Modulation of FAK, Akt, and p53 by Stress Release of the Fibroblast-Populated Collagen Matrix

Mark A. Carlson, M.D.,* Michael T. Longaker, M.D.,† and Jon S. Thompson, M.D.*

*Department of Surgery, University of Nebraska Medical Center and the Omaha VA Medical Center, Omaha, Nebraska; and †Department of Surgery, Stanford University, Stanford, California

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Background. Fibroblast survival in a three-dimensional collagen matrix is dependent in part upon the rigid anchorage of the matrix to tissue culture plastic. We hypothesized that focal adhesion kinase (FAK) and protein kinase B (Akt) would be activated and that the p53 level would be low in the rigidly anchored (attached) collagen matrix; loss of anchorage (detachment) was hypothesized to have the opposite effects.

Materials and methods. Human foreskin fibroblasts were cultured in attached bovine collagen matrices for 48 h before detachment as free-floating matrices. At various time points postrelease, matrix lysates were blotted for the proteins of interest, and the terminal deoxynucleotidyltransferase-mediated dUTP nick-end label assay was performed on both whole matrices and cytospin preparations. Irradiated monolayer fibroblasts were used as positive controls for the amount of p53 protein.

Results. Terminal deoxynucleotidyltransferase-mediated dUTP nick-end label positivity in attached versus detached matrices (at 24 h post detachment) was 0.7 ± 0.3 versus 5.3 ± 1.7% (P < 0.05, unpaired t test). FAK and Akt were phosphorylated (activated) in the attached matrix; there was a near complete loss of both activated forms within 4 h of matrix detachment. Irradiated monolayer fibroblasts had increased levels of p53, mdm2, and p21. In contrast, the p53, mdm2, and p21 levels were just at the level of detection in the attached matrix, but were induced 5- to 10-fold within 2–4 h after matrix detachment.

Conclusions. FAK and Akt are activated in the attached fibroblast-populated collagen matrix whereas the p53 level is relatively low; matrix detachment downregulates FAK and Akt activity and induces p53. The state of mechanical anchorage of the collagen matrix regulates the survival of embedded fibroblasts through a mechanism which may involve FAK. © 2004 Elsevier Inc. All rights reserved.

Key Words: fibroblast populated collagen matrix; focal adhesion kinase; protein kinase B; Akt; p53; mdm2; p21.

INTRODUCTION

Excisional wounding of the dermis in mammals results in the generation of granulation tissue, which fills the wound defect; the wound subsequently is closed through the processes of wound contraction and epithelialization [1]. Immediately after excision, the number of wound cells present is essentially zero; rapid wound cell proliferation occurs subsequently, followed by the prolonged maturation and contraction phase, during which the hypercellular granulation tissue gradually regresses secondary to apoptosis [2], with scar as the end result. The wound cell population is tightly regulated during this progression, and hence the control of wound cell proliferation and survival is highly relevant for appropriate healing.

Because in vivo models of granulation tissue are complex and difficult to work with, in vitro models such as the fibroblast-populated collagen matrix (FPCM) have been developed to study the behavior of fibroblasts in a three-dimensional matrix [3, 4]. The three-dimensional collagen matrix can model in vivo cell–extracellular matrix (ECM) interactions more closely than two-dimensional (monolayer) culture systems [5]. Both fibroblast proliferation [6, 7] and survival [8, 9] are upregulated in the FPCM by attachment of the matrix to a rigid substratum (i.e., tissue culture plas-
tic). Detachment (release) of the collagen matrix from its substratum results inhibits the fibroblast cell cycle and induces apoptosis. The effect of matrix release on the cell cycle appears to be via a block in the extracellular-regulated kinase pathway at the level of the small GTP-binding protein Ras [7, 10, 11]. The mechanism involving release-induced apoptosis appears to be dependent on inhibition of an ECM-regulated phosphatidylinositol 3-kinase (PI-3-kinase)/Akt pathway [12].

