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Gene expression of fibroblast matrix proteins is altered by indomethacin

A. Mauviel†, V.-M. Kähäri×, J. Heino×, M. Daireaux†, D.J. Hartmann*, G. Loyau† and J.-P. Pujol†

†*Laboratoire de Biochimie du Tissu Conjonctif, Université de Caen, CHU, Côte de Nacre, F-14040 Caen Cedex, France,* ×*Department of Medical Biochemistry, University of Turku, Turku, Finland* and **Centre de Radioanalyse, Institut Pasteur, Lyon, France*

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The role of indomethacin in the regulation of extracellular matrix synthesis was studied in dermal fibroblast cultures. Indomethacin (10 μM) blocked totally the prostaglandin secretion and markedly increased the synthesis of collagen. In parallel, measurement of fibronectin, type I and type III procollagen mRNA levels showed a substantial increase under the action of indomethacin. On the other hand, indomethacin did not modify the mRNA levels of dermatan sulfate proteoglycan core protein. Measurement of collagen production estimated as the amount of collagenase digestible protein and by specific radioimmunoassay indicated a good correlation with the corresponding mRNA levels. These results suggest that indomethacin can regulate the extracellular matrix deposition at a transcriptional level.

Indomethacin; Collagen; Fibronectin; Dermatan sulfate proteoglycan core protein; Prostaglandin E_2; Extracellular matrix

1. INTRODUCTION

It is generally accepted that metabolites of the arachidonic cascade play major roles in inflammation, asthma and platelet functions. Pro-inflammatory effects have been attributed to several mediators of this system, among which prostaglandins formed via the cyclooxygenase pathway. Conversely, the effectiveness of nonsteroidal anti-inflammatory drugs (NSAIDs) is mainly due to inhibition of cyclooxygenase, preventing the transformation of arachidonic acid to the stable prostaglandins [1].

Indomethacin is certainly one of the most widely used NSAIDs. A number of reports has documented the effect of this drug on several aspects of inflammation: chemotaxis of monocytes and polymorphonuclear leukocytes, production of superoxide anions, cellular immune function, destruction of joint tissue [2]. However, very few studies deal with the eventual action of indomethacin on the matrix synthesis, which is known to undergo marked changes in several inflammatory diseases. An increased synthesis of collagen in granuloma of rats treated with indomethacin has been reported [3,4]. Similarly, the in vitro production of collagen was increased in callus tissue from rats after in vivo treatment with indomethacin [5]. However, others have observed an inhibition of collagen synthesis in rat granuloma after local injection of indomethacin [6].

Using indomethacin as a control to block prostaglandin formation in studies on the in vitro effect of interleukin 1, it has been previously observed that the drug enhanced collagen synthesis [7,8]. Therefore, we planned the present study to investigate the effect of indomethacin added to fibroblast cultures on the expression of collagens I and III, fibronectin and dermatan sulfate proteoglycan (DSPG) core protein. We conclude that the drug acts as a potent stimulator of both pro-

Correspondence address: A. Mauviel, Laboratoire de Biochimie du Tissu Conjonctif, Université de Caen, CHU, Côte de Nacre, F-14040 Caen Cedex, France

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collagen and fibronectin mRNA levels whereas it does not affect mRNA amounts of DSPG core protein. These findings suggest a differential modulation of indomethacin on extracellular matrix deposition which could be due to inhibition of prostaglandin synthesis.

2. MATERIALS AND METHODS

2.1. *Cell cultures*

Fibroblasts obtained from explants of human foreskin were grown in Dulbecco's modification of Eagle's minimum essential medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (FCS), penicillin (100 IU/ml), streptomycin (100 μg/ml) and fungizone (2.5 μg/ml).

2.2. *Experimental procedure*

Cells were grown to confluency in 175 cm^2 flasks and preincubated for 24 h in DMEM supplemented with 10% FCS and 50 μg/ml ascorbic acid. Then the medium was replaced and fresh medium containing also 50 μg/ml β-aminopropionitrile (β-APN) was added, with or without indomethacin (10 μM). After a 24 h incubation, the medium was collected for radioimmunoassay of collagens and prostaglandin E_2, and the cells were harvested for RNA extraction.

