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# Leukotriene C4 upregulates collagenase expression and synthesis in human lung fibroblasts

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**Abstract**

Leukotriene C4 (LTC4), a mediator generated by a variety of inflammatory cells, participates in several physiological and pathological processes. It has been shown that LTC4 stimulates collagen synthesis by fibroblasts, suggesting a role in collagen turnover. However, the possible effect of this mediator on collagen degradation has not been examined. In this study we explored the role of LTC4 in the modulation of fibroblast interstitial collagenase and TIMP-1. Confluent cultures of three human normal lung fibroblast cell lines, and one derived from idiopathic pulmonary fibrosis (IPF) were exposed to LTC4 0.1, 1 and 10 nM, and to IL-1$\beta$ as positive control. Collagenase and TIMP mRNAs expression were analyzed by Northern blot followed by densitometric scanning. Immunoreactive procollagenase was detected by immunoblot, and collagenase activity was measured using [$^3$H]collagen. Our results showed that LTC4 enhanced several-fold collagenase mRNA expression in collagenase-producing fibroblasts, and induced the expression of the enzyme mRNA in collagenase-nonproducing fibroblasts, both in normal and IPF derived cell lines. LTC4 1 nM induced the highest response. Collagenolytic activity and immunoreactive collagenase paralleled collagenase mRNA expression. Interestingly, simultaneous exposure of fibroblasts to LTC4 plus IL-1 failed to show additive effects. Moreover, in two cell lines the combination resulted in a decrease of collagenase mRNA expression compared with both mediators separately. TIMP mRNA levels were not significantly modified by LTC4, nor IL1$\beta$. Our findings suggest that LTC4 plays a role in the modulation of fibroblast collagenase, and it may participate in extracellular matrix remodeling during lung inflammation.

*Keywords:* Collagenase; TIMP; Leukotriene; LTC4; Lung fibroblast

## 1. Introduction

Degradation of extracellular matrix is an essential feature in a number of physiological and pathological processes. Collagen is usually the predominant component of the extracellular matrix, being collagens type I and III the major proteins in the interstitium of the lung parenchyma [1]. Matrix metalloproteinase collagenase (MMP-1) plays a critical role in the breakdown of collagen fibers and its activity is regulated at different levels. Transcriptional regulation at the gene level, proenzyme activation normally accomplished in vivo by proteolytic cleavage, and extracellular activity controlled by specific inhibitors, mainly tissue inhibitors of metalloproteinases (TIMPs) [2,3].

The synthesis of MMP-1 as well as TIMPs is modulated by a variety of cytokines, such as growth factors and inflammatory mediators, which may up-or down-regulate their production [4,5]. Among them, two arachidonic acid derivates, prostaglandin E2 (PGE2) and prostaglandin E1 (PGE1) appear to have opposite actions regarding collagenase expression. Thus, whereas PGE2 enhances MMP-1 production in a number of systems [6,7], PGE1 decreases the synthesis of the enzyme in rabbit synoviocytes and human fibroblasts [8].

Leukotriene C4 (LTC4), a mediator generated by several inflammatory cells, including macrophages, mast cells, neutrophils and eosinophils [9], represents another molecule derived from the metabolism of arachidonic acid. This mediator participates in some

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pathological processes, and seems to play a role in the pathogenesis of several IgE-mediated hypersensitivity diseases, although most studies have been focused on asthma [10].

Recently, Phan et al. [11] have reported that LTC4 has a biological effect on fibroblasts stimulating collagen synthesis. This study suggests that in the inflammatory environment created after an injury, the secretion of LTC4 might be involved in the modulation of some fibroblast functions, particularly in those related to collagen turnover. In this context, however, the possible effect on interstitial collagenase and TIMP expression has not been examined.

The aim of the present study was to investigate the regulation of collagenase and TIMP gene expression by leukotriene C4 in lung fibroblasts. Our results revealed that 1 nM LTC4 upregulated collagenase expression and did not produce significant changes in TIMP mRNA levels except for a moderate increase in one cell line.

