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Effect of cell–cell and cell–matrix interactions on the response of fibroblasts to epidermal growth factor *in vitro*

Expression of collagen type I, collagenase, stromelysin and tissue inhibitor of metalloproteinases

Alain C. COLIGE, Charles A. LAMBERT, Betty V. NUSGENS and Charles M. LAPIÈRE*
Laboratory of Experimental Dermatology, University of Liège, CHU Sart-Tilman, Tour de Pathologie B23, B-4000 Sart-Tilman, Liège 1, Belgium

Investigations of the effect of epidermal growth factor (EGF) on the expression of four genes involved in the turnover of the extracellular matrix, collagen type I, collagenase, stromelysin and tissue inhibitor of metalloproteinases (TIMP) were performed on four strains of skin fibroblasts *in vitro*. Addition of EGF to subconfluent cultures for increasing periods of time up to 5 days induced an inhibition of procollagen α1(I) mRNA and a strong stimulation of collagenase (100-fold) and stromelysin (1000-fold) mRNAs, whereas the mRNA of TIMP was increased to a lesser extent (5-fold). After a 40 h pulse with EGF, these effects persisted for 24–48 h after withdrawal of the growth factor and slowly diminished thereafter to attain control values after several days. By culturing fibroblasts for increasing periods of time, different levels of confluence were obtained allowing for the deposition of an extracellular biomatrix. The steady-state level of collagenase and stromelysin mRNAs were profoundly depressed in confluent as against non-confluent cultures, whereas no major change for TIMP and procollagen α1(I) mRNAs was observed. Upon treatment of these cultures with EGF for 48h, the steady-state level of collagenase, stromelysin and TIMP increased, whereas procollagen α1(I) mRNA was slightly reduced. These modifications were, at least in part, dependent upon a regulation of the transcription rate, as suggested from run-off experiments. Similar states of confluence were obtained by seeding cells at increasing densities in short-term cultures in which cell–cell contact predominated. In such culture conditions, the collagenase and stromelysin mRNAs were enhanced in high as compared to low density cultures. The response to EGF was progressively decreased for collagenase, stromelysin and, to a lesser extent, TIMP mRNAs at most densities and a complete lack of response to EGF at the highest cell density was observed. Under all culture conditions the modulation of collagenase mRNA was paralleled by similar modifications of enzyme activity. These results emphasize the importance of the cell–cell contacts and cell–matrix interactions in the expression of the genes coding for metalloproteinases or their inhibitor and their modulation by growth factors.

INTRODUCTION

Mechanisms of growth, differentiation and repair involve both the synthesis and the degradation of the extracellular matrix. These processes are modulated by growth factors, cytokines and cell–cell and cell–matrix interactions [1].

Collagen synthesis is regulated *in vivo* [2–4] as well as *in vitro* by a variety of factors as different as culture conditions [5,6], products of procollagen processing [7], ascorbic acid [8], growth factors [9,10] etc. Degradation is also highly regulated. Tissue procollagenase synthesis is inducible and enhanced by interaction with matrix components [11] or by such agents as phorbol esters [12], cytochalasin B [13], the Ca^{2+} ionophore A23187 [14] and growth factors [15–17]. Most of these regulatory events exert also a control on proteins involved in the activation of collagenase [18]. Among them, stromelysin, a proteolytic enzyme that, besides its broad substrate specificity for a number of glycoproteins and proteoglycans [1], is also able to activate procollagenase [19]. Tissue inhibitor of metalloproteinases (TIMP) suppresses this activity as well as that of collagenase and gelatinase [20].

In the present study, we investigated the effect of epidermal growth factor (EGF) on the expression of four genes involved in the turnover of the extracellular matrix, namely those coding for procollagen type I, procollagenase 1, prostromelysin and TIMP. Experiments were devised to determine the effect of cell–cell or cell–matrix interactions on the cell response to EGF.

MATERIALS AND METHODS

Materials

Foetal-calf serum (FCS) was from Gibco and Boehringer, bisbenzimid H33258 {2-[2-(4-hydroxyphenyl)-6-benzimidazoyl]6-(1-methyl-4-piperazyl)benzimidazol} was from Hoechst, and acrylamide, *NN'*-methylenebisacrylamide and *NNN'N'*-tetramethylethylendiamine were from Bio-Rad Laboratories. X-ray films Royal X-Omat S were from Kodak; EGF from mouse submaxillary glands was from Sigma and [α-^{32}P]dCTP and [α-^{32}P]UTP were from New England Nuclear. The random-priming DNA-labelling kit was from Boehringer.

