Modulation of Human Dermal Fibroblast Extracellular Matrix Metabolism by the Lymphokine Leukoregulin

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Abstract. The effect of leukoregulin, a 50-kD lymphokine with unique antitumor properties, was studied in vitro on several fibroblast functions. Leukoregulin did not inhibit fibroblast proliferation, as measured by cell enumeration and [^{3}H]thymidine incorporation, and had no cytotoxic effect in terms of increased membrane permeability detected by trypan blue exclusion, two of the major leukoregulin actions on tumor cells. Leukoregulin induced a dose-dependent decrease in collagen synthesis, demonstrated by decreased [^{3}H]proline incorporation into collagenase-digestible protein, as early as 6 h after the addition of the lymphokine to human fibroblasts. Leukoregulin inhibited the synthesis of both type I and type III collagen, as measured by SDS-PAGE and by specific radioimmunoassay. Neutralizing antibodies to interleukin-1α, interleukin-1β, tumor necrosis factor-α, and interferon-γ failed to alter the effect of leukoregulin on collagen synthesis, attesting that leukoregulin action was not due to contamination by these cytokines. Inhibition of collagen synthesis occurred concomitantly with increased secretion of prostaglandin E_2 and a transient rise in intracellular cyclic AMP content, peaking at 6 h. However, blocking prostaglandin synthesis with indomethacin did not counteract inhibition of collagen synthesis by leukoregulin, demonstrating independence of this action of leukoregulin from cyclooxygenase metabolites. Leukoregulin also stimulated glycosaminoglycan production in a dose-dependent manner, affecting the synthesis of hyaluronic acid as the major fibroblast-derived extracellular glycosaminoglycan. In addition, secretion of neutral proteases (collagenase, elastase, caseinase) was increased. These observations indicate that leukoregulin is able to regulate synthesis of molecules critical to the deposition of the extracellular matrix by nontransformed nonmalignant fibroblasts.

LEUKOREGULIN (LR)[1] is a lymphokine synthesized and secreted by activated human lymphocytes, that inhibits proliferation of and kills tumor cells, increases their sensitivity to natural killer lymphocyte cytotoxicity and produces a rapid and reversible increase in tumor cell membrane permeability (Ransom et al., 1985; Barnett and Evans, 1986; Evans et al., 1987). LR is a glycoprotein with a molecular size of ~50 kD and several isoelectric forms, one with a pI near 5.1 and another near 7.0 (Evans et al., 1989). Unlike many other lymphokines, which primarily regulate cells within the immune system, LR has no identified immunoregulatory role for cells in the normal immune system. Instead, LR prevents carcinogen- and radiation-induced transformation of normal cells and has cytostatic and cytotoxic actions on abnormal target cells as part of its anticancer action (reviewed in Evans, 1987).

Fibroblasts are mesenchymal cells that play a critical role in embryonic morphogenesis, would repair, and tissue remodeling by synthesizing and organizing connective tissue components. It is well established that fibroblasts do not function autonomously but, instead, respond to numerous molecular signals from different cells of the immune system (e.g., T lymphocytes, macrophages, platelets) and other sources (reviewed in Postlethwaite and Kang, 1988). Numerous soluble leukocytic mediators including interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and transforming growth factor-β (TGF-β) are present in the sites of chronic inflammation or would healing, that have been infiltrated by leukocytes. The ability of these cytokines to modify connective tissue cell metabolism has been well documented, both in vitro and in vivo (reviewed in Postlethwaite and Kang, 1988; Freundlich et al., 1986). However, a number of studies suggest that other still undefined factors are involved with a balance between several cytokines controlling fibroblast recruitment and matrix deposition during wound healing. At the present time, it is

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1. *Abbreviations used in this paper*: ECM, extracellular matrix; GAG, glycosaminoglycan; IFN-γ, interferon-γ; IL-1, interleukin-1; LR, leukoregulin; PGE_2, prostaglandin E_2; Suc(Ala)$_3$ NA, succinyl trialanine paranitroalanine; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

not known whether LR is present among these factors and no study has examined if LR has a role in the regulation of fibroblast function.

The control of extracellular matrix (ECM) turnover involves both deposition of structural components such as collagen, fibronectin, and proteoglycans, and their degradation by secreted proteases. In this work, we report that LR modulates the synthesis of matrix components, inhibiting collagen and increasing glycosaminoglycan production. This effect is paralleled by an increase in extracellular collagenase, elastase and neutral protease activities, a transient rise in intracellular cyclic AMP and a release of prostaglandin E_2 (PGE_2). These LR-induced changes in fibroblast metabolism occur without change in cell proliferation or plasma membrane permeability and indicate the potential of LR to regulate fibroblast function.

Materials and Methods

Cell Cultures

Fibroblasts obtained from explanted infant foreskins were grown in Dulbecco's modified of Eagle's medium (DMEM; Gibco, Paisley, UK) supplemented with antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone) and 10% heat-inactivated FCS (Gibco). The cells were grown in a 5% CO_2 atmosphere and experiments were performed on different cell strains between fourth and eighth passage.

Leukoregulin Preparation

Leukoregulin with a pI of 5.1 and a molecular mass of 50 kD was purified from human peripheral blood leukocytes as previously described (Evans et al., 1989). In brief, normal human lymphocytes were stimulated with phytohaemagglutinin (leukoagglutinin isomer; Sigma Chemical Co., St. Louis, MO), for 48 h and LR was purified using sequential diafiltration, anion exchange, isoelectric focusing and high performance molecular sieving liquid chromatography. 1 U of LR equals that amount causing a 50% increase in the plasma membrane permeability of 1×10^6 K562 erythroleukemia cells/ml during a 2-h period (Barnett and Evans, 1986).

