Omega-3 Fatty Acids Enhance Ligament Fibroblast Collagen Formation in Association with Changes in Interleukin-6 Production (44467)

KURT D. HANKENSON,*[,2] BRUCE A. WATKINS,† INGRID A. SCHOENLEIN,* KENNETH G. D. ALLEN,‡ AND JOHN J. TUREK*[,1]

Department of Basic Medical Sciences and †Department of Food Science, Lipid Chemistry and Molecular Biology Laboratory, Purdue University, West Lafayette, Indiana 47907; and ‡Department of Food Science and Human Nutrition, Colorado State University, Fort Collins, Colorado 80523

Abstract. Altering dietary ratios of *n*-3 and *n*-6 polyunsaturated fatty acids (PUFA) represents an effective nonpharmaceutical means to improve systemic inflammatory conditions. An effect of PUFA on cartilage and bone formation has been demonstrated, and the purpose of this study was to determine the potential of PUFA modulation to improve ligament healing. The effects of *n*-3 and *n*-6 PUFA on the *in vitro* healing response of medial collateral ligament (MCL) fibroblasts were investigated by studying the cellular coverage of an *in vitro* wound and the production of collagen, PGE_2, IL-1, IL-6, and TNF. Cells were exposed to a bovine serum albumin (BSA) control or either eicosapentaenoic acid (EPA, 20:5*n*-3) or arachidonic acid (AA, 20:4*n*-6) in the form of soaps loaded onto BSA for 4 days and wounded on Day 5. AA and EPA improved the healing of an *in vitro* wound over 72 hr. EPA increased collagen synthesis and the overall percentage of collagen produced, but AA reduced collagen production and total protein. PGE_2 production was increased in the AA-treated group and decreased in the EPA-treated group, but was not affected by wounding. IL-1 was not produced at the time point evaluated, but TNF and IL-6 were both produced, and their levels varied relative to the PUFA or wounding treatment. There was a significant linear correlation ($r^2 = 0.57$, $P = 0.0045$) between IL-6 level and collagen production. These results demonstrate that *n*-3 PUFA (represented by EPA in this study) positively affect the healing characteristics of MCL cells and therefore may represent a possible noninvasive treatment to improve ligament healing. Additionally, these results show that MCL fibroblasts produce PGE_2, IL-6, and TNF and that IL-6 production is related to MCL collagen synthesis. [P.S.E.B.M. 2000, Vol 223]

Ligament injuries are a common cause of pain and disability (1). Some ligaments, such as the anterior cruciate ligament (ACL), have little intrinsic healing capability (2), whereas others, such as the medial collateral ligament (MCL), mount a fairly effective repair response (3, 4). Even though the MCL heals, a lengthy period is generally required for the ligament to remodel to the point of comparable original strength (3, 5). Additionally, the repair process can result in ligament contraction due to immobility and lack of use (6). Attempts to reveal exogenous agents that could be used to improve ligament healing have evaluated a number of growth factors, such as PDGF, TGFβ, and bFGF. The addition of these factors, alone and in combination, stimulated *in vitro* cell proliferation and/or matrix production by ACL and MCL cells in culture (7, 8) and by MCL explants (9, 10). Similar studies, extended *in vivo*, have been moderately successful (11, 12); however, there are complicating factors, such as the delivery method of the

This research was supported by USDA Animal Health Project #IND072017V.

[1] To whom requests for reprints should be addressed at Purdue University, Department of Basic Medical Sciences, 1246 Lynn Hall, W. Lafayette, IN 47907-1246. E-mail: jjt@vet.purdue.edu

[2] Author's current address is Department of Biochemistry, University of Washington, Box 357350, Seattle, WA 98195.

Received March 3, 1999. [P.S.E.B.M. 2000, Vol 223]
Accepted August 10, 1999.

0037-9727/00/2231-0088$14.00/0

growth factor to the injured site, dosage requirements, timing of administration, and the quality of the healed tissue. Therefore, it is beneficial to investigate the potential use of alternative agents that could be administered easily to a patient to modulate ligament healing.

Dietary *n*-3 and *n*-6 polyunsaturated fatty acids (PUFA) are essential nutrients that represent a class of systemic modulatory agents that affect eicosanoid (13) and cytokine (14) production, and influence expression of some membrane receptors (15, 16) or activity of cell activation enzymes such as protein kinase C (PKC) (17). Dietary supplementation with *n*-3 PUFA has been studied extensively with regard to inflammatory conditions, such as, rheumatoid arthritis (RA) (18, 19). Kremer *et al.* (20) showed that supplementation of the diet with *n*-3 fatty acids decreased clinical signs of RA and that cytokine production by synovial macrophages was decreased. The effects of *n*-3 PUFA on connective tissue healing have not been studied extensively. However, *in vitro* studies with various cell types indicate that PUFA have mixed effects on cell proliferation or migration, either decreasing, increasing, or having no effect depending on the cell type and the PUFA type (21–23). Moreover, *n*-6 PUFA enrichment of chondrocytes in culture reduced collagen synthesis (24), which suggests that PUFA also influence matrix production.

In this study, the ability of PUFA to modify cell migration into an *in vitro* wound and collagen production was used as a model of ligament cellular response to injury. We hypothesized that the administration of *n*-6 PUFA to ligament cells would decrease collagen synthesis and that *n*-3 PUFA would have the opposite effect. The production of interleukin-1 (IL-1), IL-6, tumor necrosis factor (TNF), and prostaglandin E_2 (PGE_2) was measured to determine if MCL fibroblasts produced these inflammatory products and if PUFA could modulate these mediators. The production of these agents by MCL fibroblasts has not been demonstrated previously.