Cell culture

Fibroblasts cultures were established from skin explants as previously described [21] or obtained from the National Institute of Aging (Camden, NJ, U.S.A.) Aging Cell Repository. The cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) FCS and 50 μg of ascorbic acid/ml and used between passages 4–12. Confluent cultures were

Abbreviations used: EGF, epidermal growth factor; TIMP, tissue inhibitor of metalloproteinases; DMEM, Dulbecco's modified Eagle medium; FCS, foetal-calf serum; NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulphonyl fluoride; PBS, phosphate-buffered saline (composition is given in the text); 1 × SSC, 0.15 M-NaCl/0.015 M-sodium citrate.

* To whom correspondence should be sent.

harvested by treatment with 0.1% trypsin/0.02% EDTA and subcultured at a 1:3 split ratio.

Monolayers on plastic at different levels of confluence were obtained either by culturing for increasing periods of time or by seeding at increasing cellular densities in DMEM supplemented with 50 μg of ascorbic acid/ml, 10% dialysed decomplemented FCS in the absence or presence of EGF (25 ng/ml). The medium was renewed every 24 h. Every experimental condition was tested at least in duplicate.

Collagenase assay

The collagenase assay was performed on conditioned medium as described by Bailly *et al.* [22], with some modifications. Briefly, acid-soluble type I collagen purified from fetal- or newborn-calf skin was labelled by [^{3}H]acetylation as described by Gisslow & McBride [23]. The specific radioactivity was 1.5×10^6 c.p.m./mg. The native state of the labelled collagen was assessed by trypsin digestion, which released a maximum of 15% of the radioactivity. The conditioned media were first dialysed against 3 M-KSCN to dissociate collagenase from complexes with serum inhibitors as described by Abe & Nagai [24]. The activation of latent collagenase was performed by incubation with 100 μg of trypsin/ml of culture medium for 10 min at 37 °C, followed by addition of a 5-fold excess of soya-bean trypsin inhibitor. The activated medium were incubated for 16 h at 25 °C with 16000 c.p.m. of [^{3}H]collagen in the presence of 25 mM-*N*-ethylmaleimide (NEM) and 0.5 mM-phenylmethanesulphonyl fluoride (PMSF). The dissociation by KSCN coupled to the activation by a high concentration of trypsin and by NEM during the incubation permitted the recovery of 70% of the collagenase activity, as measured from control experiments performed with medium conditioned by fibroblasts without FCS and containing active collagenase, then supplemented with 10% FCS and treated as described above. The enzymic reaction was stopped by adding 10 mM-EDTA and ethanol at a final concentration of 18% (v/v). After being left for 30 min at room temperature, the samples were centrifuged at 9000 *g* for 20 min. Under these conditions the uncleaved substrate was totally precipitated, whereas more than 95% of the cleaved fragments remained in the supernatant. The measurement of the radioactivity in the supernatant was performed by liquid-scintillation spectrometry in a Rackbeta (LKB) instrument. Parallel samples containing 10 mM-EDTA added before incubation were similarly handled, providing blank values that were subtracted from the values obtained for the active medium. Blank values never exceeded 10% of the total radioactivity in the test. One unit of collagenase is defined as the amount of enzyme that degrades 1 μg of collagen in 16 h at 25 °C. The specificity of the collagenase cleavage was checked by SDS/PAGE, performed by the technique of Laemmli [25].

DNA determination

The cells were washed in phosphate-buffered saline (PBS; 0.05 M-KH_2PO_4/Na_2HPO_4/0.15 M-NaCl, pH 7.2), sonicated and heated at 60 °C for 1 min before performing the measurements by a fluorimetric method using the bis-benzimid H33258 reagent [26].

Isolation and analysis of RNA

The cell layers were solubilized in 5 M-guanidium isothiocyanate/5 mM-sodium citrate (pH 7.0)/0.1 M-2-mercaptoethanol/5.5% *N*-sodium dodecylsarcosinate. Total RNA was isolated by using the discontinuous CsCl-gradient method as described by Chirgwin *et al.* [27].

For Northern-blot analysis, total RNA was processed as described by Lehrach *et al.* [28] and modified by Fourney *et al.* [29]. Briefly, 5 μg of the RNA of representative samples were freeze-dried, resuspended in the Mops/formaldehyde buffer, and fractionated in a 1.2%-agarose gel. The transfer of RNA from the gel to the nylon membrane was achieved by capillary action.

For slot-blotting, the RNA was denatured at 65 °C for 15 min in 3 vol. of 6.15 M-formaldehyde and 10 × SSC (1 × SSC is 0.15 M-NaCl/0.015 M-sodium citrate), serially diluted at the desired concentration and slotted on a nylon membrane using a filtration manifold (Schleicher und Schüll). The nylon filters were u.v.-irradiated, prehybridized and hybridized [27] with 2×10^7 c.p.m. of the probes labelled to high activity (about 1×10^9 c.p.m./μg of DNA) by the random-priming DNA-labelling method.

