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Collagenase gene expression in fibroblasts is regulated by a three-dimensional contact with collagen

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Collagenase activity in fibroblasts is regulated by cytokines and the interaction with the extracellular matrix. In this study we demonstrate that fibroblasts cultured within a three-dimensional collagen gel show a strong induction of collagenase gene expression. In addition to increased de novo synthesis most of the secreted enzyme was found to be activated leading to a high collagenolytic activity and complete degradation of collagen matrices after removal of fetal calf serum. Collagen I gene expression was found to be reduced under these conditions. These data suggest a specific modulation of cellular metabolism in response to contact with a three-dimensional collagenous matrix resulting in the divergent regulation of collagen and collagenase.

Collagenase synthesis; Collagen gel; Extracellular matrix; Fibroblast

1. INTRODUCTION

Remodelling of the extracellular matrix (ECM) during tissue repair of connective tissue requires controlled degradation and deposition of connective tissue proteins and plays a crucial role. Degradation of collagen is initiated by highly specific metalloproteinases. Products of the initial cleavage can then be attacked by other proteases. Cleavage of interstitial collagens (types I–III) occurs by a distinct metalloprotease, collagenase [1–3]. This enzyme is produced by several cell types including macrophages [4] and fibroblasts [5] and cleaves triple helical collagen such that fragments of one and three quarter length of the entire molecule are released [6]. Since collagenase represents the key enzyme in the degradation of connective tissue, its activity is tightly controlled. It is synthesized as inactive precursor, which can be activated by proteolytic cleavage [7,8]. In addition, several specific inhibitors are known, which are present in tissue and serum [9]. Recently, a clone for human collagenase has been identified and characterized [10,11], which facilitates studies of the expression of collagenase on a molecular level. Collagenase was found to be induced by several cytokines [12–14], and after UV-irradiation or transformation of fibroblasts [15–17]. In this communication we provide evidence that both, collagenase gene expression and activation, depend on the three-dimensional contact of fibroblasts with a collagenous extracellular matrix.

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Abbreviations: PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum

2. METHODS

Fibroblasts were obtained by outgrowth of cells from skin biopsies of healthy human adults. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, sodium ascorbate (50 μg/ml), glutamine (300 μg/ml), penicillin (400 U/ml) and streptomycin (50 μg/ml) in plastic Petri dishes in the moist atmosphere of a CO_2 incubator (5% CO_2, 95% air) at 37°C [18]. Confluent monolayers were propagated by trypsinization (0.1% trypsin, 0.02% EDTA) and replating at 1:2 dilution. For experiments fibroblasts were used in passages 4–8. Collagen gels were prepared as previously described [19,20] by combining 1.6 ml of

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1.5 × concentrated medium containing fetal calf serum with 0.4 ml of collagen solution (type I collagen from calf skin, 3 mg/ml in 0.1% sterile acetic acid) and 0.4 ml of cell suspension (1.5×10^5/ml). For isolation of RNA, 150 mm dishes and 10 times the volume were used, containing 15 mg collagen and 1×10^7 cells per gel [20]. For measuring collagenase activity collagen gels and cells in Petri dishes were washed free of serum with PBS and incubated 12 more hours in DMEM without serum. Medium and cell layer of cell containing collagen gels were separately assayed for the presence of collagenase. Cells or gels were extracted with 300 μl Tris buffer (20 mM Tris, 0.17 M NaCl, 5 mM $CaCl_2$, 50 mM ε-caproic acid, pH 7.6) by freezing and thawing three times, and centrifuged (5 min, 15000 × g). Latent collagenase was activated by trypsin. 120 μl of the supernatant were incubated for 10 min at 37°C with 5 μl of trypsin solution (50 μg/ml in Tris-buffer). The reaction was stopped by adding 5 μl soybean trypsin inhibitor solution (200 μg/ml in Tris buffer). Samples to be analyzed for already active collagenase were prepared by adding trypsin and trypsin inhibitor to another 120 μl supernatant, but in reverse order. Medium was similarly treated but was not frozen and thawed. These samples were serially diluted twofold in Tris buffer and assayed for active collagenase by measuring the release of radioactivity from ^{125}I-collagen type I (labeled by the Bolton and Hunter reagent [21]), attached to the plastic of microtitre plates as previously described [22]. Data recorded are per cent collagen released by 50 μl of 1/10 diluted sample, read from a curve in which collagen degradation was plotted against sample dilution.