## 2. Material and methods

### 2.1. Materials

F-12K medium and fetal calf serum were obtained from Gibco Laboratories (Grand Island, NY). Human interleukin-1-$\beta$, recombinant (IL-1$\beta$), leukotriene $C_4$ (LTC4), trypsine, and soybean trypsin inhibitor were from Sigma (St. Louis, MO). Nytran transfer membranes were from Schleicher and Schuell (Keene, NH). Multiprime DNA labeling kit, [$^{32}$P]dCTP (specific activity $200 \cdot 10^6$ dpm/$\mu$g, and [$^3$H]acetic anhydride 50 mCi/mmol were from Dupont-New England Nuclear (Boston, MA). All other chemicals were of the purest grade available.

### 2.2. Cell culture

A fibroblast cell strain (NAC) derived from normal human lung was obtained in our laboratory as described elsewhere [12]. Briefly, NAC was derived from an individual having lobectomy for removal of a primary lung tumor; no morphological evidence of disease was found in the tissue sample used for fibroblast isolation. Lung fibroblasts were isolated by trypsin dispersion and fibroblast cell strain was established in F-12K medium supplemented with 10% fetal calf serum. In addition, two human normal lung fibroblast cell lines, CCD-11 Lu and CCD-25 Lu, and one derived from idiopathic pulmonary fibrosis, LL-29, were obtained from the American Type Culture Collection. The cells were cultured under standard conditions, in T-25 cm$^2$ Falcon flasks, using F-12K medium supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 $\mu$g/ml) and amphotericin B (0.25 $\mu$g/ml). When fibroblasts reached early confluence the medium was replaced with serum free F-12K medium containing LTC4 0.1, 1 or 10 nM, interleukin-1 (IL-1) 250 U, or IL-1 plus the different concentrations of LTC, and the cells were incubated for 48 h. Conditioned media were collected and stored at $-20$°C until assayed for collagenase activity and Western blot analysis.

### 2.3. RNA isolation and Northern blot

Total cellular RNA was isolated by the acid guanidinium thiocyanate/phenol chloroform extraction method [13]. Total RNA (10 $\mu$g/lane) was fractionated on a 1% agarose gel containing 0.66 M formaldehyde. Ribosomal RNA was visualized with ethidium bromide and the fractionated RNA was transferred onto Nytran transfer membranes by capillary blotting overnight. The membranes were air-dried and baked at 80°C for 2 h. The membranes were prehybridized at 42°C for 16 h in $5 \times$ SSC, 50% formamide, $5 \times$ Denhardt's solution and 0.5% SDS, containing 100 $\mu$g/ml of denatured salmon sperm DNA. Hybridization was carried out at 42°C for 16 h in hybridization buffer containing 50% dextran sulfate plus heat denatured $^{32}$P-labeled probe. The membranes were washed in $2 \times$ SSC 0.1% SDS at room temperature three times for 5 min, followed by $0.1 \times$ SSC 0.1%SDS at 55°C twice for 15 min. After drying, the membranes were exposed to Kodak X-AR film at $-70$°C with an intensifying screen. Equal loading of RNA samples was monitored by assessing the mRNA level of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). cDNA clones for human procollagenase, human TIMP, and GAPDH were obtained from the American Type Culture Collection. The probes were radiolabeled with a [$^{32}$P]dCTP to specific activity of $200 \cdot 10^6$ dpm/$\mu$g. Autoradiographs were scanned by computerized densitometry (Hoefer Scientific Instruments; GS365W Electrophoresis Data System).