After hybridization, the filters were washed twice at room temperature in 2 × SSC/0.1% SDS and six times at 65 °C (twice in 2 × SSC/0.1% SDS; once in 1 × SSC/0.1% SDS and three times in 0.3 × SSC/0.1% SDS), dried, and exposed to X-ray film. The densitometric scanning of the slot-blot autoradiograms was performed for a large range of serial dilutions and different times of exposition with a LKB Ultroscan XL instrument. The amount of applied RNA was normalized to the signal obtained with a 28 S rRNA probe.

The cDNAs of prostromelysin (p SL 51-4.3) and TIMP (p TIMP I 332.1-3) were generously given by Dr. G. Goldberg (Washington University School of Medicine, St. Louis, MO, U.S.A.), whereas that of collagenase (GEM 3 COLL K4) was kindly provided by Dr. P. Herrlich (Institute of Genetics and Toxicology, Karlsruhe, Germany). The cDNA of procollagen α1(I) (Hf677) was a gift from Dr. F. Ramirez (Mount Sinai School of Medicine, New York, NY, U.S.A.). The cDNA specific for 28 S rRNA was as previously described [30].

Transcription-rate assays

The relative rate of transcription measurements were performed on nuclei prepared as described by Greenberg & Ziff [31]. The nuclear-run-off transcripts were labelled mainly as described by Thompson & Rosner [32] by using 500 μCi of [α-^{32}P]UTP (3000 Ci/mmol) per sample. Nuclear RNA was isolated as described by Chirgwin *et al.* [27] and harvested in water. Fractions of RNA representing equal amounts of radioactivity were incubated in 0.2 M-NaOH for 10 min on ice, diluted with an equal volume of 2 M-ammonium acetate and mixed with the hybridization solution [50% formamide/5 × SSC/2.5 × Denhardt's/1 mM-EDTA/tRNA (250 μg.ml)] before hybridization. For binding to nylon filters, cDNAs were treated with 30 mM-NaOH (final concn.) at 65 °C for 30 min, added of an equal volume of 2 M-ammonium acetate and spotted on to the membranes (500 ng of cDNA/slot). λ-phage DNA digested with *Hin*dIII was used as a negative control. Filters were prehybridized for 3 h and hybridized for 45 h at 50 °C. Blots were washed twice in 0.3 × SSC/0.1% SDS/1 mM-EDTA for 30 min at room temperature, treated with ribonucleases (RNAase A, 10 μg/ml; RNAase T1, 2 μg/ml) in 2 × SSC for 30 min at 37 °C and further washed twice in 2 × SSC at 37 °C before being exposed to Kodak X-Omat S films. The intensity of the signals was recorded by using the 2D Gelscan 5.0 software of the LKB Ultroscan XL.

RESULTS

The effects of EGF (25 ng/ml) was tested on normal skin fibroblasts at confluence after increasing periods of treatment up to 5 days. In control cultures, no significant change in cell number or modification of the various tested mRNAs was noted during the 5 days of observation (Fig. 1*a* and 1*b*). In parallel cultures treated with EGF, proliferation of contact-inhibited fibroblasts was not induced upon addition of the growth factor, since similar DNA contents were measured (results not shown).

