

Distinct patterns of collagen gene expression are seen in normal and keloid fibroblasts grown in three-dimensional culture

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Summary

The purpose of this study was to examine collagen gene expression in various types of scar fibroblasts as well as normal fibroblasts in a novel three-dimensional culture system and to compare them with those in a monolayer culture system. Cells in three-dimensional culture formed multiple layers within the self-produced dense extracellular matrix and formed a dermis-like structure. In monolayer culture, both normal and scar fibroblasts continued to express high levels of mRNA for pro α 1(I) and pro α 1(III) collagens. However, in three-dimensional culture, the mRNA levels gradually declined in normal fibroblasts. In contrast, mRNA levels remained high in keloid and hypertrophic scar fibroblasts. Atrophic scar fibroblasts demonstrated similar changes to normal fibroblasts in three-dimensional culture. When we compared mRNA expression in fibroblasts from the centre and the edge of hypertrophic scar, cells from the centre showed a persistently decreased level of collagenase mRNA expression. These results suggest that the mRNA expression pattern of pro α 1(I) and pro α 1(III) collagens varies depending on the culture system. Fibroblasts from keloids and hypertrophic scar may have a defective system of down-regulation in extracellular matrix metabolism.

Fibroblasts play important parts in the production and degradation of extracellular matrix (ECM), being in the maintenance of both the physiological properties and the structure of the tissue. As fibroblasts *in vivo* are surrounded by ECM proteins, fibroblasts in conventional monolayer culture are considered to have adapted to their artificial conditions, which are quite different from the *in vivo* situation. Nevertheless, monolayer culture has commonly been used in scientific studies because of its simplicity. Attempts have been made to establish *in vitro* culture systems that mimic tissue-like structures such as collagen gel,¹ but the environmental conditions thus created are still artificial in that the fibroblasts are embedded in artificial collagens. Recently, we reported a novel fibroblast culture system supplemented with L-ascorbic acid 2-phosphate (Asc 2-P).² The addition of Asc 2-P to the conventional culture medium induced dermal fibroblasts to form a dermis-like structure via the accumulation of collagens without any need to pretreat the plastic dish. In this culture system, cells form multiple layers within the self-produced dense ECM. We ascertained that the principal disaccharide units and neutral insoluble collagens produced by normal human dermal fibroblasts in this culture system were similar to those found in normal human dermis.^{2,3} Using electron

microscopy, we also revealed an accumulation of mature collagens in the extracellular space.²

Previous studies have demonstrated increased levels of mRNA for pro α 1(I) and pro α 1(III) collagens,^{4–7} an increased type I/III procollagen mRNA ratio⁴ and decreased expression of collagenase^{8,9} in hypertrophic scar or keloid fibroblasts in monolayer culture systems. As our novel three-dimensional culture system provides culture conditions that are more physiological for fibroblasts, studies of the molecular biological aspects of this culture system are of great interest. In this study, we investigated the expression of the genes for pro α 1(I) and pro α 1(III) collagens in normal human dermal fibroblasts in three-dimensional culture and compared it with gene expression in monolayer culture. We also examined the expression of these genes in various types of scar fibroblasts.

Materials and methods

Cell cultures

Normal fibroblasts were obtained from the skin of five normal individuals of varying ages. Keloid, hypertrophic scar and atrophic scar tissue specimens were obtained by skin biopsy or surgical removal after obtaining

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Table 1. Profile of tissue specimens

