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IL-1 Rescues Scleroderma Myofibroblasts from Serum-Starvation-Induced Cell Death

Theresa Z. Kirk and Maureen D. Mayes[1]
Rheumatology Division, Wayne State University School of Medicine, Detroit, Michigan 48201

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Normal senescent adult human fibroblasts are relatively insensitive to the effects of serum deprivation in culture. In contrast, the myofibroblast phenotype, which is predominant in scleroderma-affected skin and cultured fibroblasts from scleroderma-affected skin, shows signs of deterioration within 3 days of serum starvation. The addition of IL-1 (5 ng/ml) prevents this deterioration. We propose that IL-1 is a factor promoting myofibroblast longevity with consequent fibrosis in scleroderma skin. © 1999 Academic Press

The myofibroblast is a fibroblast phenotype common to wound healing and fibrosis and characterized by the expression of α-smooth muscle actin (α-SM actin) (for review, see Ref. 1). In the process of wound healing, myofibroblasts disappear by apoptosis when wound closure is complete (2). In contrast, myofibroblasts appear to persist in fibrocontractive diseases, such as scleroderma (3). The continued presence of myofibroblasts in the fibrotic lesion has been proposed to be the result of a lack of a factor(s) which induce apoptosis or cell death and/or the continued presence of factor(s) which enable myofibroblast survival.

Growth factors act by stimulating cell proliferation and/or preventing cell death. IL-1 is a pleiotropic cytokine, synthesized primarily by monocytes and macrophages, which has been demonstrated to induce apoptosis in some cell types while increasing cell viability in others. Stimulated monocytes from scleroderma patients release IL-1 in greater amounts than do monocytes from controls (4, 5). Monocytic infiltration is seen in scleroderma skin (6) and scleroderma fibroblasts have been demonstrated to be more sensitive to the effects of IL-1β than normal fibroblasts (7). In this study we investigated the effect of IL-1 on the viability of cultured (myo) fibroblasts from normal human adults and from affected skin of scleroderma patients.

[1] To whom correspondence should be addressed. Fax: (313) 577-1938. E-mail: mmayes@oncgate.roc.wayne.edu.

METHODS

Patient selection. Patients were diagnosed with scleroderma according to ACR criteria. Normal volunteers served as controls.

Fibroblast cultures. Fibroblasts were grown out from skin punch biopsies from the forearm. The growth medium was DMEM (Gibco) supplemented with glutamine and penicillin/streptomycin. Fibroblasts were routinely passaged 1:2. Additional controls were fibroblasts derived from unaffected scleroderma skin and neonatal fibroblasts. Neonatal foreskin fibroblasts were purchased from Clonetics and cultured following the methods suggested by Clonetics.

Cytokines and antibodies. Human recombinant IL-1 β was purchased from Pharmingen and human recombinant TGFβ_1 from Boehringer-Mannheim. Monoclonal antibody against α-SM actin was purchased from Boehringer-Mannheim and monoclonal antibody against human N-cadherin (13A9) was kindly provided by Margaret Wheelock, Medical College of Ohio (Toledo OH), and Karen Knudsen, Lankenau Medical Research Center (Wynnewood, PA).

Western blotting. Equivalent amounts of cells were plated in each well of a 6- or 12-well culture plate and used when confluent. For serum-free conditions, cells were rinsed once with DMEM. After the experimental incubation period, lysates of fibroblast cultures were prepared by removing the cell media and rinsing the cell layer twice with PBS, adding boiling lysis buffer to the cell layer and scraping the bottom of the well. Lysates were transferred to Eppendorf tubes and boiled an additional 5 min. The lysis buffer consisted of 0.25 M Tris, 4% SDS, 10% glycerol. Lysates were stored at −60°C until use. Mercaptoethanol was added to a final concentration of 5% in thawed lysates. Concentrated bromophenol blue was also added to the lysates and the lysates boiled for an additional five minutes before loading on the gel. Equivalent amounts of protein (approximately 5 μg) were loaded on each lane of a 7.5 or 10% polyacrylamide gel. Western blotting for α-SM actin and N-cadherin was performed according to standard methods. Immunoreactive protein bands were detected using the Amersham ECL kit.

Determination of cell number. An equivalent number of cells were plated in each well of a 96-well plate. At least three wells were used for each experimental condition. Cells were used when confluent. The cells used in serum-free conditions were rinsed once with DMEM before adding the experimental medium. After the experimental incubation period cell number was estimated by the MTT assay essentially as described by Mosmann (8).