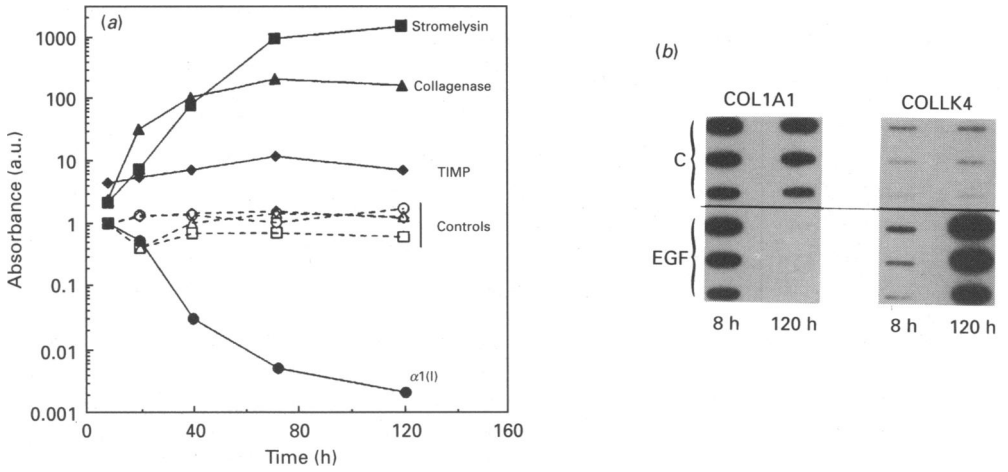

Fig. 1. Time course of EGF-induced effects on confluent fibroblasts

(*a*) Confluent fibroblasts (strain Mo..) were incubated in the absence (open symbols) or presence (closed symbols) of EGF (25 ng/ml). At the indicated time, the steady-state level of mRNA for stromelysin (■), collagenase (▲), TIMP (◆) and collagen α1(I) (●) were measured by slot-blotting as described in the Materials and methods section. Results are expressed in arbitrary units (a.u.) on a logarithmic scale taking the values of the controls at 8 h as unity. Each point is the mean value obtained from at least two separate experiments. (*b*) As an illustration, representative slot-blot autoradiograms obtained with the α1(I) collagen (COL1A1) and collagenase (COLLK4) cDNA after 8 and 120 h of induction with EGF ('EGF') as compared with non-induced control cultures ('C').

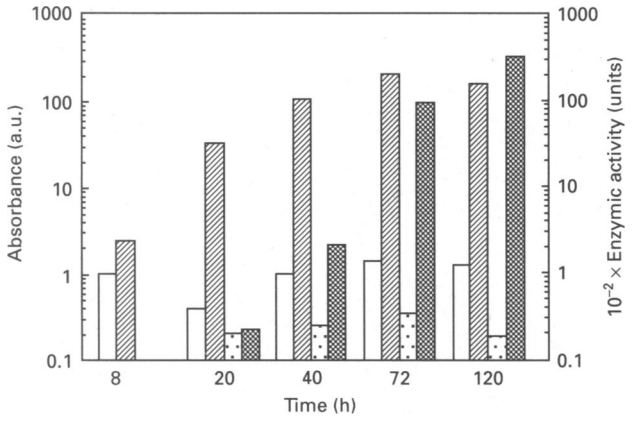

Fig. 2. Time course of EGF-induced collagenase mRNA expression and collagenase activity

Conditioned medium of the cultures used for measuring the mRNA content described in Fig. 1 were utilized. Results are expressed in units of enzymic activity for control (⊡) and EGF-treated (⊠) cultures. For comparative purposes, collagenase mRNA measurement, in arbitrary units (a.u.) of absorbance, are illustrated for control (□) and EGF-treated (▨) cells. Control at 8 h is taken as unity.

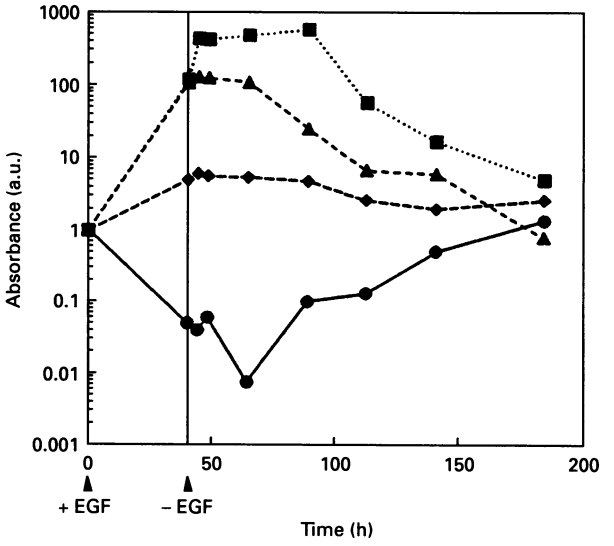

Fig. 3. Time course of the disappearance of the EGF-induced modifications

After a 40 h pulse with EGF (25 ng/ml), the cultures were extensively washed and incubated for increasing periods of time without EGF before measuring the mRNA level (a.u.) of α1(I) collagen (●), collagenase (▲), TIMP (◆) and stromelysin (■).

In these cultures, significant variations in the steady-state level of the four tested mRNAs already occurred between 8 and 20 h and dramatically expanded up to 5 days. Whereas procollagen α1(I) mRNA level was decreased by several orders of magnitude after 5 days, the collagenase mRNA level (±100-fold) and the stromelysin mRNA (±1000-fold) were strongly increased. The mRNA steady-state level for TIMP was less affected. The pre-translational modifications of procollagen α1(I) and collagenase were expressed at the protein level. Indeed, we previously showed that EGF decreased procollagen synthesis in monolayer cultures of skin fibroblasts [33]. The increased procollagenase mRNA level was paralleled by an enhanced enzyme activity released in the culture medium, although with a lag period of at least 20 h (Fig. 2). The collagenase was released in the medium in a latent form activable by KSCN, trypsin and NEM. In subsequent experiments, a 2-day induction by EGF was selected because of its ability to induce pronounced effects on the phenotype of the cells without promoting significant proliferation.

