RESEARCH COMMUNICATION

Leukoregulin down-regulates type I collagen mRNA levels and promoter activity in human dermal fibroblasts, and counteracts the up-regulation elicited by transforming growth factor- β

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Leukoregulin (LR), a T-cell-derived growth factor, modulates fibroblast functions *in vitro* [Mauviel, Rédini, Hartmann, Loyau & Pujol (1991) *J. Cell Biol.* 113, 1455–1462]. In the present study, incubation of human dermal fibroblasts with LR (0.1–2 units/ml) resulted in decreases in the mRNA steady-state levels for $\alpha 1(I)$, $\alpha 2(I)$ and $\alpha 1(III)$, but not $\alpha 2(V)$, collagen genes. LR also down-regulated $\alpha 2(I)$ collagen promoter activity in transient cell transfections of control cells as well as those incubated with transforming growth factor- β , a potent up-regulator of collagen type I gene expression. Thus LR is a strong inhibitor of type I collagen gene expression, acting at the level of transcription.

INTRODUCTION

Regulation of connective-tissue formation is under rigorous control of a number of soluble mediators which act in concert to ensure tissue integrity during homeostasis, development and repair. Alterations in the balance between the action of these factors or aberrant response of fibroblasts to regulators of matrix gene expression may lead to abnormal deposition of connective tissue. Among these factors, interleukin (IL)-1, tumour necrosis factor (TNF)- α , interferon (IFN)- γ and transforming growth factor (TGF)- β have been detected in inflammatory sites and in healing wounds, and extensive studies both *in vivo* and *in vitro* have established their essential role in tissue remodelling (for reviews, see Dinarello, 1988; Krane *et al.*, 1990; Massagué, 1990). Specifically, these cytokines have been shown to control connective-tissue cell recruitment and proliferation, as well as synthesis of the extracellular-matrix components. For example, TGF- β is a potent inducer of type I collagen gene expression, and the up-regulation takes place primarily at the transcriptional level (Ignatz & Massagué, 1986; Rossi *et al.*, 1986; Kähäri *et al.*, 1990).

Type I collagen is the major structural component in the skin, bones and tendons, and it is the major collagen type produced by dermal fibroblasts in culture (for reviews, see Prockop & Kivirikko, 1984; Krane, 1984). It has been proposed that abnormal regulation of collagen synthesis in keloids preferentially affects type I collagen (Abergel *et al.*, 1987). The role of TGF- β as the principal factor inducing collagen gene expression which leads to tissue fibrosis has been suggested by the observation that TGF- β expression often parallels increased type I collagen gene expression in fibrotic lesions (Nakatsukasa *et al.*, 1990; Peltonen *et al.*, 1990; Broekelmann *et al.*, 1991). By contrast, IL-1, which increases the expression and production of proteinases capable of degrading the extracellular-matrix components, may have an important role in tissue destruction in chronic inflammation (for

review, see Dinarello, 1988). IL-1 is also capable of modulating the synthesis of extracellular matrix macromolecules, such as collagens (Goldring & Krane, 1987; Kähäri *et al.*, 1987; Postlethwaite *et al.*, 1988; Mauviel *et al.*, 1988, 1991a) and proteoglycans (Yaron *et al.*, 1987; Heino *et al.*, 1988).

