Normal Human Primary Fibroblasts Undergo Apoptosis in Three-Dimensional Contractile Collagen Gels

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Apoptosis of primary fibroblasts was observed in vivo during wound healing in skin and is expected to occur in other organs as well; however, the environmental signal for induction of apoptosis in fibroblasts and the putative influence of cell–matrix interactions on the regulation of apoptosis remain to be identified. Here we provide evidence for the role of fibrillar collagen in this process, and demonstrate that normal human primary fibroblasts embedded in contractile collagen gels undergo apoptosis as shown by the appearance of cytoplasmic histone-associated DNA fragments starting at day 1 of culture with a peak around days 2–4. This induction of apoptosis in primary fibroblasts seems to be specific for contractile collagen gels, because apoptosis of primary fibroblasts was neither observed in cells grown on culture dishes or on plastic dishes coated with collagen, nor observed in cells seeded in either anchored collagen gels or contractile fibrin gels. We therefore conclude that a distinct environment such as a contractile collagen matrix determines the susceptibility of normal primary fibroblasts to apoptosis.


During normal embryonic and adult development, cells that are unnecessary or deleterious appear to be eliminated by a process referred to as apoptosis or programmed cell death. Apoptosis can be distinguished from necrosis by distinct morphologic characteristics (e.g., chromatin condensation, nuclear fragmentation, plasma membrane blebbing, and cell shrinkage) as well as by genomic DNA cleavage via endonucleases (for review see Majno and Joris, 1995). Recently, it became apparent that in most systems apoptosis seems to be dependent on de novo protein synthesis leading to the characterization of several pro- and anti-apoptotic genes (Nagata and Golstein, 1995; Korsmeyer, 1995).

Apoptosis has been described both in vitro and in vivo in several tissues and cell types; however, little is known about the induction of apoptosis in fibroblasts. In vitro there is good evidence that primary fibroblasts can undergo apoptosis: myofibroblasts suffer apoptosis in granulation tissue evolving into a scar (Desmoulière et al, 1995). On the other hand it has been demonstrated that primary fibroblasts in vitro do not undergo apoptosis following serum withdrawal or irradiation (Frisch and Francis, 1994; McKenna et al, 1996); however, serum-deprived fibroblasts, transfected with c-myc, die if c-myc is activated (Harrington et al, 1994). Besides c-myc, rho-p21 is another example of a potent inducer of apoptosis, whereas bcl-2, v-abl, E1B, or ras prevent activation of the apoptotic pathway in rodent fibroblasts, even in the presence of an otherwise deadly stimulus (Jiménez et al, 1995).

Yet, the question of which environmental conditions make primary fibroblasts susceptible to apoptosis is unresolved. Because cell–matrix interactions have major effects on several biologic features such as organomorphogenesis during embryonal development, wound healing, tumor invasion, metastasis, and fibrosis (Folkman and Moscona, 1978; Adams and Watts, 1993; Langholz et al, 1995), we hypothesize that extracellular matrix components might also act as regulatory elements of apoptosis in fibroblasts (Meredith et al, 1993; Frisch and Francis, 1994; Ruoslalhi and Reed, 1994). Further evidence in favor of this hypothesis has recently been published. It has been shown that apoptosis is induced in epithelial cells by the disruption of interactions with their extracellular matrix (Frisch and Francis, 1994; Boudreau et al, 1995). Furthermore, in human melanoma cells grown in collagen gels the expression of α4β3 integrin suppresses apoptosis (Montgomery et al, 1994).