Materials and Methods

Primary Cell Culture and Fatty Acid Enrichment. MCL fibroblasts were harvested and cultured according to modified procedures of Ross *et al.* (25) from three groups of six pigs as described previously (26). Tissues were collected in accordance with institutional animal care and use guidelines. Briefly, the periligamentous tissues were removed, the ligaments rinsed in Dulbecco's Modified Eagles Medium (DMEM) (Sigma, St. Louis, MO) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B, and cubed into 1-mm pieces. The ligament pieces were digested for 6 hr with collagenase (Sigma Type 1 a 350 U/ml) in a 37°C shaker bath. The cells freed from the tissue by digestion were collected by centrifugation, rinsed with DMEM medium, and resuspended in DMEM plus 15% fetal bovine serum (FBS) (Hyclone, Logan UT). The cells were grown in a 25-cm^2 culture flask (Corning, Corning, NY) and were frozen for later experimental use after reaching confluency in 9–14 days.

Frozen cells were quick-thawed, plated at a density of 1.0×10^5 cells/cm^2, and grown to confluence in 25-cm^2 flasks. For experimental procedures, these cells were removed by trypsin treatment and then plated at a density of 1.0×10^4 cells/cm^2. Upon reaching confluence on Day 7, fibroblasts were treated with one of three different experimental media, (i) BSA, (ii) AA, or (iii) EPA, for 4 additional days prior to further manipulation or data collection. The control medium consisted of DMEM with 5% FBS and 20 μ*M* bovine serum albumin, hence forth referred to as BSA or control PUFA treatment. The PUFA-enriched cells were cultured in DMEM + 5% FBS plus either 60 μ*M* arachidonic acid (AA, 20:4, *n*-6) (Nu-Chek-Prep, Elysian, MN), or 60 μ*M* eicosapentaenoic acid (EPA, 20:5, *n*-3) (generous gift from Scotia Pharmaceutical, Sterling, Scotland). AA and EPA, used in this study as representative *n*-6 and *n*-3 PUFA, are the primary substrates for the cyclooxygenase enzyme. The PUFAs were converted to soaps and bound to essentially fatty acid–free BSA (Sigma, St. Louis, MO) (24). The concentration of the PUFA treatment was determined from previous cell culture experiments (24), and 60 μ*M* was a level that provided for adequate fatty acid enrichment of cell membranes without any negative effects on cell growth.

Fatty Acid Analysis. To demonstrate that the cells metabolized the enriched PUFA effectively, cellular lipids were harvested and fatty acids evaluated. Cells were harvested, pelleted, and suspended in 100% methanol and quenched with liquid N_2 prior to freezing at –70°C. Lipids were extracted, and fatty acid methyl esters (FAME) were prepared using 14% boron trifluoride in methanol (Alltech Associates Inc., Deerfield, IL). FAME were analyzed using capillary gas chromatography as previously described (24).

***In Vitro* Wound Assay.** A well-described *in vitro* wounding assay was used to evaluate the migration and proliferation of MCL fibroblasts (27, 28). This assay does not mimic the complex process of wound healing; rather the cellular coverage of an *in vitro* cell-free zone is measured to evaluate the combined effects of cell proliferation and migration. A 0.5-mm-wide wound, the entire diameter of a 35-mm culture plate, was created in a confluent layer of MCL fibroblasts by streaking a sterile pipet tip across the culture. The migration of fibroblasts into the wound was measured every 12 hr for 72 hr. Multiple photographs of the wound were obtained by phase contrast microscopy at 100x, and the mean area of coverage (μm^2) for each plate was determined with image analysis software (Optimas 6.0, Bothell, WA).

A duplicate set of plates was used to evaluate cytokine and prostaglandin production and collagen synthesis. In these plates, a portion of the media was collected at 12 hr, and then replaced with fresh media containing the same PUFA composition. At 24 hr postwounding the cells were

exposed to 5 μCi of ^{3}[H]-proline (Amersham, Oakbrook, IL) for collagen synthesis determination.

Cytokine Bioassays. Bioassays for TNF-like activity, IL-6, and IL-1 were performed as previously described (29). To normalize the cytokine production on a per cell basis, the MCL cells were washed in HBSS and digested with 1 *N* ammonium hydroxide and 0.2% Triton X-100 for total cellular DNA quantitation (30) after collecting the culture supernatant fluids. Murine fibroblasts (L929) were used to assay for TNF-like activity. Dilutions of fibroblast culture supernatant fluid were placed in wells containing L929 murine fibroblasts with growth medium containing actinomycin-D. After incubation for 20 hr at 37°C, the fluid in the wells was discarded, and 5 mg/ml of MTT in Hank's balanced salt solution (HBSS) added to each well. After 3 hr, 100 μl of a solution of SDS in N,N dimethylformamide pH 4.7 was added to each well and incubated at 37°C overnight. The absorbance at 550 nm was then read in a microplate reader.

The IL-6 bioassay was performed using B9 cells (31). The cells were grown in Iscove's Modified Dulbecco's medium supplemented with 10% fetal calf serum (FCS), 4 pg/100 ml of mouse recombinant IL-6, and 50 m*M* 2-mercaptoethanol (2-ME). Dilutions of culture supernatant fluid were added in octuplicate to 96-well culture plates containing B9 cells. After 72 hr of incubation, 20 μl of 5 mg/ml of MTT in HBSS was added to each well, and then 6 hr later 100 μl of SDS-DMF. The absorbance at 550 nm was recorded after an overnight incubation at 37°C.

A mouse plasmacytoma cell line (T1165.17) that proliferates in response to IL-1 was used to assay for IL-1 activity. The IL-1 receptor on the T1165.17 cell line was blocked using a monoclonal antibody (LA 15.6) to the IL-1 receptor (32). The cells were grown in minimal essential medium supplemented with 10% FCS, 50 m*M* 2-ME, and 20 pg/ml of IL-1. Cells were seeded in 96-well plates (5×10^5/ml), and samples were assayed in quadruplicate. Four wells were blocked with antibody (1 μg/ml) 30 min before addition of samples, and four wells were unblocked. Diluted samples were added and incubated for 18 hr, and then MTT was added as described for the IL-6 bioassay. Six hours later SDS-DMF was added, and the plates were incubated and absorbance measured as described for the IL-6 assay. The difference in absorbance between blocked and unblocked wells, corrected for control levels, determined the amount of IL-1 activity in the MCL culture medium.

