Synthesis of Interleukin 1β, Tumor Necrosis Factor-α, and Interstitial Collagenase (MMP-1) Is Eicosanoid Dependent in Human Osteoarthritis Synovial Membrane Explants: Interactions with Antiinflammatory Cytokines

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ABSTRACT. Objective. To determine the level of leukotriene B4 (LTB4) synthesized and released by synovium of patients with osteoarthritis (OA), and to study the role of lipoxygenase (LO)/cyclooxygenase (COX) products on proinflammatory cytokine and interstitial collagenase (MMP-1) synthesis.

Methods. Human OA synovial explants were cultured in the presence of lipopolysaccharide (L) and the ionophores ionomycin (I) and thapsigargin (T) (LIT) for 72 h at 37°C, and LTB4 released into the culture medium was measured in the absence or presence of a COX-2-specific inhibitor, NS-398, or the 5-LO activating protein inhibitor Bay-x-1005. Increasing concentrations of LTB4 (10^-9 to 10^-6 M) were incubated with explants for 24 h at 37°C, and interleukin 1β (IL-1β) and tumor necrosis factor-α (TNF-α) in the conditioned medium were quantitated by ELISA. The effect of endogenous eicosanoids on basal and induced levels of IL-1β, TNF-α, and MMP-1 synthesis was examined by incubating explants in the presence of NS-398 and Bay-x-1005. The effect of antiinflammatory cytokines rhIL-4, IL-10, and IL-13 on basal and LTB4 dependent stimulation of IL-1β/TNF-α synthesis was studied under titration conditions.

Results. Physiologically relevant concentrations (10^-10 to 10^-9 mol/l) of LTB4 were produced in the presence of LIT. Bay-x-1005 abrogated LTB4 release, while NS-398 was without effect. LTB4 stimulated IL-1β and TNF-α synthesis with an EC50 of 190 ± 35 and 45 ± 9 nmol/l, respectively. Significant concentrations of IL-1β and TNF-α were released (100–200 and 500–600 pg/ml, respectively). Basal and LIT induced IL-1β and TNF-α production were inhibited by Bay-x-1005 in a dose dependent manner, while the addition of NS-398 caused a potent stimulatory effect. The preferential COX-2 inhibitor also induced MMP-1 synthesis in a manner essentially identical to the proinflammatory cytokines. The antiinflammatory cytokine IL-4 blocked LTB4 dependent stimulation of IL-1β and TNF-α synthesis. In contrast, IL-10 markedly stimulated both cytokines when incubated alone or in the presence of LTB4 where the effect was additive.

Conclusion. Endogenous and locally produced eicosanoids regulate proinflammatory cytokine and MMP-1 synthesis under basal and stimulated conditions in vitro, with leukotrienes and prostaglandins having opposite effects in general. The clinical use of antiinflammatory drugs that inhibit eicosanoid synthesis requires an appreciation of their relative capacity to inhibit LO/COX in order to predict their effect on the synthesis of proinflammatory cytokines and matrix metalloproteases. IL-10 stimulated proinflammatory cytokine synthesis in our ex vivo culture system.

