends were allowed to

retract. In the remaining 64 rats, the ACL of the left knee was exposed through medial parapatellar arthrotomy and lacerated adjacent to the tibial insertion according to the method used in dogs described by O'Donoghue et al. [31].

Next, 32 rats with a lacerated MCL and 32 rats with a lacerated ACL were housed in individual cages under normal atmospheric pressure (Groups MC and AC, respectively), while the remaining 64 rats were exposed to 100% oxygen at 2.5 atmospheres absolute for 2 h for 5 days a week beginning on the day of surgery (Groups MH and AH, respectively) [26]. A P-5100S cylindrical pressure chamber (Hanyuda Iron Works, Tokyo, Japan) with a volume of 15.2l was used for the administration of HBO (Fig. 1).

Rats were sacrificed by intraperitoneal injection of a lethal dose of pentobarbital, with eight animals from each group euthanized at 3, 7, 14 and 28 days postoperatively. For animals euthanized at 28 days, HBO was only administrated during the first 2 weeks.

After macroscopic examination of the surgically lacerated ligament, bilateral MCLs were harvested from Groups MC and MH, and bilateral ACLs were harvested from Groups AC and AH.

Extraction of total RNA

Total RNA was extracted using RNeasy Mini (QIAGEN GmbH, Hilden, Germany). The quantity and quality of extracted RNA were determined by absorbency at 260 nm.

Semi-quantitative reverse transcription (RT)-polymerase chain reaction (PCR)

Reverse transcription of total RNA and PCR was performed using an RNA LA PCR Kit (AMV) version 1.1 (Takara Bio, Shiga, Japan) in accordance with the instructions of the manufacturer. Total volume of the RT reaction was 20 μl and final concentrations of reagents in the reaction mixture were: RNA PCR buffer, 1×; $MgCl_2$, 5 mM; deoxynucleoside triphosphate mixture, 1 mM; random 9mers, 2.5 μM; avian myeloblastosis virus (AMV) reverse trancriptase, 0.25 units/μl; RNase inhibitor, 1 U/μl; diethyl pyrocarbonate-treated H_2O; and total RNA. Reactions were performed in a GeneAmp 9600 thermal cycler (Perkin Elmer, MA, USA) at 30°C for 10 min, 42°C for 30 min, 99°C for 5 min, and 5°C for 5 min, consecutively. Total volume of the PCR reaction was 100 μl. Final concentrations of reagents in the reaction mixture were: LA PCR™ Buffer II, 1×; $MgCl_2$, 2.5 mM; TaKaRa LA Taq™, 2.5 U/100 μl; forward and reverse primers, 0.2 mM each; and diethyl pyrocarbonate-treated H_2O. Reaction mixtures were incubated in the GeneAmp thermal cycler with the following standard protocol: 1 cycle of 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 90 s, then 1 cycle of 72°C for 5 min and cooling to 5°C. For quantification, PCR products were electrophoresed through a 1.0% agarose gel with ethidium bromide. The density of PCR products forming bands was then measured using a gel documentation system (Gel Doc 2000, BioRad and Quantity One; BioRad Laboratories, CA, USA). Targeted genes were type I and type III procollagens, MMPs (MMP-2, -9 and -13), and TIMPs (TIMP-1 and -2). Primers used in this study were designed according to previously published gene sequences and are listed in Table 1. As an internal standard, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was amplified from the same samples, electrophoresed and analyzed in the same ways. Standardized densitometry values defined as the ratio of target gene to GAPDH were determined.

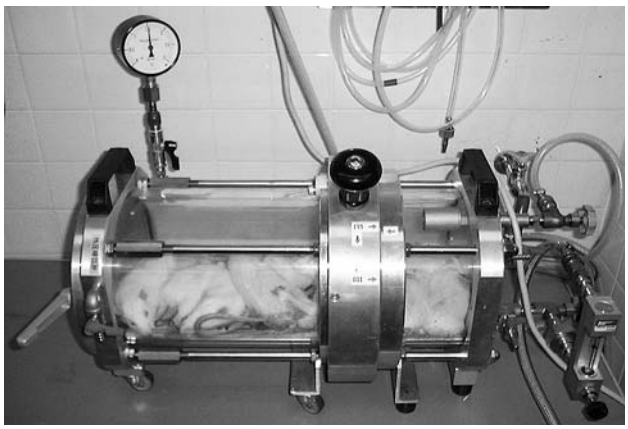

Fig. 1 Hyperbaric oxygen chamber (P-5100S. Hanyuda Iron Works Ltd., Tokyo, Japan)

Data analysis

Unpaired Student *t* tests were used for statistical analyses. For all statistical analyses, values of $P < 0.05$ were considered significant.