Prostaglandin E_2 and Collagen Production. The concentration of PGE_2 in the culture medium was measured by radioimmunoassay (33), and fibroblast collagen production was measured as previously described (26). Briefly, 24 hr following wounding, the culture medium was removed, and the cells were washed and incubated with 5 μCi of ^{3}[H]proline (Amersham, Oakbrook, IL) in the same initial culture medium. After 24 hr, the media and cells were collected separately. The cells were pelleted, and the supernatant was combined with the media fraction. The cells were lyzed, and then 0.75 ml of lysate combined with the media fraction and 0.25 ml was used for total DNA determination (30). The combined cell and media fractions were precipitated with trichloroacetic acid (TCA). The acid insoluble precipitate was then rinsed several times with TCA to remove free ^{3}[H]proline. The precipitate was redissolved in 0.5 *M* NaOH, buffered to pH 7.2, and incubated for 6.5 hr with protease-free collagenase (Sigma Type VII Collagenase). Following the digestion, TCA was again added to precipitate the acid-insoluble proteins. The supernatant containing ^{3}[H]proline was counted on a scintillation counter (Packard 1900 TR, Meriden, CT), and collagen production was determined by the DPM/μg DNA. The amount of noncollagenous protein (NCP) was determined by re-dissolving the acid-insoluble precipitate in 0.5 *M* NaOH and then counting that fraction for DPM/μg DNA. Total Protein (TP) was calculated by summing the values determined for collagen with the values determined for NCP.

Statistical Analysis. Data were analyzed using both one-way and two-way ANOVA procedures of SAS (SAS Institute, Cary, NC). A Duncan's Multiple Range Test ($\alpha = 0.05$) was performed to analyze significant main and interaction effects. Correlation analysis was completed using Prism software (GraphPad, San Diego, CA).

Results

Fatty Acid Analysis of MCL Fibroblasts. Analysis of the lipid content of MCL cells that were exposed to either AA or EPA treatments for 4 days revealed significant alterations in the levels of varying cellular fatty acids (Table I). Cells that were treated with the *n*-6 fatty acid AA had significantly increased levels of *n*-6 fatty acid metabolites

Table I. Fatty Acid Composition (Mean ± SD) of Fibroblast Polar Lipids (Area %)

Fatty acid	Treatments		
	BSA	AA	EPA
14:0	0.62 ± 0.11	0.53 ± 0.11	0.44 ± 0.12
16:0	12.80 ± 2.52	9.31 ± 2.32	9.78 ± 0.12
18:0	21.98 ± 3.59	14.09 ± 2.61	16.10 ± 3.78
total 18:1	13.08 ± 1.68^a	8.32 ± 1.62^b	8.98 ± 1.89^b
t-18:2*n*-6	2.10 ± 0.46	1.89 ± 0.92	1.73 ± 0.28
18:2*n*-6	18.11 ± 1.87^a	12.12 ± 0.50^b	18.45 ± 4.21^a
20:3*n*-6	2.37 ± 0.44	2.64 ± 0.47	2.41 ± 0.37
20:4*n*-6	14.25 ± 3.23^a	33.57 ± 8.62^b	7.78 ± 5.76^a
20:5*n*-3	ND	ND	16.47
22:4*n*-6	4.77 ± 1.60^a	10.18 ± 0.81^b	2.01 ± 0.69^c
22:5*n*-3	2.96 ± 1.56^a	2.29 ± 1.06^a	12.17 ± 2.84^b
22:6*n*-3	2.46 ± 0.31^a	1.56 ± 0.22^b	1.05 ± 0.31^b
total *n*-6	39.78 ± 7.47^a	58.68 ± 5.85^b	30.65 ± 3.84^a
total *n*-3	4.6 ± 2.98^a	3.32 ± 1.96^a	29.69 ± 9.09^b
n-6:*n*-3 ratio	13.72 ± 11.71^a	24.30 ± 17.14^a	1.12 ± 0.49^b

Note. Values ($n = 9$) within rows having different superscripts are significantly different ($p < 0.05$) as determined by one-way ANOVA and Duncan's Multiple Range Test. BSA, bovine serum albumin; AA, arachidonic acid; EPA, eicosapentaenoic acid; ND, not detected.

relative to BSA and EPA enriched cells, whereas the *n*-3 fatty acid content for cells treated with EPA was significantly increased over the BSA control and the AA groups. This resulted in a fatty acid ratio of *n*-6:*n*-3 in the EPA group (1.12) that was significantly different ($P < 0.05$) from that of the BSA (13.78) and AA groups (24.30). The magnitude of alteration in the *n*-6:*n*-3 ratio with *n*-3 PUFA supplementation is similar to what has been achieved both *in vitro* in chondrocytes (24) and *in vivo* in chicken cortical bone (33), murine macrophages (34), and human serum (35) under altered levels of *n*-3 and *n*-6 PUFA.

***In Vitro* Wounding.** The administration of PUFA increased the coverage of the cell-free zone. Both AA and EPA PUFA treatments resulted in a significant increase ($P < 0.05$) in wound coverage compared with the control at every time point analyzed for the 72 hr of the assay (Fig. 1). At 72 hr AA treated cells had a 21% greater coverage, and EPA had a 17% greater coverage than the BSA control.

IL-1 Production. There was no detectable IL-1 production at the 12-hr time point of the wounding assay. However, several samples taken following 24 hr of exposure did have a low level of IL-1 production (data not shown).

TNF Production. The interaction effect of PUFA administration with wounding was significant at $P < 0.05$ (Fig. 2). In nonwounded cells, EPA administration significantly increased ($P < 0.05$) levels of TNF production compared with either BSA or AA enriched cells. In wounded EPA-enriched cells, there was a significant ($P < 0.05$) decrease in TNF compared to the nonwounded cells. This decrease eliminated the difference observed with EPA in nonwounded cells and resulted in no significant differences in TNF production among PUFA treatments for the wounded cells.

