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Release of Mechanical Tension Triggers Apoptosis of Human Fibroblasts in a Model of Regressing Granulation Tissue

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In an *in vitro* model of granulation tissue, early passage human diploid fibroblasts under mechanical tension showed little or no apoptosis. Release of mechanical tension triggered an apoptotic response that occurred within 3–6 h and reached a plateau by 24 h. The percentage of apoptotic cells (~15%) remained constant up to 7 days, and after 3 days, total cell number declined. Identification of mechanical unloading as a stimulus for apoptosis, without application of pharmacologic or genetic intervention, is a novel observation that permits us to model similar events that occur during wound healing. Studies on the mechanism regulating apoptosis under these conditions established that the apoptotic response does not require differentiation of cells into myofibroblasts but is governed by a combination of mechanical tension and growth factors in the collagen matrix.

Key Words: **apoptosis; wound repair; mechanical tension.**

INTRODUCTION

Newly formed tissue that fills a wound during the early stages of healing (granulation tissue) contains densely packed fibroblasts along with mononuclear cells in a rapidly developing capillary network. Unlike fibroblasts of dermis that are quiescent and stationary, fibroblasts of granulation tissue are proliferative and motile [1, 2]. During repair, tensile force develops within the wound matrix. This force normally is relieved through a combination of biosynthetic activity and wound contraction [3]. Fibroblasts in the granulation tissue are responsible for biosynthesis of the new connective tissue matrix and also differentiate into myofibroblasts. Myofibroblasts contain actin stress fibers and α-smooth muscle actin and have been implicated in wound contraction [4].

As normal repair progresses, the cells of granulation tissue disappear over a period of weeks, and the matrix takes on the histological features of scar [1–3]. If granulation tissue fails to regress, then fibrocontractive disease can result [5–7]. *In vivo* studies suggest that the regression of granulation tissue occurs by apoptosis [8–12], but the parameters governing this response are not understood. Moreover, it is not known if there is a difference in the propensity of fibroblasts and myofibroblasts to undergo apoptosis [4].

To learn more about fibroblast activation and regression during wound repair, we have studied cells cultured in collagen matrices using the stress–relaxation model [13] (Fig. 1). In the first stage of this two-step model, cells contract collagen matrices attached to culture dishes. Contraction becomes isometric as the matrix resists the pull of the cells, and the mechanical load that develops results in polarized cell morphology and formation of prominent actin stress fibers [13, 14]. Fibroblasts in the mechanically loaded matrix resemble proliferating cells of granulation tissue [15], and the isometric tension is equivalent to that found in skin wounds [16, 17]. In the second stage of the stress–relaxation model, mechanical unloading is accomplished by releasing the attached matrices. Isotonic contraction follows, accompanied by rapid desensitization of growth factor receptors [18–20] and loss of stress fiber organization [13, 14, 20].

In vivo, a catastrophic drop of mechanical tension across granulation tissue fibroblasts does not occur; rather, the change takes place gradually over days to weeks. The *in vitro* model, therefore, provides an opportunity to analyze within a convenient time frame conditions believed to be analogous to starting and final states. Consistent with this idea, after mechanical unloading, fibroblasts become quiescent [13] and disappear from the matrix [21] as occurs during regression of granulation tissue.

Since apoptosis was implicated in regression of granulation tissue (see above), we wanted to determine if apoptosis might also account for regression of fibroblasts in collagen matrices after switching from mechanically loaded to unloaded conditions. Herein we report that fibroblasts in mechanically loaded collagen matrices showed little or no apoptosis. After switching

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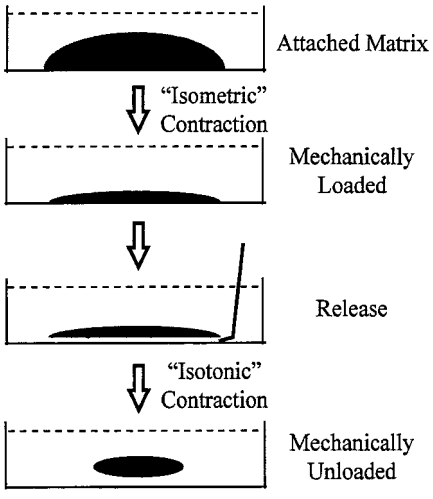

FIG. 1. Stress–relaxation model of collagen matrix contraction. Contraction of attached matrices becomes isometric as matrix anchorage to the underlying culture dish prevents matrix diameter from decreasing. Release results in isotonic contraction in which the external mechanical load is essentially zero. See text for details.

cells to mechanically unloaded conditions, apoptosis occurred within 3–6 h and reached a plateau by 24 h. The percentage of apoptotic cells remained constant up to 7 days, and after 3 days, total cell number began to decline. Identification of mechanical unloading as a stimulus for apoptosis, without application of pharmacologic or genetic intervention, is a novel observation that permits us to model similar events that occur during wound healing. Studies on the mechanism regulating apoptosis under these conditions established that the apoptotic response does not require differentiation of cells into myofibroblasts but is governed by a combination of mechanical tension and growth factors in the collagen matrix. (This work has been reported previously in abstract form [22].)

METHODS

Materials. Dulbecco's modified Eagle medium (DMEM) and trypsin/EDTA solution were obtained from Gibco BRL (Gaithersburg, MD). Fetal bovine serum (FBS) was obtained from Intergen Company (Purchase, NY). Vitrogen "100" collagen was obtained from Collagen Corp. (Palo Alto, CA). Terminal deoxynucleotidyl transferase (TdT), dUTP, biotin-16–dUTP, and RNase (DNase-free) were obtained from Boehringer Mannheim (Indianapolis, IN). Fluorescein–avidin (DCS) was obtained from Vector Labs (Burlingame, CA). Bovine serum albumin (BSA, Fraction V) and HRP-conjugated goat anti-mouse IgG H + L were obtained from ICN Inc. (Aurora, OH). Transforming growth factor β1 (TGF-β1) was obtained from Calbiochem (La Jolla, CA). Propidium iodide and mouse monoclonal anti-α-smooth muscle actin were obtained from Sigma Chemical Company (St. Louis, MO). FITC-conjugated goat anti-mouse IgG was obtained from Zymed (San Francisco, CA). Monoclonal anti-actin antibody was obtained from Amersham Life Sciences (Arlington Heights, IL). RITC-conjugated phalloidin was obtained from Molecular Probes Inc. (Eugene, OR). Fluoromount G was obtained from Southern Biotechnology Associates (Birmingham, AL).

