Contraction-Dependent Apoptosis of Normal Dermal Fibroblasts

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The mechanisms underlying the contraction-dependent apoptosis of primary fibroblasts are of prime importance in understanding anchorage-dependent survival/apoptosis of dermal fibroblasts. As integrins are essential extracellular matrix receptors in fibroblasts, their role in anchorage-dependent apoptosis/survival of fibroblasts was analyzed. Primary human fibroblasts displayed a marked reduction of apoptosis in mechanically relaxed collagen matrices in the presence of adhesion-blocking antibodies against $\alpha_1\beta_1$ or $\alpha_2\beta_1$. Anti-$\alpha_v\beta_3$ antibodies had a considerably weaker effect. In additional experiments RD cells, which lack α_2 integrin, displayed no apoptosis in mechanically relaxed collagen matrices. Their susceptibility to apoptosis was restored after transfection with functional α_2 integrin, and it could be blocked again by adhesion-blocking antibodies against $\alpha_2\beta_1$ integrin. Therefore we conclude that apoptosis of human primary fibroblasts in contractile collagen matrices is – at least in part – inhibited by adhesion-blocking anti-integrin antibodies, suggesting that the mode of apoptosis in this case is different from anoikis. Further, apoptosis in a mechanically relaxed collagen matrix could be abrogated by depolymerization of F-actin using cytochalasin D and also by disturbing actin–myosin interaction using 2,3-butanedione monoxime, indicating a possible dependence of apoptosis on mechanical forces and/or cell shape. ***Key words: apoptosis/cell–matrix interaction/cytochalasin D/fibroblastic cell/integrin. J Invest Dermatol 116:686–692, 2001***

Tissue regeneration during wound healing is a complex biologic process. Much attention has been paid to factors that control cell proliferation and synthesis of components of the extracellular matrix (Gailit and Clark, 1994; Raghow, 1994). In contrast, little is known about the equally important process of removal of cells (for instance myofibroblasts in granulation tissue evolving into a scar), which are no longer needed as wound healing proceeds. *In vivo* there is good evidence that this happens at least in part via apoptosis (Desmoulière *et al*, 1995).

Culturing fibroblasts within a three-dimensional environment reconstituted mainly of collagen I fibrils has proven to be a valuable tool to study a variety of cellular functions during wound healing under conditions that resemble the *in vivo* situation more closely than fibroblast monolayer cultures (Bell *et al*, 1979; Grinnell, 1994). Furthermore, we have recently shown that human fibroblasts undergo apoptosis when seeded into such a three-dimensional mechanically relaxed collagenous matrix (Fluck *et al*, 1998). This induction of apoptosis is specific for contractile collagen gels, as apoptosis does not occur in mechanically stressed (i.e., noncontractile) collagen gels or in contractile fibrin gels (Fluck *et al*, 1998).

The mechanisms underlying this contraction-dependent apoptosis are of major importance in understanding anchorage-dependent survival/apoptosis during wound healing. Therefore, we investigated the involvement of integrins, which are known to mediate cell–cell and cell–matrix interactions and to act as mechanoreceptors transmitting mechanical signals to the actin cytoskeleton (Wang *et al*, 1993). There are many examples that gene expression and cell differentiation are regulated through adhesive interactions with the extracellular matrix (Juliano and Haskill, 1993). It has also been shown that integrin-mediated adhesion to extracellular matrix proteins is required for the survival of many cell types (Meredith and Schwartz, 1997). The β_1 integrin receptor subunit, in combination with α_1 or α_2 subunits, plays an important role in controlling gel contraction and expression of collagen type I and matrix metalloproteinase 1 collagenase (Langholz *et al*, 1995). Montgomery *et al* (1994) demonstrated that variant melanoma cells, lacking the α_v subunit of $\alpha_v\beta_3$ integrin, rapidly progressed to apoptosis within three-dimensional dermal collagen. On the other hand, transfection of these cells with an α_v cDNA restored $\alpha_v\beta_3$ expression and prevented apoptosis.

It was previously shown that fibroblasts, which have lost contact with their surrounding extracellular matrix, undergo reversible growth arrest (Folkman and Moscona, 1978). Furthermore, other cell types such as epithelial (Frisch and Francis, 1994) and endothelial (Meredith *et al*, 1993) cells undergo apoptosis when displaced from their physiologic extracellular matrix. This anchorage-dependent apoptosis was later termed ''anoikis'' (Frisch and Ruoslahti, 1997), and it has been hypothesized that it represents a safety mechanism *in vivo* to prevent detached cells from reattaching to inappropriate matrices and growing dysplastically. Although one would presume, however, that integrins provide the initial signals governing anchorage-dependent apoptosis, this has not been studied in detail yet (for review see Aplin *et al*, 1998).

In this study we investigated the putative role of distinct integrins in the regulation of extracellular-matrix-mediated apoptosis of

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fibroblasts in an *in vitro* model. In addition, using the actin-depolymerizing agent cytochalasin D and the inhibitor of actin–myosin interaction 2,3-butanedione monoxime (BDM), we analyzed whether this apoptosis is contraction dependent.

MATERIALS AND METHODS

Cells Primary human skin fibroblast cultures were established from biopsies taken from healthy volunteers. RD cells, lacking functional α_2 integrins (Schiro *et al*, 1991), were obtained from ATCC (CCL 136). The integrin α_2-encoding cDNA (Takada and Hemler, 1989) cloned into the expression vector pFneo (Chan *et al*, 1991) was stably transfected into RD cells. After selection with 0.6 mg per ml G418, $\alpha_2\beta_1$ expressing cells were stained with the monoclonal anti-α_2 antibody A2IIE10 and a fluorescein isothiocyanate labeled antimouse IgG antibody, and finally enriched using fluorescence activated cell sorting (FACS). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 50 mg per ml sodium ascorbate, 300 mg per ml glutamine, 100 U per ml penicillin, and 100 mg per ml streptomycin under 5% CO_2 and at 37°C. For all experiments fibroblasts from passages one to five were used after harvesting by trypsinization [0.025% trypsin, 0.02% ethylenediamine tetraacetic acid in phosphate-buffered saline (PBS)].