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Osteoarthritis (OA) is the most common of the rheumatic diseases and it is idiopathic, notwithstanding the compelling evidence that distinct forms of OA are inherited as dominant Mendelian traits1. Increased cartilage degradation and secondary synovitis are key events in the pathogenesis of the disease, and it appears that the synovitis is fundamental to the appearance and progression of cartilage lesions1,2. In part, this is due to the secretion of proinflammatory mediators that increase cartilage catabolism. Prototypic among these factors are interleukin 1β (IL-1β) and tumor necrosis factor-α (TNF-α), which are present and elaborated in OA...
joint tissues. These cytokines have been shown to suppress the synthesis of cartilage matrix macromolecules while stimulating that of extracellular matrix destructive metalloproteases (MMP) (e.g., MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-13). In addition, they stimulate the synthesis and release of lipid mediators, derived from cyclooxygenase (COX) and lipoxygenase (LO) metabolism of arachidonic acid, that are etiologically associated with arthritic and inflammatory diseases. Indeed, increased levels of prostaglandins (PG) and leukotrienes (LT) have been detected in synovial tissue and fluid in OA and rheumatoid arthritis (RA), likely released by infiltrating monocyte/macrophages, mast cells, neutrophils, and human polymorphonuclear leukocytes (PMN). Proof of this exists in studies describing the antirheumatic and antiinflammatory properties of drugs (nonsteroidal antiinflammatory drugs, NSAID) that block COX/LO activity. Recent studies in animal models and clinical trials have convincingly shown the potential of drugs that inhibit LO derived LT in the treatment of rheumatic diseases. Presumably, by reducing production of eicosanoids and LT, which are known to function in an intracrine, paracrine, and autocrine fashion, leukocyte infiltration, vasodilatation, chemotaxis, pyresis, thrombosis, bronchoconstriction, and platelet aggregation will be inhibited. What has not been fully established is the mechanistic rationale at the cellular and molecular level for the therapeutic efficacy of these classes of medications. Recent studies have indicated that LT, acting through positive feedback loops, increase the synthesis of IL-1 in explants of human synovial tissue. Further, we have recently shown that LTB4 upregulates COX-2 expression through both transcriptional activation and posttranscriptional message stabilization. Stimulation of the nuclear factor-kB (NF-kB) signaling cascade by LTB4 has been documented and is likely responsible for mediating the LTB4 dependent transcriptional effects on COX-2 and IL-1.

In terms of the development and resolution of inflammation, relatively little is known about the interrelationship between the so-called antiinflammatory cytokines (e.g., IL-4, IL-10, IL-13), proinflammatory cytokines, and the LO system. Recent evidence has shown significant quantities of IL-4 and IL-10 in OA and RA synovia produced by infiltrating T cells. In some studies the Th2 cell derived cytokines stimulated LTB4 synthesis and release from PMN and macrophages through the activation of LTB4 hydroxy-lase and/or 5-LO. Conversely, in isolated ionophore activated monocyte/macrophages, there was a general inhibitory effect by IL-4 and IL-13 on LTB4 release induced by IL-1 or interferon-γ. Further, IL-4 and IL-10 were shown to inhibit IL-1B synthesis by freshly released adherent rheumatoid synovial cells, while IL-13 suppressed IL-1B and TNF-α expression and synthesis in OA synovium.

We examined the effects of exogenously added (paracrine) and endogenous levels (intra/autocrine activity) of LTB4 and PGE2 on IL-1B, TNF-α, and MMP-1 synthesis/release by OA synovial explants. Further, we investigated whether IL-4, IL-10, and IL-13 can mitigate LTB4 action and cell signaling by studying their effects on LTB4 induced IL-1B/TNF-α synthesis by OA synovium.

MATERIALS AND METHODS

Chemicals. Bay-x-1005 was kindly provided by Merckle GmbH (Ulm, Germany). NS-398, N-[2-(cyclohexoxy)-4-nitropheno]-methanesulfonamide, PGE2, and LTB4 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Lipopolysaccharide (LPS), ionomycin, thapsigargin, 3-isobutyl-1-methylxanthine (IBMX), and forskolin were products of Calbiochem (La Jolla, CA, USA). Human recombinant IL-18 (rhIL-18), rhIL-4, rhIL-10, rhIL-13, and rhTNF-α were purchased from R&D Systems (Minneapolis, MN, USA). HEPES buffered Dulbecco’s modified Eagle medium (HB-DMEM), heat inactivated fetal bovine serum (FBS), and an antibiotic mixture (10,000 units of penicillin G sodium, 10,000 µg of streptomycin sulfate), and DNAzol reagent were products of Gibco BRL-Life Technologies (Burlington, Ontario, Canada).