RESULTS

IL-1 preserves scleroderma fibroblast morphology. Preliminary experiments showed that scleroderma (myo)fibroblasts grown under serum-free conditions

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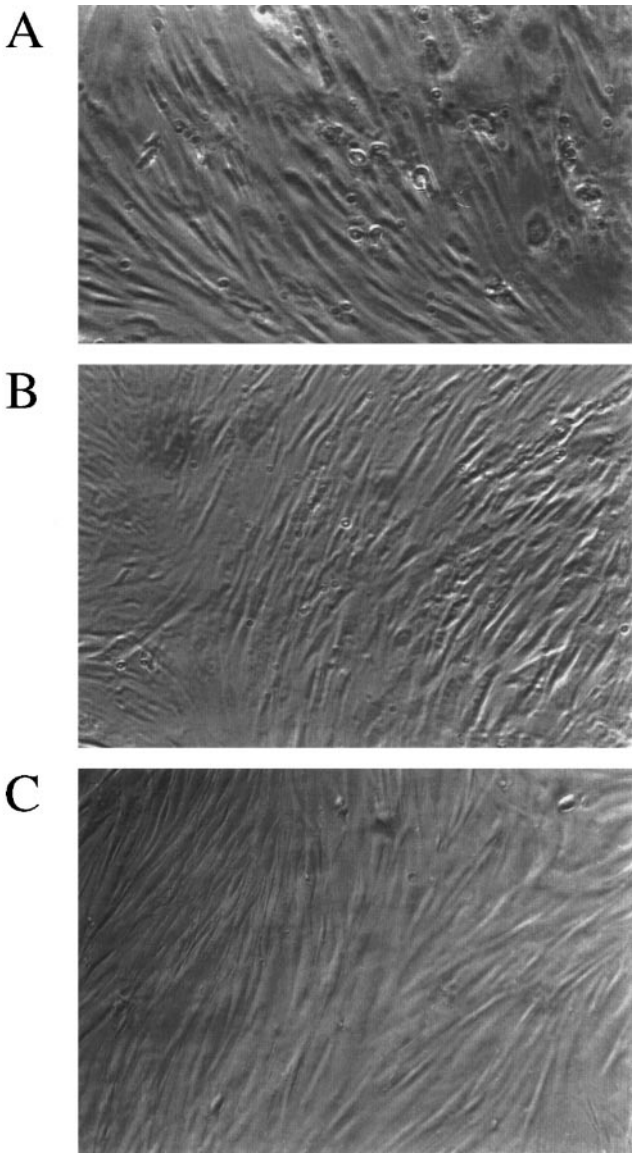

FIG. 1. Photomicrographs of confluent scleroderma fibroblasts after 3 days incubation with (A) serum-free media, (B), serum-free media with 4 ng/ml IL-1β, or (C) growth media with 10% serum.

(DMEM media supplemented with 2 mg/ml BSA) showed signs of deterioration (see Fig. 1). Cells rounded up and there were numerous small vesicles floating in the media. When IL-1 β (4 ng/ml) was added to the media fibroblasts assumed the more characteristic spindle shaped morphology. They more closely resembled cells grown in 10% serum–DMEM for 3 days. In contrast, most cultures derived from normal individuals did not show any signs of deterioration after incubation in serum-free media.

IL-1 enhances viability of scleroderma fibroblasts. The relative number of living fibroblasts present after three days incubation with 5 ng/ml IL-1β in serum-free media was generally higher than the number of cells incubated in the absence of IL-1 addition (Table 1). Scleroderma cell lines in general showed a greater difference in cell number with IL-1 addition. Some normal fibroblast strains (N1, N3, and neonatal fibroblasts) did not show any difference in cell number with IL-1. In contrast, cell strain N5 showed a dramatic difference in relative number of living cells incubated with and without IL-1. IL-1α, used in some experiments, produced results similar to those obtained with IL-1β which is to be expected since both forms of IL-1 bind to the IL-1 type I receptor.

TABLE 1

Relative Ratio of Cell Number with IL-1 Incubation Compared to Serum-Free Control

Scleroderma	Unaffected scleroderma	Controls
	3-Day incubation	
S3 1.13**	S10 1	N1 1.03
S4 1.30**	S11 1.07	N2 1.16*
S5 1.19**		N3 1.03
S6 1.13**		N4 1.12*
S7 1.25**		N5 1.72**
		Neonatal 1
	7-Day incubation	
S2 1.63**		
S4 1.60**		

* $P < 0.05$.
** $P < 0.01$.

In one experiment, the incubation time was extended to 7 days. The ratio of living cells in IL-1 treated cultures/living cells in control cultures was even greater after seven days incubation (Table 1). Also, the appearance of cells grown in serum-free media for 7 days showed increased deterioration compared to cells examined after three days while cells cultured with IL-1 maintained a normal morphology (data not shown).

IL-1 enhances viability of scleroderma fibroblasts cultured in 10% serum. Scleroderma fibroblasts grown in 10% serum–DMEM also showed morpho-

TABLE 2

Relative Ratio of Cell Number with IL-1 Compared to 10% Serum Control

Scleroderma	Unaffected scleroderma	Controls
	5-Day incubation	
S1 1.19*	S10 1	N1 1.01
S2 1.29*	S11 1.25*	N2 1.1*
S3 1.25*		
S4 1.19*		
S5 1.20*		

* $P < 0.01$.