Experimental Procedure

For the experiments on confluent cultures, cells were seeded at a density of 2×10^4 cells/cm^2 and allowed to grow to confluency (5–6 d). To study the production of collagen, the cultures were preincubated for 24 h in DMEM supplemented with 2% FCS and 50 μg/ml sodium ascorbate (Sigma Chemical Co.). Then the media were removed and fresh medium containing 50 μg/ml β-aminopropionitrile (β-APN; Sigma Chemical Co.) was added, with or without LR. The media were collected at different time points for prostaglandin E_2 assay, and the corresponding cell layers were used for cyclic AMP determination. Estimation of total collagen production was performed in 2-cm^2 dishes with 0.5 ml medium, in the presence of 2 μCi/ml [^{3}H]proline (CEA, France, 30–40 Ci/mmol) as a radioactive precursor. For determination of the amounts of type I and type III collagen and fibronectin by SDS-PAGE, radiolabeling was performed with 20 μCi/ml [^{3}H]proline in 25-cm^2 flasks (3 ml medium).

Total glycosaminoglycan (GAG) synthesis was estimated in 2-cm^2 dishes, using 2.5 μCi/ml [^{3}H]glucosamine (11 Ci/mmol; CEA, Saclay, France) and 5 μCi/ml [^{35}S] SO_4 (25 Ci/mg; Amersham International, Amersham, UK) as radioactive precursors, with no ascorbate and β-APN in the preincubation and incubation media.

Collagenase, elastase and neutral proteases activities were either assayed in the media from 75-cm^2 flasks (6 ml) or from 9.6-cm^2 dishes after 24-h incubation periods, in the absence of serum.

[^{3}H]thymidine incorporation was estimated in cells grown in 2-cm^2 dishes.

For the experiments on proliferating cells, fibroblasts were seeded at a density of 5×10^4 cells/9.6 cm^2 and allowed to grow for 4 d before preincubation.

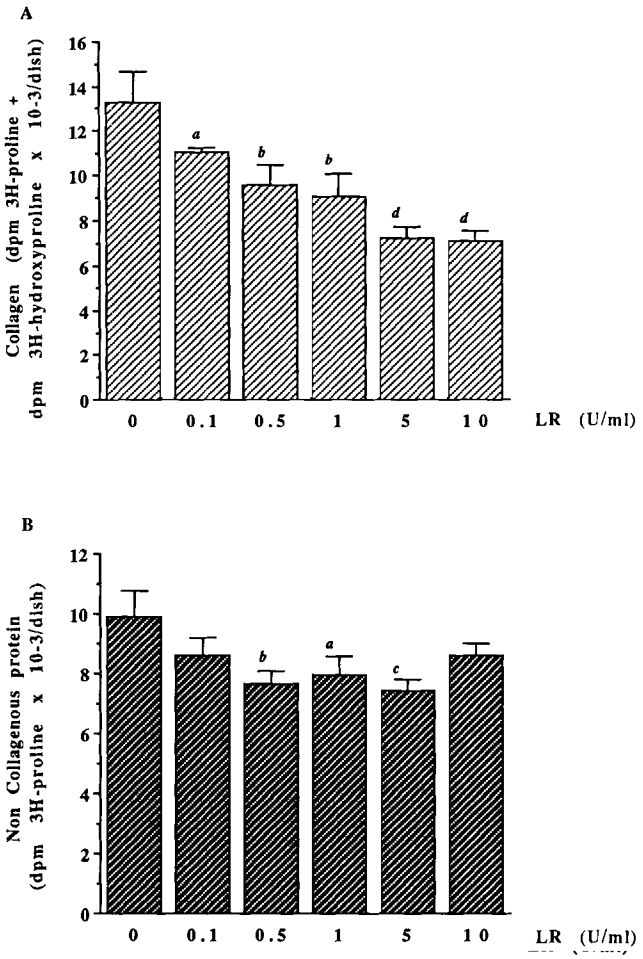

Figure 1. Effect of leukoregulin on collagen and noncollagenous protein synthesis by human dermal fibroblasts. Confluent cells were labeled for 24 h with [^{3}H]proline in the presence of varying concentrations of LR, as described in Materials and Methods. Collagen radioactivity was estimated as dpm in collagenase digestible material present in the culture medium (*A*). Noncollagenous protein represents the amount of radioactivity remaining after collagenase digestion (*B*). Results expressed as dpm $\times 10^{-3}$/dish are the mean ± SD of quadruplicate assays. *a*, $P < 0.05$; *b*, $P < 0.02$; *c*, $P < 0.01$; *d*, $P < 0.001$.

Collagen Production

Assay of Radioactive Collagen. Newly synthesized collagen was assayed in the culture medium as collagenase sensitive material (Peterkofsky and Diegelmann, 1971). We have previously shown that >90% of the newly synthesized collagen was recovered in the medium when fibroblasts are incubated with ascorbate and β-aminopropionitrile. The amount of noncollagenous protein was estimated from the radioactivity remaining after collagenase digestion (Advance Biofacture Corp., Lynnbrook, NY).

Polyacrylamide Gel Electrophoresis. Confluent cultures were first preincubated as described above and then pulsed with 20 μCi/ml [^{3}H]proline in absence or in presence of 0.1, 1, and 5 U/ml LR for 24 h. SDS-PAGE and subsequent fluorography of collagen and procollagen chains were performed as previously described (Mauviel et al., 1991). Interrupted electrophoresis was used to separate type I and type III collagen chains (Sykes et al., 1976).

Radioimmunoassay of Types I and III Collagens. RIA of collagens type I and type III were performed in the culture media as described (Magloire et al., 1986) using specific antibodies against human type I and type III collagens.