For some experiments, 5 kBq of ^{125}I-collagen were mixed into the collagen lattice, and cells were incubated for different time periods in the presence or absence of serum. Medium and collagen lattices were then collected and the radioactivtity was measured in each sample. In addition, both the material released into the medium and the collagen lattices were investigated by SDS-PAGE [23]. The collagen gels were dissolved overnight in cold 0.5% acetic acid, both medium and extract of collagen gels were diluted with an equal volume of double strength sample buffer and applied to the gel. Electrophoresis was carried out on SDS polyacrylamide slab gels (10–18% acrylamide gradient).

Total RNA was prepared from 4 M guanidinium isothiocyanate extracts of fibroblasts from collagen gels and from monolayers [24,25]. Dot blot hybridization was performed as described previously [20] with nick-translated human cDNA probes for collagenase [10] and tubulin [26], following standard protocols. For northern blot hybridization total RNA was separated by electrophoresis on a 1% formaldehyde polyacrylamide gel and transferred to nitrocellulose (BioRad) membranes [27]. Runs were calibrated with 18/28 S RNA (Boehringer).

3. RESULTS

Fibroblasts cultured within three-dimensional collagen gels showed specific reprogramming of biosynthetic activity in response to the environment. After contraction of the gels a strong induction of proteolytic activity was observed. Depletion of serum inhibitors 48 h after seeding fibroblasts into the gels resulted in slow destruction of the gels and liberation of cells. Radioactively labeled col-

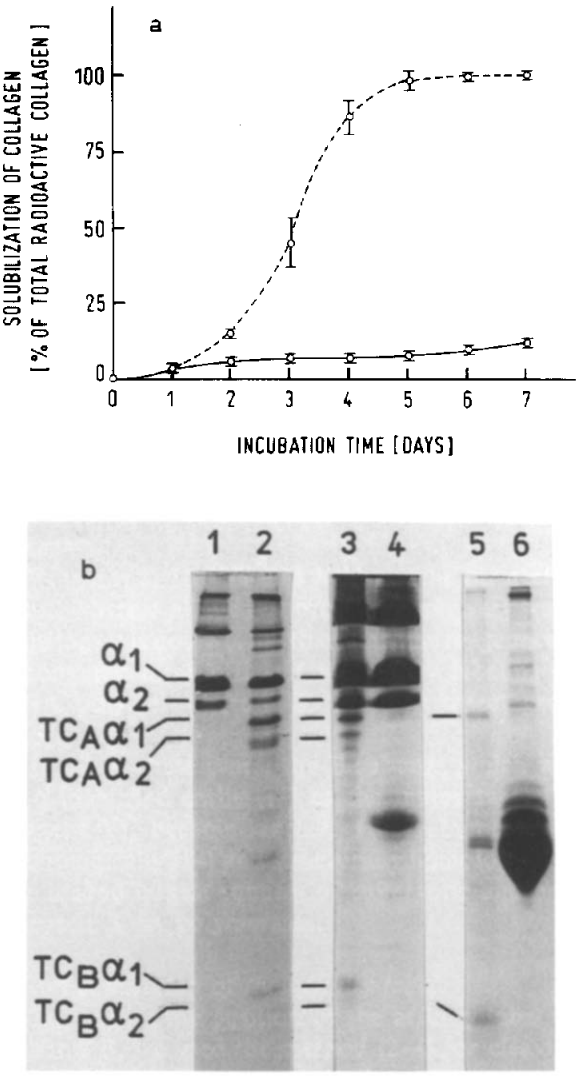

Fig.1. Degradation of ^{125}I-labeled type I collagen by fibroblasts during culture in three-dimensional collagen gels. Human skin fibroblasts were seeded into three-dimensional collagen gels. After 48 h culture the medium was removed, the gels were washed serum-free and incubated in DMEM. In controls the medium was replaced by fresh serum containing DMEM. At day one to seven medium and gels were separated, the gels were washed once with PBS and the radioactivity in the medium and collagen gels was measured using a gamma-counter. Degradation is expressed as per cent radioactivity released into the medium in the absence (dotted line) or presence (solid line) of fetal calf serum (a). Each point represents the average of triplicate estimations. Aliquots of the collagen gels (3,4) and the medium (5,6) after 5 days incubation were analyzed by SDS-PAGE (10–18% acrylamide) under reducing conditions (b). For identification of collagen fragments collagen type I (1) was partially degraded by fibroblast collagenase (2).