Specimens	Age/sex	Site	Duration of disease (years)
Normal controls	1 M	Abdomen	
	3 M	Lower leg	
	24 F	Face	
	38 F	Forearm	
	75 F	Groin	
Keloid			
K1	64 F	Chest	15
Hypertrophic scars			
	H1	58 F	Back
H2	52 M	Nape	5
Atrophic scars			
	A1	24 F	Forearm
A2	30 M	Chest	20

informed consent. The profile of the fibroblast strains used in this study is given in Table 1. In hypertrophic scar tissue, fibroblasts were sampled from the centre and the edge of the lesion. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Cells in the primary cultures were trypsinized and expanded in the same medium. For experiments, cells between passages 3 and 6 were grown in DMEM supplemented with 10% FCS in the presence or absence of 1 mmol/L Asc 2-P (Wako Pure Chemical Industries, Osaka, Japan) in 10 cm plastic dishes (5×10^5 cells/dish) and harvested for RNA extraction at the indicated times. Fibroblasts from keloid, hypertrophic scar and atrophic scar were harvested at the indicated time points up to 2.5 weeks, because the cell layer of these cells began to contract earlier than 4 weeks in three-dimensional culture.²

RNA extraction and Northern blot analysis

Cells were washed with cold phosphate-buffered saline and harvested. Total RNA was isolated by a single-step method¹⁰ using a commercially available acid guanidinium thiocyanate-phenol extraction reagent (Isogen, Nippongene, Toyama, Japan). Aliquots of 10 µg of total RNA denatured in formaldehyde were electrophoresed in 1.2% agarose-1.1 mol/L formaldehyde gels. The quality of RNA samples was checked by staining with ethidium bromide to visualize the 18S and 28S ribosomal RNA subunits under UV light. The RNA was then transferred on to a nylon membrane and cross-linked by exposure to 120 mJ/cm² of 312 nm UV radiation in a spectro UV cross-linker (Spectronics Corporation,

Table 2. mRNA levels of pro α 1(I) and pro α 1(III) collagens in normal skin fibroblasts obtained from a 24-year-old normal individual

	Incubation time (weeks)	Pro α 1 (I) Pro α 1 (III)	
Monolayer culture	1	100	100
	2	113 \pm 7	87 \pm 7
	3	115 \pm 24	103 \pm 24
	4	112 \pm 30	74 \pm 30
Three-dimensional culture	1	279 \pm 93	168 \pm 75
	2	283 \pm 69	157 \pm 84
	3	66 \pm 1	50 \pm 50
	4	58 \pm 8	19 \pm 9

The intensities of the mRNA bands were determined densitometrically. The quantity of each mRNA was normalized for the quantity of G3PDH mRNA. The ratio of each mRNA to G3PDH mRNA at 1 week in monolayer culture was set at 100. Experiments were replicated three times. Values are means \pm SEM. Statistical analyses revealed that mRNA levels of pro α 1(I) and pro α 1(III) collagens differed significantly between monolayer and three-dimensional culture ($P = 0.0001$ and $P = 0.0008$ respectively).

Westburg, NY, U.S.A.). Filters were hybridized to probes labelled with [α -³²P]dCTP by the random priming method (Gibco BRL, Gaithersburg, MD, U.S.A.). Hybridization was performed at 42 °C in a specific activity of at least 2×10^7 c.p.m./µg. After hybridization, the filters were washed twice at room temperature in $2 \times$ SSPE/0.1% sodium dodecyl sulphate (SDS) for 10 min and twice at 65 °C in $1 \times$ SSPE/0.1% SDS for 20 min, followed by two washes at 6 °C in $0.1 \times$ SSPE/0.1% SDS for 20 min. The data were scanned and densitometrically analysed with a Fujix Bas 2000 bioimaging analyser (Fuji, Kawasaki, Japan). Statistical analyses were performed using two-factor analysis of covariance.

The following human sequence-specific cDNAs were used for hybridization: a 1.4-kb-long cDNA, Hf 677-6, for pro α 1(I) collagen mRNA, a 0.9-kb-long cDNA, pH, for pro α 1(III) collagen mRNA, a 0.7-kb-long cDNA, K4, for collagenase (kindly provided by Dr A.Hatamochi),¹¹ a 0.6-kb-long cDNA, pBluescript, for tissue inhibitor of metalloproteinase-1 (TIMP-1) mRNA (kindly provided by Dr H.Sato),¹² and a 1.1-kb-long cDNA for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA purchased from Clontech (Palo Alto, CA, U.S.A.).