IL-6 Production. The interaction effect of wounding with PUFA was significant for IL-6 production (Fig. 3). In nonwounded cells, EPA enrichment significantly ($P < 0.05$) increased IL-6 production compared with either BSA- or AA-enriched cells. Cell wounding caused an overall decrease in IL-6 production for all treatments, and this effect was significant for AA- and EPA-enriched cells. This decrease with wounding eliminated significant differences in IL-6 production between the treatment groups.

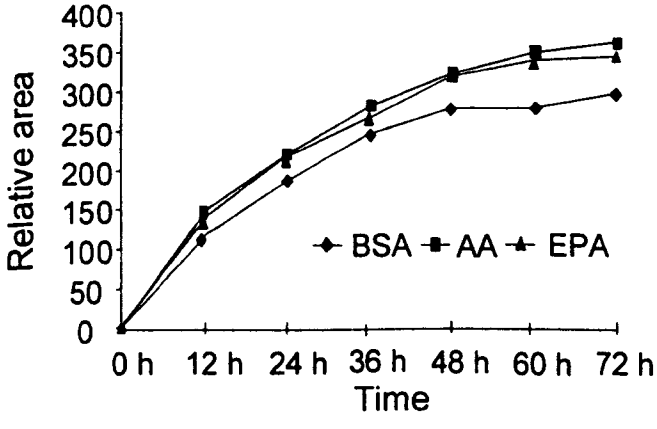

Figure 1. Healing of an *in vitro* wound (0.5-mm in width) in a monolayer of medial collateral ligament fibroblasts. The relative area of migration was evaluated every 12 hr. AA- and EPA-enriched cells had significantly increased ($P < 0.05$) wound coverage compared with the BSA control at all time points starting at 12 hr. ($n = 9$)

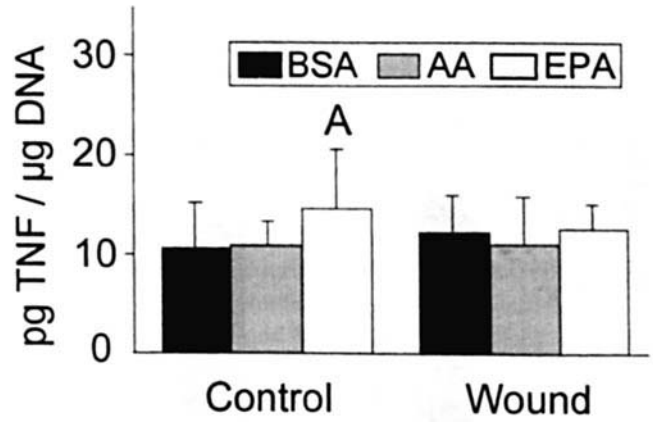

Figure 2. Tumor necrosis factor (TNF)-like bioactivity 12 hr postwounding. The letter A indicates that this value is significantly different from all other values ($P < 0.05$). ($n = 9$)

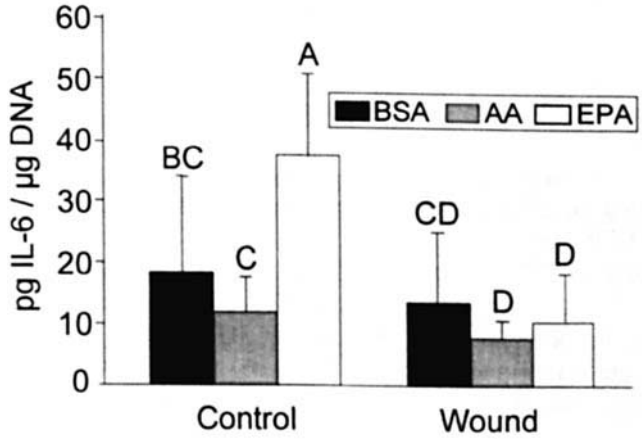

Figure 3. Interleukin-6 (IL-6) bioactivity 12 hr postwounding. Bars with different letters are significantly different ($P < 0.05$). ($n = 9$)

PGE_2 Production. Arachidonic acid increased ($P < 0.05$) PGE_2 production to 29.2 ± 6.2 ng/µg DNA from a control level of 12.0 ± 4.1 ng/µg DNA for BSA, whereas EPA decreased ($P < 0.05$) PGE_2 to 2.69 ± 0.8 ng/µg DNA. A small decrease occurred in PGE_2 production with wounding, but the effect was not significant (BSA, 10.05 ± 3.9 ng/µg DNA; EPA, 1.73 ± 0.47 ng/µg DNA; AA, 26.57 ± 9.22 ng/µg DNA). Correlation analysis between IL-6 and TNF and PGE_2 did not reveal a significant correlation between PGE_2 and cytokine production.

Collagen Production. The interaction of PUFA treatment with wounding treatment was significant at $P < 0.05$ for collagen production (Fig. 4). Under control conditions, BSA-, AA-, and EPA-treated MCL cells produced significantly ($P < 0.05$) different amounts of collagen compared with each other (EPA > BSA > AA). Wounding of the MCL monolayers decreased collagen production for the three treatment groups; however, the level of reduction was

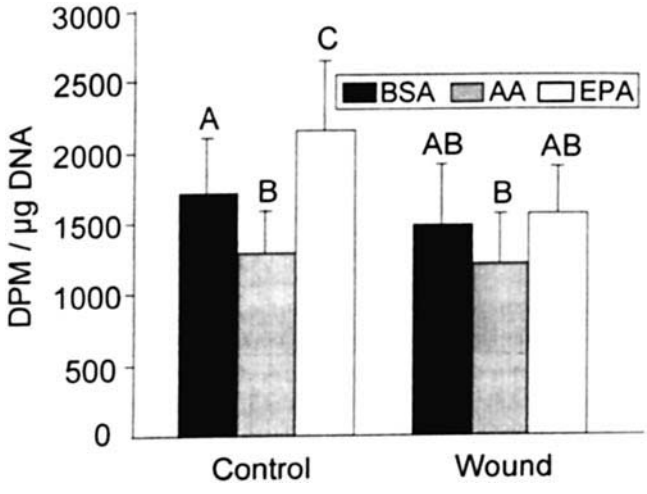

Figure 4. Collagen production 24 hr postwounding measured by the incorporation of ^{3}[H]proline into the collagenase-sensitive portion of acid-insoluble protein. Bars with different letters are significantly different ($P < 0.05$). ($n = 9$)

only significantly different from nonwounding for the EPA-treated cells.