The EGF-induced changes in the phenotype of skin fibroblasts were persistent even after withdrawal of the growth factor. After a 40 h pulse with EGF, the cultures were washed, incubated for increasing periods of time without EGF before measuring the mRNAs levels (Fig. 3). The effect of EGF persisted after its removal for 24 h for α1(I), collagenase and TIMP, and up to 48 h for stromelysin. Values close to control measurements were recovered only 6 days after removing EGF.

Four strains of human skin fibroblasts were maintained in

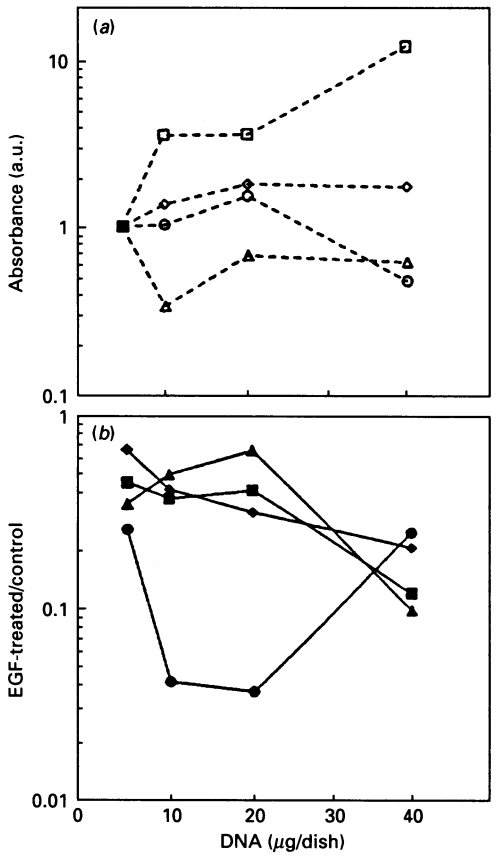

Fig. 4. Effect of confluence induced by increasing time in culture on cell response to EGF in four strains of skin fibroblasts

Two were obtained from young donors, Mo.. (●) and Ch.. (▲), and two from aged donors, Vd.. (■) and AG 5274 (◆). Fibroblasts in monolayer were cultured for increasing periods of time up to 30 days to reach increasing confluence, indicated by the DNA content (μg/dish). After a 2 days incubation with EGF (25 ng/ml), the steady-state level of α1(I) collagen mRNA was measured. The mRNA content in control cultures (*a*) were expressed in arbitrary absorbance units (a.u.) on a logarithmic scale, taking the lowest confluent cells as unity. In EGF-treated cultures (*b*) the results are expressed as the ratio between values for EGF-treated and control.

culture for increasing periods of time in order to allow for the deposition of a biomatrix. Four levels of cell density were used: non-confluent ($\sim 0.5 \times 10^6$ cells or 5 μg of DNA per dish), subconfluent ($\sim 1 \times 10^6$ cells or 10 μg of DNA per dish), confluent ($\sim 2 \times 10^6$ cells or 20 μg of DNA per dish) and hyperconfluent [$(3–4) \times 10^6$ cells or 30–40 μg of DNA per dish, depending upon cell strain]. These two last conditions were attained after 15 and 30 days of culture respectively. The steady-state level of mRNA for procollagen α1(I) as a function of increasing time in culture varied differently in the four strains of cells (Fig. 4*a*). Stromelysin mRNA was always strongly depressed in confluent as against non-confluent cultures (Fig. 5*a*). A strong repression of collagenase mRNA was also observed, with increased confluence in at least three of the four strains tested (Fig. 6*a*). Changes in the enzymic activity paralleled those observed at the mRNA level (results not shown). The steady-state level of mRNA for TIMP did not change significantly (Fig. 7*a*).

Upon treatment for 48 h with EGF (25 ng/ml) of parallel cultures at similar increasing confluency, a slight reduction of α1(I) mRNA occurred (Fig. 4*b*). A strong stimulation was observed for stromelysin, collagenase and TIMP mRNA levels