For estimation of total collagen production, the incubation was performed in 9.6 cm^2 Petri dishes using the same protocol except that 2.5 μCi/ml [^{3}H]proline (CEA, France, 30–40 Ci/mM) were added to the cultures. Each experiment was performed in triplicate.

2.3. *Radioimmunoassay of collagens type I and type III*

The amounts of both collagens were estimated in the culture medium according to the method of Magloire et al. [9], using specific antibodies against human type I and type III collagens.

2.4. *Assay of labelled collagen and non-collagenous protein*

The radioactive collagen secreted in the medium was assayed as collagenase-digestible protein [10] and expressed as dpm/μg of cell protein, measured by the method of Hartree [11]. The amount of non-collagenous protein was estimated from the radioactivity remaining after collagenase digestion.

2.5. *Prostaglandin E_2 assay*

PGE_2 was assayed in the culture medium by specific radioimmunoassay [12] with antiserum from Institut Pasteur Production (France).

2.6. *DNA probes*

The following probes were employed: pHCAL1 [13] for procollagen α1(I), pHFS3 [14] for procollagen α1(III), pHFN [15] for fibronectin, and GP40 [16], specific for dermatan sulfate proteoglycan core protein. pRGAPDH [17], a probe specific for rat glyceraldehyde-3-phosphate dehydrogenase, was used as a control.

2.7. *RNA isolation and nucleic acid hybridization*

Total cellular RNA was extracted as described [18]. Briefly, cells were lysed in 5 M guanidine thiocyanate, RNA was selectively precipitated with 4 M LiCl and extracted with phenol-chloroform. For Northern blots, 12 μg RNA of each sample were denatured with glyoxal in duplicate and fractionated electrophoretically in a 0.75% agarose gel. One set of the samples was stained with ethidium bromide to visualize the 18 S and 28 S rRNA subunits and check the preservation of RNA after extraction, the other set was transferred to Pall Biodyne A nylon membrane and immobilized by baking. Prehybridization was carried out as described by Thomas [19]. Hybridizations were performed in the prehybridization solution at 42°C for 24 h using cDNA probes labelled by nick translation with [α-^{32}P]dCTP (800 Ci/mmol, Amersham, England) to a specific activity of about 5×10^7 cpm/μg. After hybridization, filters were washed three times for 5 min in 2 × SSC/0.1% SDS at 25°C and twice for 30 min in 0.1 × SSC/0.1% SDS at 55°C. Autoradiography was performed at −70°C using Kodak X-Omat S films and scanned for quantitative determination of mRNA amount. The same filters were successively hybridized with the five probes. To remove the probes between hybridizations, the filters were washed in 50% formamide/10 mM sodium phosphate, pH 6.5, for 1 h at 65°C followed by wash in 2 × SSC/0.1% SDS at 25°C for 15 min.

3. RESULTS

3.1. *Effect of indomethacin on collagen and non-collagenous protein synthesis by confluent human dermal fibroblasts*

The concentration of 10 μM indomethacin was chosen after previous experiments showing that this drug level was required to completely inhibit the formation of prostaglandins in cultured dermal fibroblasts. The basal production of PGE_2 in our cultures, estimated in triplicate, was 3366 ± 780 pg/ml per 24 h. In the presence of 10 μM in-

Table 1

Total collagen and non-collagenous protein produced in the medium

	Collagen		Non-collagenous protein	
	dpm/μg protein	% of control	dpm/μg protein	% of control
Control	266 ± 73	100	1147 ± 95	100
Indo 10 μM	419 ± 92	158	1067 ± 70	93