Specimen selection and synovial fibroblast cultures. Synovial membranes were obtained from OA patients undergoing arthroplasty of the knee who had been diagnosed based on the American College of Rheumatology criteria (mean age 67 ± 19 yrs; M: F 1:3). Each OA synovium was aseptically dissected completely free from underlying fibrous and adipose tissue under a dissecting microscope; samples were rejected if the latter requirement could not be satisfied. Explant samples were weighed aseptically to 300 ± 50 mg, divided randomly into experimental groups, and incubated 24–36 h (washout period) in explant culture medium [HB-DMEM + 10% FBS + Pen-Strep (100 U/100 µg)] at 37°C in a humidified atmosphere containing 5% CO2,95% air prior to experiments. After experimentation, explants were extracted for genomic DNA using the DNAzol reagent per manufacturer’s instructions (Gibco-BRL) and values were used to verify tissue weights.

Eicosanoid ELISA. Measurements of PGE2, LTB4, MMP-1, IL-18 and TNF-α in conditioned medium were by ELISA according to the manufacturer’s instructions (R&D Systems). Detection limits for PGE2 and LTB4 were 39 and 3.9 pg/ml, respectively, 21 pg/ml for pro-MMP-1, while limits for IL-18 and TNF-α were 1 and 4.4 pg/ml, respectively. ELISA for pro-MMP-1 was specific for the free zymogen only.

Statistical analysis. Values were expressed as mean ± SD and mean differences between experimental groups were analyzed by 2 tailed paired Student t test or where appropriate by ANOVA with post-hoc Bonferroni multiple comparison tests. Significance was acknowledged with probability < 5%.

RESULTS

Release of LTB4 by synovial explants. Nine of 22 explant cultures spontaneously released small quantities of LTB4 (roughly 15 pg/g tissue wet weight) in culture under our incubation conditions. However, when activated with LPS and particularly with LPS in the presence of calcium flux stimulators ionomycin (10 mmol/l) and thapsigargin (20 mmol/l) (LIT), far larger amounts of LTB4 were produced (120–150 pg/g tissue wet weight, n = 4) (Figure 1). Cyclic AMP (cAMP) mimetics also increase induced LTB4 production, but were less efficient than the stimulators of calcium flux. When incubated alone, ionophore and cAMP mimetic-stimulated levels of LTB4 production were modestly but significantly different from controls. The 5-LO activating...
protein (FLAP) inhibitor Bay-x-1005 blocked LIT-induced LTB4 release by 83.79 ± 4.57% (mean ± SD) at 1 µmol/l, while at the same concentration NS-398, a preferential COX-2 inhibitor, had no effect in this regard.

Effects of LTB4 on proinflammatory cytokine production. Given the production of LTB4 from OA synovium, we performed the following experiments to examine whether LTB4 could induce the release of the major proinflammatory cytokines IL-1β and TNF-α. Exogenously added LTB4 stimulated the release of IL-1β and TNF-α in a concentration-dependent fashion with an EC50 of 190 ± 35 nmol/l and 45 ± 9 nmol/l (n = 6), respectively (Figures 2A, 2B). Under these conditions, pathophysiologically relevant concentrations of IL-1β and TNF-α were released (100–200 and 500–600 pg/ml, respectively). LPS (20 µg/ml) was added as positive control and stimulated both cytokines potently, 395 ± 65 pg/ml of IL-1β and 4500 ± 895 pg/ml of TNF-α (n = 6; Figures 2A, 2B).