To further investigate apoptosis in fibroblasts, we used in vitro test systems, which allow the systematic study of the influence of extracellular matrix components on the induction of apoptosis in fibroblasts. The cells were seeded in two different three-dimensional collagen gel systems (contractile and anchored collagen gels), compared with monolayer cultures and three-dimensional contracting fibrin gels. Culturing fibroblasts within a three-dimensional environment reconstituted mainly of collagen I fibrils has proven to be a valuable tool in studying several cellular functions under conditions that resemble the in vivo situation more closely than fibroblast monolayer cultures (Bell et al, 1979). Fibroblasts seeded in these collagen gel systems show profound differences in their mechanical features and in cell proliferation (for a review see Grinnell, 1994). In contractile collagen gels, the tension is distributed isotropically, and there is a marked decline in cellular DNA synthesis. The cells become arrested and cell regression begins. This process is accompanied by dramatic changes in fibroblast morphology (Mauch et al, 1988) and metabolism, resulting in down-regulation of type I collagen and induction of interstitial collagenase (matrix metalloproteinase-1). The latter is controlled at the transcriptional level, whereas collagen synthesis is regulated by transcriptional as well as post-transcriptional mechanisms (Mauch et al, 1989; Eckes et al, 1993). In anchored collagen matrices, on the other hand, tension is distributed anisotropically, and the cells continue to synthesize DNA and increase in cell number (for a review see Grinnell, 1994).

In this study, we provide evidence that normal human primary
fibroblasts are susceptible to apoptosis in vivo, when seeded in three-dimensional contractile collagen gels.

MATERIALS AND METHODS

Cells Normal primary human skin fibroblast cultures were established from biopsies taken from healthy volunteers. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal calf serum, 50 mg sodium ascorbate per ml, 300 mg glutamine per ml, 100 U penicillin per ml, 100 µg streptomycin per ml under 5% CO2 and at 37°C. For all experiments, cells from passages one to five were used after harvesting by trypsinization [0.025% trypsin, 0.02% ethylenediamine tetraacetic acid in phosphate-buffered saline (PBS)].

Collagen preparation Native porcine collagen I was purchased from Deutsche Gelatine Fabriken Stoess AG (Eberbach, Germany), 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 lyophilized. Finally, the collagen was dissolved in sterile 0.1% acetic acid (3 mg per ml).

Collagen gels Hydrated collagen gels were prepared according to Mauch et al. (1988). At first the acidic collagen solution was neutralized with 0.1 M NaOH. Subsequently for each experiment 2 × 10^5 fibrin gels were added to 1.5 ml aliquots of a neutralized collagen solution (final concentration 1 mg DMEM per ml, supplemented with 10% fetal calf serum, 50 mg sodium ascorbate per ml, 300 mg glutamine per ml, 100 U penicillin per ml, 100 µg streptomycin per ml, pH 7.2).

Aliquots (1.5 ml) of this cell/collagen mixture were placed in 3.5 cm culture dishes (anchored gels) or uncoated plastic dishes (contractile gels), and put in an incubator adjusted to 5% CO2 and 37°C, where the gels polymerized within 60 min.

In all systems used in this work, except anchored collagen gels, DMEM containing serum and supplements as indicated above was replaced daily to guarantee sufficient supply with nutrients, serum, and supplements. In anchored collagen gels, where the medium cannot be aspirated without destroying the gel matrix, 1.0 ml of fresh culture medium was overlaid instead and also changed daily.

Collagen coated dishes For seeding normal human primary fibroblasts on top of collagen gels, 1.5 ml of collagen solution (1 mg per ml in DMEM with standard supplements excluding fetal calf serum, pH 7.2) was polymerized on uncoated 3.5 cm plastic dishes. Subsequently 1 × 10^5 cells in DMEM containing standard supplements and 10% fetal calf serum (see above) were placed on top of the gels.

Uncoated dishes Monolayer cultures on uncoated culture dishes were prepared using standard conditions as indicated. In each experiment 1 × 10^5 cells per normal 3.5 cm culture dish were used.

Fibrin gels Fibrin gels were made according to Gillery et al (1989) with the following modifications: PolyHEMA plates were prepared by applying 2 × 4 ml of a 10 mg per ml solution of poly (2-hydroxyethyl methacrylate) (Sigma, Deisenhofen, Germany) in ethanol onto the dishes and subsequently drying under the tissue culture hood, followed by extensive PBS washes.