In addition to analyzing collagen production, we evaluated the ^{3}[H]proline incorporation into noncollagen protein (NCP), and then, in turn, the amount of total acid insoluble protein (TP) (Table II). For NCP, interacting effects were not significant, and neither PUFA nor wounding affected the level of NCP. For the TP, BSA-treated cells had significantly more protein ($P < 0.05$) than the AA group. The amount of TP for EPA-enriched cells was not significantly different from either BSA or AA. Wounding significantly ($P < 0.05$) reduced TP relative to nonwounded cells.

The interaction effect for collagen as a percentage of TP was not significant (Table II). EPA administration significantly increased ($P < 0.05$) the percentage of collagen synthesized compared with the BSA- and AA-enriched cells (Table II). Interestingly, even though there were reductions in total collagen when the cultures were wounded, this effect did not alter the percentage of collagen produced.

The results of the collagen production and the percentage of collagen were subjected to correlation analysis. The analysis compared TNF, IL-6, and PGE_2 production with both collagen and the percentage of collagen produced for the entire experiment across the three PUFA and two wounding treatment levels. There was no significant correlation between TNF and collagen production or collagen percentage ($r^2 = 0.27$ and $r^2 = 0.13$, respectively) or between PGE_2 and collagen production or percentage ($r^2 = 0.32$ and $r^2 = 0.18$, respectively). In contrast, there was a significant correlation between IL-6 and collagen production ($r^2 = 0.57$, $P = 0.0045$) and IL-6 and percentage of collagen ($r^2 = 0.43$, $P = 0.021$).

Discussion

The role that ligament fibroblasts play in both grade I/II healing (nonsystemic) and grade III healing (systemic) is complex and involves (i) cell migration/proliferation, ii) cellular communication, (iii) extracellular matrix production, and (iv) matrix degradation (36, 37). In this investigation, we studied three of these potential factors using *in vitro* assays: (i) proliferation and migration were studied by evaluating the cellular coverage of an *in vitro* wound; (ii) inflammatory mediator production was measured as a potential mechanism for cellular communication; and (iii) collagen production was evaluated as a determinant of matrix accumulation. These variables were studied in conjunction with PUFA modulation to determine if dietary fatty acids could be used to modulate ligament healing.

***In Vitro* Wounding.** Cellular coverage of an *in vitro* wound is a simple model that allows for the analysis of cell behavior when a cell monolayer is disrupted. In this study, we demonstrated that the administration of both AA and EPA significantly increased the coverage of disrupted monolayers compared with the BSA control. In this common *in vitro* assay, fibroblasts responded to fill in the cell-free gap through both proliferation and migration. Previous studies have demonstrated an influence of PUFA on cell proliferation and migration. Linoleic acid (18:2n-6) increased the proliferation of epidermal growth factor (EGF)–stimulated mouse mammary epithelial cells (38). Additionally, AA (20:4*n*-6) significantly increased 3T3 murine fibroblast proliferation compared with cells treated with two *n*-3 PUFAs (EPA and docosahexaenoic acid) (22). Although these two studies found that *n*-3 PUFA had no effect on cell growth, *n*-3 PUFA enrichment has been found to increase endothelial cell migration (21, 39). Both *n*-3 and

Table II. Collagen Production (Mean ± SD) by MCL Fibroblasts (DPM/µg DNA)

Collagen parameter	Treatments				
	BSA	AA	EPA	Control	Wound
COL*	1546 ± 392	1360 ± 489	1753 ± 600	1715 ± 532	1421 ± 542
NCP	6114 ± 2139	5538 ± 1352	5590 ± 1234	6420 ± 1880	5644 ± 1253
TP	7962 ± 2232^a	6898 ± 1507^b	7343 ± 1473^{ab}	8096 ± 2017^z	7105 ± 1369
Percentage	20.4 ± 5.9^a	19.9 ± 3.9^a	24.3 ± 5.6^b	21.3 ± 6.3	20.8 ± 5.1

* There is a significant interaction of PUFA and wounding treatments for all values ($n = 9$) in the row (see Fig. 4). Values ($n = 9$) with different superscripts (^{ab}main effect for fatty acid treatments; ^{z}wounding main effect) are significantly different ($p < 0.05$) as determined by one-way ANOVA and Duncan's Multiple Range Test. BSA, bovine serum albumin; AA, arachidonic acid; EPA, eicosapentaenoic acid; COL, collagen; NCP, noncollagenous protein; TP, total protein.

n-6 PUFA increase the activity of PKC (40) which is an important enzyme in the signaling pathways for stimulation of cell growth and migration (41). Therefore, AA and EPA may enhance MCL *in vitro* wound coverage by increasing PKC-regulated cell proliferation and/or migration.

Cytokine Production. Cytokines, which are modulated secondary to PUFA administration, play an important role in wound healing. TNF, IL-1, and IL-6 have been shown to increase nonligament fibroblast cellular proliferation and have both negative and positive effects on collagen production (42, 43); however, studies of these factors in skeletal ligament cells have not been reported.