### 2.4. Immunoblot analysis

Serum-free conditioned media were centrifuged at $3000 \times g$ at 4°C for 30 min to remove cell debris. Two mg/ml of bovine serum albumin were added and the samples were concentrated by dialisis vs. 0.008 M Tris HCl (pH 6.8), followed by lyophilization. Samples taken for Western analysis were mixed with sample buffer and aliquots of 20 $\mu$l were electrophoresed under non-reducing conditions on 10% SDS-PAGE as described by Laemmli [14]. Western transfers to nitrocellulose filter were performed at 70 V for 3 h. After the non-specific sites were blocked overnight with 4% (w/v) non-fat dry milk in PBS, the membrane was incubated with antihuman collagenase IgG (1:250 di-

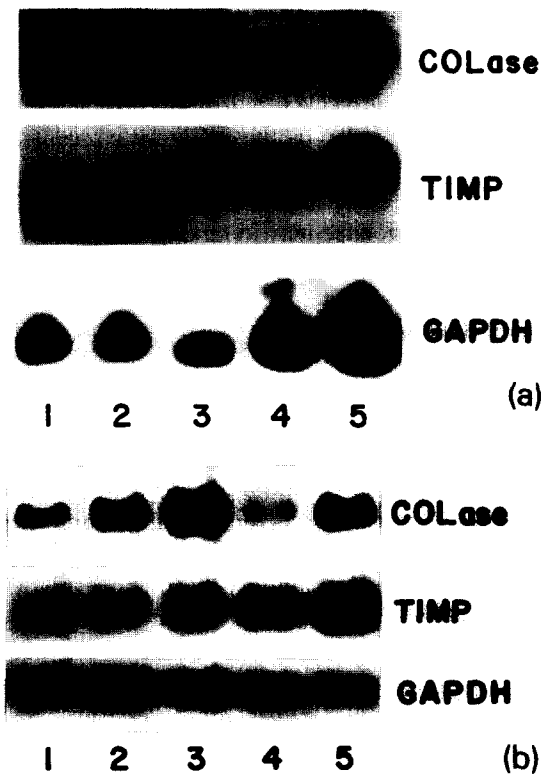



Fig. 1. Effect of LTC4 and IL-1 on collagenase and TIMP mRNAs. Fibroblasts (A: CCD-25 Lu derived from normal human lung; B: LL-29 derived from idiopathic pulmonary fibrosis) were incubated for 48 h in the presence of medium alone as control (lane 1), LTC4 0.1 nM (lane 2), LTC4 1.0 nM (lane 3), LTC4 10 nM (lane 4), and IL-1 250 U (lane 5). Ten $\mu$g of total cellular RNA extracted with guanidinium thiocyanate were electrophoresed on 1% agarose gel and transferred to Nytran membrane. The membranes were hybridized with $^{32}$P-labeled cDNA collagenase, TIMP and GAPDH probes, washed and exposed to film.

lution in PBS/1% BSA) for 2 h at room temperature. Anticollagenase antibody was kindly provided by Eugene Bauer [15]. Reaction with secondary antibody (1:500 dilution of goat anti-rabbit IgG in PBS with 1% BSA) conjugated to biotin was for 1 h at room temperature. Finally, the filter was incubated for 15–20 min at room temperature in PBS containing 0.15% $H_2O_2$, 15% (v/v) methanol, and 4-chloro-naphthol at 6 mg/ml until the color developed.

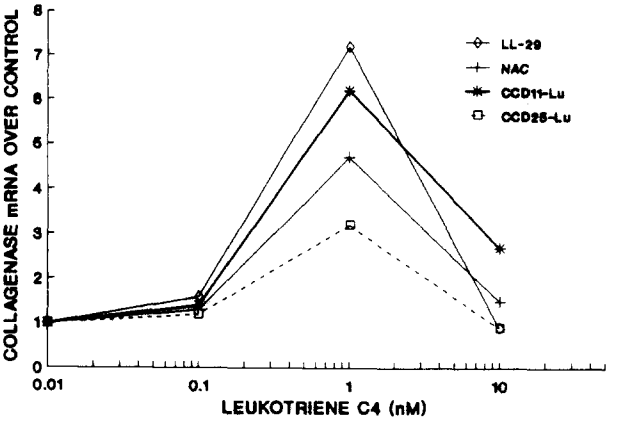
### 2.5. Assay for collagenolytic activity

Collagenase activity was measured as described elsewhere [16] by the method of Terato et al. [17] using as substrate native guinea pig skin type I collagen labeled with [$^3$H]acetic anhydride. Samples were activated by preincubation with trypsin (1–5 $\mu$g) for 10 min at 37°C followed by addition of 5-fold excess of soybean trypsin inhibitor added to inhibit further trypsin activity [18]. Incubations of activated samples with labeled collagen were carried out for 12 h at 30°C. One unit of collagenase was arbitrarily defined as that degrading 1 $\mu$g of collagen per 12 h at 30°C.