Preparation of hydrated collagen gels Native porcine collagen I was purchased from Deutsche Gelatine Fabriken Stoess (Eberbach, Germany) and purified by dialysis against 2 liters of 10 mM phosphate buffer, pH 7.2, for 5 d at 4°C with daily buffer changes. After centrifugation the collagen pellet was washed in 70% ethanol overnight and subsequently lyophilized. Finally, the collagen was dissolved (3 mg per ml) in sterile 0.1% acetic acid. Hydrated collagen gels were prepared according to Mauch *et al* (1988). At first the acidic collagen solution was neutralized with 0.1 M NaOH. Depending on the number of gels to be prepared, a neutralized collagen mix was made (final concentration 1 mg per ml collagen in 1 × DMEM, pH 7.2, supplemented with 10% FBS, 50 mg per ml sodium ascorbate, 300 mg per ml glutamine, 100 U per ml penicillin, 100 mg per ml streptomycin, and 1.33×10^5 fibroblasts per ml). Aliquots (1.5 ml) of this cell/collagen mixture, containing 2×10^5 cells each, were placed in 3.5 cm cell culture dishes (stressed gels) or uncoated plastic dishes (relaxed gels) and put in an incubator adjusted to 5% CO_2 and 37°C, where the gels polymerized within 60 min.

Cell recovery After measuring the gel areas collagen gels were washed twice with PBS and solubilized by incubation with 750 μl collagenase solution per gel on a shaker at 37°C [370 U per ml collagenase type CO (Seromed, Berlin, Germany) in PBS without additional Mg^{2+} and Ca^{2+}, containing 1% bovine serum albumin]. As soon as the gels were completely solubilized after 40 ± 10 min, the reaction was stopped with 200 μl FBS per gel, and released cells were washed twice with PBS.

Cell death detection enzyme-linked immunosorbent assay (ELISA) For quantitative determination of cytoplasmatic histone-associated DNA fragments, we used a cell death detection ELISA (Roche, Mannheim, Germany). Briefly, a tenth of the recovered cells were mixed with trypan blue, and cell number was measured with a hemocytometer. The remaining fibroblasts were lyzed in 90 μl incubation buffer (30 min, 4°C) and centrifuged at 20,000 × *g* for 15 min. The supernatant (cytoplasmatic fraction) was carefully removed and stored in aliquots at –20°C. In the immunoassay the nucleosomes contained in the sample (cytoplasm lysate of 2000 cells) bound via their histone components to an immobilized antihistone antibody. In a second incubation step anti-DNA-peroxidase bound to the DNA coiled around the nucleosomes. The amount of peroxidase retained in the immunocomplex after washing was determined photometrically at 405 nm with ABTS (2,2′-azino-di[3-ethylbenzthiazoline sulfonate (6)] diammonium salt) as substrate.

Adhesion blocking antibodies and inhibitors The following adhesion blocking monoclonal antibodies were used: 5E8D9 (Biomol, Hamburg, Germany) against integrin α_1, P1E6 (Biomol) against integrin α_2, 4B4 (Coulter, Hamburg, Germany) against integrin β_1, and 23C6 (Serotec, Wiesbaden, Germany) that recognizes the complex formed by α_v and β_3 integrins. Before their application in gel contraction assays, lyophilized antibodies were reconstituted in H_2O, and antibody preparations containing sodium azide as preservative were dialyzed against 1 liter of PBS at 4°C overnight.

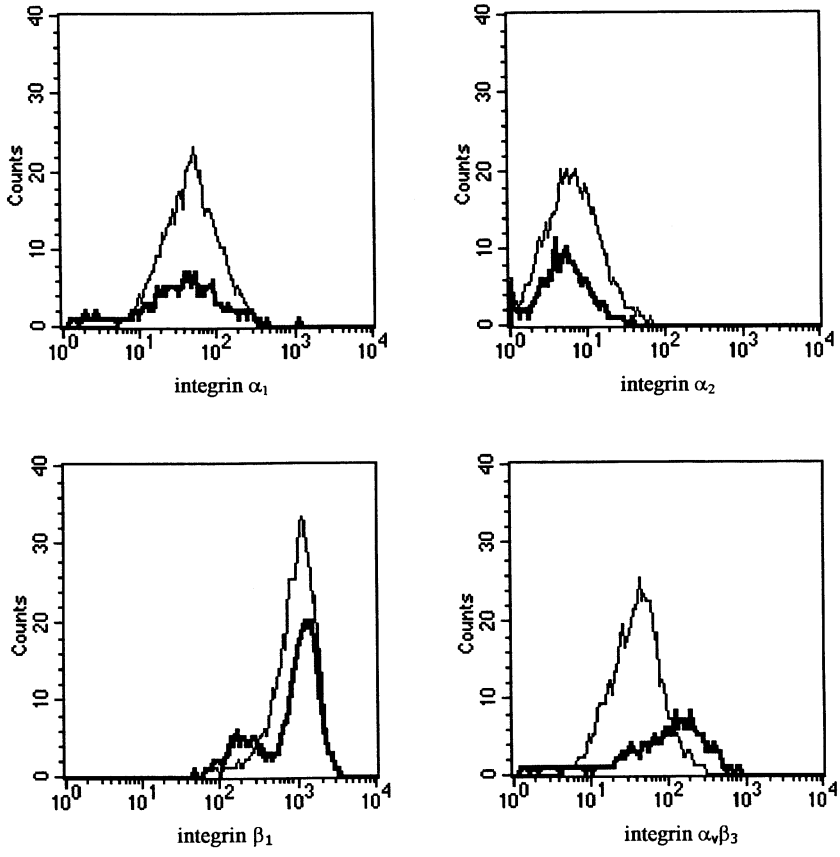

Figure 1. Surface expression of distinct integrins on fibroblasts cultured in mechanically relaxed and stressed collagen gels. Fibroblasts were cultured in three-dimensional mechanically relaxed and stressed collagen gels. After 2 d the cells were harvested and subjected to FACS analysis of the surface expression of distinct integrins (*bold lines*, integrin expression in stressed gels; *thin lines*, integrin expression in relaxed gels).