Confluent cultures were preincubated for 24 h with 50 μg/ml ascorbic acid in DMEM containing 10% FCS. Then the medium was discarded and fresh complete medium containing 50 μg/ml ascorbic acid, 50 μg/ml β-APN and 2.5 μCi/ml [^{3}H]proline was added, with or without indomethacin. After a 24 h incubation, the amounts of newly synthesized collagen and non-collagenous protein were estimated in the medium as described in section 2. Values are mean ± SE of triplicate wells

Table 2

Radioimmunoassay of types I and III collagens in supernatants of fibroblasts cultured with or without indomethacin

	Type I		Conagen type III		Total (I+III)	
	ng/ml	% of control	ng/ml	% of control	ng/ml	% of control
Control	674 ± 34	100	242 ± 18	100	916	100
Indo 10 μM	1007 ± 28	149	281 ± 13	116	1288	141

Experimental conditions were the same as for table 1, without [^{3}H]proline. After incubation, the type I and type III collagens were estimated by radioimmunoassay. Values are mean ± SE of duplicate flasks

domethacin, this level was reduced to 92 ± 21 pg/ml per 24 h which represents practically 2–3% of the control value. The effect on collagenase-digestible protein produced in such conditions is presented in table 1. Only culture medium was assayed since control experiments showed that more than 90% of the total collagen synthesized was recovered in the medium and that the relative distribution between cell layer and medium was not affected by indomethacin treatment (not shown). It was found that indomethacin increased the amount of radioactivity incorporated in the neo-synthesized collagen compared to control cultures. The effect of indomethacin on this parameter was very significant since the mean value was nearly 60% above the basal level. On the

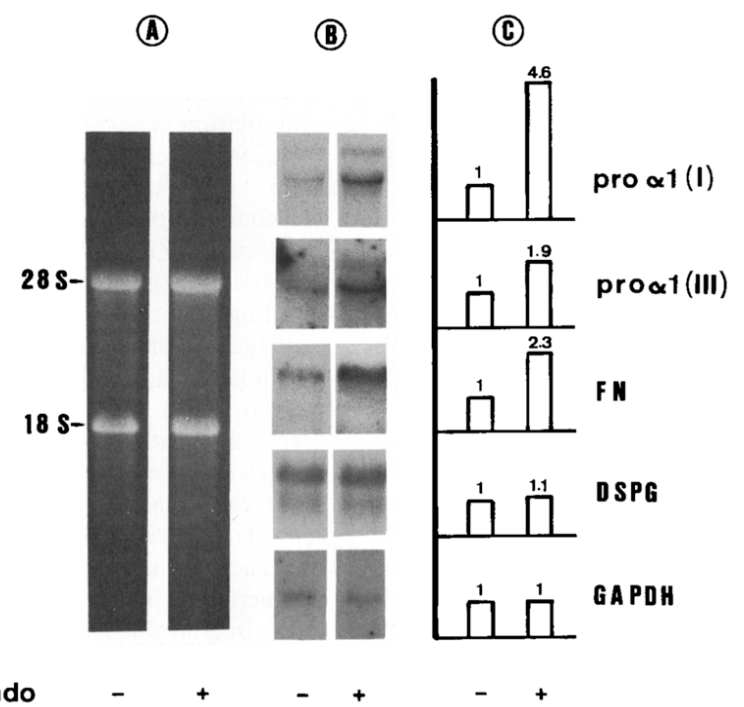

Fig.1. Measurement of the steady-state mRNA levels of pro α1(I), pro α1(III), fibronectin (FN), dermatan sulfate proteoglycan core protein (DSPG) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in human dermal fibroblasts. An equal amount of total cellular RNA (12 μg/lane) was size-fractionated in 0.75% agarose gel, and either stained with ethidium bromide (A) or transferred to nylon membrane and hybridized to nick-translated cDNA probes (B). (C) The scannings of each fluorograph. Values are relative densitometric units.

other hand, the amount of non-collagenous protein deduced from the radioactivity remaining after collagenase digestion of samples was not affected, suggesting that the effect of indomethacin was selective for collagen.