Subsequently, 2 mg fibrinogen (Tissucol®, Immuno, Heidelberg, Germany) were dissolved in 1 ml of DMEM without supplements at 37°C immediately before use and put into polyHEMA-coated 3.5 cm petri dishes. Two × 10^5 fibrin gels in 300 µl DMEM containing 130 mg Thrombin S per ml and 5 mM CaCl2 were added onto petri dishes, gently swirled during the almost immediate polymerization, and subsequently incubated with DMEM containing serum and standard supplements as indicated above.

Gel solubilization and cell recovery For cell recovery collagen gels were washed twice in PBS and solubilized by incubation with 750 µl collagenase VII (2.3 mg per ml, Biochrom, Berlin, Germany) at 37°C in PBS containing 0.5% bovine serum albumin. As soon as the gels were completely solubilized after 30 ± 10 min, the reaction was stopped with 200 µl fetal calf serum and released trypan blue solution. Cells were washed twice with PBS. One of 10 of the cells was mixed with trypan blue and counted.

Cell recovery from fibrin gels and monolayer cultures was carried out correspondingly with 0.025% trypsin and 0.02% ethylenediamine tetraacetic acid in PBS instead of collagenase VII.

Labeling of fragmented DNA with digoxigenin dioxyuridine 5’ triphosphate (DIG-ddUTP) The DNA from the recovered cells was isolated according to standard protocols (Sambrook et al, 1989) and 3' end labeled with DIG-ddUTP and terminal deoxynucleotidyl transferase (Boehringer, Mannheim, Germany). Subsequently the DIG-labeled DNA was electrophoresed in an 1.5% agarose gel, transferred onto nylon membrane, and visualized by chemiluminescence techniques (Boehringer).

Cell death detection enzyme-linked immunosorbent assay (ELISA) Recovered cells were lysed and cytoplasmatic histone-associated DNA fragments in an equivalent of 1000 cells were quantitated by a cell death detection ELISA (Boehringer) according to the manufacturer’s instructions. For better comparison, apoptotic ratios were calculated on the basis of 1000 cells per normal culture dishes. In all experiments normal human fibroblasts from three different healthy volunteers were used.

RESULTS

To evaluate different extracellular matrix conditions for their capability to induce apoptosis, we cultivated normal primary human fibroblasts in different culture systems: (i) monolayer cultures; (ii) on top of collagen gels; (iii) contractile collagen gels; (iv) contractile fibrin gels; and (v) anchored collagen gels. In all experiments normal human fibroblasts from three different healthy volunteers were used.

Fibroblasts grown as monolayer cultures or within a two-dimensional collagen matrix do not show DNA or nuclear fragmentation Fibroblasts grown as monolayer cultures started to proliferate with a duplication rate of about 2 d (Fig 1a, inset). Quantitative analyses of histone-associated DNA fragments via ELISA revealed no increase in DNA fragmentation up to day 9 (Fig 1a).

Figure 1. Fibroblasts undergo apoptosis, when seeded in three-dimensional contractile collagen gel, as demonstrated by cell death detection ELISA. Fibroblasts were cultured for the times indicated in either two-dimensional (a) or three-dimensional (b) culture systems: monolayer cultures (a), on top of collagen (b), in contractile collagen gels (c), in contractile fibrin gels (d, b), and in anchored collagen gels (c). Inset: change in cell numbers versus time. Data are means (SD of four experiments).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling-staining of apoptotic cells in gel sections After depa不明的 apoptotic cells within paraffin gel sections were labeled with an in situ cell death terminal deoxynucleotidyl transferase dUTP-fluorescein isothiocyanate (FITC) detection kit (Boehringer) according to the manufacturer’s instructions, counter-stained with 10 µg ethidium bromide per ml in PBS (10 min at 37°C), washed thoroughly with PBS, and examined with a fluorescence microscope (Axioskop, Zeiss, Oberkochen, Germany) using standard excitation/emission filter combinations.

Detection of fragmented nuclei in gel sections Gel sections were depara不明定, rinsed shortly in 4,6-diamidino-2-phenylindole (DAPI) solution (1 µg per ml in methanol), and stained for 10 min at 21°C with the same solution. Subsequently sections were washed thoroughly with PBS, and fragmentation of nuclei in apoptotic cells (Collins et al, 1992) was visualized with a fluorescence microscope.