No detectable IL-1 was produced at the 12-hr time point; however, the cytokines TNF and IL-6 were present 12 hr postwounding, and their levels were varied depending on the treatments. TNF production was downregulated by PGE_2 *via* a cAMP-dependent pathway (44), and PGE_2 was reduced by EPA by competing with AA for the enzyme cyclooxygenase. Thus, for the EPA-treated MCL cells, the higher level of TNF production in nonwounded cells was expected since EPA decreased PGE_2. IL-6 production was low with AA treatment, which was unexpected since PGE_2 can induce IL-6 production (45). As for TNF, EPA significantly elevated IL-6 release over the BSA control and AA-treated cells, which suggests that EPA modulates cytokine responses in MCL cells.

Tissue injury is known to mediate cytokine release (46), and it was thought that MCL wounding might result in a generalized increase in cytokine production by the MCL cells, but this was not observed. Wounding did not change TNF production by control or AA-treated cells and resulted in decreased TNF production in EPA-enriched cells. Wounding also decreased IL-6 production in all of the PUFA treatment groups, indicating that *in vitro* injury of MCL cells is a negative effector of *in vitro* cytokine production. Since wounding decreased cytokine production without a concurrent change in the amount of PGE_2, wounding downregulation was independent of PGE_2. This decrease in cytokine production could be due to the release of factor(s), such as PDGF or bFGF, from injured cells, or alternatively, an intercellular signaling event generated at the time of wounding could be propagated directly from cell to cell across the confluent monolayer (47). *In vitro* denudation of endothelial cells is associated with the generation of an intercellular calcium flux that extends well into the zone of noninjured cells *via* gap junction communication (48).

Collagen Production. Previously, Watkins *et al.* (24) established that linoleic acid, an *n*-6 PUFA and PGE_2 precursor, reduced avian chondrocyte collagen synthesis. In our experiments, AA, another PGE_2 precursor, also reduced collagen synthesis. Conversely, EPA, an *n*-3 fatty acid, which interferes with PGE_2 production, upregulated collagen production. Interestingly, this is not an increase in relative total protein, but rather a significant increase in the percentage of collagen produced. Thus, fatty acid administration directly affects collagen production by cultured MCL cells, with the *n*-6 PUFA, AA, decreasing collagen synthesis and the *n*-3 PUFA, EPA, increasing collagen synthesis.

The PUFA effects on collagen production in this study are likely partially mediated by PGE_2 since increased PGE_2 decreases procollagen mRNA transcription (49). However, PGE_2 alterations do not completely explain the variation in collagen production observed with wounding. Collagen production drops with wounding despite the fact that PGE_2 levels remain unchanged from control conditions, suggesting a controlling influence with wounding that over-rides the effect of prostaglandins. In this case, the dominant influence may be related to IL-6, since alterations in collagen production that are associated with both PUFA treatments and wounding are significantly correlated with IL-6.

IL-6 increases collagen transcription (50) and induces the synthesis of tissue inhibitors of metalloproteinases (51). Thus, we hypothesize that the influence of PUFA on collagen synthesis is through an IL-6–associated pathway that is partially prostaglandin-mediated. When IL-6 levels are high in nonwounded EPA cultures in association with low PGE_2, collagen is increased, but when IL-6 levels drop with monolayer disruption, collagen production decreases in spite of maintained low levels of PGE_2.

Alternative mechanisms unrelated to PGE_2 and IL-6 may also be responsible for decreasing collagen production in denuded cultures. For instance, wounding may induce the release of growth factors such as bFGF, which negatively affects collagen production (52). Additionally, collagen synthesis could be decreased in the wounded monolayers due to the propagation of a cell-cell signal originating from the injured cells, as previously discussed (47). Finally, since it is recognized that proliferating and migrating fibroblasts have reduced collagen production (53), this could account for a portion of the decreased collagen production with wounding.

In summary, we have established that either *n*-3 or *n*-6 PUFA administration to ligament cells improves coverage of an *in vitro* wound. However, only *n*-3 PUFA increased collagen production as opposed to *n*-6 PUFA which decreased collagen formation. Dietary supplementation with *n*-3 PUFA may represent a potential treatment to improve ligament healing postinjury. Increasing the oral intake of *n*-3 PUFA either in capsular form or by ingesting an increased amount of foods enriched with *n*-3 PUFA is known to increase serum and tissue PUFA levels, including bone and cartilage (33, 35), and likely would result in altered ligament PUFA levels. Increased oral *n*-3 PUFA intake could therefore be used to enhance cell ingress in the early stages of ligament injury and then promote collagen synthesis through the intermediate and later stages of healing. Further, in our study, we have demonstrated the production of PGE_2, TNF, and IL-6 by MCL cells and have confirmed that their levels are modulated secondary to PUFA treatment. Neither PGE_2 nor TNF are apparently associated with

wounding or collagen production; however, IL-6 levels are decreased in association with cell disruption and significantly correlated with collagen synthesis. IL-6 likely plays a role in regulating the collagen alterations that are associated with both PUFA treatment and monolayer wounding in MCL fibroblasts. For future studies, the interactions of IL-6 with growth factors such as TGF-β, bFGF, and IGF-I should be investigated. Growth factors stimulate *in vitro* cell proliferation and stimulate or inhibit matrix production by cultured cells (7, 8, 52), and there is also evidence that dietary lipids can modulate IGF-I *in vivo* (54).

The cell line and antibody used for IL-1 analysis were generously supplied by Dr. Andrew Glasebrook, Eli Lilly and Co., Indianapolis, IN. Approved as journal paper number #15867 of the Purdue Agricultural Experiment Station.