Collagen culture. Fibroblasts from human foreskin specimens (<10 passages) were maintained in Falcon 75-cm^2 tissue culture flasks in DMEM supplemented with 10% FBS. In experiments to stimulate differentiation of fibroblasts into myofibroblasts, 5 ng/ml TGF-β1 was added to the cultures at 2- to 3-day intervals for 5–6

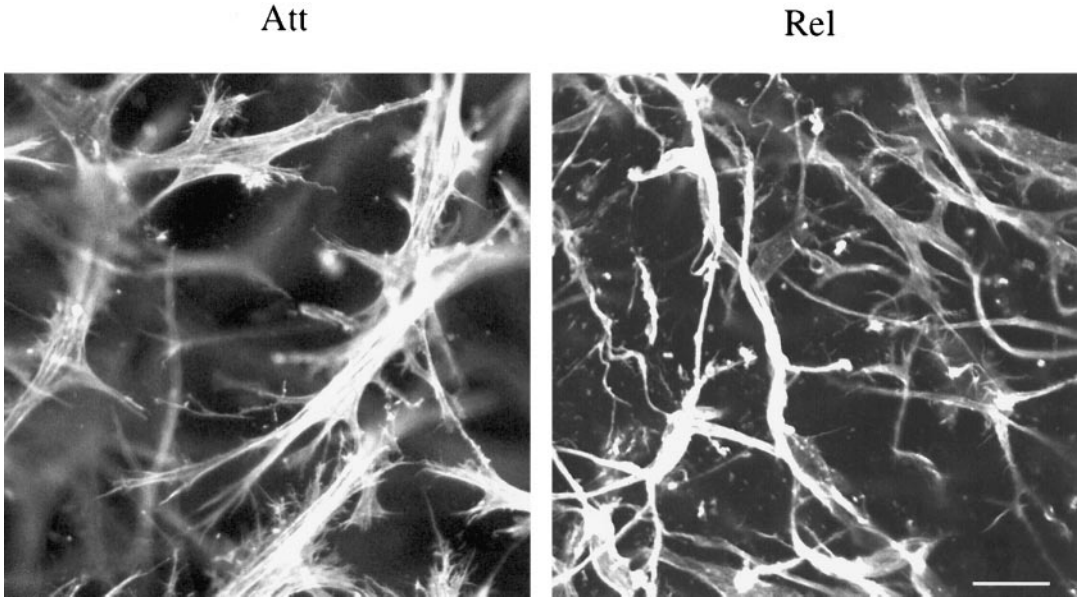

FIG. 2. Actin organization observed by confocal microscopy in attached and released collagen matrices. Fibroblasts were cultured in attached collagen matrices for 48 h (Att) or in matrices that were attached for 24 h and released for 24 h (Rel). The matrices were fixed and stained by RITC–phalloidin to determine actin organization. Observations were made by confocal microscopy. Bar, 22 μm.

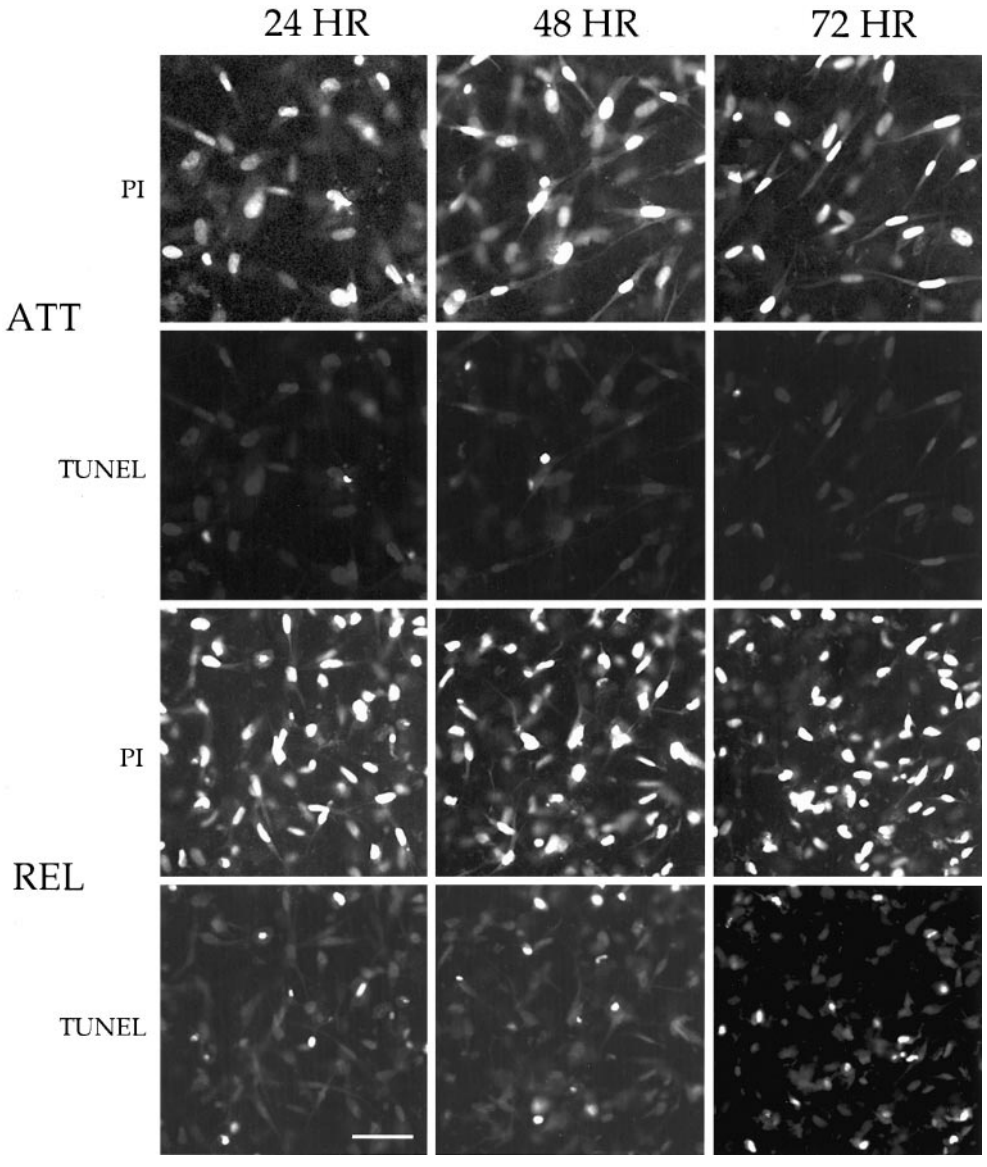

FIG. 3. Apoptosis of fibroblasts observed by confocal microscopy in attached and released collagen matrices. Fibroblasts were cultured in attached collagen matrices for the time periods shown (ATT) or in matrices that were attached for 24 h and then released for the time periods shown (REL). The matrices were fixed and stained to determine PI- and TUNEL-positive cells. Observations were made by confocal microscopy. Bar, 56 μm.

days [7, 23]. Fibroblasts were harvested from monolayer culture with 0.25% trypsin/1 mM EDTA solution followed by DMEM/10% FBS.

Collagen matrix contraction. The collagen matrix stress–relaxation model has been described previously [13, 20] and is outlined in Fig. 1. Briefly, hydrated collagen matrices were prepared from Vitrogen "100" collagen. Neutralized collagen solutions (1.5 mg/ml) contained fibroblasts in DMEM without serum. Aliquots (0.2 ml; 2×10^5 cells) of the cell/collagen mixtures were prewarmed to 37°C for 4 min and then placed in 24-well culture plates. Each aliquot occupied an area outlined by a 11-mm-diameter circular score within a well. Polymerization of collagen matrices required 60 min at 37°C. Subsequently, the polymerized matrices were cultured with 1.0 ml of DMEM/10% FBS and 50 μg/ml ascorbic acid for 1–2 days to allow cells and matrices to become mechanically loaded. To initiate mechanical unloading, attached matrices were gently released from the underlying culture dish with a spatula, after which the matrices were incubated at 37°C for the times indicated in the experiments.