## 3. Results

### 3.1. Effect of LTC4 on collagenase mRNA

To analyze the role of LTC4 on the expression of interstitial collagenase and TIMP mRNAs, confluent fibroblasts were incubated in the presence of three different concentrations of LTC4. Fibroblasts stimulated with IL-1$\beta$ and fibroblasts in serum free media were used as controls. Consistent with prior observations, the four fibroblast cell lines exhibited different



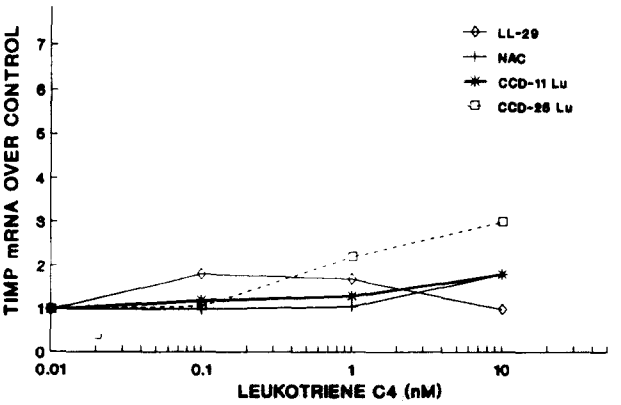




Fig. 2. Effect of different concentrations of LTC4 on collagenase and TIMP mRNA levels. Human lung fibroblasts were incubated for 48 h in the presence of medium alone as control, and LTC4 0.1 nM, 1.0 nM, and 10 nM. Ten $\mu$g of total cellular RNA extracted with guanidinium thiocyanate were electrophoresed on 1% agarose gel and transferred to Nytran membrane. The filters were hybridized with $^{32}$P-labeled cDNA collagenase, TIMP and GAPDH probes, washed and exposed to film. The autoradiograms were scanned by densitometer and the values were standardized in relation to corresponding GAPDH mRNA levels.

patterns of basal interstitial collagenase mRNA expression, and are exemplified in Fig. 1. Thus, whereas CCD-25 Lu (Fig. 1A) and CCD-11 Lu cell lines exhibited a considerable steady-state level of MMP-1 mRNA, LL-29 (Fig. 1B) and NA cell strains displayed a faint signal of MMP-1 mRNA. The addition of LTC4 resulted in a marked enhancement of the collagenase mRNA levels in the fibroblasts cell lines independently of the basal level of expression. Incubation with IL-1$\beta$ (250 U) produced a similar increase in collagenase mRNA to that observed with 1 nM LTC4 (Fig. 1).

To quantify the effect of the varying concentrations of LTC4 in the expression of collagenase mRNA levels, the autoradiograms were scanned by densitometer and the values were standardized in relation to corresponding GAPDH mRNA levels. One nM LTC4 induced the higher increase in collagenase mRNA expression in the four cell lines studied (Fig. 2, upper panel). Incubation with 10 nM LTC4 resulted in a moderate upregulation of MMP-1 mRNA in two cell lines, while it returned to basal levels in the other two. The increase in collagenase mRNA level induced by IL-1 varied from 2.5- to 9-fold in the four cell lines studied.

The combination of IL-1 with the different concentrations of LTC4 resulted in heterogeneous responses. Fig. 3 exemplifies the results obtained when IL-1 plus 1 nM LTC4 were used. The combination fails to show additive effects on the expression of collagenase mRNA levels. Moreover, in two cell lines, the combination resulted in a decrease of collagenase mRNA expression compared with both mediators separately.