To address the effect of endogenously produced eicosanoids on cytokine production, Bay-x-1005 was added in increasing concentrations and caused a decrease in basal levels of IL-1β and TNF-α by a maximum of 39.95 ± 14.66% and 46.79 ± 13.8% (n = 4), respectively, at 1 µmol/l (Figure 3A). In contrast, NS-398, a preferential COX-2 inhibitor, stimulated the release of both IL-1β (maximum 372.6 ± 127.19%, n = 4) and TNF-α (maximum 198.57 ± 12.12%, n = 4) over controls at therapeutically relevant concentrations (0.01 to 1 µmol/l) (Figure 3B). In these experiments, basal release of IL-1β and TNF-α varied between 14.9 and 34.5 pg/300 mg wet weight. When the membrane explants were exposed to LIT stimulation, Bay-x-1005 inhibited IL-1β (maximum 54.28 ± 8.38%, n = 4)
Figure 3. Role of endogenous production of LTB₄ and PGE₂ on IL-1β, TNF-α, and MMP-1 release in human synovial explants in culture. Explants were incubated 24 h at 37°C under basal (A, B) or LIT [LPS (20 µg/ml), ionomycin (10 nmol/l), thapsigargin (20 nmol/l)] (C, D, E) stimulatory conditions in the absence or presence of increasing concentrations (A, C) of Bay-x-1005 (0.01, 0.1, 1, 10 µmol/l or 0.0036, 0.036, 0.36, 3.6 µg/ml), or (B, D, E) NS-398 (0.01, 0.1, 1, 10 µmol/l or 0.0031, 0.031, 0.31, 31 µg/ml). The concentrations of IL-1β, TNF-α, and MMP-1 were measured in conditioned medium by ELISA. Statistical analysis, ANOVA and Bonferroni post-hoc test (A) IL-1β, F = 11.94, p = 0.0001; control vs 0.01 µmol/l Bay-x-1005, no t significant (NS); TNF-α, F = 10.98, p = 0.0002; control vs 0.01 µmol/l Bay-x-1005, NS; (B) IL-1β, F = 23.24, p < 0.0001; control vs 10 µmol/l NS-398, NS; TNF-α, F = 29.04, p < 0.0001; control vs 10 µmol/l NS-398, NS; (C) IL-1β, F = 31.41, p < 0.0001; control vs 0.01 µmol/l Bay-x-1005, NS; TNF-α, F = 29.45, p < 0.0001; control vs 0.01 µmol/l Bay-x-1005, NS; (D) IL-1β, F = 27.25, p < 0.0001; control vs 10 µmol/l NS-398, NS; TNF-α, F = 14.47, p < 0.0001; control vs 10 µmol/l NS-398, NS; (E) F = 11.13, p < 0.0001 [overleaf].
and TNF-α (maximum 51.56 ± 6.99%, n = 4) release, while NS-398 stimulated IL-1β (maximum 411.93 ± 66.46%, n = 4) and in particular TNF-α (maximum 295.4 ± 92.55%, n = 4) release, as illustrated in Figures 3C and 3D. The nonselective COX inhibitor naproxen had stimulatory effects similar to NS-398 (data not shown). In the presence of LIT, values were between 1850 and 6300 pg/300 mg wet weight for IL-1β and 3300–9650 pg/300 mg wet weight for TNF-α.

Interestingly, NS-398 stimulated proMMP-1 release from LIT activated membrane explants, reaching a zenith of 191 ± 41.5% (n = 7) at 0.1 µmol (Figure 3E); Bay-x-1005 had no effect in this regard (data not shown). With respect to MMP-1, nonstimulated tissue released between 58 and 587 ng/300 mg wet weight, while in the presence of LIT we obtained between 110 and 905 ng/300 mg wet weight.

In companion measurements using the same culture medium, basal concentrations of PGE2 were totally unaffected by Bay-x-1005 (control, 17,298 ± 1218 vs 10 µmol/l of Bay-x-1005, 17,460 ± 1634 pg PGE2/100 mg tissue wet weight, n = 4). As expected, NS-398 inhibited basal PGE2 production (control, 17,298 ± 1218 vs 1 µmol/l of NS-398, 536 ± 101 pg PGE2/100 mg tissue wet weight, n = 4). The IC50 for Bay-x-1005 suppression of LIT stimulated LTβ4 production was 0.33 ± 0.21 µmol/l (n = 4); Vmax of the reaction was 147 ± 39 pg LTβ4/g tissue wet weight. The IC50 for NS-398 suppression of LIT stimulated PGE2 production was 12.2 ± 3.6 nmol/l (n = 4); the total amount of PGE2 released in the presence of LIT was 6.89 ± 0.35 µg/100 mg tissue wet weight, while the addition of 0.01, 0.1, 1.0, and 10.0 µmol/l of NS-398 reduced this amount to 4.01 ± 0.21, 0.25 ± 0.04, 0.02 ± 0.01, and 0.0189 ± 0.007 µg/100 mg tissue wet weight, respectively (n = 4).