Cytospin sample preparations. Fibroblasts were harvested from collagen matrices (or in some cases from coverslips) by treatment with 0.25% trypsin/1 mM EDTA at 37°C for 10 min (100 μl/matrix), followed by treatment in a shaking incubator for 30–45 min with 5 mg/ml collagenase in 130 mM NaCl, 10 mM Ca acetate, 20 mM Hepes buffer, pH 7.2 (400 μl/matrix). Enzyme activity was stopped with FBS (50 μl), and the cells were collected by centrifugation for 2 min at 2000 rpm (Beckman Microfuge). Cell pellets were suspended in 0.2 ml DMEM/10% FBS and collected by cytospin (Shandon Cytospin 3) for 6 min at 1000 rpm onto 12 mm circular No. 1 glass coverslips.

Apoptosis assay. Collagen matrix samples and cytospin samples were analyzed for apoptosis using the TdT-mediated dUTP nick end

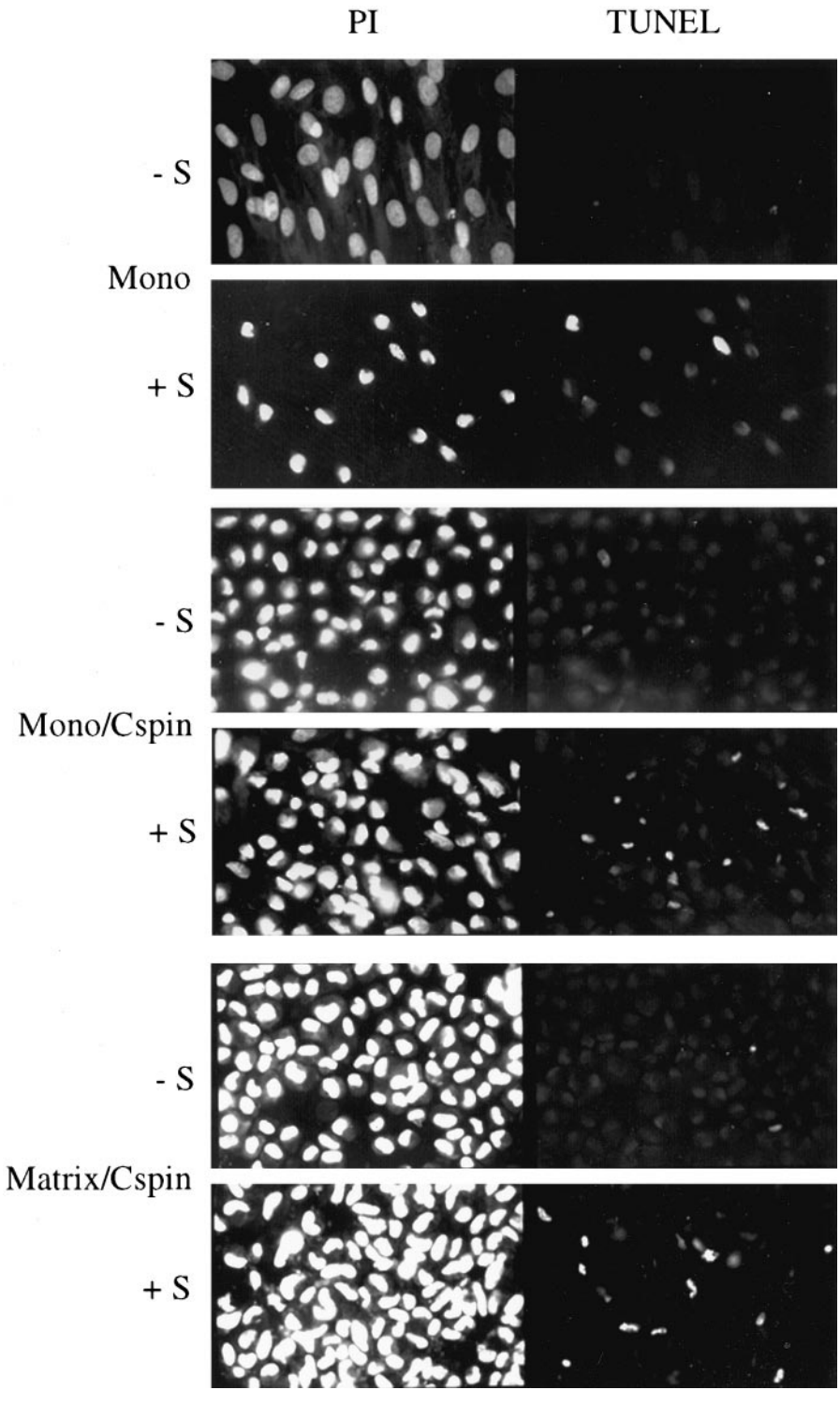

FIG. 4. Apoptosis of fibroblasts in monolayer and collagen matrices treated with staurosporine. Fibroblasts were cultured on coverslips or in attached collagen matrices for 24 h. Subsequently, the samples were treated with 1 μM staurosporine for 4 h. Samples of monolayer cultures were either fixed and stained by PI and TUNEL (Mono) or harvested and collected by cytospin and then fixed and stained by PI and TUNEL (Mono/Cspin). Attached matrix samples were harvested and collected by cytospin and then fixed and stained by PI and TUNEL (Mono/Cspin).

labeling (TUNEL) method using the Boehringer Mannheim TUNEL assay kit according to the manufacturer's recommendations. Initially, the cells were fixed with 3% paraformaldehyde in PBS (150 mM NaCl, 3 mM KCl, 6 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.2) for 15 min at 22°C, washed three times with PBS, and then incubated further with PBS containing 0.2% Triton X-100 for 10 min at 22°C.

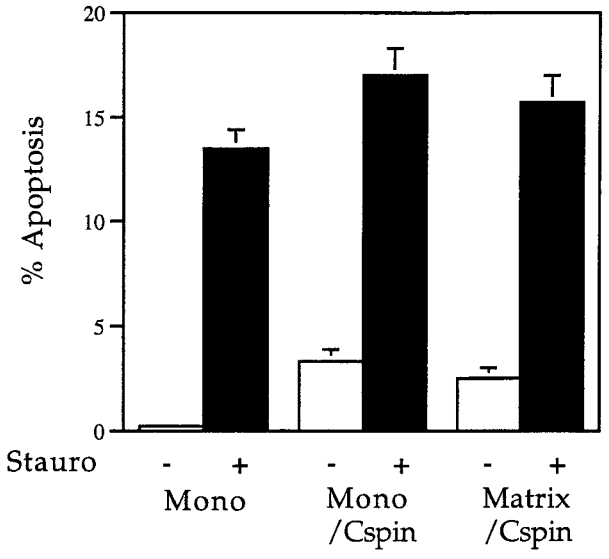

FIG. 5. Extent of staurosporine-induced apoptosis. Details are the same as in the legend to Fig. 4 except that the number of PI- and TUNEL-positive cells was determined and percentage of apoptosis (TUNEL/PI) was calculated.