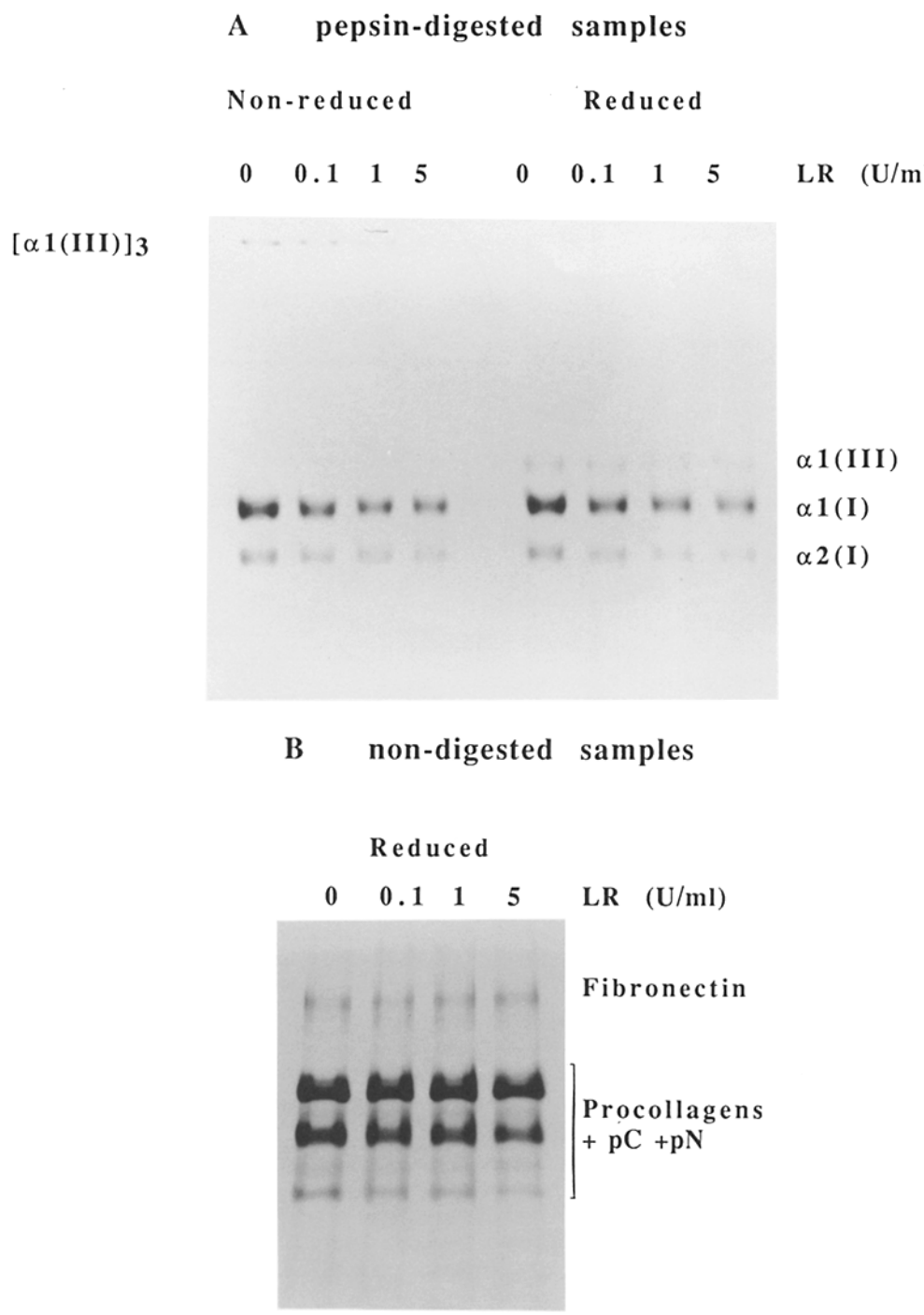

Figure 2. Fluorograph of SDS-PAGE analysis of extracellular proteins synthesized in control and leukoregulin-treated human dermal fibroblasts. Confluent cells were labeled for 24 h with [^{3}H]proline as described in Materials and Methods, in the absence or presence of 0.1, 1, and 5 U/ml LR. Proteins from the medium were separated electrophoretically in 6% acrylamide gels containing SDS and 2 M urea, with (*A*) or without (*B*) pepsin digestion. Reduction of collagen type III trimers with mercaptoethanol (0.1 M) occurred during interruption of the electrophoresis. Bands corresponding to procollagens, collagens, and fibronectin chains are indicated.

Glycosaminoglycan Production

Assay of Total Labeled Glycosaminoglycans. The total amount of labeled GAG was estimated by precipitation of media and cell extracts with cetylpyridinium chloride (1% [wt/vol]; Merck, Darmstadt, Germany) and ethanol (5 vol) as previously described (Wasteson et al., 1976). The final pellet was dissolved in 0.5 ml 75 mM NaCl and the radioactivity of an aliquot was assayed by liquid scintillation counting.

Ion-exchange Chromatography of Labeled Proteoglycans. 25-cm^2 flasks of confluent fibroblast cultures were labeled for 24 h with [^{3}H]glucosamine (2.5 μCi/ml) and [^{35}S]sulfate (5 μCi/ml) in presence or in absence of LR (1 U/ml). Proteoglycans extracted by 4 M guanidinium-HCl (Oegema et al., 1975) from cell layers and media, were dialyzed for 24 h at 4°C against 0.1 M Tris-HCl, 7 M urea pH 7.5 containing 50 mM EDTA, 1 mM PMSF, 10 mM *N*-ethylmaleimide, 100 mM 6-aminohexanoic acid, and 10 mM benzaminidium chloride (Sigma Chemical Co.) as protease inhibitors. Then the samples were applied to a 1 × 10 cm DEAE-Sephacel column (Pharmacia, Uppsala, Sweden) equilibrated with the same buffer. The column was washed with 15 ml of the buffer and eluted with a linear 0–1 M NaCl gradient. Fractions of 0.4 ml were collected and assayed by liquid scintillation counting.