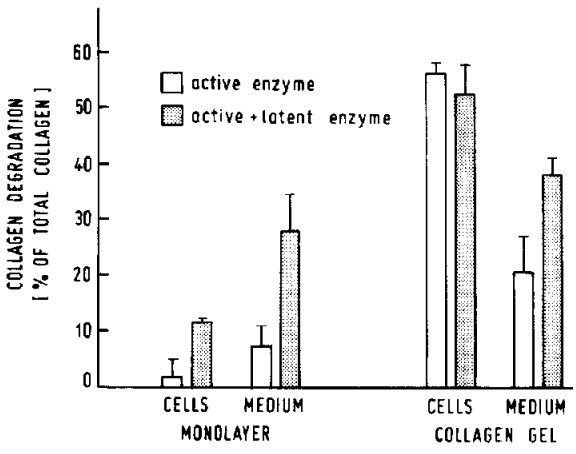

Fig.2. Activity of collagenase in fibroblasts cultured for 48 h as monolayers or within three-dimensional collagen gels. Fibroblasts were seeded into Petri dishes or into collagen gels (2×10^5 cells). After 48 h cell layer and collagen gels were washed serum-free twice with PBS and incubated for 24 h in DMEM containing all supplements but no serum. After the incubation period medium and cells or collagen gels were collected separately. For measurement of the activity of collagenase, cells and collagen gels were extracted with Ca^{2+} containing Tris buffer. Collagenase activity was measured by determining release of radioactivity from ^{125}I-collagen type I adsorbed to plastic microtitre plates. Samples were assayed with or without activation of procollagenase by trypsin (50 μg/ml, 10 min, 37°C). Data recorded are per cent collagen released by 50 μl of 1/10 diluted sample after incubation for 16 h at 27°C (100% is approx. 50 ng collagen).

lagen gels were dissolved, releasing more than 95% of the radioactivity into the medium within 5 days (fig.1a). Electrophoretic analysis revealed the presence of collagen fragments of three quarters (TC_A) and one quarter (TC_B) lengths of the molecules (fig.1b). Presence of serum prevented degradation of the collagen gels.

For quantification of collagenase activity, proteins were extracted from medium and from cells in Petri dishes or collagen gels. Incubation of fibroblasts in collagen gels for 48 h resulted in increased total activity of the enzyme compared to monolayer cultures. The activity was enhanced, both in medium and in the collagen gels, but much more activity was found in the gel than in the medium. In addition, most of the enzyme was present in active form in the gel system whereas in monolayer culture only poor activation of the enzyme had occurred (fig.2).

Dot blot hybridization of serial dilutions of cytoplasmic RNA with various cDNA probes allowed quantification of the mRNA levels encoding the proteins including mammalian collagenase. The data obtained provided evidence that increased enzyme activity measured at the protein level in fibroblasts cultured within collagen gels originated from elevated levels of collagenase mRNA (fig.3a). Induced gene expression of collagenase was found in the gels already after 9 h. The mRNA levels were