Subsequently, samples were preincubated with TdT reaction buffer containing 2.5 mM $CoCl_2$ and 0.3% Triton X-100 for 10 min at 22°C followed by 2 h at 37°C in the same buffer containing 2 units/μl TdT and 12 μM μdUTP mixture (2:1 dUTP:biotinylated 16-dUTP). At the end of the incubations, the samples were rinsed three times with PBS and then incubated with fluorescein–avidin solution (20 μg/ml in PBS) for 10 min at 4°C in the dark followed by rinsing three times with PBS. The samples were counterstained by propidium iodide (PI) (3 μg/ml in PBS containing 20 μg/ml DNase-free RNase) for 30 min at 37°C. After mounting the samples on glass slides with Fluoromount G, observations and photographs of cytospin preparations were made using a Zeiss IIIRS fluorscence microscope, and observations of collagen matrices were made using a Bio-Rad MRC 1024 laser sharp confocal microscope.

TUNEL- and PI-stained cells were counted as follows. Photographs were prepared of three to five fields in which cells were uniformly distributed (e.g., Figs. 4 and 6). Percentage of apoptosis was calculated as the number of TUNEL-positive cells divided by the number of PI-positive cells. In cell recovery experiments, total cell number was determined using a hemacytometer. Each data point was measured in duplicate or triplicate and every experiment was repeated two or more times. Data points and error bars in the figures represent averages and standard deviations. Where error bars cannot be seen, the standard deviation was smaller than the data points.

Analysis of actin and α-smooth muscle actin. Actin and α-smooth muscle actin levels were determined by immunoblotting. Samples (2×10^5 cells/0.1 ml buffer) were mixed for 15 min on ice with lysis buffer, i.e., DPBS (1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 150 mM NaCl, 3 mM KCl, 6 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.2) containing 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM AEBSF, 1 mM Na_3VO_4, 1 mM Na_2MoO_4, and 0.5% NP40. Samples were centrifuged for 10 min at 11,000*g* (Eppendorf microcentrifuge Model 5415C), and the supernatants were dissolved in 4× reducing sample buffer (250 mM Tris, 4% SDS, 40% glycerol, 20% mercaptoethanol, 0.04% bromophenol blue) and boiled for 5 min. Samples were subjected to SDS–PAGE using 7.5% acrylamide minislab gels and transferred to PVDF membranes. The membranes were blocked with 3% BSA, 0.05% Tween 20 in 50 mM Tris–HCl, 150 mM NaCl, pH 7.2) for 1 h, followed by the incubation with either anti-actin antibody (1:2000) or anti-α-smooth muscle actin monoclonal antibodies (1:1000) for 2 h and then by HRP-conjugated goat anti-mouse IgG H + L (1:10000) for 1 h. The membranes were developed by the ECL system (Amersham) according to the manufacturer's protocol.

Distribution of actin and α-smooth muscle actin was determined by immunofluorescence. Cell on coverslips were fixed 15 min at 22°C with 3% paraformaldehyde in DPBS, washed 2 × 10 min with DPBS, blocked for 30 min with DPBS containing 1% BSA and 1% glycine, and then permeabilized for 10 min with DPBS containing 0.2% NP-40. Subsequently, the samples were incubated for 1 h at 37°C with DPBS containing monoclonal anti-α-smooth muscle actin (1:20), washed five times with DPBS, and incubated for 30 min at 37°C with FITC-labeled goat anti-mouse IgG (1:100) followed by five additional washes with DPBS. Second staining was performed using RITC-conjugated phalloidin (0.8 units/ml) for 30 min at 37°C followed by six washes with DPBS. After mounting the samples on glass slides with Fluoromount G, observations and photographs were made using a Zeiss IIIRS fluorescence microscope, and observations of collagen matrices were made using a Bio-Rad MRC 1024 laser sharp confocal microscope.

RESULTS

TUNEL Staining of Fibroblasts in Collagen Matrices after Mechanical Unloading

Switching fibroblasts from mechanically loaded to unloaded conditions results in cell quiescence and regression [13, 21]. Figure 2 shows typical confocal microscopic images of the changes in the actin cytoskeleton that accompany this transition. Fibroblasts in attached collagen matrices assembled prominent stress fibers within 24 h (Fig. 2, Att). After release of these matrices from the underlying substratum, the cells remained spread, but reorganization of the actin cytoskeleton resulted in disappearance of stress fibers (Fig. 2, Rel).

To learn whether apoptosis accounted for cell regression after mechanical unloading, TUNEL staining was used to detect apoptotic cells with propidium iodide as a counterstain for total cells. Figure 3 shows the typical confocal microscopic appearance of fibroblasts after various times in attached or released matrices. Few fibroblasts in attached (ATT) matrices became TUNEL positive, whereas numerous TUNEL-positive nuclei were detected in cells in released (REL) matrices.

Determination of Apoptosis Using Cytospin Cell Preparations

To determine apoptotic indexes, fibroblasts were harvested from collagen matrices and collected by cytospin on culture slips, and then analyzed by the TUNEL method. Studies in which apoptosis was induced pharmacologically by treatment of fibroblasts with staurosporine [24] were carried out to ensure that the harvest/cytospin procedure permitted an accurate determination of apoptotic index and to assess whether the mechanically loaded collagen matrix itself had any nonspecific repressive effect on apoptosis.