Protease Assays

Substrate Gels. For visual localization and determination of molecular masses of bands with enzyme activity, aliquots of culture medium (50 μl)

Table I. Effect of Neutralizing Antibodies Against TNF-α, IL-1α, IL-1β, or IFN-γ on Leukoregulin-induced Inhibition of Fibroblast Collagen Synthesis

	Noncollagenous protein (dpm [^{3}H]-proline/dish)	Collagen (dpm [^{3}H]proline + dpm [^{3}H]-hydroxyproline/dish)
Control	7,163 ± 395 (100)	11,287 ± 1,132 (100)
LR (*1 U/ml*)	5,621 ± 308* (78)	5,492 ± 833‡ (49)
TNF-α (*10 ng/ml*)	5,057 ± 324‡ (71)	5,636 ± 522‡ (50)
Anti-TNF-α (*50 μg/ml*)	6,244 ± 220§ (87)	9,586 ± 1,198§ (85)
TNF-α + anti-TNF-α	6,201 ± 377§ (87)	9,824 ± 400§ (87)
LR + anti-TNF-α	4,627 ± 263* (65)	5,043 ± 352‡ (45)
IL-1α (*10 U/ml*)	6,002 ± 418* (84)	7,000 ± 270‡ (62)
Anti-IL-1α (*100 μg/ml*)	6,254 ± 511§ (87)	10,491 ± 1,119§ (93)
IL-1α + anti-IL-α	7,504 ± 508§ (105)	9,525 ± 701§ (84)
LR + anti-IL-1α	6,556 ± 320§ (92)	6,446 ± 570‖ (57)
IL-1β (*10 U/ml*)	6,169 ± 398* (86)	7,881 ± 898* (70)
Anti-IL-1β (*100 μg/ml*)	6,722 ± 409§ (94)	10,168 ± 457§ (90)
IL-1β + anti-IL-1β	6,796 ± 539§ (95)	10,468 ± 888§ (93)
LR + anti-IL-1β	5,622 ± 176§ (78)	6,656 ± 775‖ (59)
IFN-γ (*10^4 U/ml*)	4,752 ± 619‡ (66)	4,561 ± 170‡ (40)
Anti-IFN-γ (*1:100 dilution*)	7,936 ± 810§ (111)	11,962 ± 1,012§ (106)
IFN-γ + anti-IFN-γ	6,642 ± 913§ (93)	8,643 ± 745* (77)
LR + anti-IFN-γ	6,253 ± 527§ (87)	6,184 ± 701‖ (55)

Collagen synthesis was measured by ^{3}[H]-proline incorporation after 24-h incubation as described in Materials and Methods. Results are the mean ± SD of quadruplicate assays. Numbers in brackets correspond to the percentage of control.
* $P < 0.05$.
‡ $P < 0.001$.
§ Not significant.
‖ $P < 0.01$.

were electrophoresed on SDS-substrate gels containing 1 mg/ml of gelatin (Overall et al., 1989). After removal of SDS with Triton X-100, neutral protease activity was revealed by incubation of the gels for 48 h at 37°C in 0.1 M Tris-HCl buffer, pH 7.4 containing 10 mM $CaCl_2$, followed by staining in 0.5% Coomassie blue in 30% propanol-2/10% acetic acid and destaining in 5% methanol/7.5% acetic acid.

Elastase Activity. Succinyl trialanine paranitroalanine ($Suc(Ala)_3NA$) was used as a synthetic substrate for the determination of elastase activity (Szendroi et al., 1984). Enzyme units are defined as nmol of nitroalanine released per hour, assuming a molar extinction coefficient of E_M = 8,800.

Collagenase and Caseinase Activities. Collagenase and caseinase activities were assayed using ^{3}H-acetylated collagen (1 mg/ml, 1.9 × 10^5 cpm/mg) and ^{3}H-acetylated casein (1 mg/ml, 1.13 × 10^6 cpm/mg) as substrates (Cawston and Barrett, 1977).

Prostaglandin E_2 Assay

PGE_2 was assayed in the culture medium by specific radioimmunoassay (Dray et al., 1975) with antiserum from Institut Pasteur Production (Marnes la Coquette, France).

Cyclic AMP Assay

After incubation, the cells were washed three times with cold PBS and kept overnight in 80% ethanol. The cells were scraped and debris was removed by centrifugation. The ethanolic supernatants were evaporated and the residue resuspended in 0.05 M Tris–4 mM EDTA, pH 7.5. cAMP content was estimated using a specific radioimmunoassay kit (Amersham International).