To study the effect of these antibodies on gel contraction and apoptosis by fibroblasts, trypsinized cells were preincubated with 15 μg per ml of either anti-α_1 and anti-β_1, or anti-α_2 and anti-β_1, or anti-$\alpha_v\beta_3$ antibodies for 30 min at 37°C, 5% CO_2. Subsequently, contractile collagen gels were prepared as described above and supplemented in excess with 5 μg per ml of the respective antibodies (Langholz *et al*, 1995). Every second day fresh medium and antibodies as indicated were added to the cells.

FACS analyses of the cells were performed on a FACS Calibur (Becton Dickinson, San Jose, CA). In addition to the antibodies mentioned above phycoerythrin-coupled anti-α_2 integrin clone 12F1-H6 (Pharmingen, San Diego, CA) and phycoerythrin-coupled rat-antimouse IgG_1 clone X56 (Becton Dickinson) were used.

Cytochalasin D (Sigma, Deisenhofen, Germany), an inhibitor of actin polymerization (Cooper, 1987), was dissolved as 2 mM stock in dimethylsulfoxide (DMSO; Sigma). For inhibitor studies it was diluted down to 0.5 nM with DMEM. BDM (Sigma), which disturbs the actin–myosin interaction by reducing fiber tension (McKillop *et al*, 1994), was dissolved as 5 M stock in DMSO. For inhibitor studies it was diluted to a final concentration of 20 mM in cell culture medium.

Affinity staining of the actin cytoskeleton Collagen gels prepared as described above were washed with PBS and fixed with 3 ml of freshly prepared 4% formaldehyde in PBS for 30 min at room temperature. Subsequently the gels were rinsed three times for 5 min with 3 ml PBS. Until staining fixed gels were stored at 4°C in PBS containing 0.05% sodium azide (Serva, Heidelberg, Germany). Gel segments were cut out and incubated for 60 min at room temperature in the dark with 100 μl tetramethylrhodamine isothiocyanate (TRITC) labeled phalloidin (0.05 μg per ml, Sigma) in PBS. Subsequently, the gel seqments were washed three times for 5 min with PBS and mounted on glass slides with vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA). For fluorescence microscopy a Nikon Eclipse E800 microscope equipped with a Basler A113 digital camera and the Lucia 4.51 software on a Windows NT workstation was used.

RESULTS

Antibodies directed against $\alpha_1\beta_1$, $\alpha_2\beta_1$, or $\alpha_v\beta_3$ integrins attenuate collagen gel contraction and reduce apoptosis of fibroblasts cultured in mechanically relaxed collagen gels Fibroblasts cultured in a contractile, mechanically relaxed collagen matrix undergo apoptosis in a time-dependent manner as described earlier (Fluck *et al*, 1998), and in order to further investigate this phenomenon we studied the role of distinct integrins in apoptosis induction in fibroblasts.

First, the presence of $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_v\beta_3$ integrins on the surface of primary fibroblasts released from mechanically relaxed and stressed collagen gels was confirmed by FACS analysis (**Fig 1**). These experiments showed that the surface expression of α_1, α_2, and β_1 integrins is slightly elevated in fibroblasts cultured in a mechanically stressed matrix. In contrast to this, the fluorescence signal for integrin $\alpha_v\beta_3$ was slightly elevated in mechanically relaxed gels and simultaneously fewer cells in these gels were positive. So, although the integrin expression was slightly elevated in mechanically stressed gels as opposed to relaxed gels, their expression in both culture systems was approximately equivalent.

Then primary fibroblasts were cultured in three-dimensional collagen matrices in the presence of adhesion-blocking antibodies directed against $\alpha_1\beta_1$, $\alpha_2\beta_1$, or $\alpha_v\beta_3$ integrins, and as control either without any, or with an irrelevant anti-β_2, antibody. Antibodies directed against $\alpha_1\beta_1$ integrins showed a delayed onset as well as a pronounced decrease in gel contraction, and the effect of antibodies against $\alpha_2\beta_1$ was even stronger (**Fig 2*a***, *inset*). In contrast, anti-$\alpha_v\beta_3$ antibody had no effect on gel contraction compared with the control.

For the same experiments the respective time courses of apoptosis in the presence or absence of anti-integrin antibodies were determined by extraction and quantitation of cytoplasmic nucleosome-size DNA using a sandwich ELISA. Apoptosis reached its maximum between day 2 and day 4 in all experiments (**Fig 2*a***), and cell numbers decreased (data not shown). Interestingly, there was a marked difference in the extent of apoptosis in cells incubated with distinct antibodies. Blocking of $\alpha_1\beta_1$ or $\alpha_2\beta_1$ integrin by adhesion blocking antibodies nearly halved the extent of apoptosis at day 2 (**Fig 2*a***), whereas incubation with an antibody against $\alpha_v\beta_3$ integrin resulted in a 30% reduction of apoptosis. Moreover, when mechanically stressed collagen gels were used instead of relaxed collagen gels, apoptosis of fibroblasts did not occur either in the absence or in the presence of the respective antibodies (**Fig 2*b***).