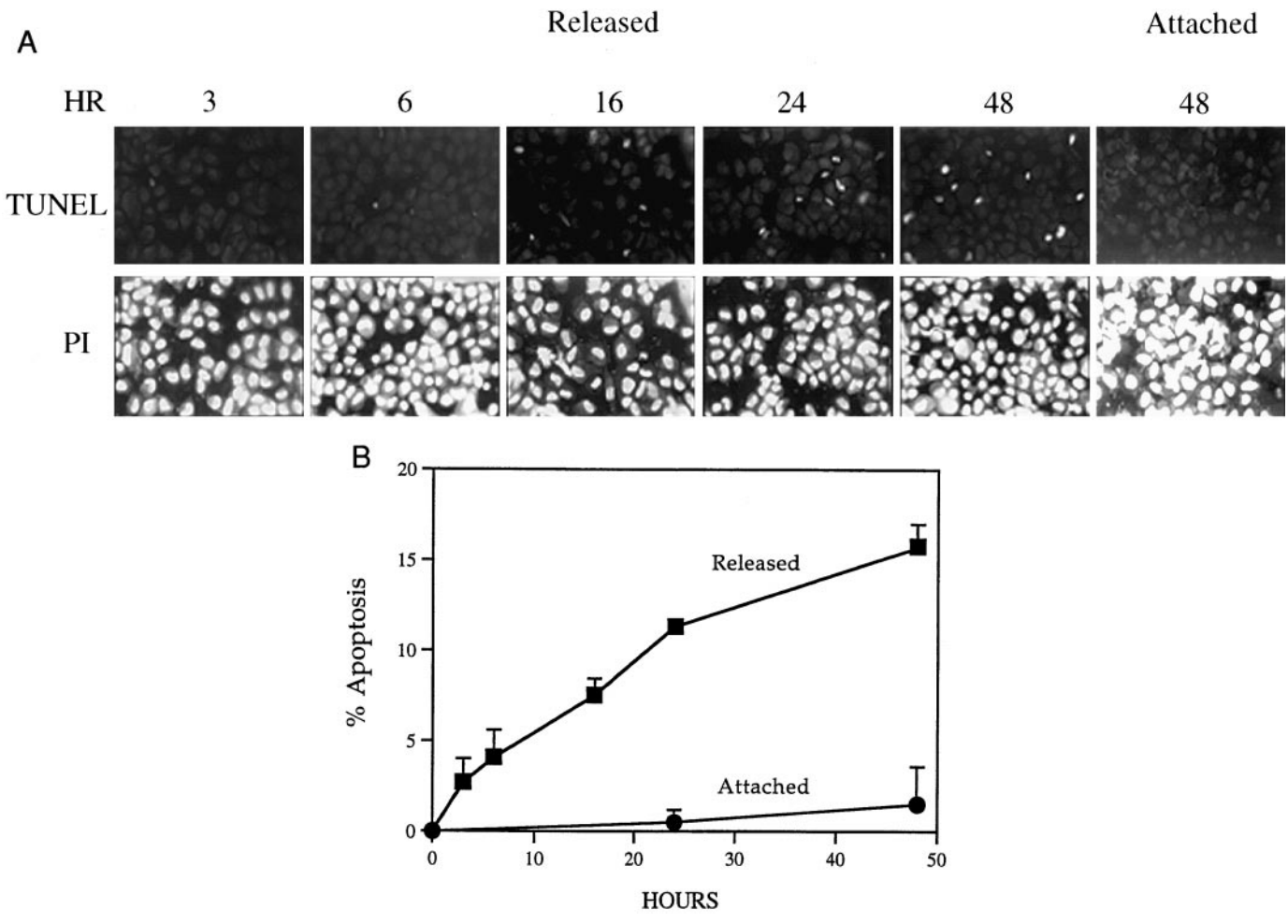

FIG. 6. Onset of apoptosis following release of collagen matrices. Fibroblasts were cultured in attached collagen matrices for 24 h and then released for the times indicated. (A) Cells were harvested from the matrices, collected by cytospin, and stained by TUNEL and PI. (B) The number of PI- and TUNEL-positive cells was determined, and percentage apoptosis (TUNEL/PI) was calculated.

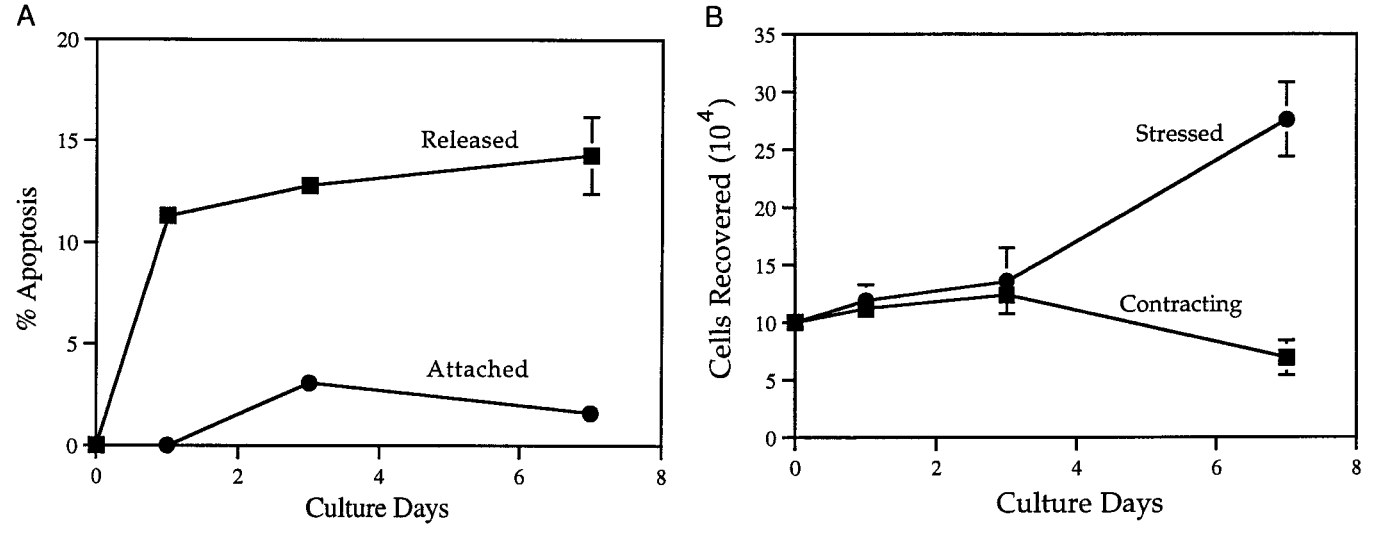

FIG. 7. Extent of apoptosis and cell recovery following release of collagen matrices. Fibroblasts were cultured in attached collagen matrices for the times indicated or in attached matrices for 24 h and then released for the times indicated. (A) Cells were harvested from the matrices, collected by cytospin, and stained by TUNEL and PI. The number of PI- and TUNEL-positive cells was determined, and percentage of apoptosis (TUNEL/PI) was calculated. (B) Same as A except the total number of cells recovered from the matrices was determined.