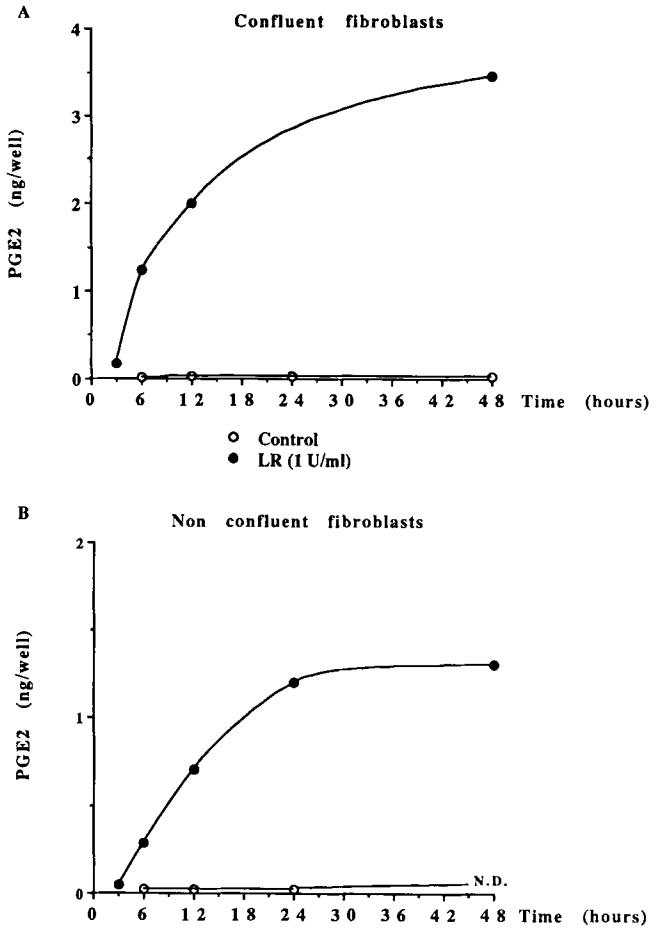

Figure 3. Effect of leukoregulin on PGE_2 production by confluent and proliferating human dermal fibroblasts. Fibroblast cultures were incubated as described in Materials and Methods with or without 1 U/ml LR. PGE_2 was assayed by RIA in the culture medium at different time points. Results, expressed as nanograms of PGE_2 per well, are the mean of triplicate determinations.

Cell Enumeration and [^{3}H]thymidine Incorporation

For cell enumeration, the cells were washed with PBS, detached with trypsin (0.25%) and counted in an electronic counter (Hycell Inc., Houston, TX).

DNA replication was estimated as follows. Nonconfluent and confluent fibroblast cultures were incubated with or without 1 U/ml LR for different time periods. [^{3}H]thymidine (2 μCi/ml) was either added at the beginning of the incubation or during the last 6 h. Cells were then washed three times with cold PBS and blocked by the addition of cold 5% TCA overnight. The cell layers were washed three times with 5% TCA and dissolved in 0.2 N NaOH before measurement of [^{3}H]thymidine incorporation by liquid scintillation.

Cytokine Antibodies

Rabbit anti-human IL-1α and anti-human TNF-α, sheep anti-human IL-1β, and mouse anti-human IFN-γ were immunoglobulins G1 purified by caprylic acid precipitation.

Each antibody was added to the culture media either alone or in combination with its corresponding recombinant antigen (cytokine) as a control of its neutralizing activity, and together with LR in order to ensure the specificity of LR action on fibroblast collagen synthesis.

Statistical Analysis

All the results presented are expressed as mean ± SD unless indicated otherwise. Student's *t* test was used to evaluate the difference of the means between groups.

Results

Effect of Leukoregulin on Fibroblast Collagen Synthesis

The effect of leukoregulin on fibroblast collagen synthesis was tested by three alternative methods: incorporation of [^{3}H]proline into collagenase-sensitive peptides, SDS-PAGE, and radioimmunoassay. Fig. 1 illustrates the effect of 0.1, 0.5, 1, 5, and 10 U/ml of human LR on the incorporation of [^{3}H]proline into collagen and noncollagenous protein in the culture medium during a 24-h incubation of confluent human dermal fibroblasts. LR exerted a dose-dependent inhibition of collagen synthesis (Fig. 1 *A*). Maximal inhibition by LR (−50%, $P < 0.001$) was observed at 5 U/ml and then reached a plateau. In contrast, the production of noncollagenous protein was only slightly reduced (maximum −24%, $P < 0.01$, Fig. 1 *B*). No alteration in cell morphology was observed with phase contrast microscopy when cells were treated with LR; moreover, trypan blue exclusion revealed no change in cell membrane permeability, even with 10 U/ml LR.

To estimate the kinetics of LR-induced decrease of collagen production, the cells were incubated with or without 1 U/ml LR and [^{3}H]proline incorporation into collagenase digestible protein was measured at different time points (6, 12, 24, 48, and 72 h). The inhibitory effect exerted by LR could be detected as early as 6 h after addition of the cytokine (−30%, $P<0.01$). This inhibitory effect of LR could not be attributed to an inhibition of cell proliferation by LR since neither cell counts nor [^{3}H]thymidine incorporation during these incubation periods revealed any significant difference between control and LR-treated cultures (not shown).

The inhibitory effect of LR on the amounts of secreted collagen as measured by assay of collagenase-sensitive protein was confirmed by the electrophoretic analysis of pepsin-resistant material (Fig. 2 *A*). Densitometric analysis of the fluorographs showed inhibition of both type I and III collagen. The amounts of newly synthesized procollagens present in the medium were also reduced by LR (Fig. 2 *B*), suggesting that the inhibition of collagen synthesis is not due to a modification of the maturation of precursor molecules. Moreover, this effect is also not due to an impaired secretion since no parallel accumulation of collagen in the cell layer could be detected after LR treatment (not shown). Fibronectin production was also reduced, in both secreted and cell-associated fractions. In contrast, the synthesis of α1(V) collagen chains, as revealed on fluorographs obtained after a long time exposure of the gels, was almost unaffected by LR (not shown).

Inhibition of collagen synthesis by LR was further confirmed by specific radioimmunoassay. The extent of inhibition reached 39% ($P < 0.02$) for type I collagen and 32% ($P < 0.05$) for type III collagen after 24 h incubation with 1 U/ml LR. These data obtained with a labeling-independent quantitative method exclude the possibility of an altered intracellular pool of proline by LR that would result in the dilution of the isotopes and to a subsequent decreased incorporation into protein.

To be sure that the effect of LR was not due to a contamination of the preparation by other cytokines, we used antibodies against IL-1α, IL-1β, TNF-α, and IFN-γ, the major cytokines cytokines known to decrease fibroblast collagen production in vitro (Jimenez et al., 1984; Mauviel et al., 1988*a*, 1991). As can be seen in Table I, the antibodies neutralized the effect of their respective antigens (cytokines) but failed to block the inhibition exerted by LR, supporting the specificity of LR inhibition of fibroblast collagen synthesis.