Recently, we have identified leukoregulin (LR), a 50 kDa glycoprotein isolated from supernatants of phytohaemagglutinin-stimulated leucocytes (Ransom *et al.*, 1985), as a novel modulator of fibroblast anabolic and catabolic functions *in vitro* (Mauviel *et al.*, 1991b, 1992). LR was originally described as a cytokine with unique cytotoxic and cytostatic effects on tumour cells. It was shown to increase tumour-cell sensitivity to natural killer and lymphokine-activated killer lymphocyte cytotoxicity (Furbert-Harris & Evans, 1989), and to produce a rapid and transient increase in plasma-membrane permeability of the target cells (Barnett & Evans, 1986). The latter effect facilitates the uptake of anti-tumour antibiotics (Evans & Baker, 1988). Moreover, LR prevents radiation-induced as well as carcinogen-induced transformation of normal cells, and kills tumour cells (Evans & DiPaolo, 1988). Taken together, these actions have proved that LR is a natural anti-cancer agent *in vitro*. Its effects on connective-tissue metabolism include the induction of prostaglandin E_2 and glycosaminoglycan production by fibroblasts, the inhibition of production of types I and III collagen, and the induction of collagenase gene expression (Mauviel *et al.*, 1991b, 1992). Even though the presence of LR in lymphocytic-tissue infiltrates has not been established as yet, one could speculate that LR is a potential modulator of connective-tissue remodelling, and interacts with other cytokines to potentiate or antagonize their effects on the extracellular-matrix deposition.

To investigate further the mechanism by which LR modulates fibroblast functions *in vitro*, we have studied its effects on the expression of the genes encoding fibrillar collagen types I, III and V.

Abbreviations used: CAT, chloramphenicol acetyltransferase; DME medium, Dulbecco's modification of Eagle's medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN, interferon; IL, interleukin; LR, leukoregulin; TGF, transforming growth factor; TNF, tumour necrosis factor.
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MATERIALS AND METHODS

Cell cultures

Normal human dermal fibroblasts were cultured by explanting tissue specimens obtained during surgical operations, and utilized in passages 3–8. The cells were maintained in Dulbecco's modified Eagle's (DME) medium supplemented with 10% (v/v) fetal-calf serum, 2 mM-glutamine and antibiotics.

Cytokines and growth factors

LR, of pI 5.1 and molecular mass approx. 50 kDa, was purified from phytohaemagglutinin-stimulated human blood leucocytes as described elsewhere (Evans *et al.*, 1989). In brief, normal human lymphocytes were stimulated with phytohaemagglutinin (Sigma Chemical Co., St. Louis, MO, U.S.A.) for 48 h, and LR was purified by sequential diafiltration, anion exchange, isoelectric focusing, and high-performance molecular-sieving liquid chromatography. One unit of LR was defined as the amount of activity causing 50% increase in the plasma-membrane permeability of K562 human erythroleukaemia cells (1×10^6 cells/ml) during a 2 h incubation (Barnett & Evans, 1986).

TGF- β 1, purified from bovine bone, kindly provided by Dr. David R. Olsen (Celtrix Laboratories, Palo Alto, CA, U.S.A.), was used in this study, and is referred to as TGF- β .

Northern analyses

Adult skin fibroblasts in confluent monolayer cultures were incubated with or without LR and subjected to isolation of total RNA as previously described (Chirgwin *et al.*, 1979). RNA was analysed by Northern hybridization with 32 P-labelled cDNA probes (Sambrook *et al.*, 1989) for α 1(I), α 2(I), α 1(III) and α 2(V) collagen chains [Chu *et al.* (1982), Myers *et al.* (1981), Chu *et al.* (1985) and Weil *et al.* (1987) respectively], and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control (Fort *et al.*, 1985). The [32 P]cDNA-mRNA hybrids were detected by autoradiography, and the steady-state levels of mRNA were quantified by scanning densitometry using a He-Ne laser scanner at 633 nm (LKB Produkter, Bromma, Sweden).