Results

Macroscopic findings

In Groups MH and MC, the gap between retracted ends of the lacerated MCL was filled with scar tissue in each animal, reaching a maximum amount by 14 days

Table 1 Primer information

Gene name	Primer sequences	Size (bp)	References
Procollagen α2 (I)	F: 5′-TGTTCGTGGTTCTCAGGGTAG-3′ R: 5′-TTGTCGTAGCAGGGTTCTTTC-3′	254	Nicoletti [29]
Procollagen α1 (III)	F: 5′-CGAGGTAACAGAGGTGAAAGA-3′ R: 5′-AACCCAGTATTCTCCGCTCTT-3′	349	Power [33]
MMP-2	F: 5′-CTATTCTGTCAGCACTTTGG-3′ R: 5′-CAGACTTTGGTTCTCCAACTT-3′	309	Wells [44]
MMP-9	F: 5′-AAATGTGGGTGTACACAGGC-3′ R: 5′-TTCACCCGGTTGTGGAAACT-3′	299	Wells [44]
MMP-13	F: 5′-GCCCTGAATGGGTATGACAT-3′ R: 5′-GCATGACTCTCACAATGCGA-3′	324	Wells [44]
TIMP-1	F: 5′-GACCTGGTCATAAGGGCTAAA-3′ R: 5′-TGCAGCTGCTCCCCGGTGCAC-3′	216	Ishidoya [19]
TIMP-2	F: 5′-TGCAGCTGCTCCCCGGTGCAC-3′ R: 5′-TTATGGGTCCTCGATGTCGAG-3′	590	Sava [36]
GAPDH	F: 5′-ACCCCCAATGTATCCGTTGT-3′ R: 5′-TACTCCTTGGAGGCCATGTA-3′	299	Wells [44]

postoperatively in both groups. Healing MCL appeared to have more scar tissue in Group MH than in Group MC at each time point, with maximum difference at 7 days (Fig. 2).

In Groups AH and AC, none of the lacerated ACLs united macroscopically (Fig. 3). Remnants of the lacerated ACL were swollen at 3 days, decreasing in size thereafter. Little, if any, difference was apparent on macroscopic findings between Groups AH and AC and no scar tissue formation was observed macroscopically in either specimen.

Semi-quantitative RT-PCR

In Groups MH and MC, no significant difference was seen in gene expressions for unoperated MCL between groups or over time. In addition, no significant differences in gene expression of unoperated ACL were seen over time or between Groups AH and AC.

For lacerated MCL, significantly increased gene expression of type I procollagen was shown at 14 days postoperatively in Group MC and at 7 and 14 days in Group MH compared with unoperated MCL (Fig. 4). Gene expression of type I procollagen was greater in Group MH than in Group MC at 7 and 14 days postoperatively, with a significant difference at 7 days (Fig. 4). Significantly increased gene expression of type III procollagen was shown at 3, 7 and 14 days postoperatively in both groups (Fig. 4). No significant difference in gene expression of type III procollagen was seen between groups at each time interval (Fig. 4).

Regarding gene expression for MMPs, significantly increased gene expression of MMP-2 was noted at 7 and 14 days postoperatively in both groups (Fig. 5).