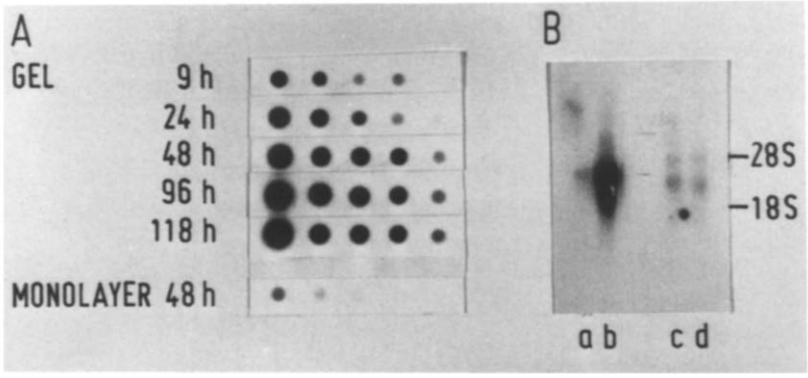

Fig.3. Expression of collagenase by cells in collagen gels or monolayers. Quantification of cytoplasmic collagenase and tubulin mRNA levels in fibroblasts cultured in monolayer and in three-dimensional collagen gels. 0.185, 0.37, 0.75, 1.5 and 3 μg of total RNA isolated from fibroblasts grown for different times in monolayer culture and contracted collagen gels were blotted onto nitrocellulose and hybridized with ^{32}P-labeled nick-translated human collagenase and tubulin cDNA probes (A). For northern blot analysis, 2 μg of total RNA were separated in a 1% denaturing formaldehyde agarose gel, blotted and hybridized with the radioactively labeled cDNA probes specific for human collagenase (a,b) and tubulin (c,d). Positions of 18 S and 28 S RNA markers are shown at right (B).

Table 1

Quantification of mRNA levels for collagenase and tubulin in cells cultured for various lengths of time as monolayers or within three-dimensional collagen gels

Cells in	mRNA for	
	Collagenase	Tubulin
Monolayer, 48 h	1.0	1.0
Collagen gel, 9 h	3.1	1.0
24 h	6.5	1.0
48 h	23.0	1.0
96 h	28.0	1.0
118 h	30.0	1.0

Expression of collagenase in three-dimensional collagen gels as function of time. Fibroblasts were cultured within three-dimensional collagen gels and total RNA was isolated as described in section 2. Serial dilutions (0.185, 0.37, 0.75, 1.5 and 3 μg) of total cytoplasmic RNA were dotted onto nitrocellulose. Filters were hybridized after baking with ^{32}P-labeled nick-translated cDNA probes. After autoradiography blots were quantitated by densitometry

maximal after 4 days with 30 times the amount found in confluent monolayers (table 1). In addition, production of collagenase mRNA was induced specifically since tubulin mRNA was synthesized after contraction of the gels in constant amounts. Specificity of induction was further demonstrated by northern blot analysis where strong induction of the 4.1 kb mRNA species to eight times the values of monolayer culture was noticed in collagen gels whereas synthesis of the two mRNA species for tubulin was not affected by both different culture conditions (fig.3b).

4. DISCUSSION

Collagenase activity in human skin fibroblasts is closely controlled and depends on the influence of cell growth and cell-cell contacts [29]. It can also be regulated by several cytokines which play important roles during wound healing and inflammation [12–14]. Control is exerted at various levels during biosynthesis of collagenase and extracellularly by regulation of the activation of the latent enzyme. We show in this study that the three-dimensional contact of fibroblasts with collagen fibrils results in induced gene expression and at the same time activation of procollagenase. As shown before, culture of human skin fibroblasts within a three-dimensional collagen gel induces characteristic changes of several cellular functions [20]. The specificity of this process is indicated by the unaltered levels of mRNA for tubulin, fibronectin and type VI collagen [20,30], whereas mRNA levels for interstitial collagens I and III are strongly reduced. Induction of collagenase gene expression was much more rapid than the other events described previously [20] and collagenase mRNA was found to be elevated already after 4 h. It is not clear yet, whether this is entirely due to enhanced transcription or whether it also involves an alteration of the mRNA half-life, but there is certainly increased translation and secretion of the enzyme. Furthermore, the released enzyme is activated which indicates that proteases which are required for processing of procollagenase are also present during reprogramming of the fibroblast metabolism in response to contact with collagen fibrils. Similar observations have been previously reported when soluble collagens had been added to the culture medium of synovial cells [28]. Most of the activated enzyme was found in the matrix and not in the culture medium as in monolayer culture, probably due to the high affinity of activated collagenase to collagen fibrils [31]. Divergent regulation of collagen and collagenase gene expression may not only result from cellular contact with fibrillar collagen but has also been observed after treatment with TNF or IF-γ [32] and in rabbit synovial fibroblasts under certain culture conditions [33]. The divergent regulation of collagen and collagenase gene expression could play an important role during remodelling of connective tissue where rapid removal of collagen fibrils is necessary.

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