1. Woo SL-Y, Ohland KJ, McMahon PJ. Biology healing and repair of ligaments. In: Finerman GAM, Noyes FR, Eds. Biology and Biomechanics of the Traumatized Synovial Joint: The knee as a model. Rosemont, IL: AAOS, pp241–273, 1992.
2. Hefti FL, Annetrudi K, Fasel J, Morscher EW. Healing of the transected anterior cruciate ligament in the rabbit. J Bone Joint Surg Am **73**:373–383, 1995.
3. Frank C, Amiel D, Woo SL-Y, Akeson W. Normal ligament properties and ligament healing. Clin Orth Rel Res **196**:15–25, 1985.
4. Clayton ML, Miles JS, Abdulla M. Experimental investigations of ligamentous healing. Clin Orthop **61**:146–153, 1968.
5. Akeson WH, Garfin S, Amiel D, Woo SL-Y. Para-articular connective tissue in osteoarthritis. Semin Arthritis Rheum **18**:41–50, 1989.
6. Akeson WH, Woo SL-Y, Amiel D, Couts RD, Daniel D. The connective tissue response to immobility: Biochemical changes in periarticular connective tissue of the immobilized rabbit knee. Clin Orthop **93**:356–362, 1973.
7. Schmidt CC, Georgescu HI, Kwoh CK, Blomstrom GL, Engle CP, Larkin LA, Evans CH, Woo SL-Y. Effect of growth factors on the proliferation of fibroblasts from the medial collateral and anterior cruciate ligaments. J Orthop Res **13**:184–190, 1995.
8. Scherping SC Jr., Schmidt CC, Georgescu HI, Kwoh CK, Evans CH, Woo SL-Y. Effect of growth factors on the proliferation of ligament fibroblasts from skeletally mature rabbits. Connect Tissue Res **36**:1–8, 1997.
9. Murphy PG, Loitz BJ, Frank CB, Hart DA. Influence of exogenous growth factors on the synthesis and secretion of collagen types I and III by explants of normal and healing rabbit ligaments. Biochem Cell Biol **72**:403–409, 1994.
10. Lee J, Green MH, Amiel D. Synergistic effect of growth factors on cell outgrowth from explants of rabbit anterior cruciate and medial collateral ligaments. J Orthop Res **13**:435–441, 1995.
11. Letson AK, Dahners LE. The effects of combinations of growth factors on ligament healing. Clin Orthop **308**:207–212, 1994.
12. Batten ML, Hansen JC, Dahners LE. Influence of dosage and timing of application of platelet-derived growth factor on early healing of the rat medial collateral ligament. J Orthop Res **14**:736–741, 1996.
13. Abeywardena MY, McLennan PL, Charnock JS. Differences between *in vivo* and *in vitro* production of eicosanoids following long-term dietary fish oil supplementation in the rat. Prost Leukot Essent Fatty Acids **42**:159–165, 1991.
14. Meydani SN. Modulation of cytokine production by dietary polyunsaturated fatty acids. Proc Soc Exp Biol Med **200**:189–193, 1992.
15. Awazu M, Yared A, Swift LL, Hoover RL, Ichikawa I. Dietary fatty acid modulates glomerular atrial natriuretic peptide receptor. Kidney Int **42**:265–271, 1992.
16. Opmeer FA, Adolfs MJP, Bonta IL. Regulation of prostaglandin E_2 receptors *in vivo* by dietary fatty acids in peritoneal macrophages from rats. J Lipid Res **25**:262–268, 1984.
17. May CL, Southworth AJ, Calder PC. Inhibition of lymphocyte protein kinase C by unsaturated fatty acids. Biochem Biophys Res Commun **195**:823–828, 1993.
18. Geusens P, Wouters C, Nijs J, Jiang Y, Dequeker J. Long-term effect of omega-3 fatty acid supplementation in active rheumatoid arthritis: A 12-month, double-blind, controlled study. Arth Rheum **37**:824–829, 1994.
19. Kremer JM. Effects of modulation of inflammatory and immune parameters in patients with rheumatic and inflammatory disease receiving dietary supplementation of *n*-3 and *n*-6 fatty acids. Lipids **31** (Suppl):S243–S247, 1996.
20. Kremer JM. Studies of dietary supplements with omega-3 fatty acids in patients with rheumatoid arthritis. In: Nelson GJ, Ed. Health Effects of Dietary Fatty Acids. Champaign, IL: American Oil Chemists' Society, pp223–233, 1991.
21. Kanayasu T, Morita I, Nakao-Hayashi J, Ito H, Murota S. Enhancement of migration in bovine endothelial cells by eicosapentaenoic acid pretreatment. Atherosclerosis **87**:57–64, 1991.
22. Danesch U, Weber PC, Sellmayer A. Differential effects of *n*-6 and *n*-3 polyunsaturated fatty acids on cell growth and early gene expression in Swiss 3T3 fibroblasts. J Cell Physiol **168**:618–624, 1996.
23. Sellmayer A, Danesch U, Weber PC. Effects of different polyunsaturated fatty acids on growth-related early gene expression and cell growth. Lipids **31**(Suppl):S37–S40, 1996.
24. Watkins BA, Xu H, Turek JJ. Linoleate impairs collagen synthesis in primary cultures of avian chondrocytes. Proc Soc Exp Biol Med **212**:153–159, 1996.
25. Ross SM, Joshi R, Frank CB. Establishment and comparison of fibroblast cell lines from the medial collateral and anterior cruciate ligaments of the rabbit. *In Vitro* Cell Dev Biol Anim **26**:579–584, 1990.
26. Hankenson KD, Turek JJ. Porcine anterior cruciate ligament fibroblasts are similar to cells derived from the ligament of the head of the femur, another nonhealing intra-articular ligament. Connect Tissue Res **40**:13–21, 1999.
27. Nagineni CN, Amiel D, Green MH, Berchuck M, Akeson WH. Characterization of the intrinsic properties of the anterior cruciate and medial collateral ligament cells: An *in vitro* cell culture study. J Orthop Res **10**:465–475, 1992.
28. Witkowski J, Yang L, Wood DJ, Sung KL. Migration and healing of ligament cells under inflammatory conditions. J Orthop Res **15**:269–277, 1997.
29. Turek JJ, Li Y, Schoenlein IA, Allen KGD, Watkins BA. Modulation of macrophage cytokine production by conjugated linoleic acids is influenced by the dietary (*n*-6):(*n*-3) fatty acid ratio. J Nutr Biochem **9**:258–266, 1998.
30. Downs TR, Wilfinger WW. Fluorometric quantitation of DNA in cells and tissues. Anal Biochem **131**:538–547, 1983.
31. Aarden LA, De Groot ER, Schaap OL, Lansdorp PM. Production of hybridoma growth factor by monocytes. Eur J Immunol **17**:1411–1416, 1987.
32. Karavodin LM, Glasebrook AL, Phelps JL. Decline in growth and antibody production of established hybridomas and transfectomas with addition of interleukin 6. Eur J Immunol **19**:1351–1354, 1989.
33. Watkins BA, Shen CL, Allen KGD, Seifert MF. Dietary (*n*-3) and (*n*-6) polyunsaturates and acetylsalicylic acid alter *ex vivo* PGE_2 biosynthesis, tissue IGF-I levels, and bone morphometry in chicks. J Bone Mine Res **11**:1321–1332, 1996.
34. Lokesh BR, Hsieh HL, Kinsella JE. Peritoneal macrophages from mice fed dietary (*n*-3) polyunsaturated fatty acids secrete low levels of prostaglandins. J Nutr **116**:2547–2552, 1986.
35. Vidgren HM, Agren JJ, Schwab U, Rissanen T, Hanninen O, Uusitupa MI. Incorporation of *n*-3 fatty acids into plasma lipid fractions, and erythrocyte membranes and platelets during dietary supplementation