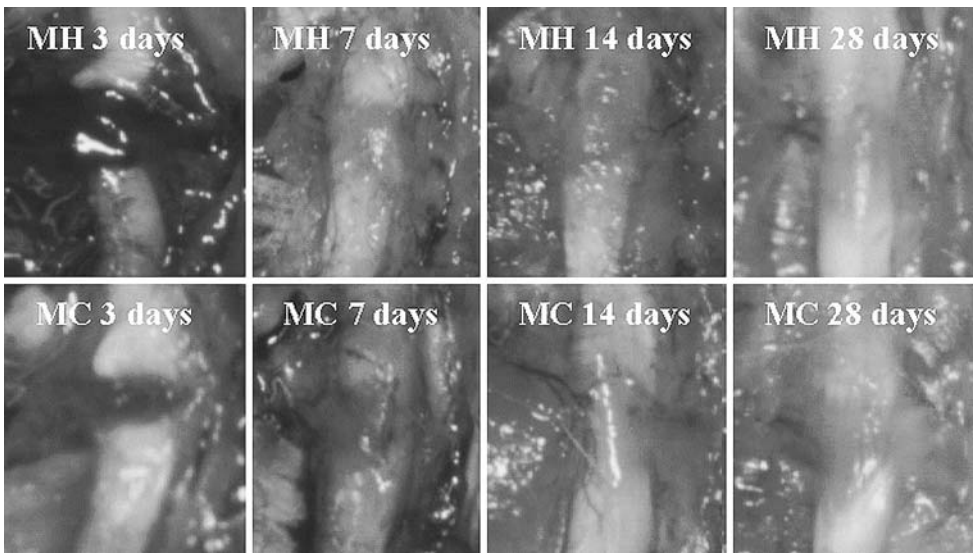

Fig. 2 Macroscopic findings of injured MCLs. Photographs on the *top row* are for Group MH and the *bottom row*, for Group MC

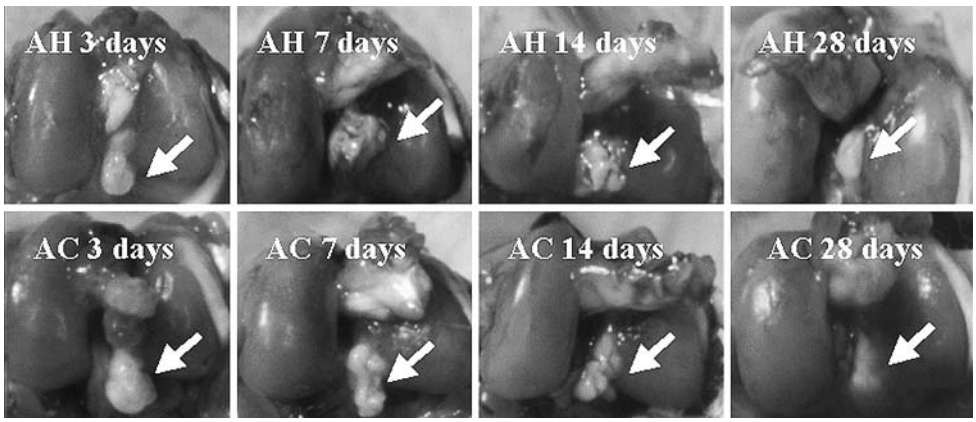

Fig. 3 Macroscopic findings of injured ACLs. *Arrows* indicate the remnants of the lacerated ACLs. Photographs on the *top row* are for Group AH and the *bottom row* for Group AC

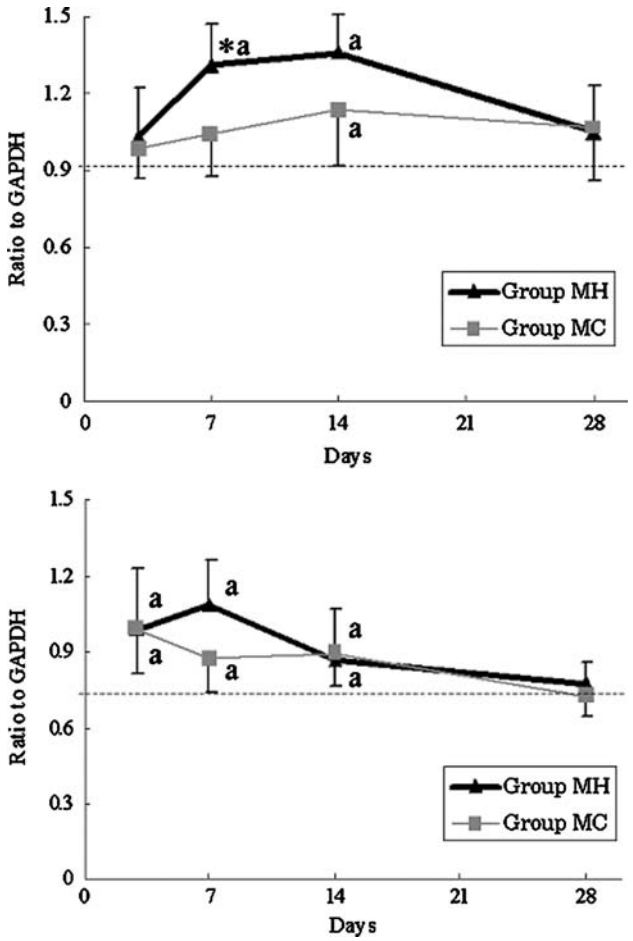

Fig. 4 Gene expression of procollagens in MCL. *Top* Type I procollagen. *Bottom* Type III procollagen. *Broken line* indicates average value in unoperated MCL. *a* $P < 0.05$ versus unoperated MCL. *$P < 0.05$ between the groups