### 3.2. Effect of LTC4 on TIMP mRNA

TIMP-1 gene was actively expressed in basal conditions as determined at the mRNA level (Fig. 1). When calculations of relative effect of LTC4 and IL-1 were equalized with respect to GAPDH mRNA, TIMP-1 mRNA expression remained essentially without significant changes in three out of the four cell lines (Fig. 2, lower panel). In CCD-25 Lu cell strain, however, 1.0 and 10 nM LTC4 produced an increase in TIMP mRNA expression. Interestingly, in this cell line IL-1 displayed a 3.8-fold increase, which was similar to that observed with 10 nM LTC4. The combination of IL-1 and LTC4 did not show any additive effect, nor induced any significant change in TIMP mRNA expression compared to basal levels in all the cell lines.

### 3.3. Collagenase immunoblot analysis

To examine the LTC4 induction at the protein level, immunoreactive collagenase was analyzed in the conditioned media of LL-29 and CCD-11 Lu cell strains under basal conditions, and after 1 nM LTC4. The results confirmed those of Northern blot analysis showing non-detectable enzyme in steady state conditions in LL-29 fibroblasts and the induction of a doublet at approximately 52 kDa after LTC4 and IL-1 stimulation (Fig. 4, lanes 1–3). CCD-11 Lu also displayed an increase in immunoreactive protein with LTC4 incubation (Fig. 4, lanes 4 and 5).

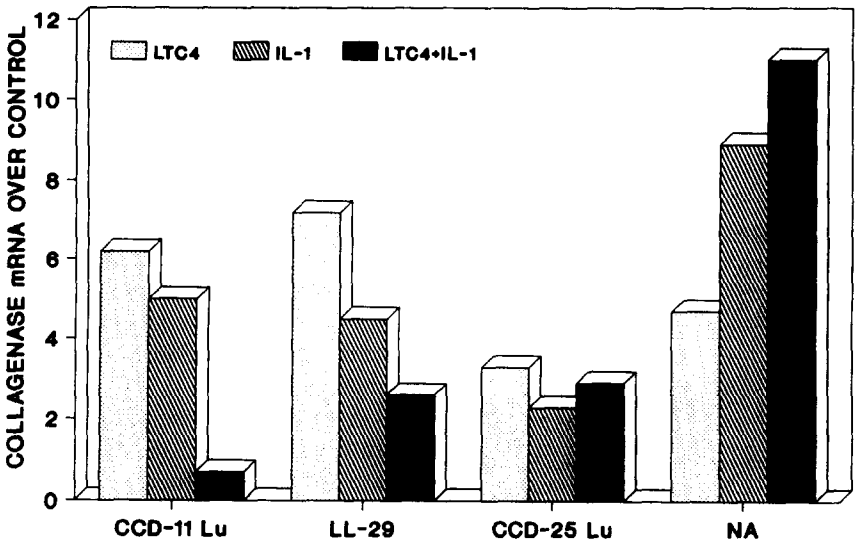



Fig. 3. Effect of LTC4, IL-1 U, and the combination of both mediators on collagenase mRNA expression. Three fibroblast cell lines derived from normal human lungs (CCD-11 Lu, CCD-25 Lu, and NAC), and one derived from idiopathic pulmonary fibrosis (LL-29) were incubated in the presence of medium alone as control, LTC4 1.0 nM, IL-1 250 U, and LTC4 1.0 nM plus IL-1 250 U. Total RNA was isolated and analyzed by Northern blot. Collagenase mRNA transcripts were scanned by densitometer and the values were standardized in relation to corresponding GAPDH mRNA levels.