Results

Collagen mRNA levels in normal fibroblasts in monolayer and three-dimensional cultures

The levels of mRNA at 1 week were higher in

Table 3. mRNA levels of pro α 1(I) collagen in normal skin fibroblasts from four normal individuals

	Incubation time (weeks)	Age/sex			
		1/M	3/M	38/F	75/F
Monolayer culture	1	100	100	100	100
	2	98	531	718	126
	3	118	715	813	91
	4	105	1111	678	85
Three-dimensional culture	1	79	447	509	133
	2	72	523	575	133
	3	47	707	528	92
	4	14	371	345	79

Each mRNA level was expressed as in Table 2.

Table 4. mRNA levels of pro α 1(III) collagen in normal skin fibroblasts from four normal individuals

	Incubation time (weeks)	Age/sex			
		1/M	3/M	38/F	75/F
Monolayer culture	1	100	100	100	100
	2	131	556	200	126
	3	268	562	228	99
	4	195	448	206	103
Three-dimensional culture	1	150	793	183	107
	2	56	745	200	105
	3	34	718	201	98
	4	12	275	169	78

Each mRNA level was expressed as in Table 2.

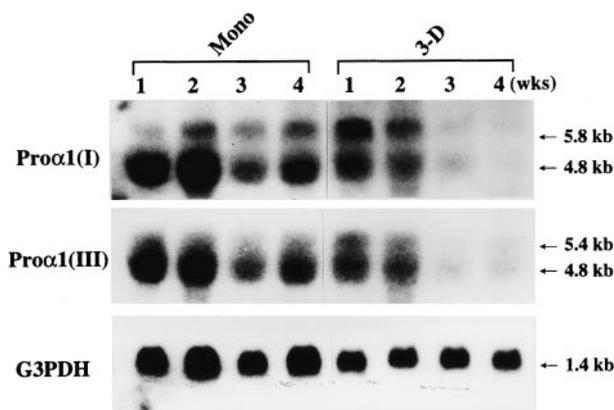


Figure 1. Northern blot analysis of pro α 1(I) collagen, pro α 1(III) collagen and G3PDH mRNA in normal fibroblasts obtained from a 24-year-old woman. Total RNA was isolated from monolayer and three-dimensional cultures after 1, 2, 3 and 4 weeks' incubation.

three-dimensional culture than in monolayer culture in cells from all but one case (1-year-old boy, pro α 1(I) collagen). Table 2 shows the results of three separate experiments on normal control cells from a 24-year-old woman. Other cells demonstrated similar expression patterns (Tables 3 and 4). mRNA levels for both pro α 1(I) and pro α 1(III) collagen gradually declined in three-dimensional culture, whereas they increased or remained almost unchanged in monolayer culture. The result from a representative blot (24-year-old woman) is shown in Fig. 1. The expression patterns of collagenase and TIMP mRNA varied among normal human dermal fibroblasts both in monolayer and in three-dimensional cultures, but expression was detectable at all incubation times.

Table 5. mRNA levels of pro α 1(I) and pro α 1(III) collagens in atrophic scar fibroblasts in three-dimensional culture

Incubation time (weeks)	Pro α 1 (I)		Pro α 1 (III)	
	A1	A2	A1	A2
1	100	100	100	100
1.5	99 \pm 38	159 \pm 31	87 \pm 43	108 \pm 2
2	67 \pm 9	120 \pm 14	61 \pm 11	101 \pm 3
2.5	65 \pm 17	128 \pm 9	54 \pm 27	78 \pm 13

Each mRNA level was expressed as in Table 2. Experiments were replicated three times. Values are means \pm SEM. Cell strains A1 and A2 are described in Table 1.