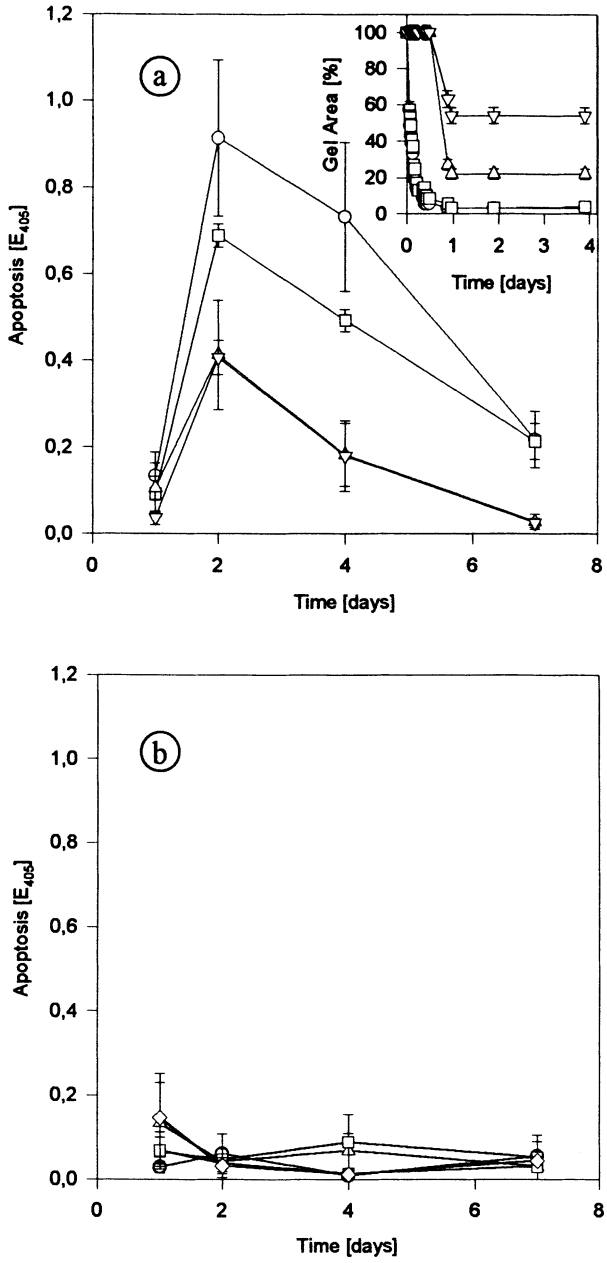

Figure 2. Adhesion-blocking anti-integrin antibodies reduce contraction-dependent apoptosis of primary fibroblasts embedded in a three-dimensional collagen matrix without inducing anoikis. Fibroblasts were cultured in the presence of antibodies against integrins $\alpha_1\beta_1$ (△), $\alpha_2\beta_1$ (▽), or $\alpha_v\beta_3$ (□), and as control either without (○) or with (◇) irrelevant anti-β_2 antibodies, in three-dimensional mechanically relaxed (*a*) and stressed (*b*) collagen gels. After distinct incubation times the cells were counted and lyzed, and nucleosome-size DNA in the cytoplasmatic fraction of lyzed fibroblasts was measured with a cell death detection ELISA. Data are means (± SD) of a representative experiment run in triplicate. (*a*) *inset*: Fibroblasts were embedded and cultured in mechanically relaxed collagen gels in the absence (○) or presence of antibodies directed against the integrins $\alpha_1\beta_1$ (△), $\alpha_2\beta_1$ (▽), or $\alpha_v\beta_3$ (□). At distinct time points gel areas were measured. Data are means (± SD) of a representative experiment run in triplicate.

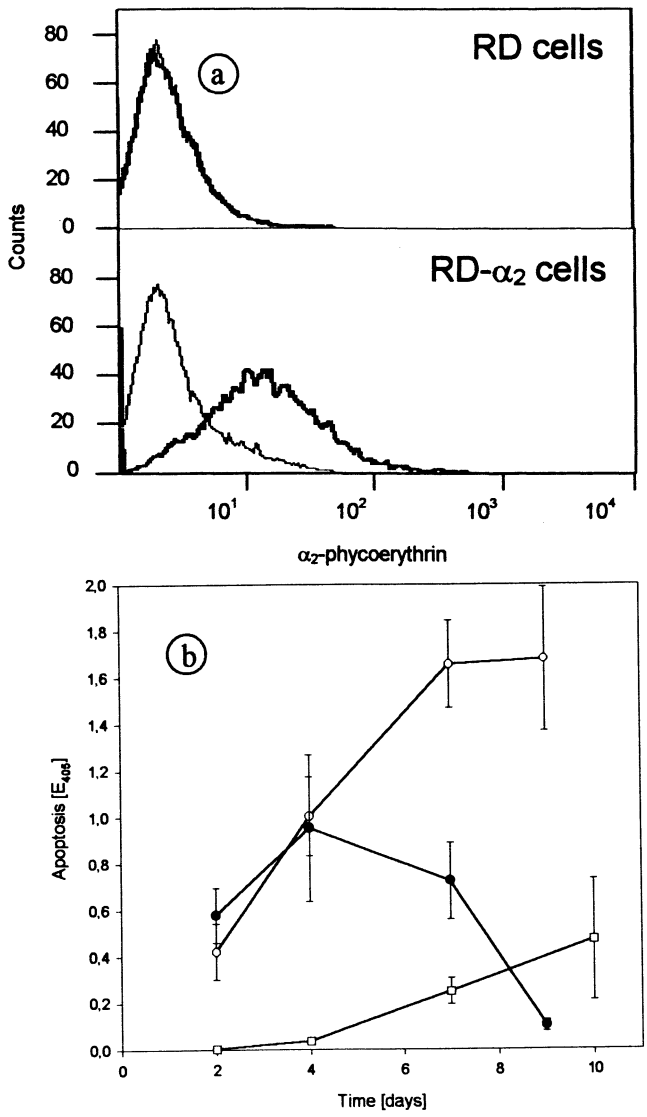

Figure 3. Contraction-dependent apoptosis of α_2-deficient RD cells can be restored by transfection of α_2 integrin. (*a*) FACS analysis of α_2 expression in RD cells (*upper panel*) and retransfected RD cells (*lower panel*). *Bold line*, integrin α_2 signal; *thin line*, unlabeled control. (*b*) RD cells lacking α_2 integrin (□) and stably retransfected RD cells were embedded in contractile collagen gels either in the presence (●) or absence (○) of antibodies against $\alpha_2\beta_1$ integrins. After distinct incubation times the cells were counted and lyzed, and nucleosome-size DNA in the cytoplasmatic fraction of lyzed cells was measured with a cell death detection ELISA. Data are means (± SD) of a representative experiment run in triplicate.

A_2-deficient RD cells do not significantly undergo contraction-dependent apoptosis in relaxed collagen gels, and susceptibility to it is restored after retransfection with α_2 integrin To further investigate the involvement of α_2 integrin in apoptosis, RD cells that lack functional α_2 integrin (**Fig 3*a***, *upper panel*) were cultured in mechanically relaxed collagen gels. The time course of apoptosis, as determined by cell death detection ELISA, is shown in **Fig 3(*b*)**. Interestingly, in α_2-deficient RD cells a slight increase of apoptosis with time in contractile gels up to day 10 could be observed. In order to find an explanation for this increase, we checked by FACS the expression of other integrins that might be responsible for this (**Fig 4**). There was virtually no difference in α_1 and $\alpha_v\beta_3$ integrin expression, whereas β_1 was slightly elevated in RD cells compared with α_2-transfected RD cells.