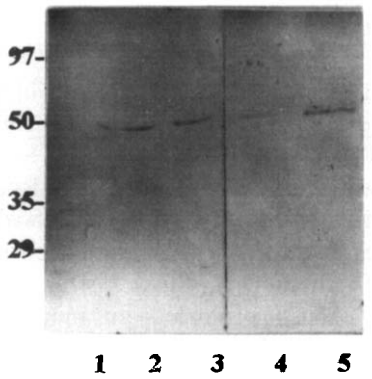



Fig. 4. Western blot analysis of procollagenase induced by LTC4 in human lung fibroblasts. Early confluent cultures of lung fibroblast cell lines were stimulated for 48 h with 1 nM LTC4 or 250 U IL-1$\beta$. Conditioned media were applied to polyacrylamide gels (SDS-PAGE) under non-reducing conditions. After electrophoresis the proteins were electrotransferred to nitrocellulose filter and probed with anti-collagenase antibody. Unstimulated LL-29 (lane 1) and CCD-11 Lu (lane 4) fibroblasts. Stimulated with 1 nM LTC4 (LL-29: lane 2, and CCD-11 Lu: lane 5). LL-29 stimulated with 250 U IL-1$\beta$ (lane 3). Molecular mass markers are shown on the left side.

### 3.4. Collagenolytic activity

The effect of 1 nM of LTC4 on collagenase production was also analyzed measuring collagenase activity. Conditioned media derived from confluent lung fibroblasts activated with trypsin were used. Limited proteolysis by trypsin results in activation of procollagenase which is inactive in its native state [18]. It is shown in Table 1 that under basal conditions, NAC and LL-29 fibroblast cell strains conditioned media did not exhibit collagenolytic activity. By contrast, collagenase activity was demonstrable when cells were stimulated with LTC4. On the other hand, unstimulated CCD-25 Lu and CCD-11 Lu displayed collagenolytic activity which was increased by LTC4 1 nM. In all cell lines IL-1 used as positive control revealed a similar collagenolytic activity as LTC4 stimulated cells.

Table 1
Effect of LTC4 and IL-1 on collagenolytic activity

| Cell line | Collagenolytic activity (units) | | |
|---|---|---|---|
| | Basal | LTC4 (1 nM) | IL-1$\beta$ |
| NAC | 0.2±0.1 | 28.7±3.4 | 30.4±3.2 |
| CCD-25 Lu | 18.5±2.8 | 29.4±4.2 | 28.4±2.0 |
| LL-29 | 0 | 19.2±5.2 | 22.1±2.4 |
| CCD-11 Lu | 23.2±1.4 | 39.6±4.1 | 31.3±3.7 |

Confluent lung fibroblasts were incubated for 48 h alone, and with LTC4 1.0 nM, or IL-1$\beta$ 250 U. The conditioned media were preincubated with trypsin (1–5 $\mu$g) for 10 min at 37°C followed by a 5-fold excess of soybean trypsin inhibitor. Collagenase activity was assayed using as substrate native guinea pig skin type I collagen labeled with [$^3$H]acetic anhydride. Incubations of activated samples with labeled collagen were carried out for 12 h at 30°C. One unit of collagenase degrades 1 $\mu$g of collagen per 12 h at 30°C. Results are the mean ± S.D. of two determinations.

## 4. Discussion

Independently of etiology, lung inflammation is characterized by the accumulation of a variety of immune and inflammatory cells, usually including macrophages, mast cells, neutrophils and eosinophils, most of them capable of synthesizing biological mediators of arachidonic acid.

Leukotrienes, produced through the 5-lipoxygenase pathway of arachidonate metabolism, represent a family of potent mediators of hypersensitivity and inflammation [9]. Leukotriene C4 in particular has been considered as playing a major role in asthma, evoking bronchospasm through smooth muscle contraction, and increasing vasopermeability and mucus secretion [19]. However, studies concerning other putative roles of LTC4 in the inflammatory microenvironment, as well as its possible participation in other inflammatory disorders, are scanty.

We have previously demonstrated that increased levels of LTC4 are usually observed in bronchoalveolar lavage of a variety of chronic interstitial lung diseases, a group of disorders characterized by diffuse interstitial inflammation and fibrosis [20]. Although the possible role of LTC4 in these disorders is unclear, the finding that this mediator has a stimulatory effect on fibroblast collagen synthesis [11] suggests that it may play a role in collagen metabolism during inflammation. Interstitial collagen turnover, however, involves a stringently regulated balance between the deposition of the structural molecule, its degradation by specific collagenases, and the interaction among these metalloproteinases and their natural tissue inhibitors (TIMPs).