**Pro and antiinflammatory cytokine interactions in LTβ4 treated human OA synovial explants.** The antiinflammatory T cell derived cytokine IL-4 reversed the stimulatory effect of LTβ4, in terms of both IL-1β and TNF-α, in a concentration dependent fashion with a maximum inhibition of 83% at 10 ng/ml (Figures 4A, 4B); IL-4 had no significant effect on basal levels of these 2 cytokines. In contrast, IL-10 at 100 pg/ml stimulated IL-1β and TNF-α release and, when coincubated with LTβ4, did so in a concentration dependent manner (Figures 4A, 4B). IL-13 also modestly reversed the LTβ4 stimulatory pattern at lower concentrations, while at 10 ng/ml the cytokine produced an additive effect with LTβ4 (data not shown).

**DISCUSSION**

The involvement of lipoxygenase products in the pathophysiology of OA has been implied from studies using experimental OA and animal models. Leukotriene B4 was detected in significant quantities in the synovial fluid and joint tissues of untreated control animals, and treatments with chemical inhibitors of LO, FLAP, and/or LTA4 hydrolase were shown to inhibit the severity of the disease15,29. Validation of the concept has also come from studies with OA patients where the presence of LTβ4 in synovial fluid was conspicuous and 5-LO inhibitors proved efficacious in remediating disease symptomology10-13,30. This study shows that human OA synovial explants have the capacity to produce significant quantities of LTβ4 when the tissue is treated with calcium ionophores, confirming previous studies5,10,31. Cells derived from synovial tissue express 5-LO and FLAP mRNA and 5-LO metabolites were detected in the conditioned culture medium32. It should be remembered that the OA synovium is replete with proliferating type A and B synoviocytes, and in the subintimal layer macrophages, mast cells, plasma cells, T and B lymphocytes, endothelial cells (blood vessels), and neutrophils may be present. To a degree, these changes may likely be the manifestation of LTβ4 induced chemotaxis of blood-borne and synovial fluid neutrophils that will degranulate and release destructive lysosomal enzymes. Also, LTβ4 can affect leukocyte adhesion to capillary walls, thus cell trafficking is affected to the point where cells may no longer migrate1,14,33.

The LTβ4 detected in our culture supernatants was inhibited by the FLAP inhibitor Bay-x-1005 but was refractory to NS-398, a preferential COX-2 inhibitor. Previous studies measured calcium ionophore (A23187) activated LTβ4 release from synovial explants10,31; however, we found a synergistic relationship between mixed ionophores (ionomycin/thapsigargin) and LPS that gave much more stimulation than ionophores alone. It is now well established that increases in cellular calcium favor the translocation of 5-LO from cytoplasmic stores to its membrane docking protein (FLAP), where it can then interact with its substrate, arachi-
donic acid (AA). However, we reasoned that the addition of LPS would stimulate MAP kinase dependent phosphorylation of cytosolic phospholipase A2 (cPLA2), with the resultant increase in cellular levels of AA driving the forward reaction toward 5-LO/COX metabolites34-37.

Further, 5-LO promoter activity, gene expression, and LTB4 release are enhanced by substances that augment the transcriptional activity of early growth response factor (Egr-1/Krox24), and cAMP mimetics (e.g., IBMX and forskolin) are known to do so38,39. Parenthetically, inhibitors of cAMP formation block leukotriene synthesis and release in certain cell types33. This cAMP dependent mechanism is not as productive as activation by calcium ionophores; however, it does provide another signaling paradigm by which leukotriene synthesis may be upregulated.