After transfection with α_2 integrin RD cells expressing functional α_2 integrin (**Fig 3*a***, *lower panel*) showed, in contrast to α_2-deficient RD cells, a marked increase of apoptosis within this time period under the same culture conditions (**Fig 3*b***). This effect could be abrogated again by addition of adhesion-blocking antibodies against $\alpha_2\beta_1$ integrin (**Fig 3*b***).

Disturbance of cytoskeletal integrity reduces apoptosis in primary fibroblasts cultured in a mechanically relaxed collagen matrix Contraction-dependent apoptosis may be inhibited not only by disturbing the cells' contact to their extracellular matrix, but also by inhibiting the intracellular force-generating system. Therefore, inhibitor studies were performed with cytochalasin D, which affects the actin cytoskeleton and thereby the cells' ability to build up contraction forces. In addition, we investigated the effect of BDM, which inhibits myosin ATPase and thereby hampers cellular contractility. At a concentration of 0.5 nM cytochalasin D already disturbed the cytoskeletal organization and effectively destroyed stress fibers, which are usually formed in fibroblasts under mechanical tension during culture in stressed collagen gels but are absent in mechanically relaxed collagen gels (**Fig 5*c–f***). Notably, this concentration did not induce apoptosis by itself, neither in mechanically stressed collagen gels, where usually no apoptosis occurs (Fluck *et al*, 1998), nor in relaxed collagen gels (**Fig 5*a***). But it was still capable of effectively inhibiting contraction in relaxed gels, which reached only about 53% of the initial area (data not shown). Concomitant with the inhibition of contraction apoptosis was completely abrogated (**Fig 5*a***). Likewise, 20 mM BDM alone did not induce apoptosis in primary fibroblasts, but rather suppressed apoptosis in contractile, mechanically relaxed gels (**Fig 5*b***).

DISCUSSION

In a three-dimensional collagenous and contractile matrix primary dermal fibroblasts undergo apoptosis, but not when cultured on collagen (two-dimensional), or in three-dimensional contracting fibrin gels, or in noncontractile collagen gels. After demonstrating the necessity of these three prerequisites (Fluck *et al*, 1998), we now investigated the dependence of apoptosis on matrix contraction in more detail.

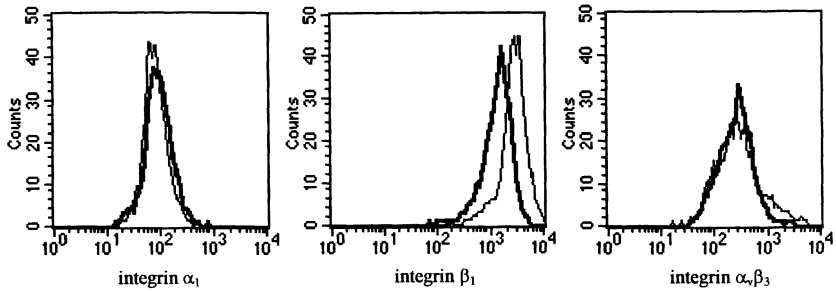

Figure 4. Expression of integrins α_1, β_1, and $\alpha_v\beta_3$ on α_2-deficient RD cells and on RD cells stably retransfected with α_2 integrin. RD cells and stably retransfected RD cells were harvested from monolayer cultures and analyzed by FACS for the surface expression of distinct integrins (*bold lines*, integrin expression in α_2-expressing RD cells; *thin lines*, integrin expression in α_2-deficient RD cells).