Here we analyzed the influence of LTC4 on the expression of lung fibroblast interstitial collagenase and TIMP-1. We have previously found that under basal conditions there are collagenase producing and non-producing lung fibroblast cell strains, unrelated to disease state [12]. In the present study our results clearly demonstrated that the exposure of fibroblasts to LTC4-induced collagenase expression in non-producing cells and increased it in collagenase-producing fibroblasts, while the expression of TIMP was relatively unaffected. There were no differences in the response to LTC4 exposure among the fibroblast cell line derived from fibrotic lung compared with that of normal lungs.

A consistent observation was that 1 nM LTC4 concentration was the most effective in inducing collagenase stimulation. Increased collagenase mRNA ranged from 3- to 7-fold, and was similar to that found with IL-1$\beta$ which resulted in a stimulation between 2.5- and 8-fold. Interestingly, the combination of IL-1 with the different concentrations of LTC4 did not produce additive effects on collagenase expression. Moreover, in two cell lines, the combination resulted in a decrease

of the transcript encoding collagenase compared to that obtained with both mediators separately.

The levels of secreted immunoreactive collagenase under LTC4 stimulation were observed to correspond to levels of collagenase mRNA, and zymogen activation induced by trypsin revealed collagenolytic activity in the conditioned media derived from fibroblasts expressing mRNA for interstitial collagenase.

The concentrations at which LTC4 stimulated collagenase expression are similar to that reported by Phan et al. [11] concerning collagen synthesis, and consistent with the possibility that this observation may be physiologically relevant. Since activated inflammatory cells are able to release large amounts of leukotrienes in vitro [21], it can be assumed that in sites of injury and inflammation the presence of this mediator might be participating through fibroblast stimulation in matrix remodelation.

The mechanism(s) by which LTC4 could be upregulating collagenase expression was not determined. It is well documented that collagenase levels are enhanced by the increase of intracellular calcium. Thus, in rabbit synovial fibroblasts calcium ionophore A23187, which increases intracellular calcium levels, stimulates collagenase expression [22]. Likewise, it has been reported that electroporation induced an increase in collagenase expression which was suppressed by EGTA, suggesting that $Ca^{2+}$ might be mediating the transduction of this effect [23]. Taking into account that leukotrienes are able to produce intracellular $Ca^{2+}$ mobilization in mesenchymal cells [24,25], it can be hypothesized that the upregulation of collagenase by LTC4 might be related to a rise in intracellular calcium.

TIMP-1 expression at the RNA level was evaluated in parallel with that of collagenase in view of the potentially important regulatory role exerted by this inhibitor on the activity of the enzyme. Neither LTC-4 nor IL-1 significantly affected TIMP expression with the exception of one cell line in which interestingly both mediators induced an approximately 4-fold increase. In this context, different results have been reported concerning the role of IL-1 on TIMP expression; thus while in human skin and rheumatoid fibroblasts [26,27] IL-1 did not produce significant changes, in fetal lung fibroblasts and human uterine cervical fibroblasts IL-1 induced a clear TIMP upregulation [28,29]. Therefore, in contrast to its potent effect on the production of metalloproteinases, IL1-$\beta$ has variable effect on the production of TIMP. Our results showed the same variability with IL-1 and with LTC4, since whereas in three out of four lung fibroblast cell lines LTC4 and IL-1 stimulation did not have any significant effect on TIMP-1 mRNA, in one cell strain both mediators enhanced the expression of the inhibitor. Since the different effect of the mediators on TIMP production was observed in lung fibroblasts, this finding suggests that it might be attributable to cell lines.

Extracellular matrix resorption and remodeling take place in many inflammatory conditions and are likely to be influenced by a complex network of cytokines, growth factors and other mediators. Our results show that leukotriene C4 may actively participate in these processes, providing fibroblasts with a collagen-degradative phenotype, preferentially increasing the release of collagenase relative to TIMP.

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