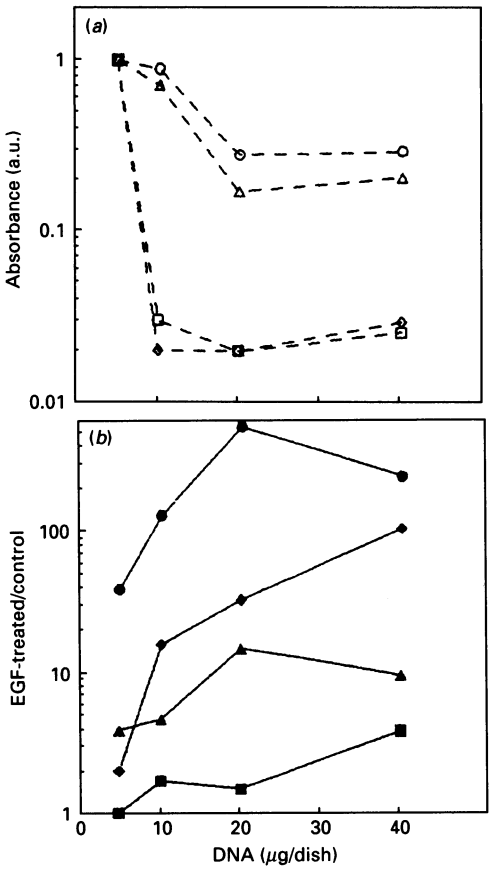

Fig. 5. Effect of confluence, induced by increasing time in culture, on the cell response to EGF for stromelysin mRNA

Details are as for Fig. 4.

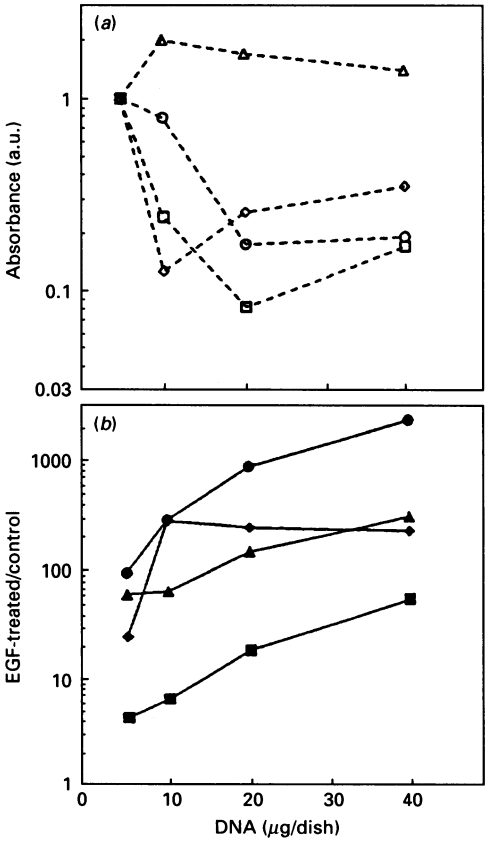

Fig. 6. Effect of the state of confluence, induced by increasing time in culture, on the cell response to EGF for collagenase mRNA

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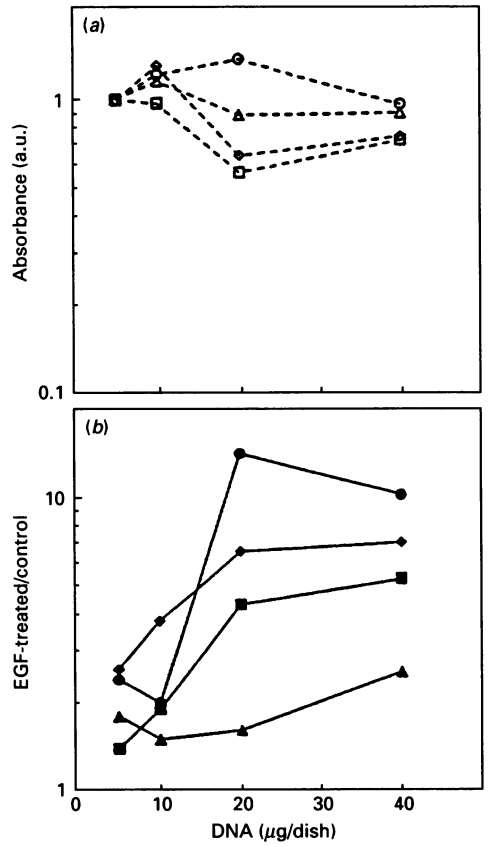

Fig. 7. Effect of the state of confluence, induced by increasing time in culture, on the cell response to EGF for TIMP mRNA

Details are as for Fig. 4.

(Figs. 5*b*, 6*b* and 7*b*). It increased as a function of cell density. The magnitude of the response to EGF was, however, somewhat variable in the four tested strains. The modifications of collagenase mRNA level were also reflected at the post-translational level by a marked accumulation of the latent form of the enzyme in the medium (results not shown).

The effects of EGF on the rate of transcription of α1(I) and collagenase genes was measured in hyperconfluent cells of strain 'Mo..'. EGF decreased the number of run-off transcripts of the α1(I) collagen chain by a factor of 3, whereas the number of collagenase transcripts was increased by a factor of 2.5 (Fig. 8). The absence of hybridization with the *Hin*dIII-digested λ-phage DNA demonstrated the specificity of the hybridization. These results suggest that EGF acts, at least partly, on the transcription rate of α1(I) and collagenase genes.