Significantly increased gene expressions were noted at 7 days for MMP-9 and at 7 and 14 days for MMP-13 in Group MC, whereas no significant increase in gene expressions of these MMPs was seen postoperatively in Group MH (Fig. 5). Gene expressions of MMPs tended to be lower in Group MH than in Group MC, with significant differences in gene expression of MMP-13 at 7 days postoperatively (Fig. 5).

Increased gene expressions of TIMP-1 and -2 were shown postoperatively in both groups, with significant differences at 3, 7 and/or 14 days (Fig. 6). Gene expressions of TIMPs tended to be greater in Group MH than in Group MC, but the difference was not significant (Fig. 6).

For lacerated ACL, increased gene expression of type I procollagen was shown in both groups compared with unoperated ACL, with significant differences at 3, 7, 14 and/or 28 days postoperatively (Fig. 7). Gene expression of type I procollagen tended to be greater in Group AH than in Group AC at each interval, with a significant difference at 28 days (Fig. 7). Increased gene expression of type III procollagen was shown in both groups, with significant differences at 3 days in Group AC and at 3, 7, 14 and 28 days in Group AH (Fig. 7). No significant difference in gene expression of type III procollagen was seen between groups at each time interval (Fig. 7).

Significantly increased gene expressions of MMP-2, -9 and -13 were shown at 3, 7 and/or 14 days postoperatively in both groups (Fig. 8). Gene expressions of MMPs in Group AH were basically equivalent to those in Group AC (Fig. 8).

Significantly increased gene expression of TIMP-1 was shown at 3 days in Group AC and in each time interval in Group AH (Fig. 9). Gene expression of TIMP-1 was significantly greater in Group AH than in Group AC at 7, 14 and 28 days postoperatively (Fig. 9). Significantly increased gene expression of TIMP-2 was shown in each time interval in Group AH, whereas increased gene expression of TIMP-2 in Group AC was not significant except for at 14 days (Fig. 9). Gene expression of TIMP-2 was significantly greater in Group AH than in Group AC at 3, 7 and 14 days postoperatively (Fig. 9).