Transient transfections of cultured cells

Human neonatal foreskin fibroblasts in late-exponential growth phase were transfected with 10 μ g of plasmid DNA (pMS-3.5/CAT), which contains 3.5 kb of 5'-flanking DNA region of the human α 2(I) collagen gene linked to the chloramphenicol acetyltransferase (CAT) reporter gene (Boast *et al.*, 1990), or with an α 2(V) collagen promoter/CAT gene construct (kindly provided by Dr. F. Ramirez, Mount Sinai School of Medicine, New York, NY, U.S.A.). The cells were co-transfected with a RSV promoter/ β -galactosidase reporter gene construct for determination of transfection efficiency (Sambrook *et al.*, 1989). The specificity of the LR effect was assessed by transfecting parallel cultures with pSV₂CAT, a construct containing SV40 early-region promoter and SV40 enhancer, linked to the CAT gene (Gorman *et al.*, 1982). The transfections were performed with the calcium phosphate/DNA co-precipitation method (Graham & Van der Eb, 1973) followed by a 1 min 15%-glycerol shock. After the glycerol shock, the cells were placed in medium supplemented with 10% fetal-calf serum, or 1% in the experiments where TGF- β was added. After 3 h of incubation, LR and/or TGF- β were added, and the incubation was continued for 40 h. The cells were then harvested and lysed by three cycles of freeze-thawing in 100 μ l of 0.25 M-Tris/HCl, pH 7.8. The protein concentration of each extract was determined with a commercial assay kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.), and identical samples (5–10 μ g) were used for the CAT

assay, with [14 C]chloramphenicol as substrate (Gorman *et al.*, 1982).

RESULTS AND DISCUSSION

Effects of LR on type I, III and V collagen mRNA steady-state levels

Adult human skin fibroblasts in confluent cultures were incubated for 24 h with various concentrations of LR. Steady-state levels of collagen type I, III and V mRNA were then estimated by Northern hybridizations. The results revealed a strong inhibition of type I collagen gene expression by LR, with the maximum effect ($\sim 70\%$) being noted in the presence of 2 units/ml (Table 1). The abundance of type III collagen mRNA was also decreased, but to a lesser extent, the maximal inhibition ($\sim 50\%$) being noted with 2 units of LR/ml. By contrast, the expression of the α 2(V) collagen gene was not affected by LR, at any of the concentrations tested (Table 1). These results are in accordance with our previous data showing decreased synthesis of type I and III collagen, but no alteration in the production of type V collagen by LR in fibroblast cultures, as estimated by SDS/PAGE characterization and quantification of the corresponding α chains (Mauviel *et al.*, 1991b).

The action of LR was studied further by measuring type I collagen mRNA levels at different time points after the addition of LR (1 unit/ml). As shown in Table 2, 30% inhibition was observed at 6 h of incubation with LR, and the maximum inhibition, $\sim 70\%$, was achieved at 24 h. These data demonstrate that the inhibition of type I collagen synthesis by LR takes place, at least in part, at the pre-translational level.

Our results are consistent with previous demonstrations that type I and type III collagen gene expression is co-ordinately regulated in most experimental or clinical situations (Uitto & Chu, 1989). It should be noted that the inhibition of type I collagen gene expression by LR could be detected by assay of both α 1(I)- and α 2(I)-chain mRNAs. In fact, the expression of the constituent polypeptides of type I collagen, the α 1(I) and α 2(I) chains, is co-ordinately regulated in most situations. These results are in agreement with our previous data demonstrating a

Table 1. Effects of LR in various concentrations on type I, III and V collagen mRNA steady-state levels in fibroblasts

Human adult dermal fibroblasts were incubated with various concentrations of LR (0.1–2 units/ml) for 24 h in DME supplemented with 10% fetal-calf serum. Total RNA was analysed by Northern hybridization with cDNA probes for α 1(I), α 2(I), α 1(III) and α 2(V) collagen mRNAs. Quantification of the specific transcripts was performed by scanning densitometry and the values were corrected for GAPDH mRNA levels in each RNA preparation. Values are expressed as relative densitometric units, after correction for GAPDH mRNA levels in the same RNA preparations. The numbers in parentheses indicate the values relative to control cultures without addition of LR.