with fish, fish oil, and docosahexaenoic acid–rich oil among healthy young men. Lipids **32**:697–705, 1997.
36. Frank C, Schachar N, Dittrich D. Natural history of healing in the repaired medial collateral ligament. J Orthop Res **1**:179–188, 1983.
37. Laws G, Walton M. Fibroblastic healing of grade II ligament injuries: Histological and mechanical studies in the sheep. J Bone Joint Surg Br **70**:390–396, 1988.
38. Bandyopadhyay GK, Imagawa W, Nandi S. Role of GTP-binding proteins in the polyunsaturated fatty acid–stimulated proliferation of mouse mammary epithelial cells. Prostaglandins Leukot Essent Fatty Acids **52**:151–158, 1995.
39. Kanayasu-Toyoda T, Morita I, Murota S. Docosapentaenoic acid (22:5, *n*-3), an elongation metabolite of eicosapentaenoic acid (20:5, *n*-3), is a potent stimulator of endothelial cell migration on pretreatment *in vitro*. Prostaglandins Leukot Essent Fatty Acids **54**:319–325, 1996.
40. Holian O, Nelson R. Action of long-chain fatty acids on protein kinase C activity: Comparison of omega-6 and omega-3 fatty acids. Anticancer Res **12**:975–980, 1992.
41. Yamamura S, Nelson PR, Kent KC. Role of protein kinase C in attachment, spreading, and migration of human endothelial cells. J Surg Res **63**:349–354, 1996.
42. Mauviel A, Heino J, Kahari VM, Hartmann DJ, Loyau G, Pujol JP, Vuorio E. Comparative effects of interleukin-1 and tumor necrosis factor-α on collagen production and corresponding procollagen mRNA levels in human dermal fibroblasts. J Invest Dermatol **96**:243–249, 1991.
43. Choi I, Kang HS, Yang Y, Pyun KH. IL-6 induces hepatic inflammation and collagen synthesis *in vivo*. Clin Exp Immunol **95**:530–535, 1994.
44. Taffet SM, Singhel KJ, Overholtzer JF, Shurtleff SA. Regulation of tumor necrosis factor expression in a macrophage-like cell line by lipopolysaccharide and cyclic AMP. Cell Immunol **120**:291–300, 1989.
45. Hinson RM, Williams JA, Shacter E. Elevated interleukin 6 is induced by prostaglandin E_2 in a murine model of inflammation: Possible role of cyclooxygenase-2. Proc Natl Acad Sci U S A **93**:4885–4890, 1996.
46. Laskin DL, Pendino KJ. Macrophages and inflammatory mediators in tissue injury. Ann Rev Pharmacol Toxicol **35**:655–677, 1995.
47. Sammak PJ, Hinman LE, Tran PO, Sjaastad MD, Machen TE. How do injured cells communicate with the surviving cell monolayer? J Cell Sci **110**:465–475, 1997.
48. Tran PO, Hinman LE, Unger GM, Sammak PJ. A wound-induced $[Ca^{2+}]_i$ increase and its transcriptional activation of immediate early genes is important in the regulation of motility. Exp Cell Res **246**:319–326, 1999.
49. Varga J, Diaz-Perez A, Rosenbloom J, Jimenez SA. PGE_2 causes a coordinate decrease in the steady-state levels of fibronectin and types I and III procollagen mRNAs in normal human dermal fibroblasts. Biochem Biophys Res Commun **147**:1282–1288, 1987.
50. Duncan MR, Berman B. Stimulation of collagen and glycosaminoglycan production in cultured human adult dermal fibroblasts by recombinant human interleukin 6. J Invest Dermatol **97**:686–692, 1991.
51. Sato T, Ito A, Mori Y. Interleukin 6 enhances the production of tissue inhibitor of metalloproteinases (TIMP) but not that of matrix metalloproteinases of human fibroblasts. Biochem Biophys Res Commun **170**:824–829, 1990.
52. Kato S, Muraishi A, Miyamoto T, Fox JC. Basic fibroblast growth factor regulates extracellular matrix and contractile protein expression independent of proliferation in vascular smooth muscle cells. *In Vitro* Cell Dev Biol Anim **34**:341–346, 1998.
53. Coppock DL, Kopman C, Scandalis S, Gilleran S. Preferential gene expression in quiescent human lung fibroblasts. Cell Growth Differ **4**:483–493, 1993.
54. Watkins BA, Shen CL, McMurtry JP, Xu H, Bain SD, Allen KG, Seifert MF. Dietary lipids modulate bone prostaglandin E_2 production, insulin-like growth factor-I concentration and formation rate in chicks. J Nutr **127**:1084–1091, 1997.