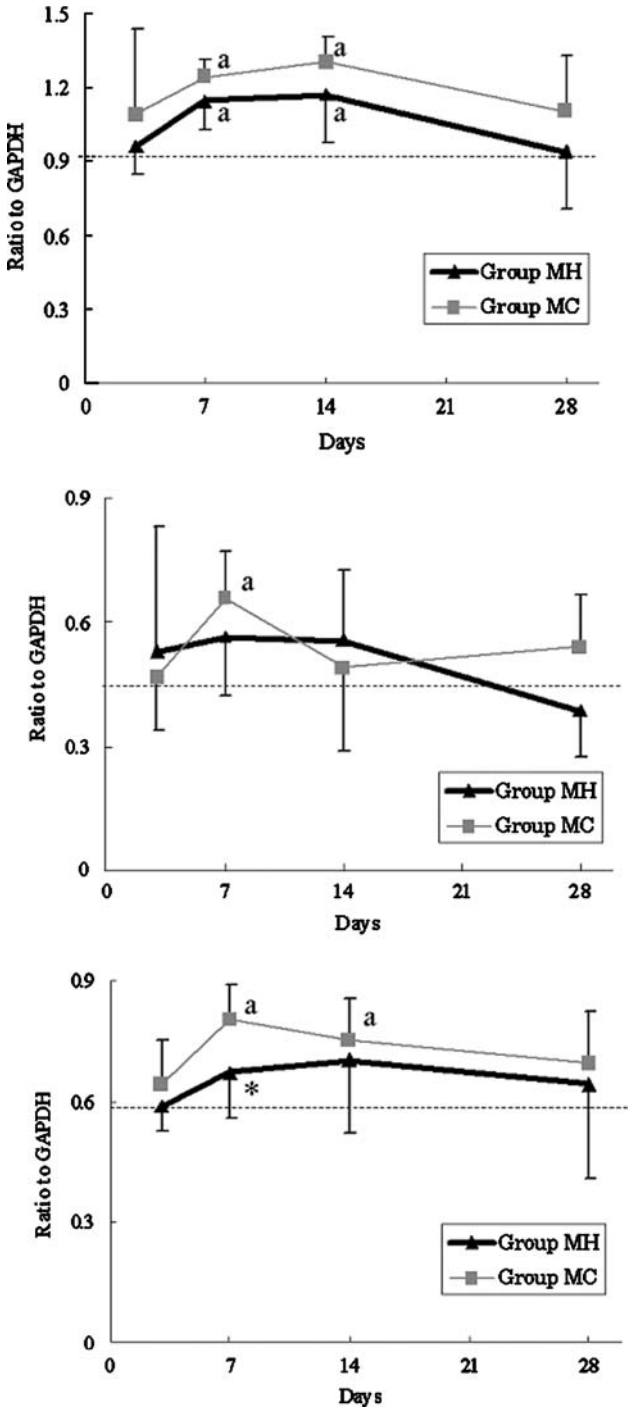

Fig. 5 Gene expression of MMPs in MCL. *Top* MMP-2. *Center* MMP-9. *Bottom* MMP-13. *Broken line* indicates average value in unoperated MCL. *a* $P < 0.05$ versus unoperated MCL. *$P < 0.05$ between the groups

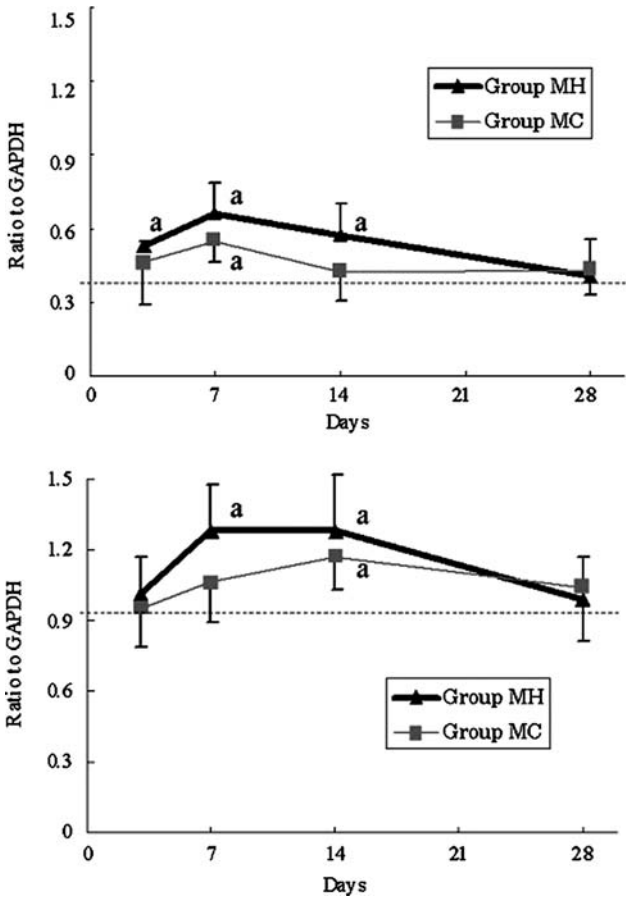

Fig. 6 Gene expression of TIMPs in MCL. *Top* TIMP-1. *Bottom* TIMP-2. *Broken line* indicates average value in unoperated MCL. *a* $P < 0.05$ versus unoperated MCL

Discussion

The purpose of this study was to investigate whether and how the administration of HBO affects the gene expressions of procollagens, MMPs and TIMPs in injured ACL in comparison with injured MCL. We examined gene expressions of these proteins to assess structural protein synthesis and the degenerative process, as gene expression is the initial event in protein production and changes as affected by alterations in a milieu, such as administration of HBO, are likely to be detected even in the early phase after ligament injury.