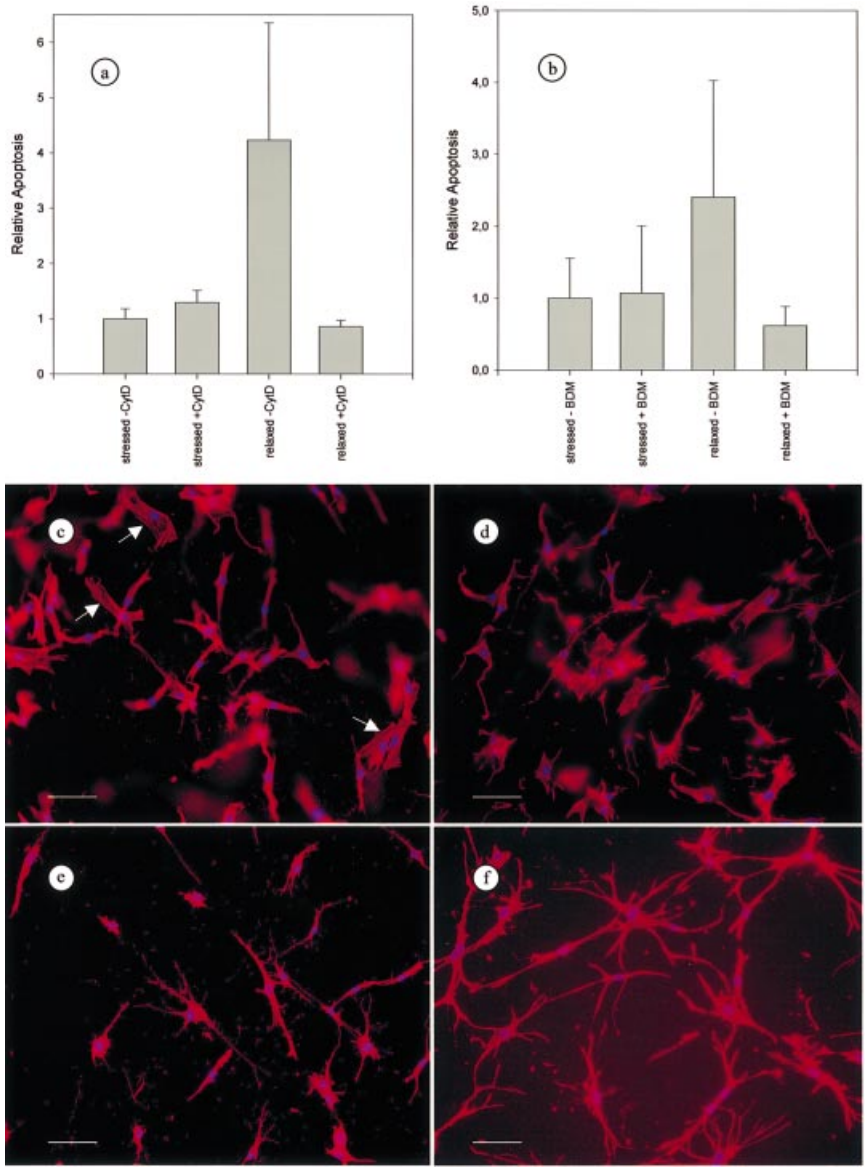

Figure 5. Disturbance of cytoskeletal integrity reduces apoptosis in primary fibroblasts cultured in a mechanically relaxed collagen matrix. Fibroblasts were cultured for 2 d in mechanically stressed and relaxed gels either in the presence or absence of 0.5 nM cytochalasin D or 20 mM BDM. Subsequently, the cells were harvested and lyzed, and nucleosome-size DNA in the cytoplasmatic fraction of lyzed cells was measured with a cell death detection ELISA relative to controls without any inhibitor (*a*, *b*). In addition, gels were fixed with formalin and the actin cytoskeleton of collagen-embedded cells was affinity stained with phalloidin-TRITC; arrows point to stress fibers (*c–f*; *scale bars*: 25 μm). Data are means (± SD) of a representative experiment run in triplicate.

Integrins are well-known receptors mediating cell–cell and cell–matrix interactions. Meredith *et al* (1993) and Frisch and Francis (1994) showed that normal endothelial and epithelial cells undergo apoptosis when the cells are detached from their substrate with RGD peptides or attachment is prevented by growing the cells in suspension. Meredith *et al* (1993) demonstrated in addition that attachment of endothelial cells to a substrate coated with anti-integrin antibodies protected against apoptosis, whereas antibodies against nonintegrin cell surface molecules were ineffective. In certain cell types, however, only specific ligated integrins suppress apoptosis, even though other integrins could mediate attachment (Howlett *et al*, 1995). Therefore, the same integrin may mediate a different signal transduction in different cell types and promote apoptosis in one cell type and prevent apoptosis in the other cell type.

In this study an *in vitro* system, recently established by us, was used, which is based on the observation that fibroblasts undergo apoptosis, as determined by DNA laddering, TUNEL, cell death ELISA, and nuclear morphology, when cultured in three-dimensional mechanically relaxed, contractile collagen matrices (Fluck *et al*, 1998). This system allows the systematic investigation of distinct integrins in the induction of apoptosis in human fibroblasts.

In a first series of experiments we analyzed the expression of α_1, α_2, β_1, and $\alpha_v\beta_3$ integrins on the surface of fibroblasts cultured in a three-dimensional matrix of fibrillar collagen I of different mechanical resistance. FACS analyses revealed that the surface expression of α_1, α_2, and β_1 integrins was approximately equivalent between cells cultured either in stressed or in relaxed collagen gels; at the most their expression was slightly elevated in mechanically stressed gels but not in relaxed gels. In contrast to this, expression of integrin $\alpha_v\beta_3$ was slightly elevated in mechanically relaxed gels. These findings together with the concept of anchorage-dependent survival (Frisch and Ruoslahti, 1997) therefore suggest that disturbance of the cell–matrix contact would result in increased apoptosis in mechanically stressed rather than in relaxed gels.

In a subsequent series of experiments we consequently investigated the effect of different adhesion-blocking antibodies against these integrins on gel contraction and apoptosis of dermal fibroblasts cultured in these culture systems. Our experiments clearly revealed that the incubation of normal human primary fibroblasts with antibodies directed against $\alpha_1\beta_1$ or $\alpha_2\beta_1$ reduced gel contraction compared with control fibroblasts without the addition of antibodies. This result was considered as control for the biologic efficacy of the respective antibodies and integrins, as it is in

accordance with findings of Langholz *et al* (1995). Moreover, we demonstrate that incubation with an antibody directed against the vitronectin receptor integrin $\alpha_v\beta_3$, which is expressed by the fibroblasts, does not alter gel contraction compared with untreated control fibroblasts.

In the same experiments the time courses of apoptosis were determined. Apoptosis reached its maximum between day 2 and day 4 in all experiments. This result for primary fibroblasts is in accordance with our previous findings (Fluck *et al*, 1998) and served as a control for the validity of our system. Interestingly, incubation of normal human primary fibroblasts with adhesion-blocking antibodies directed against $\alpha_1\beta_1$, $\alpha_2\beta_1$, or $\alpha_v\beta_3$ integrins significantly reduced apoptosis in mechanically relaxed (i.e., contractile) collagen gels. This effect was more pronounced with anti-$\alpha_1\beta_1$ and $\alpha_2\beta_1$ antibodies, and it was smaller for anti-$\alpha_v\beta_3$ antibodies, which were shown not to influence gel contraction. Experiments in which adhesion-blocking antibodies against integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ were applied simultaneously to increase this effect were – albeit interesting – not performed, as this would have also increased the number of nonadherent cells, which simply fall through the collagen matrix, to an unacceptably high level. On the other hand, using mechanically relaxed, contractile fibrin gels (Fluck *et al*, 1998) and mechanically stressed (i.e., noncontractile) collagen gels (**Fig 2*b***), no apoptosis was detectable, either in the presence or in the absence of the respective antibodies. As a consequence, the adhesion-blocking antibodies used in these experiments obviously did not induce apoptosis of dermal fibroblasts by disturbing cell–matrix contact. Rather they reduced apoptosis by inhibiting matrix contraction in this system. This is an interesting phenomenon, as it is not obvious at the first glance how it may be possible for a cell to survive expressing a pro-apoptotic collagen receptor. It may be possible, however, that (i) the different integrin signals are redundant and the signal of a single integrin type is not sufficient to trigger apoptosis. It may also be possible that (ii) alterations of mechanical forces, which the cells experience within the three-dimensional meshwork of extracellular matrix molecules, rather than integrin ligation/extracellular matrix contact, induce apoptosis. It is further possible that (iii) signals from different integrin types have distinct functions, e.g., gel contraction or perception of matrix tension, and that only the simultaneous integration of all the signals triggers apoptosis. The latter could also explain why blocking $\alpha_v\beta_3$ reduces apoptosis by 30% but has no observable effect on gel contraction.