In addition to the well described proinflammatory effects of LTB4 and leukotrienes in general, our data suggest that the cytokines IL-1β and TNF-α are also under the control of locally produced LTB4. The stimulation of IL-1β synthesis by LTB4 in synovial explants has been described40,46, and we now also report a potent effect on TNF-α (IC50 45 nmol/l). This LTB4 dependent effect was confirmed by blocking endogenous levels of LTB4 with the FLAP inhibitor Bay-x-1005, resulting in downregulation of basal cytokine secretion. Under LPS stimulatory (inflammatory) conditions, the drug could reduce cytokine levels by about 45–50%, again implying a role for leukotrienes under simulated inflammatory conditions in vitro and confirming the notion that a significant portion of liberated AA is metabolized by the LO pathway in OA synovial membrane explants. However, more compelling are the present data showing just how important the COX pathway metabolites may be in the regulation of proinflammatory cytokine and MMP-1 release. NS-398 (and naproxen) stimulated the basal and induced release of both cytokines and MMP-1 in a very pronounced fashion, and it is tempting to conclude that prostaglandins, particularly PGE2, function as a homeostatic bioregulator in both nonpathological and pathological (OA) conditions. Our results support our previous experiments and those of others that showed the inhibitory effects of exogenous and endogenous PGE2 on TNF-α expression and synthesis and IL-1β maturation and release in isolated human macrophages40-43. The concomitant coordinated synthesis of IL-1β/TNF-α and MMP-1 in the face of COX-2 inhibition provides further support for a strong relationship between proinflammatory cytokines and matrix destructive metalloproteases in the pathophysiology of OA44-46.

The most successful gene therapy approaches for treating experimental OA make use of gene transfer technology that codes for proteins that block proinflammatory cytokine activity47. Examples would be genes coding for IL-1 receptor antagonist (IL-1RA) and soluble receptors for TNF-α and IL-1β. Furthermore, delivery and expression of genes encoding transforming growth factor-β, IL-4, IL-10, and IL-13 have also been shown to be effective particularly in RA models. The effectiveness of these so-called “antiinflammatory” cytokines derives from their potent inhibition of lipid inflammatory mediators and proinflammatory cytokine synthesis47,48-51. Nevertheless, there are conflicting reports about the effects of IL-4, IL-10, and IL-13 on LTB4 release and their efficacy in blocking TNF-α/LTB4 production, as noted. Our data confirm the inhibitory effects of IL-

Figure 4. Regulation of IL-1β (A) and TNF-α (B) synthesis by antiinflammatory cytokines in LTB4 treated human synovial explants. Explants were incubated 24 h at 37°C in the absence (control, C) or presence of LTB4 (190 nmol/l for A and 45 nmol/l for B), rhIL-4 (100 pg/ml), rhIL-10 (100 pg/ml), or LTB4 in the presence of increasing concentrations of rhIL-4 (1–10,000 pg/ml) or rhIL-10 (1–10,000 pg/ml). Concentrations of IL-1β (A) and TNF-α (B) were measured in the conditioned medium by ELISA. (A) Student t test: p < 0.0001 and p < 0.008 vs control; ANOVA: LTB4, LTB4 + rhIL-10 (1–10,000 pg/ml), F = 50.58, p < 0.0001, Bonferroni post-hoc tests, LTB4 vs LTB4 + rhIL-10 (1–100 pg/ml), NS; LTB4, LTB4 + rhIL-4 (1–10,000 pg/ml), F = 28.95, p < 0.0001, Bonferroni, LTB4 vs LTB4 + rhIL-4 (1 pg/ml), NS; (B) p < 0.001 and p < 0.0001 vs control; ANOVA: LTB4, LTB4 + rhIL-10 (1–10,000 pg/ml), F = 81.25, p < 0.0001, Bonferroni post-hoc tests, LTB4 vs LTB4 + rhIL-10 (1, 10 pg/ml), NS; LTB4, LTB4 + rhIL-4 (1–10,000 pg/ml), F = 98.5, p < 0.0001.
4 on proinflammatory cytokine synthesis but clearly indicate that IL-10, particularly in the presence of LTB4, is a potent activator in this regard. Couple the latter findings to the fact that IL-10 has been shown to increase the number of TNF-α cell surface receptors on macrophages isolated from OA synovial membranes and fluid8,2, and one may question the strategy of using IL-10 as an antirheumatic treatment.

There is now wide agreement that proinflammatory cytokines, particularly IL-1β and TNF-α, play a cardinal role in the pathophysiology of arthritic diseases. Endogenous and ambient leukotrienes are powerful stimulators of proinflammatory cytokine synthesis and may occupy a prominent place in the hierarchy of mediators implicated in the inflammatory cascade. The development of pharmacological agents that inhibit leukotriene synthesis could thus be useful to treat OA and inflammatory arthritic conditions.

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REFERENCES