To determine whether a two-dimensional collagenous matrix induces apoptosis, proliferation and apoptosis rates of normal primary fibroblasts, seeded on top of collagen gels, were measured. Yet, we observed no major differences on principal, neither in growth rate (Fig 1a, inset) nor in DNA fragmentation (Fig 1a), as compared with monolayer cultures. In particular, no apoptosis was detected up to day 9 in this system.
Figure 2. DNA fragmentation in fibroblasts isolated from contractile collagen gels is revealed by electrophoretic size fractionation. DNA fragmentation is time dependent with its onset after 0.2 d and a maximum around day 4. It occurs only in fibroblasts seeded in contractile collagen gels, but not in monolayer cultures. Fibroblasts were cultured on culture dishes as monolayers (Mo) or in contractile collagen gels for 0.2 d, 1 d, 4 d, or 7 d. Their DNA was isolated, 3' end labeled with DIG-ddUTP, analyzed by electrophoresis in a 1.2% agarose gel, and visualized by chemiluminescence techniques. Two separate and independent experiments showed identical results.

Figure 3. Fibroblasts seeded in contractile collagen gels are susceptible to apoptosis, as shown by DNA and nuclear fragmentation using terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling and DAPI staining. Fibroblasts were either grown as monolayer cultures (a-c) or seeded in three-dimensional contractile collagen gels (d-f) and in anchored collagen gels (g-i) for 4 d. Monolayer cultures and paraffin sections of the collagen gels, respectively, were first labeled with ddUTP-FITC (a, d, g, yellow-green immunofluorescence), followed by a staining with ethidium bromide (b, e, h, red staining) in order to visualize the nuclei of identical regions. Exposure time and processing were identical for these photomicrographs. In addition, fragmented DNA was labeled with ddUTP-FITC in paraffin sections of contracting collagen gels. After 1 d FITC-labeled cells could scarcely be detected (data not shown). On day 4 of cultivation, however, we found that the majority of all visible cells were labeled with FITC (Fig 3d). Although most of the fibroblasts were located near to the contractile gel’s periphery, there was no difference in the ratio of apoptotic and viable cells in the inner and outer part of the gel (data not shown). The microscopic observation of DAPI-stained fragmented nuclei (Fig 3f) gives further independent evidence that the mode of cell death here is apoptosis.
Fibroblasts seeded in contractile fibrin gels or anchored collagen gels do not undergo apoptosis. Additional experiments were performed to test the hypothesis that induction of apoptosis is dependent on collagen as the major matrix component and that a three-dimensional noncollagenous matrix (e.g., fibrin) is unable to induce apoptosis in fibroblasts. For this purpose normal primary fibroblasts were seeded in contractile fibrin gels. Although the fibroblasts contracted the fibrin gels to an extent comparable with collagen gels, they did not proliferate and did not show any enhanced DNA fragmentation (Fig 1b) in this system.

Finally, normal primary fibroblasts were grown in anchored collagen gels, which are not contractile. Fibroblasts seeded in this system started to proliferate with a duplication rate of about 2 d (Fig 1b inset), and no increase in histone-associated DNA fragments and fragmented nuclei was observed (Fig 1b, Fig 3g,b).

DISCUSSION

Healing of wounds, as well as any tissue regeneration related to organomorphogenesis, tumor invasion, metastasis, inflammation, and fibrosis, are complex biologic processes that result in remodelling of the tissue. Much attention has been paid to factors that control cell proliferation and activation of extracellular matrix synthesis (Gailit and Clark, 1994). In contrast, very little is known about mechanisms that are necessary for downregulation of activated cells or for their removal when they are no longer needed.

Cell elimination could be the result of terminal differentiation, e.g., fibroblasts responsible for wound contraction start to express α-smooth muscle actin and differentiate into myofibroblasts. Elimination of cells could also be mediated by degenerative necrosis or through apoptosis. There is, however, only limited information available about the regulation of apoptosis in fibroblasts and the putative influence of environmental conditions operative in this event.