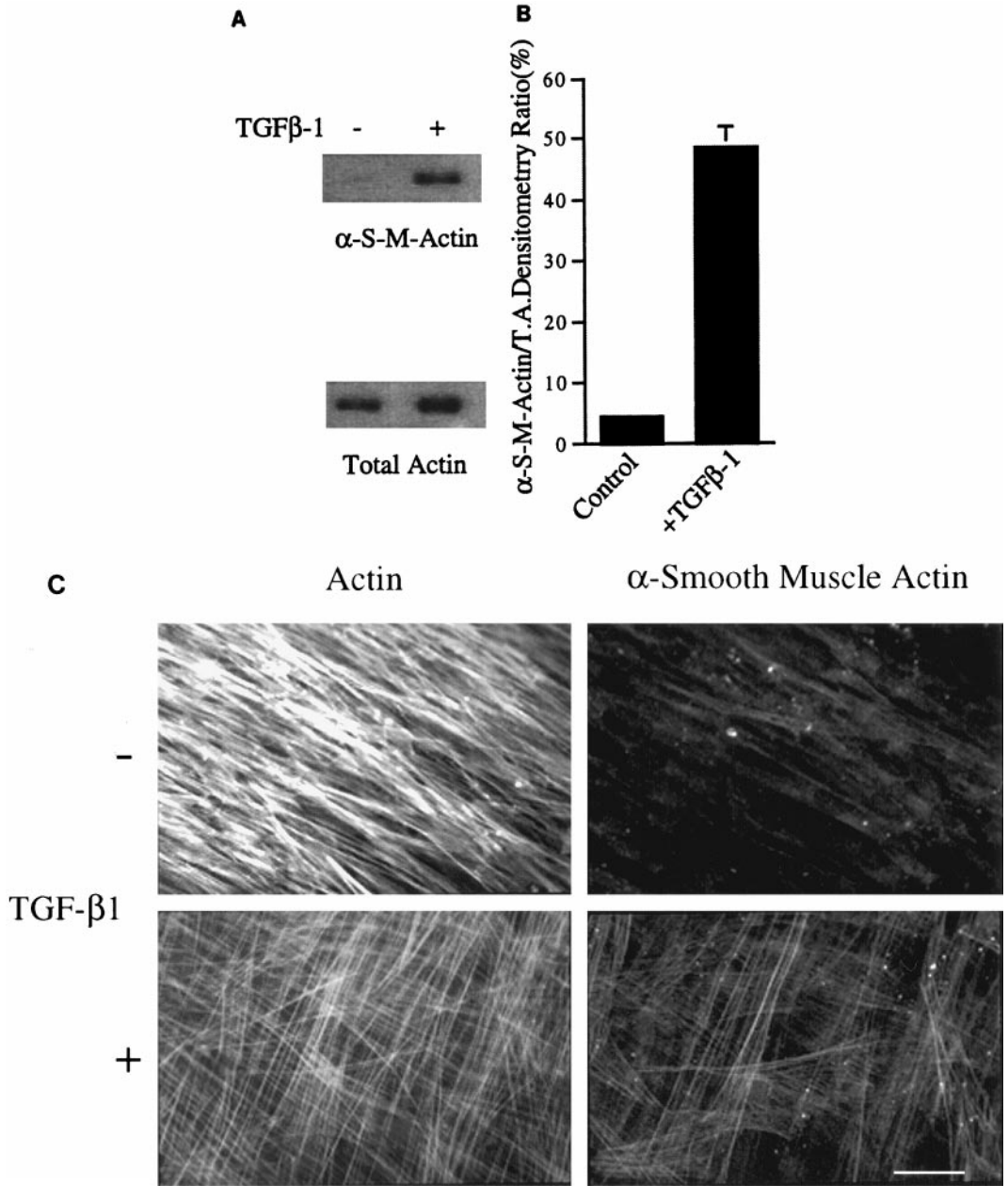

FIG. 8. Expression of α-smooth muscle actin in TGF-β1-treated fibroblasts. Fibroblasts were cultured for 6 days with or without 5 ng/ml TGF-β1 as indicated. (A) Cell extracts were subjected to SDS–PAGE and immunoblotting with anti-α-smooth muscle actin and total actin antibodies. (B) The results in A were quantified using ImageQuant with a Molecular Probes personal densitometer. (C) Cells were fixed and stained by antibodies to detect against α-smooth muscle actin or with RITC–phalloidin to detect total actin. Bar, 12 μm.

Figure 4 shows the typical appearance of fibroblasts that were incubated 4 h with or without 1 μM staurosporine and analyzed using the TUNEL/PI method under three different conditions. In the first set (Mono), cells were in monolayer culture during both staurosporine treatment and TUNEL/PI staining. In the second set (Mono/Cspin), cells were in monolayer culture during staurosporine treatment as above, but then harvested and collected by cytospin before TUNEL/PI staining. In the third set (Matrix/Cspin), cells were treated with staurosporine in attached collagen matrices, harvested, collected by cytospin, and stained as above.

Staurosporine caused a marked increase in the number of TUNEL-positive cells. Direct counts of the coverslips indicated that within 4 h staurosporine treatment induced apoptosis in approximately 15% of the cells (Fig. 5, Mono), and similar results were obtained when the staurosporine-treated cells were harvested from mono-

layers and collected by cytospin before TUNEL staining (Fig. 5, Mono/Cspin). Therefore, the cell harvest/cytospin procedure provides an accurate measure of apoptotic index. In addition, the apoptotic index of fibroblasts in collagen matrices (Fig. 5, Matrix/Cspin) was similar to that observed when staurosporine was used to treat cells in monolayer culture. Consequently, the mechanically loaded collagen matrix did not exert any repressive effect on susceptibility to cell death.

It should be noted that cytospin treatment itself resulted in a low level of background apoptotic staining (~3%). This increase was not consistent from day to day and probably reflects variability in the cell population and the assay method.

Regulated cell death is typically accompanied by caspase activation and, in related experiments, we sought to measure caspase activity in cell extracts using the Clontech CPP32 protease assay kit. When fibroblasts were treated for 4 h with staurosporine in monolayer culture, we detected a 10-fold increase in caspase-associated fluorescence. When, however, fibroblasts were treated with staurosporine in collagen matrices, there was no more than a twofold increase in fluorescence (data not shown) even though the extent of cell death was similar (see Fig. 5). Given the limited number of cells per collagen matrix (~2×10^5), extracts from 5–10 matrices were required per sample for the biochemical assays, and it is possible that the high ratio of collagen to cellular protein interfered with the assay.

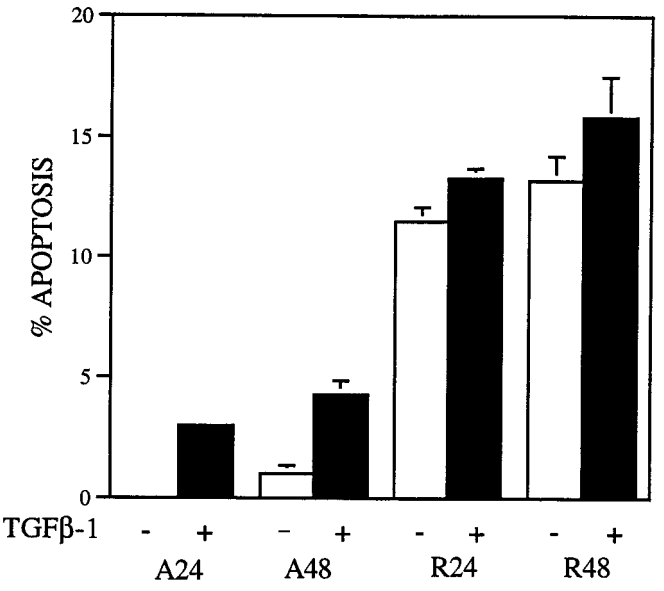

FIG. 9. Extent of apoptosis of fibroblasts in attached and released collagen matrices with and without prior treatment of the cells by TGF-β1. Fibroblasts pretreated with (open bars) or without (closed bars) TGF-β1 as indicated in the legend to Fig. 8 were cultured in attached collagen matrices for 24 (A24) or 48 h (A48) or in matrices that were attached for 24 h and then released for 24 (R24) or 48 h (R48). Cells were harvested from the matrices, collected by cytospin, and stained by TUNEL and PI. The number of PI- and TUNEL-positive cells was determined, and percentage of apoptosis (TUNEL/PI) was calculated.

Time Course of Cell Entry into Apoptosis and Cell Regression

Using the cytospin method together with TUNEL/PI staining, the time course of cell entry into apoptosis was determined. Typical results are shown in Figs. 6A and 6B, where it can be seen that the number of apoptotic fibroblasts began to increase within 3–6 hr after release of attached matrices. Figure 7A shows an example of a longer term experiment indicating that up to 7 days after release, the percentage of apoptotic cells was relatively constant (~15%). Parallel measurements shown in Fig. 7B, indicated that the number of cells recoverable from the released matrices decreased after 3 days. The stable apoptotic index in the released matrices over time reflects a balance between the onset of apoptosis and the loss of cells by apoptosis.