Certain primary adherent cells (especially of epithelial or endothelial origin) in monolayer culture will undergo apoptosis if the integrin-mediated attachment to the substratum is disrupted [13, 14]; this phenomenon of detachment-induced apoptosis in adherent monolayer cells also is known as “anoikis” [15]. Primary monolayer fibroblasts will go into cell-cycle arrest when if detached from the culture dish, but are relatively resistant to anoikis [16]. In monolayer primary fibroblasts, adhesion activates focal adhesion kinase (FAK), which subsequently upregulates the PI-3-kinase/Akt survival pathway and suppresses p53 [17, 18], which promotes fibroblast survival. We extrapolated these observations in monolayer culture to hypothesize that matrix anchorage in the FPCM activates FAK, which results in activation of Akt and inhibition of p53, producing a pro-survival environment. Loss of matrix anchorage was predicted to reverse this sequence, i.e., FAK and Akt inactivation and p53 induction, resulting in a pro-apoptotic environment.

MATERIALS AND METHODS

Cell Culture

The use of human tissue was approved by our institutional review board. Human fibroblasts were cultured from explants of neonatal foreskin in growth medium, which consisted of Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Inc.) supplemented with 20 mM HEPES (Sigma, St. Louis, MO), 44 mM sodium bicarbonate (Sigma), 1% Antibiotic/Antimycotic Solution (Life Technologies, Inc.), 1.5 mM Fungizone (Life Technologies, Inc.), and 10% fetal bovine serum (FBS); growth medium pH was 7.2. Cells were main-

Preparation of Collagen Matrices

Collagen matrices were prepared as previously described [8, 19]. A suspension of fibroblasts (10⁵ per mL) in solubilized bovine Type 1 collagen (Vitrogen 100, Cohesion Technologies; final concentration = 1.5 mg/mL) in DMEM (neutralized solution) was incubated at 37°C with continuous gentle agitation for 4 min before pipetting a 200-µL aliquot (containing 2 × 10⁶ cells) into each well (containing a 12-mm circular inscription) of a 24-well plate (Costar). The aliquots then were incubated for 1 h at 37°C, during which time they polymerized to form FPCMs, and then 2 mL of growth medium (supplemented with 50 µg/mL of vitamin C) was added to each well. The medium was changed 48 h later (before the initiation of any experiment). An FPCM was released by gently lifting it off the culture well with a smooth, rounded metal spatula.

Cytospin Preparations

Some of the terminal deoxynucleotidyltransferase-mediated dUTP nick-end label (TUNEL) assays were performed on cytospin preparations of the FPCMs, as previously described [8]. A matrix was washed twice with PBS and then incubated with 100 µL of 0.25% trypsin/1 mM EDTA in Hank’s balanced salt solution (Life Technologies, Inc.) for 10 min at 37°C. A 400-µL aliquot of 5 mg/mL collagenase (Type 1A, Sigma) in 130 mM NaCl, 10 mM Ca acetate, 20 mM HEPES (pH 7.2) was directly added to each well, and then the plate was placed on a rotary agitator (250 rpm) for 30–45 min at 37°C. The enzymatic digestion was quenched with 50 µL of PBS, and the cells were pelleted with a 4 min centrifugation at 600g. The cell pellet was resuspended in 200 µL of DMEM, and the cells were spun down onto Plus slides (Fisher) in a Shandon Cytospin 3 centrifuge (6 min, 1000 rpm). The cytospin preparation by itself does not have a significant impact on the apoptotic rate derived from the subsequent TUNEL assay [8].

TUNEL Assay

The TUNEL assay was performed both on whole matrices and on cytospin preparations, as previously described [8]. Whole matrices were fixed in 3% paraformaldehyde in PBS for 20 min at 22°C, rinsed three times with PBS, and then permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 30 min at 22°C. TUNEL labeling was performed using a kit (FITC In Situ Cell Death Detection; Roche), following the manufacturer’s instructions; the reaction volume was 100 µL/matrix. The matrices were rinsed three times with PBS, and then counterstained with 5 µg/ml propidium iodide (PI; Sigma) in PBS with 20 µg/mL of DNase-free RNAse (Roche) for 15 min at 22°C. The matrices were washed three times with PBS, and mounted whole onto Plus slides using Fluoromount G (Southern Biotechnology Associates) and #1 glass coverslips. Cytospin preparations were fixed in 3% paraformaldehyde in PBS for 5 min at 22°C, and then a similar sequence followed, except with smaller reactions volumes (20–25 µL/preparation). In some experiments, an attached FPCM was treated with 1 µM staurosporine (Sigma) for 4 h to generate a positive control for the TUNEL assay. Whole FPCM or cytospin preparations were visualized with a confocal microscope (Zeiss LSM 410) or a Nikon Microphot fluorescent microscope, respectively.