Collagen mRNA levels in keloid, hypertrophic scar and atrophic scar fibroblasts

In monolayer culture, keloid and scar fibroblasts from different origins revealed patterns similar to normal fibroblasts. In three-dimensional culture, both cell strains (A1 and A2) from atrophic scars exhibited an expression pattern similar to that of normal fibroblasts (Table 5). In contrast, cells from hypertrophic scars showed a steady increase in mRNA expression of pro α 1(I) and pro α 1(III) collagens. In keloid fibroblasts, mRNA levels reached a peak at 2 weeks and rapidly declined by 2.5 weeks (Fig. 2 and Table 6).

Collagenase and TIMP-1 mRNA levels in hypertrophic scar fibroblasts

We compared the mRNA expression of collagenase and TIMP in fibroblasts from the centre and edge of hypertrophic scars from the same individuals (Table 7). The levels of collagenase mRNA expression were always low in cells from the centre, but in cells from the edge

Table 6. mRNA levels of pro α 1(I) and pro α 1(III) collagens in keloid and hypertrophic scar fibroblasts in three-dimensional culture

Incubation time (weeks)	Pro α 1 (I)			Pro α 1 (III)		
	Keloid	HS		Keloid	HS	
		H1	H2		H1	H2
1	100	100	100	100	100	100
1.5	583 \pm 156	159 \pm 41	108 \pm 14	429 \pm 92	173 \pm 34	106 \pm 19
2	1396 \pm 59	182 \pm 56	117 \pm 22	1201 \pm 137	189 \pm 37	105 \pm 20
2.5	519 \pm 130	199 \pm 34	159 \pm 26	563 \pm 214	214 \pm 27	111 \pm 25

Each mRNA level was expressed as in Table 2. Keloid and hypertrophic scar fibroblasts were obtained from the centre of the lesions. Experiments were replicated three times. Values are means \pm SEM. HS, hypertrophic scar. Cell strains of keloid, H1 and H2 are described in Table 1.

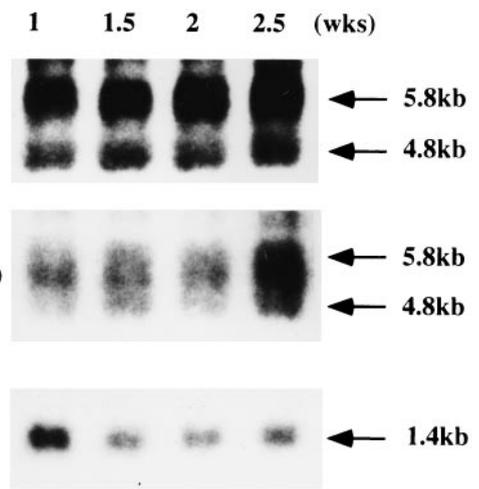


Figure 2. Northern blot analysis of pro α 1(I) collagen, pro α 1(III) collagen and G3PDH mRNA in keloid fibroblasts in three-dimensional culture. Total RNA was isolated after 1, 1.5, 2 and 2.5 weeks' incubation.

remained relatively high for 1.5 or 2 weeks before eventually decreasing. The expression of TIMP-1 mRNA was inconsistent.

Discussion

In this study, we focused on the expression of collagen mRNA in fibroblasts grown in a novel three-dimensional culture system. We found that in normal fibroblasts mRNA levels for pro α 1(I) and pro α 1(III) collagen remained high in monolayer culture, whereas they gradually decreased in three-dimensional culture. The accumulation of collagens and other ECM proteins around fibroblasts in three-dimensional culture may partly account for this observation. Similar results were obtained using a collagen gel, although the harvest time was different because collagen gels contract

Table 7. mRNA levels of collagenase and TIMP-1 in the edge and centre of hypertrophic scar fibroblasts in three-dimensional culture

Site of the lesion	Incubation time (weeks)	Collagenase		TIMP-1	
		H1	H2	H1	H2
Edge	1	100	100	100	100
	1.5	188 ± 89	89 ± 1	179 ± 74	131 ± 88
	2	121 ± 70	62 ± 11	158 ± 36	188 ± 30
	2.5	76 ± 77	53 ± 9	141 ± 37	321 ± 175
Centre	1	74 ± 9	17 ± 24	151 ± 96	119 ± 95
	1.5	93 ± 60	24 ± 46	191 ± 96	85 ± 73
	2	95 ± 72	29 ± 43	311 ± 162	152 ± 73
	2.5	97 ± 81	29 ± 35	293 ± 74	160 ± 87

Each mRNA level was expressed as in Table 2.