Few studies have examined the effect of HBO on gene expressions of procollagens in injured ligaments or tendons [20, 21, 26]. Ishii et al. [20, 21] examined expression of the type I procollagen gene in lacerated patellar tendons in rats by Northern blot hybridization, and found that the level of gene expression was increased 7–14 days after laceration by HBO. Mashitori et al. [26] examined type I procollagen gene expression in lacerated MCL of rats using in situ hybridization study, and reported that type I procollagen gene expression is significantly increased at 7 and 14 days under administration of HBO. In the current study, serial changes of type I procollagen gene expression in injured MCL of the control group (Group MC) were

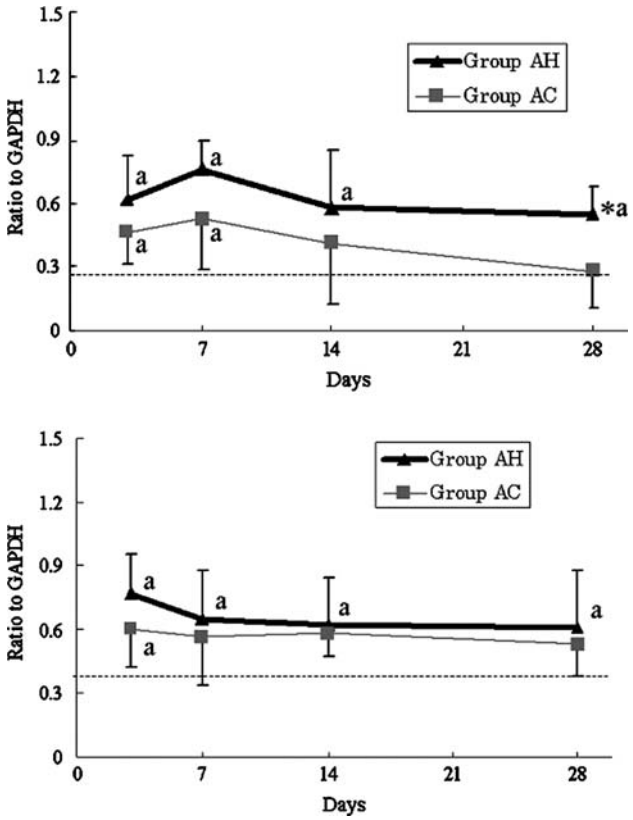

Fig. 7 Gene expression of procollagens in ACL. *Top* Type I procollagen. *Bottom* Type III procollagen. *Broken line* indicates average value in unoperated ACL. *a* $P < 0.05$ versus unoperated ACL. *$P < 0.05$ between the groups