	LR (units/ml)				
	0	0.1	0.5	1	2
α 1(I) mRNA	8.9 (100)	3.3 (38)	3.2 (36)	4.2 (48)	2.9 (32)
α 2(I) mRNA	6.6 (100)	2.9 (45)	2.7 (42)	3.2 (49)	2.4 (37)
α 1(III) mRNA	1.5 (100)	1.5 (100)	1.0 (70)	1.2 (84)	0.7 (45)
α 2(V) mRNA	3.9 (100)	4.2 (108)	3.3 (85)	4.6 (117)	4.0 (103)

Table 2. Time-dependent inhibition of the $\alpha 2(I)$ collagen mRNA steady-state levels by LR

Adult human dermal fibroblasts in early confluent cultures were incubated with LR (1 unit/ml) for different time periods, up to 48 h, as indicated in Table 1. The abundance of $\alpha 2(I)$ collagen mRNA levels was then determined by Northern hybridization, as described in the Materials and methods section. Values are expressed as relative densitometric units, after correction for GAPDH mRNA levels in the same RNA preparations. The numbers in parentheses indicate the inhibition relative to control cultures before addition of LR.

	Time of incubation (h)			
	0	6	24	48
$\alpha 2(I)$ mRNA	7.9 (100)	5.8 (73)	2.4 (30)	2.4 (30)

Fig. 1. Effect of LR on the $\alpha 2(I)$ collagen promoter activity in transient cell transfections

Human neonatal foreskin fibroblasts were transfected with a human type I collagen promoter/CAT reporter gene construct pMS-3.5/CAT, together with a RSV promoter/ β -galactosidase reporter gene construct, as described in the Materials and methods section. At 3 h after glycerol shock, the cells were exposed to various concentrations of LR (0.1–2 units/ml) in medium containing 10% fetal-calf serum. After further incubation for 40 h, the cells were harvested and CAT activity was determined in duplicate cultures. The Figure shows a representative autoradiogram of the CAT assay, depicting the separation of acetylated (AC) and unacetylated (C) forms of [14 C]chloramphenicol by t.l.c. Quantification of CAT activity, expressed as the percentage of [14 C]chloramphenicol acetylated, is indicated at the bottom of the Figure, and is the mean value from the corresponding duplicate samples, after correction for β -galactosidase activity in the same samples, as an index of transfection efficiency.

decrease in the synthesis of both pro $\alpha 1(I)$ and pro $\alpha 2(I)$ chains in fibroblasts incubated with LR (Mauviel *et al.*, 1991b).

Effects of LR on $\alpha 2(I)$ collagen promoter activity

Transient transfection experiments with a human type I collagen promoter/CAT reporter gene construct pMS-3.5/CAT were performed to examine whether LR down-regulates collagen type I gene expression through inhibition of the transcription at the promoter level. CAT activity, an index of the promoter activity, was assayed in the cells after 40 h of incubation following

Fig. 2. Effect of LR on TGF- β -induced up-regulation of the $\alpha 2(I)$ collagen promoter activity

Human neonatal foreskin fibroblasts were transfected with a human collagen type I promoter/CAT reporter gene construct pMS-3.5/CAT, together with a RSV promoter/ β -galactosidase reporter gene construct, as described in Fig. 1. At 3 h after the glycerol shock, the cells were exposed to 5 ng of TGF- $\beta 1$ /ml, in the absence or presence of 1 unit of LR/ml, in medium containing 1% fetal-calf serum. No factors were added to the control cultures (CTL). After additional incubation for 40 h, the cells were harvested and CAT activity was determined in duplicate cultures. The Figure shows a representative autoradiogram of the CAT assay, depicting the separation of acetylated (AC) and unacetylated (C) forms of [14 C]chloramphenicol by t.l.c. Quantification of CAT activity, expressed as the percentage of [14 C]chloramphenicol acetylated, is indicated in the Figure, and is the mean value from the corresponding duplicate samples, after correction for β -galactosidase activity in the samples, as an index for transfection efficiency.