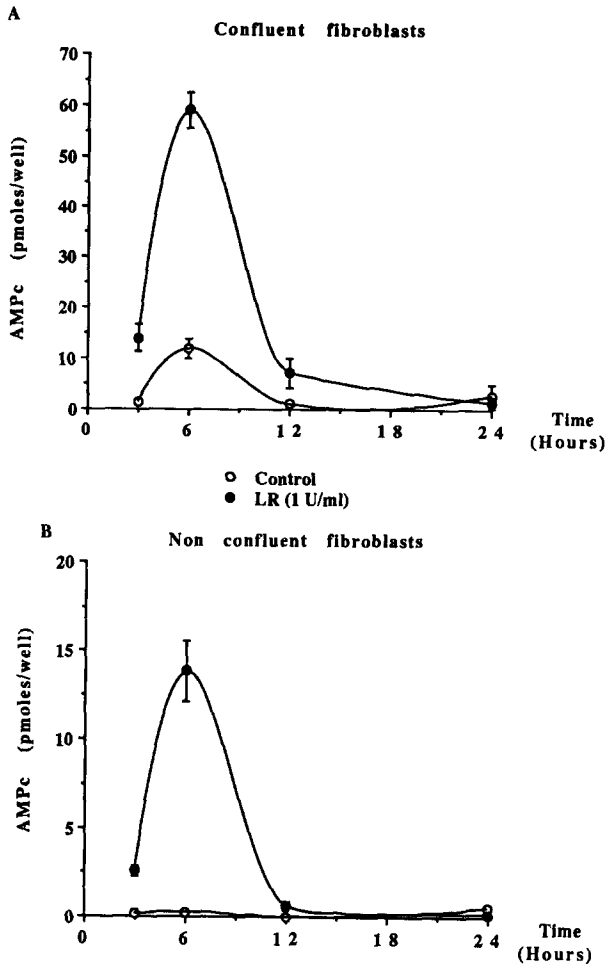

Figure 4. Effect of leukoregulin on intracellular cyclic AMP levels in confluent and proliferating human dermal fibroblasts. Fibroblast cultures were incubated as described in Materials and Methods with or without 1 U/ml LR. Intracellular cAMP was assayed in parallel to PGE_2 production (Fig. 3). Results, expressed as picomoles of cAMP per dish, are the mean ± SD of triplicate determinations.

Effects of Leukoregulin on Prostaglandin E_2 and Cyclic AMP Production by Fibroblasts

To investigate the mechanism of fibroblast activation by LR, and to determine if the inhibition of collagen synthesis could be related to arachidonate metabolism, we assayed the accumulation of PGE_2 in the culture medium in relation to intracellular cyclic AMP contents.

As can be seen in Fig. 3, LR (1 U/ml) markedly enhanced PGE_2 production by fibroblasts, in both confluent (Fig. 3 *A*) and nonconfluent cultures (Fig. 3 *B*), with a maximal effect during the first 12 h. PGE_2 was increased >10 times after 6 h stimulation by LR. LR also induced a transient rise in intracellular cAMP (Fig. 4), with a peak at 6 h in both confluent (Fig. 4 *A*) and nonconfluent (Fig. 4 *B*) cultures.

Since the depression of collagen synthesis induced by LR occurred concomitantly with a very large increase of PGE_2 released into the culture medium, and since PGE_2 has been

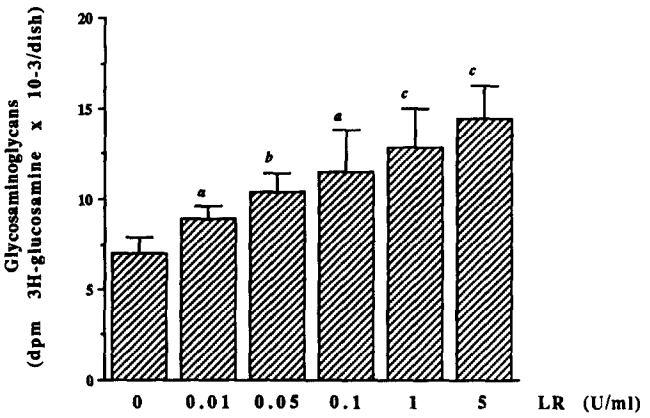

Figure 5. Effect of leukoregulin on total glycosaminoglycan production by human dermal fibroblasts. Confluent cells were labeled for 24 h with [^{3}H]glucosamine as described in Materials and Methods. GAG radioactivity was estimated as dpm in pronase-digested CPC precipitated material present in the culture medium. Results expressed as dpm $\times$ 10^{-3}/dish are the mean $\pm$ SD of quadruplicate assays. (*a*) $P < 0.05$; (*b*) $P < 0.02$; (*c*) $P < 0.01$.

reported to inhibit fibroblast collagen synthesis (Varga et al., 1987), we studied the effect of 1 U/ml LR in the presence of 10 μM indomethacin, a concentration known to almost totally abolish fibroblast prostaglandin synthesis (Mauviel et al., 1988*b*). Indomethacin did not however reduce the inhibitory effect of LR on collagen synthesis (not shown), suggesting that this effect of LR is not prostaglandin dependent.

Effect of Leukoregulin on Fibroblast Glycosaminoglycan Synthesis

Treatment of fibroblasts over a 24-h period with various concentrations of LR resulted in a significant increase in [^{3}H]-glucosamine incorporation into GAG secreted in the medium, which represent about 90% of total GAG synthesized by fibroblasts (Fig. 5). 0.05 U/ml LR was sufficient to markedly augment GAG production (+30%, $P < 0.02$). A dose-dependent increase was observed and 5 U/ml led to twofold greater [^{3}H]glucosamine incorporation than in control cultures ($P < 0.001$).