3.2. *Relative proportion of collagens type I and type III produced in the presence of indomethacin*

To further characterize the effects of indomethacin on fibroblast collagen biosynthesis, the amounts of type I and type III collagens secreted in the medium over a 24 h period were determined by specific radioimmunoassay. As shown in table 2, indomethacin produced a clear-cut increase in the proportion of type I collagen whereas type III collagen level was elevated to a lesser extent. As a consequence of this effect, the relative proportion of collagens released in the medium was altered (% of type III: 21.8 in indomethacin-treated cultures versus 26.4 in controls).

3.3. *Levels of mRNA for procollagens, fibronectin and DSPG core protein in fibroblasts treated with indomethacin*

We further determined the amounts of mRNA corresponding to four major proteins of the fibroblast extracellular matrix, including type I and type III collagens, in order to detect an eventual effect of indomethacin at the transcriptional level. Total RNA was isolated, size-fractionated by Northern blot and sequential hybridizations performed. The results presented in fig.1 show that the expression of GAPDH gene was unaffected by exposure to indomethacin and could serve as a control. In contrast, the mRNA level for type I procollagen increased substantially in response to indomethacin. The amount of type III procollagen mRNA was also elevated, albeit to a lesser extent. The steady-state level of fibronectin mRNA was similarly increased after exposure to indomethacin. The drug, however, did not affect DSPG core protein mRNA.

4. DISCUSSION

The results presented here demonstrate that indomethacin is capable of stimulating the production of type I and type III collagen by dermal fibroblasts in culture. Hybridization of total RNA isolated from control and indomethacin-treated cultures with cDNA probes for types I and III procollagens demonstrated that the drug induced a related increase in the steady-state levels of mRNA for these proteins. A similar increase was also observed for fibronectin mRNA level. These results suggest that the increase in collagen production under the effect of indomethacin is a result of the corresponding increase in the respective mRNA levels. For the moment, we do not know whether this effect is due to enhanced transcription or decreased degradation of these mRNAs. Interestingly, it has been recently reported that addition of exogenous PGE_2 to fibroblast cultures induced an inhibition of types I and III collagens and fibronectin production which paralleled a decrease in the steady-state levels of the corresponding mRNAs [20]. Our present observations corroborate this finding as far as the indomethacin blockade of prostaglandin production could suppress an inhibitory effect of PGE_2 on the transcriptional activity. However, it cannot be excluded that other cellular processes besides inhibition of prostaglandin synthesis may contribute to the stimulation of collagen and fibronectin production. An interference with the GTP binding protein, for example, has been suggested as a mechanism by which NSAIDs could inhibit aggregation of human neutrophils [21]. The present study is consistent with most of the investigations dealing with the in vivo effect of indomethacin on collagen synthesis which concluded to a stimulatory action [3–5]. These data concern both experimental granuloma and callus tissue from femoral fractures. Furthermore, the stimulatory effect has been also observed in vitro using fracture callus tissue from indomethacin-treated rats [5]. Therefore, it is likely that the stimulation of collagen synthesis produced in vivo by indomethacin involves the cellular effect observed here on fibroblast cultures.

Human skin fibroblasts in culture produce small proteoglycans but the major sulfated product is a small size dermatan sulfate proteoglycan [16]. Using a cDNA probe specific for the core protein mRNA of this small DSPG, we have demonstrated in the present study that indomethacin did not affect the transcriptional activity for this protein.

This finding suggests that synthesis of the core protein of DSPG is regulated by processes different from those of collagen and fibronectin synthesis. Since this small proteoglycan binds specifically to collagen and could be involved in the formation of collagen fibrils [22,23], the differential modulation exerted by indomethacin may lead to alteration of the extracellular matrix composition with impaired physical properties as a consequence.

In conclusion, indomethacin has been identified as a NSAID capable of stimulating collagen and fibronectin transcripts levels, probably via inhibition of prostaglandin synthesis. This effect may be a critical event of the matrix metabolism in patients receiving indomethacin.

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