the glycerol shock. As shown in Fig. 1, clearly detectable CAT activity was present in control cells incubated in DME medium containing 10% fetal-calf serum. A dose-dependent decrease in the $\alpha 2(I)$ collagen promoter activity was noted in the presence of LR (Fig. 1), in a similar manner as was noted by assay of the corresponding mRNA (Table 1). Approx. 50% inhibition of the promoter activity was observed with 1 unit of LR/ml and reached ~75% with 2 units/ml (Fig. 1). Parallel transfections with pSV₂CAT showed little, if any, effect on the CAT activity by LR, attesting to the specificity of the effect observed with the human collagen promoter construct. These data suggest that the inhibition of $\alpha 2(I)$ collagen gene expression, as detected at the mRNA level, is mediated primarily by decreased transcriptional activity of the corresponding promoter. Since the expression of $\alpha 1(I)$ and $\alpha 2(I)$ genes was co-ordinately regulated by LR, as determined at the mRNA level (see Table 1), it is conceivable that the $\alpha 1(I)$ collagen promoter is also under similar transcriptional control to the $\alpha 2(I)$ promoter. On the other hand, transient transfection experiments with an $\alpha 2(V)$ collagen promoter/CAT construct showed no effect by LR on the promoter activity (results not shown), confirming the results obtained at the mRNA level (Table 1).

Since TGF- β has been shown to be a potent up-regulator of type I collagen gene expression, it was decided to examine whether LR could counteract the stimulatory effect exerted by TGF- β on fibroblast collagen gene expression. For this purpose, fibroblasts were transfected with the pMS-3.5/CAT construct as described above, and incubated with TGF- β , in either the

presence or the absence of LR. As expected, TGF- β (5 ng/ml) exerted a strong stimulatory effect on the $\alpha 2(I)$ collagen promoter activity (9.5-fold stimulation over the activity in control cells; Fig. 2). This level of activation of the type I collagen promoter is in agreement with values published in the literature (Rossi *et al.*, 1988; Kähäri *et al.*, 1990). When LR (1 unit/ml) was added together with TGF- β , the stimulatory effect was attenuated, and the CAT activity was increased only 2.5-fold over control values (Fig. 2). This represents a $\sim 75\%$ decrease in the enhancing effect of TGF- β . A similar effect of LR was also observed at the mRNA level. However, in the latter case, LR was added to the cultures 1 h before addition of TGF- β , and the inhibition of the TGF- β effect was complete.

Several other cytokines, including TNF- α , IFN- γ and IL-1, have been previously shown to counteract TGF- β -elicited up-regulation of type I collagen gene expression (Heino & Heinenen, 1990; Kähäri *et al.*, 1990). Furthermore, TNF- α and IFN- γ either alone or synergistically inhibit constitutive expression of the type I collagen gene in fibroblasts (Scharfetter *et al.*, 1989; Kähäri *et al.*, 1990; Mauviel *et al.*, 1991a). However, the mechanisms of inhibition appear to be distinct. TNF- α acts primarily at the transcriptional level, whereas IFN- γ decreases the mRNA steady-state levels by destabilization of the transcripts at the post-transcriptional level (Kähäri *et al.*, 1990). The results of our study indicated that the action of LR on type I collagen gene expression is primarily mediated at the promoter level. Previous studies have established, however, that LR is distinct from TNF- α , IFN- γ or IL-1, as judged by the inability of specific neutralizing antibodies to these cytokines to block the effects of LR on fibroblast collagen synthesis (Mauviel *et al.*, 1991b). Thus LR is a unique cytokine with potent antagonistic effects on TGF- β -induced collagen gene expression. It should be noted that LR is able to induce collagenase gene expression in fibroblasts, and this effect is also mediated at the transcriptional level (Mauviel *et al.*, 1992). Consequently, LR may contribute to the cytokine network which maintains the balance between the synthesis and degradation of collagen in physiological situations.

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