Radiation Treatment

Subconfluent monolayer fibroblasts in 10-cm round plates were placed into a Mark I-22 Irradiator (J. L. Shepherd and Associates) with a 137Cs source, which had been calibrated within 6 months of the experiments to deliver an average dose of 3,190 rad in 5 min. The dose ranged from 2160 to 4020 rad, depending on the position within the radiation canister.

Immunoprecipitation and Immunoblotting

At the indicated time points post-release, matrices were placed on ice and washed three times with ice-cold PBS and dounced (50 strokes) in ice-cold lysis buffer (500 µL per 18 matrices) containing 50 mM Tris (pH 7.4; Sigma), 150 mM NaCl, 1% NP40 (Sigma), 1% sodium deoxycholate (Sigma), 0.1% SDS (Sigma), 5 mM EDTA (Sigma), 50 mM sodium fluoride (Sigma), 0.1 mM sodium vanadate (Sigma), 1 mM AEBSP (Sigma), 1 mg/mL pepstatin A (Sigma), and 1 mg/mL leupeptin (Sigma). Crude lysates were incubated on ice for 30 min and centrifuged at 25,000g for 30 min at 4°C. A 50-µL aliquot of the supernatant was saved for the DNA assay, and the remainder was boiled for 4 min in reducing buffer. Sodium dodecyl sulfate
Polyacrylamide gel electrophoresis was performed on 7% polyacrylamide gels as previously described; the proteins were transferred to PVDF membrane and then blotted with antibodies to FAK (clone C20; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-Tyr397-FAK (clone 44–624; Biosource International), α-tubulin (Sigma), Akt (clone H-136; Santa Cruz), phospho-Ser473-Akt (Cell Signaling), p21 (clone F5; Santa Cruz), or mdm2 (clone SMP14; Santa Cruz). Protein bands were visualized using Enhanced Chemiluminescence (ECL; Amersham). For p53, a 200-μL aliquot of each clarified lysate was combined with 2 μL of anti-p53 antibody (clone Ab-7, from sheep; Calbiochem) overnight on a clinical rotator at 4°C. The antibody was precipitated with 50 μL of protein G-agarose beads for 1 hr at 4°C, and the beads were boiled in reducing buffer (25 μL) for 4 min. The boiled volume was run on sodium dodecyl sulfate polyacrylamide gel electrophoresis as above, and the PVDF membrane was blotted with anti-p53 (clone 80, from mice; BD Transduction Labs). Densitometry was performed with an image analysis program (ImageJ, http://rsb.info.nih.gov/ij/).

DNA Assay

The DNA assay was adopted from a standard protocol [20]. Clarified lysate (10 μL) was added to 5 ng/mL bisbenzimide (Hoechst 33258; Sigma) in PBS (final volume 2 mL; performed in triplicate), and fluorescence was read on a spectrophotometer (excitation/emission of 356/458 nm) against DNA standards of 0–1 μg/mL.

Data Analysis

TUNEL-positive and PI-labeled nuclei were quantified within ImageJ; the apoptotic index (% apoptotic) was the number of TUNEL-positive nuclei in a 10x micrograph divided by the total number of nuclei (PI-labeled), × 100. A minimum of eight micrographs from each condition in each experiment was counted to generate a mean apoptotic index. Data were analyzed with analysis of variance and the unpaired t test.

RESULTS AND DISCUSSION

Stress Release of the FPCM Induces Fibroblast Apoptosis

Previous work indicated that mechanical detachment of the prestressed FPCM would induce fibroblast apoptosis [8, 9, 21, 22]; our first intention with these investigations was to replicate this result. Confocal microscopy was performed on whole FPCMs which were attached, released for 24 h, or treated with staurosporine (Fig. 1A–F). The latter is a broad-spectrum kinase inhibitor that can induced apoptosis in a wide range of cells, including primary dermal fibroblasts [8]. Qualitatively, there was minimal to nil apoptosis in the attached FPCM. Both staurosporine and matrix release induced cell death. The increase in apoptotic index after matrix release was quantified in cytospin preparations (Fig. 1G). Immediately after release, the index was approximately 0.5% in both the attached and released conditions; 24 h later, the index had risen 10-fold in the released matrix. These data were consistent with previously published data.
Stress Release of the FPCM Downregulates FAK and Akt

Phosphorylation of FAK on Tyr-397 (the autophosphorylation site) and of Akt on Ser-473 has been shown to correlate closely with kinase activity and signal transduction through these enzymes [23, 24]. The effect of FPCM attachment and release on total versus phosphorylated FAK and Akt is shown in Fig. 2. Both enzymes were phosphorylated in the attached matrix; within 2–4 h after release; however, this activity dropped and was almost undetectable by 8 h. The level of total FAK and Akt also began to decrease in the released matrix 8–24 h after detachment (Fig. 2A and C). These data indicate that FAK and Akt are activated in the attached FPCM, but become inactivated several hours after stress release.