Experiments were replicated three times. Values are means ± SEM.

Statistical analyses revealed that mRNA levels of collagenase differed significantly between the edge and the centre in H2 ($P=0.0001$) but not in H1 ($P=0.1415$). TIMP-1 mRNA levels were not significantly different between the edge and the centre in either H1 or H2 ($P=0.0916$ and $P=0.1303$ respectively).

rapidly.¹ For example, Mauch *et al.*¹ compared mRNA levels for pro α 1(I) and pro α 1(III) collagens in monolayer and contracted collagen gels after 48 h incubation. They also reported that collagen synthesis by *in vivo* fibroblasts from normal skin was lower than in monolayer cultures. Our results suggest that fibroblasts in three-dimensional culture may adapt to their microenvironment, as occurs *in vivo*, and there may be a close interaction between fibroblasts and ECM.¹³

It is of note that in monolayer culture we could observe no significant difference in the expression pattern of collagen mRNA among fibroblasts of different origins. The most striking feature in three-dimensional culture was the fact that collagen mRNA expression was not decreased but maintained at a high level in keloid and hypertrophic scar fibroblasts. Keloid fibroblasts exhibited an apparent peak at 2 weeks and the mRNA levels fell rapidly by 2.5 weeks. This change might be associated with the contraction of the cell layer at 2.5 weeks.

Increased levels of mRNA for pro α 1(I) and pro α 1(III) collagens in hypertrophic scar and keloid fibroblasts have been demonstrated in monolayer culture.^{4,5} Furthermore, decreased expression of collagenase in hypertrophic scars and other fibrotic diseases has been reported.^{8,9,14} In these regards, our results of three-dimensional culture accord well with previous results. We observed that, in three-dimensional culture, the cell layer of scar fibroblasts contracted faster than that of normal fibroblasts. At present, we do not have enough data to explain this phenomenon. However, our preliminary experiments demonstrated a significant

increase in collagenase mRNA expression in scar fibroblasts at the time of or just before contraction without apparent change in the level of TIMP mRNA.

Northern blot analysis revealed two mRNA species of pro α 1(I) and pro α 1(III) collagens. These bands changed in parallel in both culture systems. It is of interest that in three-dimensional culture keloid fibroblasts showed a relative increase in the upper band (5.8 kb) at the expense of the lower band (4.8 kb) of pro α 1(I) compared with normal fibroblasts (Fig. 2). The biological functions of the different mRNA species are still unknown;¹ however, abnormal collagen synthesis in keloid might be in part caused by the altered expression of different collagen mRNA species. To confirm this, further studies are required.

This is the first study demonstrating that long-term incubation in three-dimensional culture decreases collagen mRNA expression in normal and atrophic scar fibroblasts but not in hypertrophic scar or keloid fibroblasts. The excessive collagen deposition that occurs in keloid or hypertrophic scars could in part be explained by increased collagen gene expression and decreased collagenase gene expression. There may be a defect(s) in the negative feedback mechanism of keloid and hypertrophic scar fibroblasts. Recently, it was reported that collagen and collagenase gene expression are differentially regulated by integrins.¹⁵ Although we have not studied integrins, it is possible that abnormality of integrin expression or signal transduction by integrins is related to the altered ECM metabolism in keloid or hypertrophic scar.

It is well recognized that fibroblast functions are finely regulated by ECM and cytokines.¹³ The mechanism of abnormal expression of collagen and collagenase genes remains to be elucidated. In this context, three-dimensional culture would be a better *in vitro* tool to investigate connective tissue metabolism by producing *in vitro* conditions that are more physiological for fibroblasts.

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