To characterize the types of proteoglycans synthesized in the presence of LR, fibroblasts were incubated with [^{3}H]-glucosamine and [^{35}S]sulfate, in presence or absence of 0.1 U/ml LR, a concentration shown to increase by 80% the [^{3}H]glucosamine incorporation into cetylpyridinium chloride-precipited GAG (Fig. 5). Ion-exchange chromatography profiles of secreted proteoglycan eluted from DEAE-Sephacel show that LR strongly augmented [^{3}H]glucosamine incorporation in a peak eluting at 0.2 M NaCl (Fig. 6) which was sensitive to hyaluronidase digestion (not shown) and thus corresponds to hyaluronic acid. The incorporation of [^{3}H]glucosamine into glycoproteins eluted with the starting buffer was also strongly stimulated by LR. In contrast, [^{35}S]-sulfate incorporation into secreted sulfated GAG (eluted at 0.5 M NaCl) was almost unaltered by LR.

Effect of Leukoregulin on Extracellular Protease Activities

To determine the eventual role of LR on catabolic functions

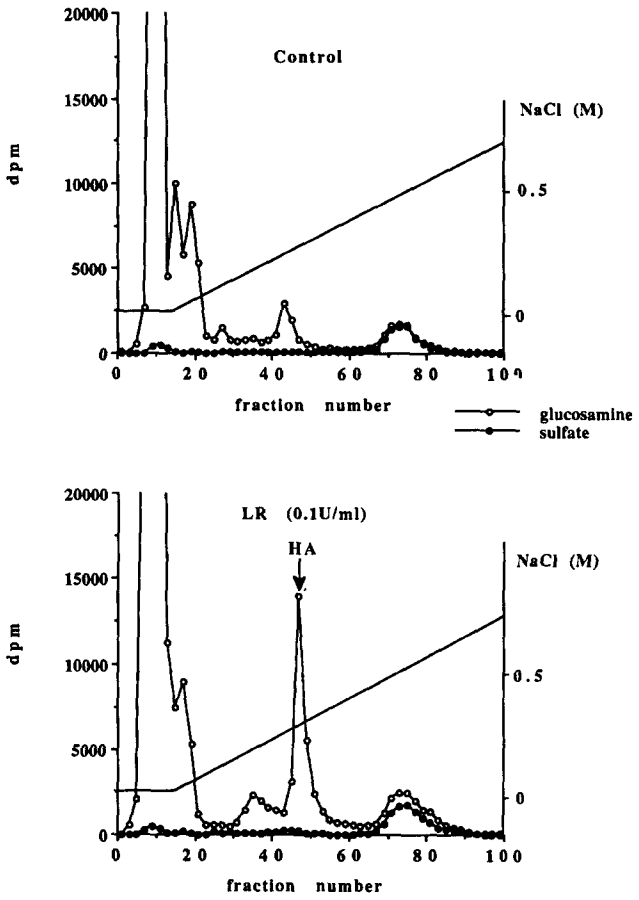

Figure 6. Ion-exchange chromatography profiles of proteoglycans secreted by control and leukoregulin-treated human dermal fibroblasts. Confluent cells were labeled for 24 h with [^{3}H]glucosamine and [^{35}S]SO_4 as described in Materials and Methods. Samples were applied to DEAE-Sephacel columns equilibrated with 0.1 M Tris-7 M urea pH 7.5, and eluted by a 0–1-M NaCl gradient. Radioactivity of 0.4-ml fractions was counted by liquid scintillation.

of fibroblasts, media from LR-treated fibroblast cultures (1 U/ml during 24 h) were tested for protease activities.

A time course of fibroblast secreted proteases was analyzed by SDS–substrate PAGE using gelatin as a substrate (gelatin zymogram). Gelatinolytic activity was detected as cleared bands against the stained gelatin background. As can be seen in Fig. 7, the major protease activity appearing in control cultures was ~70 kD, corresponding to 72-kD progelatinase (Overall et al., 1989). Two pairs of bands were also detected, the larger ~100–110 kD, which may correspond to the native form of the 72-kD progelatinase (Overall et al., 1989), and the smaller ~45–50 kD, which corresponds to both stromelysin or interstitial 45-kD collagenase, even if the ability of these two enzymes to degrade gelatin substrates is limited (Chin et al., 1985; Okada et al., 1986). A faint band of ~30 kD was detected, corresponding to a subtype of stromelysin (Okada et al., 1986). Treatment of fibroblasts with 1 U/ml LR led to an increased expression of both 45- and 30-kD protease activities, which could be detected after 6 and 12 h, respectively. No increase in the amount of the 70- and 110-kD proteases was observed.

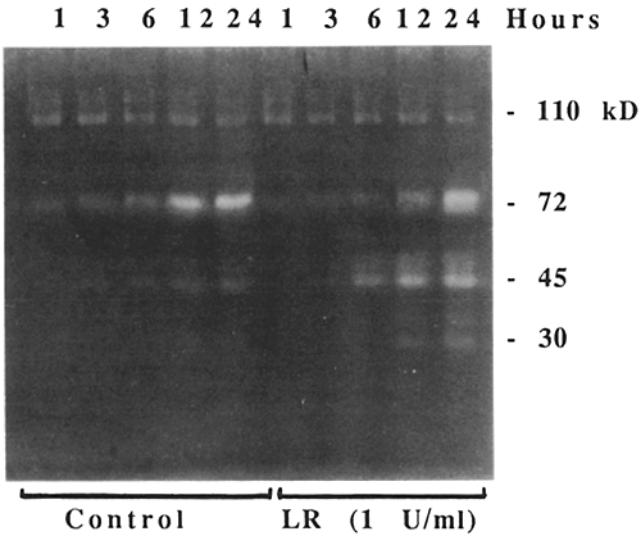

Figure 7. Gelatin-zymogram of control and leukoregulin-treated fibroblasts. Confluent fibroblast cultures were incubated for various time periods with or without 1 U/ml LR. 50-μl aliquots of supernatants were fractionated electrophoretically in a 11% acrylamide gel containing 1 mg/ml gelatin and tested for their gelatinase activity as described in Materials and Methods.