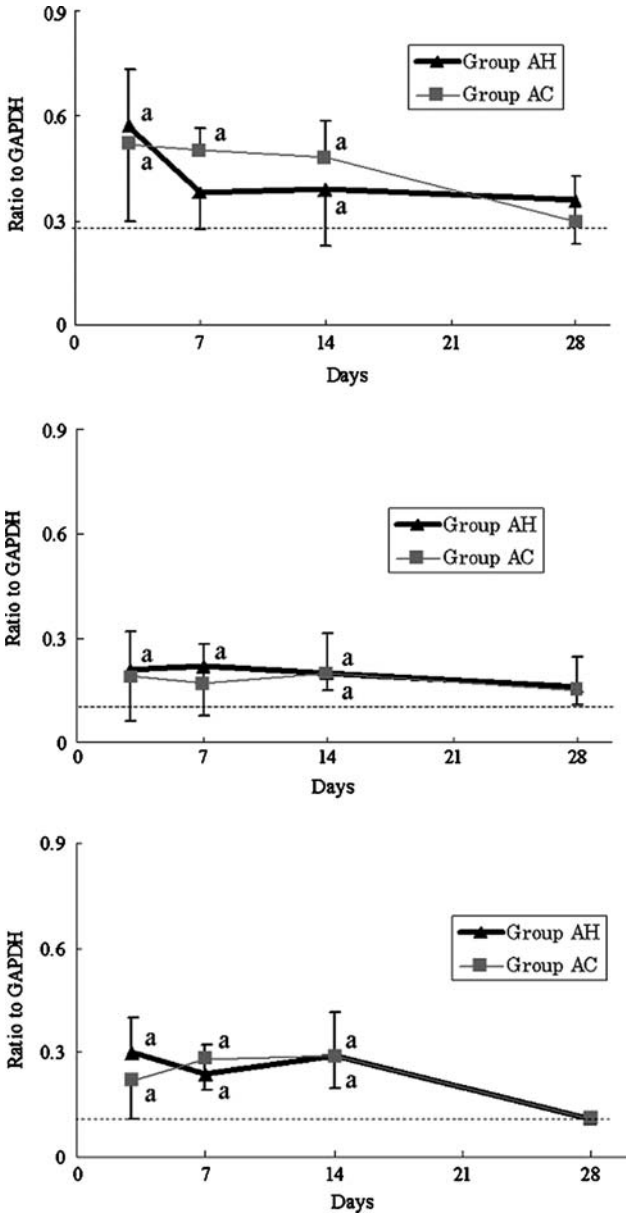

Fig. 8 Gene expression of MMPs in ACL. *Top* MMP-2. *Center* MMP-9. *Bottom* MMP-13. *Broken line* indicates average value in unoperated ACL. *a* $P < 0.05$ versus unoperated ACL

consistent with the results of previous studies [26, 35, 45]. With the administration of HBO, type I procollagen gene expression in injured MCL was significantly increased at 7 days, declining later to control levels at 28 days. This finding was also consistent with the results of our previous study [26].

The injured ACL is known to show increased gene expression of type I procollagen compared with normal ACL [25, 38, 45]. To the best of our knowledge, however, no reports have described the effect of HBO on gene expression of type I procollagen in ACL. In the current study, administration of HBO increased gene expression of type I procollagen in injured ACL with a significant difference apparent at 28 days, suggesting the effect of HBO on gene expression of type I procollagen continued longer in injured ACL than in injured MCL. The reason for this sustained effect of HBO in the injured ACL is unknown. In the injured MCL, the gap between retracted ends was filled with scar tissue as early as 3 days postoperatively, and the amount of scar tissue reached a maximum at 14 days before decreasing. In contrast, no scar tissue formation was observed macroscopically in injured ACL and lacerated ends did not unite even at 28 days in both the groups. This suggests that production of structural proteins is not a major event beyond 14 days in injured MCL, whereas fibroblasts in injured ACL may continue to attempt producing structural proteins longer with administration of HBO due to the failure of injured ACL to unite.

To the best of our knowledge, no studies have examined the effects of HBO on type III procollagen gene expression in the injured ligaments. Our results show that the difference between groups was not significant in either MCL or ACL, suggesting that HBO

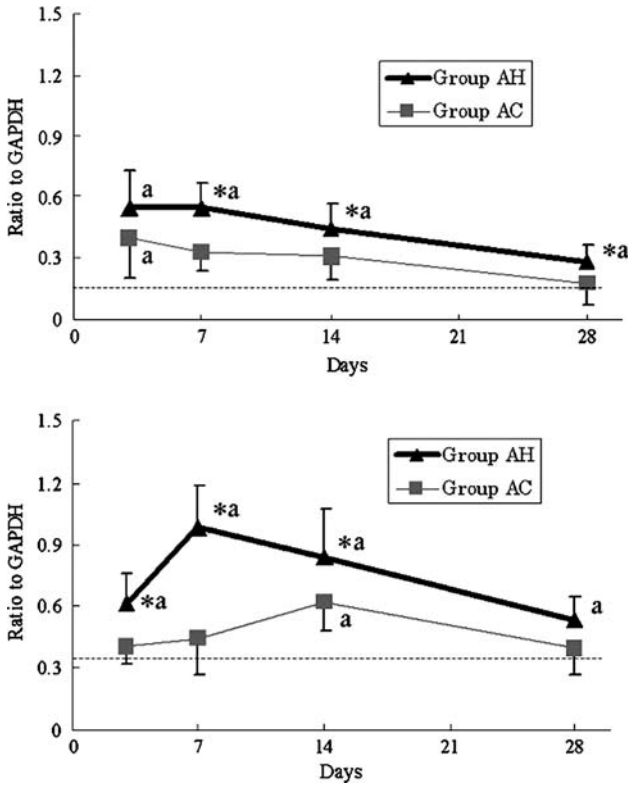

Fig. 9 Gene expression of TIMPs in ACL. *Top* TIMP-1. *Bottom* TIMP-2. *Broken line* indicates average value in unoperated ACL. *a* $P < 0.05$ versus unoperated ACL. *$P < 0.05$ between the groups

has little, if any, effect on type III procollagen gene expression in injured ligaments, compared with the effect on type I procollagen gene expression.