In order to investigate the effect of cell–cell contact on the activity of EGF in absence of biomatrix, cells were seeded at increasing densities and their phenotype analysed after a short and constant period of time in culture. Cells of the most responsive strain to EGF, Mo.., were plated at 0.4×10^6, 0.8×10^6, 2×10^6 and 4×10^6 cells/dish in order to attain within 4 days the cell density obtained in the previous series of experiments. Cells seeded at 4×10^6 per dish never acquired the shape observed at the same cell density after a long-term culture (Figs. 9*a* and 9*b*). Neither TIMP nor α1(I) mRNA level was significantly affected by cell density, except for a dramatic decrease of the latter at 4×10^6 cells per dish. A striking increase of collagenase and stromelysin mRNAs occurred in the most confluent cultures (Figs. 10*a* and 10*c*). This effect was also expressed in terms of collagenase activity (results not shown). Moreover, their response to EGF progressively decreased as

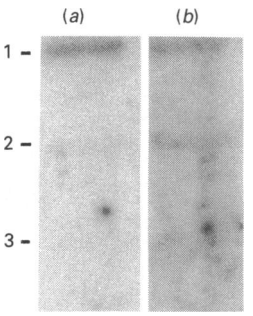

Fig. 8. Run-off experiments performed on nuclei isolated from hyperconfluent cultures (4×10^6 cells) of strain Mo.. untreated (*a*) or treated for 48 h with EGF (25 ng/ml, *b*).

The dotted DNAs (500 ng) are: 1, HF677 for α1(I); 2, COLLK4 for collagenase; and 3, *Hin*dIII-digested λ-phage DNA.

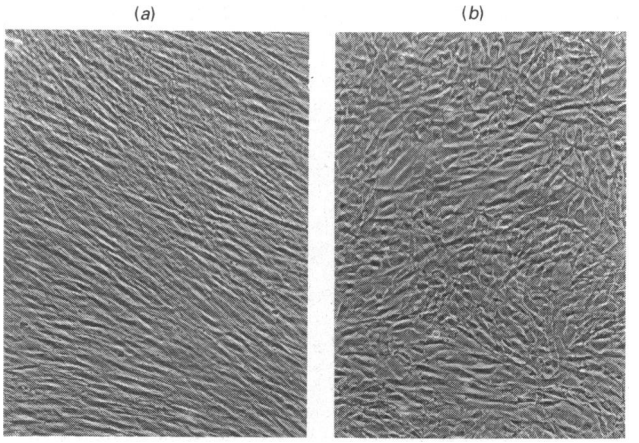

Fig. 9. Fibroblasts in cultures at high confluence (about 4×10^6 cells per dish, strain Mo..) obtained by long-term culture (*a*) or by plating a high number of cells (*b*)

Note the difference in morphology.

cellular density increased (Fig. 10*b*). At the highest cell density, a complete lack of response to EGF was noted (Figs. 10*b* and 10*c*). By adding a medium conditioned by high-density seeded cells (4×10^6) to subconfluent cultures grown from low density, an activation of the collagenase gene expression was also induced (results not shown).

DISCUSSION

Although some variations in the extent of the response occurred between the four tested strains of human fibroblasts, all the cells responded in a confluent-dependent way and to EGF by a co-ordinate increase of the mRNAs for tissue procollagenase, prostromelysin and TIMP. The steady-state level of procollagen α1(I) mRNA varied inversely, although not as systematically as in response to cell density. The parallel control of collagenase and stromelysin has been often reported [34] and seems logical, since stromelysin appears as a physiological activator of procollagenase [19]. The co-ordinate regulation of collagenase and one of its specific inhibitors, TIMP, that we and others [35,36] have observed is perhaps also significant. However, procollagenase mRNA is always increased by orders of magnitude more than TIMP. As TIMP only interacts with the activated form of collagenase, this imbalance might be important in determining the extracellular proteolysis. The parallel stimulation of synthesis of metalloproteinases secreted in a latent form and of their specific inhibitors may be potential mechanisms for a subtle and localized control of proteolysis.