Our experiments show that primary fibroblasts embedded in three-dimensional contractile fibrin gels display a bipolar shape and decrease in cell number, which is in accordance with Mauch et al (1988) and Grinnell (1994). As a new finding, our experiments clearly reveal an induction of apoptosis in primary fibroblasts, when seeded in three-dimensional contractile collagen gels. The presence of apoptosis was proven by the detection of strongly enhanced levels of fragmented low molecular weight DNA in gel electrophoresis and quantitated by an ELISA technique. In addition, with the help of a fluorescence microscope we visualized apoptotic cells in contractile collagen gel sections by labeling the DNA fragments with ddUTP-FITC and by staining the fragmented nuclei with DAPI.

The susceptibility of primary fibroblasts to apoptosis depends on a distinct environment. Based on our experiments, apoptosis of primary fibroblasts can only be induced when all of the three following conditions are given: (i) cells must be seeded in a collagenous matrix; (ii) the collagenous matrix must be three-dimensional; and (iii) the three-dimensional collagenous matrix must be contractile, emphasizing the importance of distinct features of this system (s Dorothy distribution, tension, decline in cellular DNA synthesis, cell arrest, and metabolic changes). These results are supported by the observation that apoptosis of primary fibroblasts is not induced in two-dimensional monolayer cultures either on normal culture dishes or in fibroblasts seeded on top of collagen gels, and also not in contractile fibrin gels or in anchored (i.e., noncontracting) collagen gels.

From our experiments, we conclude that the contact of fibroblasts with the surrounding three-dimensional collagenous matrix and/or distinct mechanical forces exerted in this system are inducers of apoptosis. Because integrins play an important role in cell–matrix interactions, these molecules might also mediate an apoptosis-regulating signal (Frisch and Francis, 1994; Montgomery et al, 1994; Boudreau et al, 1995). Controlling the proliferative state of cells may be another possibility to regulate susceptibility to apoptosis of normal human primary fibroblasts embedded in contractile collagen gels. In myotransfected fibroblasts apoptosis is reported to be dependent on myc-expression: if transcription of myc is activated, the cells die after serum deprivation (Harrington et al, 1994). This phenomenon seems not to result from a conflict of growth signals, but appears to be a normal physiologic aspect of c-myc function, whose execution is regulated by the availability of survival factors. Therefore, surviving fibroblasts in contractile collagen gels possibly escape apoptosis by growth arrest.

As there is no increased rate of apoptosis within contractile fibrin gels, the hypothesis that exceeding a critical cell density might be responsible for the induction of apoptosis in our system can be ruled out. Furthermore, it can be assumed that both macromolecular matrices (contractile collagen and fibrin gels) form sponge-like meshworks with diffusive spaces comparable with each other and with normal skin conditions, allowing the free access of oxygen, nutrients, and cytokines to the cells in both cases (Mauch et al, 1988; Carr and Hauge, 1990; Packer et al, 1994; Saltzmann et al, 1994). Nevertheless, cell–cell contacts or paracrine factors operative in contractile collagen gels cannot be excluded as possible inducers of apoptosis.

Recently, Desmoulière et al (1995) showed that the evolution of granulation tissue into scar tissue implies a massive decrease in cellularity, involving the disappearance of fibroblasts, endothelial cells, and pericytes. Our observation that normal human primary fibroblasts are susceptible to apoptosis in vitro provides strong evidence that the decrease in cellularity during later phases of wound healing is mediated by apoptosis. In addition, comparison of fibroblast apoptosis in contractile collagen gels in vitro and during wound healing in skin in vivo (Desmoulière et al, 1995), indicates that in both situations a contractile three-dimensional collagenous matrix is required, in vitro a contractile matrix supplemented by granulation tissue.

It can be hypothesized that a reduced apoptosis caused by an alteration of the three-dimensional collagenous matrix and/or the mechanical forces results in impaired wound healing such as hypertrophic scars and keloids.

REFERENCES

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