Apoptosis of α-Smooth Muscle Actin-Containing Fibroblasts

After they migrate into granulation tissue, fibroblasts differentiate into myofibroblasts, but the potential role of this transition in specifying apoptosis is not known [4]. We examined this issue by treating fibroblasts in monolayer culture with TGF-β, which promotes the differentiation of the fibroblasts into myofibroblasts [7, 23].

Figure 8 shows by immunoblotting (Figs. 8A and 8B) and immunofluorescence (Fig. 8C) the levels of total actin and α-smooth muscle actin before and after 6 days treatment of cells with 5 ng/ml TGF-β1. Little α-smooth muscle actin was observed in the starting cell population. After TGF-β1 treatment, however, there was a 10-fold increase in α-smooth muscle actin relative to total actin (Fig. 8B) and most of the cells showed colocalization of α-smooth muscle with actin stress fibers (Fig. 8C).

Control and TGF-β1-treated fibroblasts were cultured in attached and released collagen matrices. TGF-β1 treatment alone appeared to result in an increase in the basal apoptotic index of cells under mechanical load reaching almost 5% after 48 h. Longer times were not tested. TGF-β1 treatment also showed a slight tendency to increase induction of apoptosis triggered by mechanical unloading, but the difference was small (Fig. 9).

Apoptosis of Fibroblasts in Attached Collagen Matrices in the Absence of Serum and in the Presence of Cytochalasin

In previous studies we showed that mechanical unloading of fibroblasts in collagen matrices causes rapid desensitization of growth factor receptors [19, 25], and

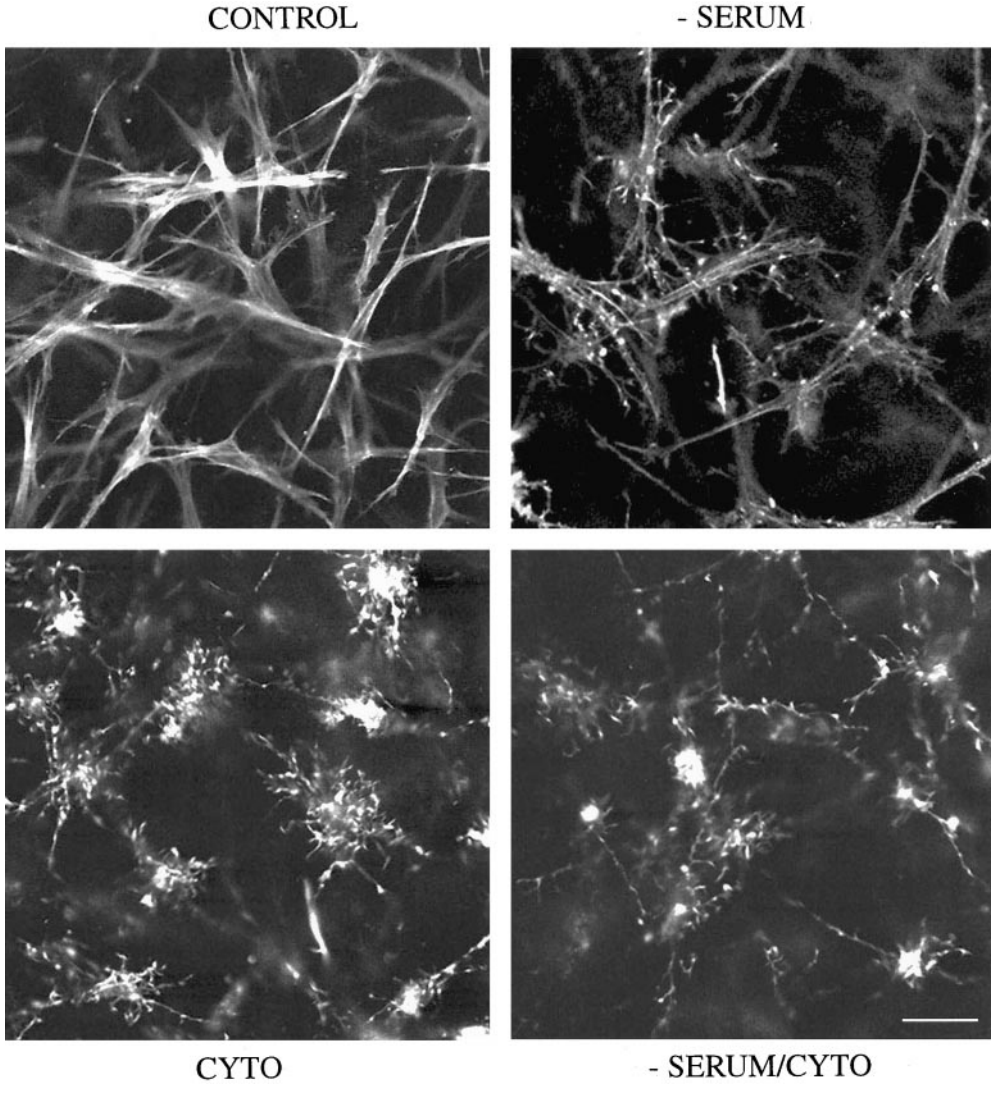

FIG. 10. Actin organization observed by confocal microscopy in fibroblasts in attached collagen matrices subjected to serum deprivation and cytochalasin D treatment. Fibroblasts were cultured in attached collagen matrices for 24 h followed by 24 h in serum-containing medium (CONTROL), serum-free medium (-SERUM), serum-containing medium plus 10 μM cytochalasin D (CYTO), or serum-free medium plus cytochalasin D (-SERUM/CYTO). The matrices were fixed and stained by phalloidin to determine actin organization. Observations were made by confocal microscopy. Bar, 39 μm.

cells become unresponsive to added growth factors [18]. Under the same conditions, fibroblasts also were shown to lose their actin stress fibers [13, 14, 20] (e.g., Fig. 2). As a first step toward learning the relationship between these changes and cell entry into apoptosis, fibroblasts in collagen matrices under mechanical load were deprived of serum to remove growth factors and/or treated with cytochalasin D to disrupt actin stress fibers.

Figure 10 shows the typical appearance of fibroblasts cultured for 24 h in attached collagen matrices and then treated during the subsequent 24 h with serum-free medium (-serum), cytochalasin D (cyto), or the combination (-serum/cyto). In serum-free medium, there was no change in overall cell morphology but a marked decrease in stress fibers. After treatment with cytochalasin D in the presence or absence of serum, cell morphology changed, and stress fibers were completely disrupted. Results from three independent experiments shown in Fig. 11 demonstrated that culturing cells either in serum-free medium or with cytochalasin D alone caused an increase in the apoptotic index. The combination of both treatments, however, was required to promote a level of apoptosis (~15%) similar to that observed in unloaded collagen matrices.