In a further series of experiments RD cells, which lack functional α_2 integrin subunits (Schiro *et al*, 1991), were embedded in this system, and the time course of apoptosis was determined. As a result, there was just a weak increase in apoptosis up to day 10. Survival of RD cells in relaxed collagen gels *per se*, however, did not imply that the absence of $\alpha_2\beta_1$ integrin could be responsible for transduction of an apoptosis-promoting signal. Therefore, control experiments were performed with retransfected RD cells expressing functional α_2 integrin. These cells underwent apoptosis at high levels again (**Fig 3*b***), providing further evidence that α_2 integrin may be responsible for transducing an apoptosis-promoting signal. By addition of anti-integrin antibodies this induction of apoptosis in α_2-transfected RD cells could be revoked again. Moreover, these results are in accordance with data that were obtained in antibody perturbation studies using primary fibroblasts. Taken together, α_2 integrin may exert an important function as transducer of a proapoptotic signal. Other integrins such as $\alpha_1\beta_1$ or $\alpha_v\beta_3$ may in part be involved in this signaling process, however, which would explain the slight increase in apoptosis of RD cells in mechanically relaxed collagen gels, as well as the incomplete inhibition of fibroblast apoptosis in such gels by adhesion-blocking anti-integrin antibodies.

All β_1 and α_v subunit containing integrins share the ability to promote the assembly of focal contacts (Giancotti, 1997). In focal contacts the extracellular matrix is attached to integrins that intracellularly directly bind to talin and α-actinin, which in turn bind to other structural proteins including vinculin, paxillin, and tensin, ultimately connecting to the actin filaments of the cytoskeleton (Aplin *et al*, 1998). Actin and myosin comprise a traction force-generating system within the cell, and disruption of the microfilaments decreases fibroblast retraction (Thoumine and Ott, 1996) and isometric tension in cell-populated collagen gels (Kolodney and Wysolmerski, 1992). When cytochalasin D, an inhibitor of F-actin polymerization, or BDM, an inhibitor of actin–myosin interaction, were applied at low doses, which did not induce cell death by themselves, to fibroblasts in relaxed collagen gels, these inhibitors revoked stress fiber formation and disturbed the cytoskeletal organization, which in turn resulted in attenuated isotonic contraction. These observations are in accordance with findings of Arora *et al* (1999), who used the same collagen gel culture systems to study the transforming growth factor β (TGF-β) mediated induction of α-smooth muscle actin in fibroblasts under different mechanical tension. They reported that TGF-β_1-induced increases of α-smooth muscle actin are dependent on the mechanical stiffness/resistance of the extracellular matrix, and that the generation of intracellular tension is a central determinant of contractile cytoskeletal gene expression. Our data presented here reveal that disturbance of cytoskeletal integrity and contractility interestingly also abrogates apoptosis in such a mechanical relaxed matrix.

Although this study does not distinguish with certainty between the possibilities of either disturbed force generation or an increased cytoskeletal stiffness (Atlas and Lin, 1978; Domnina *et al*, 1982), this observation provides further circumstantial evidence that integrin-mediated apoptosis of primary fibroblasts in three-dimensional collagen gels is contraction dependent.

The result that adhesion-blocking antibodies directed against distinct integrins reduce rather than induce apoptosis of fibroblasts in contractile collagen gels is in accordance with the fact that anoikis could not be observed in fibroblasts, in contrast to other cell types such as epithelial and endothelial cells (Ruoslahti and Reed, 1994). Furthermore, whereas contraction of a collagenous matrix triggers the disposal of fibroblasts in our system by induction of apoptosis (Fluck *et al*, 1998), the data presented here provide evidence that collagen receptors of the integrin type mediate a proapoptotic signal, but not in terms of anoikis. In our studies the fibroblasts remain attached to their extracellular matrix via redundant receptors, and blocking matrix contact of one of them results in a reduced proapoptotic signal. We assume that our observations *in vitro* may also be operative *in vivo*. Whereas mechanically stressed collagen gels resemble granulation tissue during later stages of remodeling with collagen fibers oriented parallel to fibroblasts, mechanically relaxed collagen gels are more similar to resting dermis or to very early stages of wound healing, with low resistance of the extracellular matrix to intracellular tension (Grinnell, 1994). It can be imagined that apoptotic disposal of surplus fibroblasts, for instance during late steps of wound healing and scar formation, is not primarily regulated by adherence or nonadherence, i.e., by anoikis, but rather by changes in cell shape and mechanical forces, respectively (Chen *et al*, 1997). Both appear during wound contraction and involve integrins as mechanoreceptors, which link the actin cytoskeleton to the extracellular matrix (Wang *et al*, 1993). Increased expression of α-smooth muscle actin inhibits fibroblast motility (Rønnov-Jessen and Petersen, 1996). Under physiologic conditions a reduced motility is meaningful for a fibroblast that has entered a fresh wound, both to quickly deposit new extracellular matrix material and to minimize the wound by its contraction. Once the wound is closed surplus fibroblastic cells are removed by apoptosis (Desmoulière *et al*, 1995).

The ubiquity of integrins places these molecules as primary candidates for the delivery of apoptotic or antiapoptotic signals from the extracellular matrix (Bates *et al*, 1995). The signals propagated by integrins interacting with the extracellular matrix result in the activation of several intracellular pathways (Kroemer *et al*, 1995; Richardson and Parsons, 1995; Schwartz *et al*, 1995; Parsons, 1996). It remains to be elucidated which pathway for transduction of the apoptosis-inducing signal is used by primary

fibroblasts during contraction of a three-dimensional collagenous matrix *in vitro* and during wound healing *in vivo*.