Regarding gene expressions of MMPs and TIMPs in injured ligament, several studies have reported increased gene expressions of these proteins in injured ACL and MCL [3, 17, 25, 27, 34]. In the current study, increased gene expressions of MMP-2, -9 and -13 were observed at 7 and 14 days postoperatively in the injured MCL in the control group, whereas in the injured ACL, increased gene expressions of MMP-2 and -13 were also observed at 3 days, in addition to 7 and 14 days. Increased gene expressions of MMPs in the earlier phase postoperatively in the injured ACL suggest that tissue degradation starts sooner after injury in the ACL than in the MCL.

Few studies have examined the effect of HBO on expressions of MMPs or TIMPs after injury or ischemia [41, 42]. These studies showed that HBO suppressed the expression of MMP-9 after cerebral ischemia or traumatic brain injury [41, 42]. To the best of our knowledge, however, there have been no reports describing the effects of HBO on the expressions of MMPs or TIMPs in the injured ligaments. In the current study, administration of HBO did not significantly alter gene expressions of MMPs in either the injured MCL or ACL, although gene expression of MMP-13 was decreased in the injured MCL at 7 days. This suggests that the effect of HBO on gene expressions of MMPs is minor in both injury types. In contrast, although administration of HBO did not significantly change gene expressions of TIMPs in the injured MCL, gene expressions of TIMP-1 and -2 were significantly increased by HBO in the injured ACL. Considering that TIMPs block the activities of MMPs degrading extracellular matrix [7, 8, 23], if the level of MMPs is unchanged, increased production of TIMPs suppresses the activities of MMPs, inhibiting the degradation process of the tissue. Therefore, our results suggest that HBO does not exert any obvious effect on the degradation process in the injured MCL, but inhibits the degradation process of the injured ACL by enhancing the activities of TIMPs, despite a lack of significant effects on the activities of MMPs.

Differences in the effect of HBO between the injured MCL and the injured ACL indicate that the effect of HBO on the injured ligament is ligament-specific. This difference is probably attributable to differences in the behavior of fibroblasts in the ligament regarding response to increased oxygen supply. Differences are known to exist in morphological features between fibroblasts of the MCL and those of the ACL [2]. Fibroblasts in the ACL are oval-shaped, aligned in columns between bundles of collagen fibrils and surrounded by amorphous ground substance, whereas fibroblasts in the MCL are spindle-shaped, aligned with the long axis of the ligament, and interspersed throughout the collagen fiber bundles with little or no surrounding amorphous ground substance [2]. The morphological features of fibroblasts are quite possibly interrelated with cellular function and may play a determinate role in response to injury [2]. If so, differences in the effect of HBO between the injured MCL and the injured ACL as shown in the current study may be explained by differences in fibroblast function between the MCL and ACL in terms of response to the increased oxygen supply.

In the injured ACL, HBO increased type I procollagen gene expression, suggesting that structural protein synthesis is enhanced. In addition, HBO was suggested to inhibit the degradation process of the injured ACL by enhancing the activities of TIMPs. These are the favorable effects of HBO on the injured ACL. However, no scar tissue formation was observed macroscopically and none of the lacerated ACLs united despite the administration of HBO. This indicates that

the effects of HBO are insufficient to allow the injured ACL to heal by itself.

Since the injured ACL is difficult to heal conservatively, primary repair of the ruptured ACL used to be a popular procedure to regain ACL function. However, clinical outcomes after primary repair have proven unpredictable [9, 11, 30]. ACL reconstruction using autograft is thus commonly performed to restore function of the ACL [14]. This procedure generally produces stable clinical results, but donor-site morbidity is a major debilitating adverse effect of autograft surgery. Although the success rate of primary repair of the ACL injury is not good enough, not all repaired ACLs fail to heal, and some repaired ACLs produce satisfactory function [9, 30]. If HBO therapy is used as an adjunct with primary repair of the injured ACL, the success rate of the surgery may be increased, hopefully leading to a decreased number of patients requiring ACL reconstruction.

Administration of HBO increased type I procollagen gene expression in both the injured MCL and ACL. Gene expressions of MMPs were not significantly affected in either. In addition, gene expressions of TIMPs in the injured MCL were not significantly affected, whereas gene expressions of TIMPs were increased in the injured ACL.

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