To further characterize the action of LR, we measured collagenase and neutral protease activities in fibroblast supernatants by the degradation of ^{3}H-acetylated-collagen and casein. Elastase activity was quantified using the synthetic substrate Suc(Ala)$_3$NA. As can be seen in Table II, all these proteases activities were augmented approximately to the same extent (from +20 to +30%, $P < 0.01$) by LR.

Discussion

The present study demonstrates for the first time that leukoregulin can modulate several functional aspects of fibroblast biology, including components central to the synthesis and degradation of the extracellular matrix. LR is a 50-kD lymphokine that was originally identified as a protein displaying specific regulatory activities for cells undergoing transformation to the neoplastic state or for fully neoplastic cells. Several in vitro and in vivo studies have provided evidence that ECM metabolism is controlled by a number of cytokines and growth factors acting on mesenchymal cells such as fibroblasts. Some of the mediators are produced by activated lymphocytes and macrophages present at the sites of inflammation or would repair. For example, IL-1 and TNF-α have been shown to induce production of collagenase by dermal fibroblasts or rheumatoid synovial cells, and to inhibit their collagen production (Mizel et al., 1981; Dayer et al., 1985; Mauviel et al., 1988*a*, 1988*c*, 1991). Similarly, IFN-γ has been described as a potent inhibitor of collagen synthesis (Jimenez et al., 1984). On the other hand, it has been reported that TGF-β can stimulate the synthesis of ECM components in mesenchymal cells, including fibroblasts (Ignotz and Massagué, 1986; Fine and Goldstein, 1987). It is likely that these mediators are acting in concert in vivo as elements of a balanced homeostatic network. Their relative amount of efficiency probably varies depending on whether matrix deposition is required in various remodeling situations such as wound healing. The interactions between these connective tissue modulating proteins however remain to be elucidated.

The in vitro results presented here strongly suggest that leukoregulin can be classified as a lymphokine capable of modulating the metabolism of the major matrix components, collagen and glycosaminoglycans, in dermal fibroblasts. Leukoregulin inhibits both collagen and fibronectin synthesis and stimulates hyaluronic acid production. Furthermore, LR enhances release of PGE_2, collagenase, and neutral metalloprotease activities. Similar responses are observed with IFN-γ, IL-1, and TNF-α (Jimenez et al., 1984; Mauviel et al., 1991). However, the inability of neutralizing antibodies against these cytokines to alter the LR action clearly demonstrates the specificity of LR action. Since dermal fibroblasts have been shown to express IL-1 and TNF-α mRNAs in culture, especially when they are exposed to exogenous IL-1 (Mauviel et al., 1988*d*, 1991), the question arises whether the observed effects may be due to LR-induced synthesis of these cytokines by fibroblasts. This is, however, also unlikely since the addition of neutralizing antibodies against IL-1 and TNF-α failed to block the inhibition of collagen synthesis by LR.

The molecular pathways whereby LR exerts its action on collagen and hyaluronic acid synthesis are not known. Our results obtained with the cyclooxygenase inhibitor indomethacin clearly indicate that the enhancement of PGE_2 release upon LR treatment cannot be responsible for these effects. Further studies are required to determine whether LR directly affects collagen synthesis at the transcriptional level as has been shown for IL-1 and TNF-α (Goldring and Krane, 1987; Solis-Herruzo et al., 1988; Mauviel et al., 1988*c*, 1991; Kähäri et al., 1990). We might anticipate that LR will also affect the synthesis of collagen and extracellular

Table II. Effect of Leukoregulin on Fibroblast Proteolytic Activities

	Caseinase activity (hydrolysis of ^{3}H-acetylated casein)	Collagenase activity (hydrolysis of ^{3}H-acetylated collagen)	Elastase activity (hydrolysis of Suc(Ala)$_3$N A)
	μg substrate/h/ml	*μg substrate/h/ml*	*nmol substrate/h/ml*
Control	4.1 ± 0.1 (100 ± 2)	3.2 ± 0.2 (100 ± 6)	14.8 ± 0.7 (100 ± 4)
LR (*1 U/ml*)	4.9 ± 0.2 (120 ± 5)*	4.3 ± 0.2 (134 ± 6)‡	18.8 ± 0.5 (127 ± 4)*

Confluent fibroblasts in 75-cm^2 flasks were incubated for 24 h in presence or absence of 1 U/ml LR (6 ml medium). Proteolytic activities were determined in the culture medium as described in Materials and Methods. Results are the mean ± SD of triplicate samples. % of respective controls are indicated in parentheses.
* $P < 0.02$.
‡ $P < 0.001$.

secreted proteases at the molecular level because LR has been shown to upregulate transcription of class I and II histocompatibility antigens while simultaneously inhibiting human papilloma virus early gene transcription in human cervical epithelial cells (Woodward et al., 1990).

This investigation demonstrates that LR is a protein with the ability to modulate both anabolic and catabolic functions of fibroblasts. Moreover, the induction of PGE_2 release by connective tissue cells also suggests that LR may have pro-inflammatory properties. This is the first demonstration for a potential role of this cytokine in the regulation of several functions of nontransformed nonmalignant cells that are central to tissue remodeling and to the inflammatory process. LR may be a member of a family of leukocyte-derived cytokines that control connective tissue formation or remodeling and that participate in a number of dermatological disorders in which leukocyte infiltrates are present including dermal fibrosis, keloid scars, and scleroderma.

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