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REFERENCES

Aplin AE, Howe A, Alahari SK, Juliano RL: Signal transduction and signal modulation by cell adhesion receptors: the role of integrins, cadherins, immunoglobulin-cell adhesion molecules, and selectins. *Pharmacol Rev* 50:197–263, 1998

Arora PD, Narani N, McCulloch CAG: The compliance of collagen gels regulates transforming growth factor-β induction of α-smooth muscle actin in fibroblasts. *Am J Pathol* 154:871–882, 1999

Atlas SJ, Lin S: Dihydrocytochalasin B. Biological effects and binding to 3T3 cells. *J Cell Biol* 76:360–370, 1978

Bates RC, Lincz LF, Burns GF: Involvement of integrins in cell survival. *Cancer Metastasis Rev* 14:191–203, 1995

Bell E, Ivarsson B, Merill C: Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential *in vitro*. *Proc Natl Acad Sci USA* 76:1274–1278, 1979

Chan BMC, Matsuura N, Takada Y, Zetter BR, Hemler ME: *In vitro* and *in vivo* consequences of VLA-2 expression on rhabdomyosarcoma cells. *Science* 251:1600–1602, 1991

Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE: Geometric control of cell life and death. *Science* 276:1425–1428, 1997

Cooper JA: Effects of cytochalasin and phalloidin on actin. *J Cell Biol* 105:1473–1478, 1987

Desmoulière A, Redard M, Darby I, Gabbiani G: Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. *Am J Pathol* 146:56–66, 1995

Domnina LV, Gelfand VI, Ivanova OY, Leonova EV, Pletjushkina OY, Vasiliev JM, Gelfand IM: Effects of small doses of cytochalasins on fibroblasts. Preferential changes of active edges and focal contacts. *Proc Natl Acad Sci USA* 79:7754–7757, 1982

Fluck J, Querfeld C, Cremer A, Niland S, Krieg T, Sollberg S: Normal human primary fibroblasts undergo apoptosis in three-dimensional contractile collagen gels. *J Invest Dermatol* 110:153–157, 1998

Folkman J, Moscona A: Role of cell shape in growth control. *Nature* 273:345–349, 1978

Frisch SM, Francis H: Disruption of epithelial cell–matrix interactions induces apoptosis. *J Cell Biol* 124:619–626, 1994

Frisch SM, Ruoslahti E: Integrins and anoikis. *Curr Opin Cell Biol* 9:701–706, 1997

Gailit J, Clark RAF: Wound repair in the context of extracellular matrix. *Curr Opin Cell Biol* 6:717–725, 1994

Giancotti FG: Integrin signaling: specificity and control of cell survival and cell cycle progression. *Curr Opin Cell Biol* 9:691–700, 1997

Grinnell F: Fibroblasts, myofibroblasts, and wound contraction. *J Cell Biol* 124:401–404, 1994

Howlett A, Bailey N, Damsky C, Peterson O, Bissell M: Cellular growth and survival are mediated by β_1 integrins in normal human breast epithelium but not in breast carcinoma. *J Cell Sci* 108:1945–1957, 1995

Juliano RL, Haskill S: Signal transduction from the extracellular matrix. *J Cell Biol* 120:577–585, 1993

Kolodney MS, Wysolmerski RB: Isometric contraction by fibroblasts and endothelial cells in tissue culture: a quantitative study. *J Cell Biol* 117:73–82, 1992

Kroemer G, Petit P, Zamzami N, Vayssiére J-L, Mignotte B: The biochemistry of programmed cell death. *FASEB J* 9:1277–1287, 1995

Langholz O, Röckel D, Mauch C, Kozlowska E, Bank I, Krieg T, Eckes B: Collagen and collagenase gene expression in three-dimensional collagen lattices are differentially regulated by $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins. *J Cell Biol* 131:1903–1915, 1995

Mauch C, Hatamochi A, Scharffetter K, Krieg T: Regulation of collagen synthesis within a three-dimensional collagen gel. *Exp Cell Res* 178:493–503, 1988

McKillop DFA, Fortune NS, Ranatunga KW, Geeves MA: The influence of 2,3-butanedione 2-monoxime (BDM) on the interaction between actin and myosin in solution and in skinned muscle fibres. *J Muscle Res Cell Motil* 15:309–318, 1994

Meredith JE Jr, Schwartz MA: Integrins, adhesion and apoptosis. *Trends Cell Biol* 7:146–150, 1997

Meredith JE, Fazeli B, Schwartz MA: The extracellular matrix as a cell survival factor. *Mol Biol Cell* 4:953–961, 1993

Montgomery AMP, Reisfeld RA, Cheresh DA: Integrin $\alpha_v\beta_3$ rescues melanoma cells from apoptosis in three-dimensional dermal collagen. *Proc Natl Acad Sci USA* 91:8856–8860, 1994

Parsons JT: Integrin-mediated signalling: regulation by protein tyrosine kinases and small GTP binding proteins. *Curr Opin Cell Biol* 8:146–152, 1996

Raghow R: The role of extracellular matrix in postinflammatory wound healing and fibrosis. *FASEB J* 8:823–831, 1994

Richardson A, Parsons JT: Signal transduction through integrins: a central role for focal adhesion kinase? *Bioessays* 17:229–236, 1995

Rønnov-Jessen L, Petersen OW: A function for filamentous α-smooth muscle actin: retardation of motility in fibroblasts. *J Cell Biol* 134:67–80, 1996

Ruoslahti E, Reed JC: Anchorage dependence, integrins and apoptosis. *Cell* 77:477–478, 1994

Schiro JA, Chan BMC, Roswit WT, *et al*: Integrin $\alpha_2\beta_1$ (VLA-2) mediates reorganization and contraction of collagen matrices by human cells. *Cell* 67:403–410, 1991

Schwartz MA, Schaller MD, Ginsberg MH: Integrins: emerging paradigms of signal transduction. *Annu Rev Cell Dev Biol* 11:549–600, 1995

Takada Y, Hemler ME: The primary structure of the VLA-2/collagen receptor α_2 subunit (platelet GPIa): homology to other integrins and the presence of a possible collagen-binding domain. *J Cell Biol* 109:397–407, 1989

Thoumine O, Ott A: Influence of adhesion and cytoskeletal integrity of fibroblast traction. *Cell Motil Cytoskeleton* 35:269–280, 1996

Wang N, Butler JP, Ingber DE: Mechanotransduction across the cell surface and through the cytoskeleton. *Science* 260:1124–1127, 1993