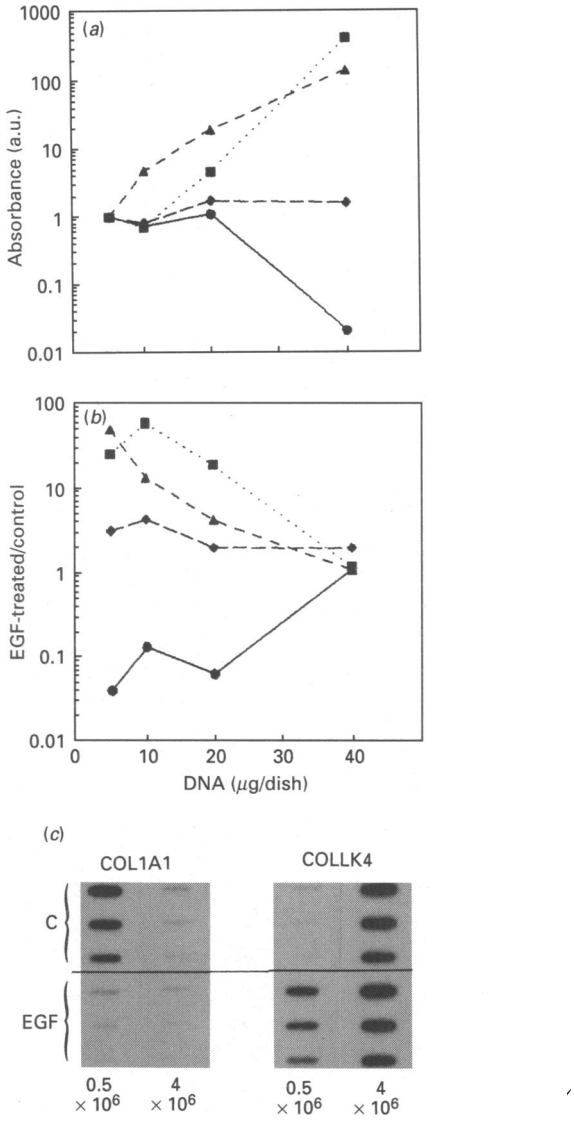

Fig. 10. Effect of confluence induced by plating cells at increasing density on cell response to EGF

Cultures of the strain Mo.. were seeded at increasing density (between 5 and 40 μg of DNA/dish) 2 days before incubation in the absence (*a*) or presence (*b*) of EGF (25 ng/ml). The mRNA in control cultures and in EGF-treated cultures are expressed as in Fig. 4 for α1(I) collagen (●), collagenase (▲), stromelysin (■) and TIMP (◆) mRNA contents. (*c*) As an illustration, representative slot-blot autoradiograms obtained with the α1(I) (COL1A1) and the collagenase (COLLK4) cDNA hybridized with the RNA extracted from cells seeded at 0.5 and 4×10^6 induced ('EGF') or not ('C') with EGF.

The data reported here demonstrate that the expression of three key molecules participating in extracellular-matrix degradation, namely procollagenase, prostromelysin and TIMP, and its modulation by EGF, were strongly dependent upon the state of confluence of the cells and the deposition of their own matrix. The steady-state level of collagenase mRNA in fibroblasts is known to be largely increased by culture in a freely retracting three-dimensional matrix of collagen [37] and down-regulated by mechanical forces [38]. Such regulation might also operate under the experimental conditions used here. Cells in long-term culture are progressively surrounded by an extracellular matrix with which fibroblasts can interact through specific receptors of the integrin family to maintain cells upon mechanical tension. EGF known to decrease cell–substrate adhesion might act by suppressing the mechanical control operated by the matrix.

The stimulating effect of EGF on the steady-state level of mRNAs coding for procollagenase, prostromelysin and TIMP occurred rapidly. Our results suggest that this regulation might operate, at least for collagenase, at a transcriptional level. This response is consistent with a c-*fos*- and c-*jun*-proto-oncogene-mediated stimulation, since these genes are readily induced by EGF and other growth factors [39–41] and were shown to play a critical role in stromelysin and collagenase expression [15,42,43]. The discrepancies observed between the modifications induced by EGF in the steady-state level of collagen and collagenase mRNAs and those recorded during run-off experiments might be related to differences in the respective mRNA stability, but also to an inefficient reinitiation of the transcription *in vitro*. The lag time observed between the mRNA stimulation and the induction of collagenase activity might be, in part, related to protein synthesis and secretion *de novo*. The persistent effect that we observed might potentially be also related to a reorganization of the cytoskeleton promoted by EGF [44,45]. Information is indeed available showing that modifications in the shape of the cells can lead to a stimulation of collagenase production [13,34] and to collagen-synthesis inhibition [46]. However, it has been demonstrated that collagenase production accompanying changes in cell shape occurs only in the presence of a biologically active cytokine, autocrine or paracrine [47]. Our data obtained with high-density-seeded cells showing an obvious altered morphology and secreting a soluble factor able to activate the collagenase gene also support these observations and could explain the high endogenous expression of collagenase under these culture conditions. As high-density-seeded cells are also unable to respond to exogenous EGF, it would suggest that the secreted factor is able to bind to, saturate and/or to down-regulate, the EGF receptors, or that this factor uses intracellular signalling pathways similar to EGF.

Altogether, these results indicate that the conditions of culture resulting in different cell–cell contacts and cell–matrix interactions are of critical importance in the modulation of the response of cells to growth factor.

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