Irradiation of Monolayer Foreskin Fibroblasts Induces p53, mdm2, and p21

To determine whether p53 was effected by stress release of the FPCM, we needed to ensure that this protein could be detected in our cells, since p53 is expressed at low levels [25]. Monolayer fibroblasts initially were treated with 1000 rad of gamma radiation, which was within the dosage range reported to produce a p53 response in human fibroblasts [26–28]; no p53 induction was observed, however, and there was no gross effect on the fibroblast morphology (phase microscopy) one week later compared to controls (data not shown). Fibroblasts then were irradiated with a dose that ranged from 1,000 to 96,000 rad; remarkably, cells receiving up to 24,000 rad were still viable and relatively normal appearing one week later (data not shown). A dose of 6000 rad (which required 9.6 min in the canister) produced no visible effect on fibroblast morphology, but p53 induction was observed; this dose was used for all subsequent irradiation experiments. To confirm p53 induction, two transcriptional targets of p53, mdm2 and p21, also were assayed with immunoblotting [29, 30]. Monolayer fibroblasts irradiated with 6000 rad demonstrated induction of p53, mdm2, and p21 within 3 h after treatment, with levels detected out to 24 h (Fig. 3A and B).

Stress Release of the FPCM Induces p53, mdm2, and p21

Having produced a positive control for p53, mdm2, and p21 induction in our cells, we next determined
whether the level of these proteins were altered after stress release of the FPCM. We used an immunoblot for tubulin as a loading control in the monolayer irradiation experiment; in preliminary experiments with the FPCM, however, the level of tubulin did not appear to be relatively constant after matrix release (data not shown). We then attempted to control for loading using Bradford-related protein assay (Bio-Rad), but this also was inaccurate because of the large amount of collagen in the whole gel lysates (data not shown). Initial blots for p53 on whole gel lysates were difficult to interpret secondary to a high level of nonspecific binding (data not shown); a readily interpretable result was obtained using a p53 immunoprecipitate/immunoblot sequence (Fig. 3C). Matrix release was associated with a 2- to 20-fold induction of p53, mdm2, and p21 within 6 h of detachment (Fig. 3C and D).

Proposed Model of ECM-Regulated Survival in the FPCM

A consistent observation in the FPCM model has been that matrix attachment to the culture well is prosurvival for the fibroblast, and that matrix detachment induces apoptosis. In this report it was demonstrated that several survival-regulating proteins are modulated during the same time frame that cell death occurs. Down-regulation of FAK and Akt, events which are both associated with anoikis in monolayer cells [31, 32], and induction of p53, which has been implicated in detachment-induced death in serum-starved monolayer fibroblasts [18], both occur in the first 6 h after FPCM release. FAK is a well-known activator/facilitator of the PI-3-kinase/Akt cell survival pathway [24]; furthermore, FAK also can suppress apoptosis by inhibiting p53 [18, 32]. A hypothesis of ECM-regulated survival in the FPCM based on all of these observations is shown in Fig. 4. In the attached condition, matrix contraction is restrained by adhesion to the underlying rigid substratum (i.e., the tissue culture well). This produces mechanical stress [33, 34], which is a known activator of integrin receptors [35, 36]. Integrin activation promotes FAK phosphorylation and activity [24]; FAK activation up-regulates Akt and inhibits p53 (the latter effect most likely is secondary to enhanced degradation [30]. The net effect on the intra-
The only FAK-regulated survival mechanism in the PI-3-kinase/Akt pathway does not appear to be an additional regulator of survival in the FPCM, because dephosphorylation of Akt. We have hypothesized that p53 is an integrin linkage, which promotes survival in part through Akt. Their results still support the concept of an ECM-attached matrix as a cell survival factor. 

Further elucidation of this model will await targeted disruption of the FPCM (see text for details).

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