DISCUSSION

The purpose of our studies was to learn if apoptosis occurred in an *in vitro* model of wound repair. In this model, little apoptosis was observed as long as the

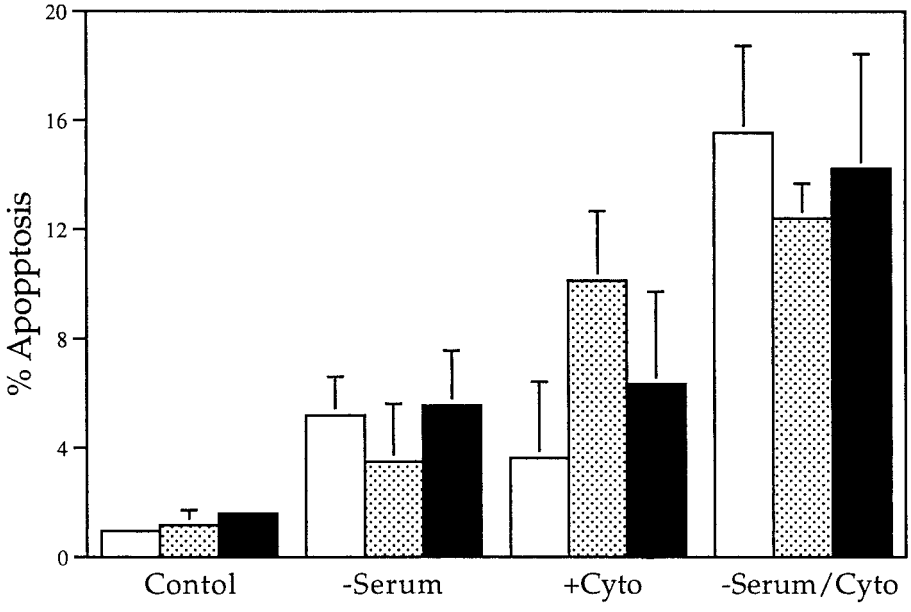

FIG. 11. Extent of apoptosis of fibroblasts in attached collagen matrices subjected to serum deprivation and cytochalasin D treatment. Details are the same as in the legend to Fig. 10 except cells were harvested from the matrices, collected by cytospin, and stained by TUNEL and PI. The number of PI- and TUNEL-positive cells was determined, and percentage of apoptosis (TUNEL/PI) was calculated. Results shown are from three independent experiments.

collagen matrix was maintained under mechanical load. Release of mechanical load triggered an apoptotic response reaching a plateau (~15% of the cell population) by 24 h and remaining constant for up to 7 days. After 3 days, the total number of cells recovered from the released matrices began to decline. The constant level of apoptotic cells in the released matrices implied that there was a balance between cells entering apoptosis and apoptotic cells that were lost. These findings resemble the regulated death of fibroblasts in granulation tissue, where the apoptotic index peaks at 9–12% [4].

Pronounced apoptosis of early passage human diploid fibroblasts triggered by mechanical unloading, without application of pharmacologic or genetic intervention, is a novel observation. Typically, normal diploid fibroblasts enter cell cycle arrest when challenged by conditions, e.g., loss of cell adhesion [26–28], that induce apoptosis in other cells types.

While our studies were in progress, Fluck *et al.* [29] reached similar conclusions using fibroblasts cultured in "contractile collagen gels," an *in vitro* model of skin originally developed for grafting [30, 31]. In contrast to our findings, however, they observed a wave of cell apoptosis that peaked 2 days after setting up the cultures. Under their experimental conditions, cells go directly from trypsinization to a quiescent state without the opportunity to recover proliferative capacity. Consequently, the occurrence of a wave of apoptosis may have been in part a response to initial cell trauma caused by trypsinization.

In trying to understand the unique features that promote apoptosis when fibroblasts are switched from mechanically loaded to unloaded collagen matrices, it is important to emphasize that cells in mechanically loaded collagen matrices were just as susceptible to staurosporine-induced apoptosis as were cells cultured in monolayer. Therefore, the collagen matrix itself does not exert a general anti-apoptotic effect on normal diploid fibroblasts.

The overriding feature accounting for apoptosis in the *in vitro* wound healing model is release of mechanical tension. A role for mechanical tension in regulating cell survival has been proposed based on studies of endothelial cell geometry [32]. Mechanical tension necessary for survival of endothelial cells *in vivo* is regulated by shear stress [33, 34]. In the case of fibroblasts in collagen matrices, changes in mechanical tension resulted in reorganization of the actin cytoskeleton when fibroblasts were switched from mechanically loaded to unloaded conditions [13, 14, 20] (e.g., Fig. 2).

While clearly important, release of mechanical tension leading to reorganization of the actin cytoskeleton was not a sufficient explanation for apoptosis of human fibroblasts in collagen matrices as indicated by the experiments in which the actin cytoskeleton of cells in attached matrices was disrupted by cytochalasin over a 24-h period. Even though the extent of cytoskeletal disruption in response to cytochalasin was much greater than that which occurred following release of tension in these matrices, the extent of the apoptotic response was less.

A second factor necessary for fibroblasts to become apoptotic in released collagen matrices may be growth factor withdrawal. Previously we showed that mechanical unloading of fibroblasts in collagen matrices abrogates growth factor responsiveness even in the continued presence of exogenous growth factors [18], and our more recent studies suggested that this change occurred as a result of desensitization of growth factor receptors [19, 25]. Other studies have implicated growth factors, e.g., serum or PDGF, in survival of fibroblasts following overexpression of c-*myc* [35, 36]. Like cytoskeletal reorganization, however, growth factor withdrawal by itself was not sufficient to obtain a complete apoptotic response, as shown by the experiments in which fibroblasts in attached collagen matrices were cultured for 24 h in serum-free medium.

While neither cytoskeletal disruption nor growth factor withdrawal alone could account for apoptosis triggered by mechanical unloading, the combination of these conditions promoted an apoptotic response equivalent to that obtained when the attached matrices were released. Therefore, release of mechanical tension together with abrogation of growth factor signaling could explain the pronounced apoptotic response in the *in vitro* granulation tissue model.

The underlying molecular mechanism that regulates fibroblast survival and death in collagen matrices has yet to be determined, but it is worth noting that cell survival depending on the PI3-kinase-AKT/protein kinase B signaling pathway [37, 38] has recently been implicated in endothelial cell response to shear stress [39]. Studies on the role of this pathway in fibroblast apoptosis in collagen matrices currently are in progress. Whatever the precise mechanism, the strength of the apoptotic signal triggered by release of tension was less robust than that induced by 1 μM staurosporine, which in 24 h caused apoptosis in almost the entire human fibroblast population (unpublished observation).

In conclusion, we have identified release of mechanical tension as a novel stimulus for apoptotic death of early passage human fibroblasts in an *in vitro* wound healing model. These findings represent an important advance in our ability to examine regulated cell death during wound repair and may contribute to the development of novel therapeutic approaches for controlling fibroproliferative disease. Since the extent of apoptosis in mechanically unloaded matrices was similar for fibroblasts treated with or without TGF-β1, it is unlikely that prior differentiation of fibroblasts into myofibroblasts is required for apoptosis in granulation tissue. Understanding parameters regulating fibroblast apoptosis in mechanically unloaded collagen matrices may help explain the ability of full thickness skin grafts and skin flaps to inhibit granulation tissue formation [40], which results from premature induction of apoptosis in the fibroblast cell population [9].

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