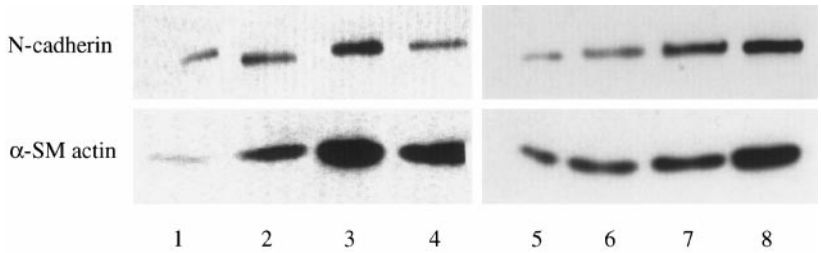

FIG. 2. Western blot for N-cadherin and α-SM actin. Lysates of scleroderma fibroblasts incubated for three days with serum-free media (1), serum-free media plus 5 ng/ml IL-1β (2), serum-free media and 2 ng/ml TGF-β (3), and media with 10% serum (4). Lysates of N5 control fibroblasts incubated for 3 days in serum-free media (5), serum-free media with 0.5 ng/ml IL-1β (6), 1 ng/ml IL-β (7), and media with 10% serum (8).

logical signs of cell loss in culture. Cells became rounded up and vesicles appeared in the media. This usually became more apparent in later passages. However, IL-1 restored viability even in these late passage (p15 or greater) fibroblasts (data not shown). Therefore, confluent fibroblast cultures were also incubated in 10% serum–DMEM (growth medium) with and without 5 ng/ml IL-1 β. Cell number was greater with the addition of IL-1β in scleroderma fibroblast cultures (Table 2).

IL-1 addition enhances expression of myofibroblast α-SM actin and N-cadherin. Fibroblast cultures consist of heterogeneous populations consisting of some combination of fibroblast and myofibroblasts (9, 10). To estimate the relative effect of IL-1 β on the myofibroblast population we made use of two protein markers of myofibroblasts: the classic myofibroblast marker (α-SM actin) and N-cadherin, whose expression levels appear to correlate with expression of α-SM actin (11, 12). Without serum both α-SM actin and N-cadherin levels tended to be lower than with 10% serum included in the media. The addition of IL-1 β to the serum-free media resulted in increased levels of these two proteins approaching that seen with 10% serum. Results from a typical scleroderma cell line are shown in Fig. 2, lanes 1–4.

TGF-β, a component of serum, is capable of transforming fibroblasts into myofibroblasts in culture (13), and is one factor contributing to fibroblast heterogeneity. The influence of IL-1β on the myofibroblast phenotype was also seen in cultures derived from normal individuals. For example, IL-1 addition to cultures of control strain fibroblasts, N5, resulted in greater cell viability (Table 1), and relatively higher levels of expression of α-SM actin and N-cadherin expression compared to the serum-free control (Fig. 2, lanes 5–8).

DISCUSSION

Il-1 has been reported to induce apoptosis of various cell types, including myofibroblasts cultured from rat lung (14) and human corneal fibroblasts (15). More recently, there have been reports of the effect of IL-1 on cell viability (16, 17). IL-1 increases the viability of osteoclasts, by a mechanism involving activation of NF-κB (18). Osteoclasts, like myofibroblasts, are cells which presumably are designed to function for a limited period of time. Osteoclasts, which under normal conditions participate in bone remodeling, can cause bone resorption if their life span is extended. Myofibroblasts, capable of synthesizing elevated levels of collagen and TIMP-1 (10), which persist after the completion of wound, healing can produce excessive scarring.

Unlike TGF-β, IL-1 is not capable of transforming fibroblasts into myofibroblasts in culture (13). Therefore, the effect of IL-1 on myofibroblast viability appears to be primarily due to a decrease in cell loss. This effect also appears to be characteristic of the myofibroblast phenotype independent of the source of the fibroblasts.

Many cell types, including fibroblasts, will readily (within 24 h) undergo apoptosis when serum-deprived (19). AKR-2B fibroblasts rapidly die by a process intermediate between apoptosis and necrosis (20). However, normal adult human fibroblasts do not show signs of apoptosis for periods of up to 4 weeks (21). This is consistent with our observations that confluent normal fibroblasts are relatively resistant to cell death in comparison to myofibroblasts. Further work is necessary to define the mechanism of myofibroblast cell death under serum-free conditions and the protective effect of IL-1.

In conclusion, myofibroblasts derived from scleroderma skin are lost in cell cultures under serum-free conditions (possibly by apoptosis) but rescued by the addition of IL-1β. One factor involved in myofibroblast longevity in scleroderma skin may be IL-1. Monocytic infiltration may provide elevated levels of IL-1. The effect of IL-1 is likely to be augmented by the relatively high sensitivity of scleroderma fibroblasts to IL-1.

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