Rigidity of Collagen Fibrils Controls Collagen Gel-induced Down-regulation of Focal Adhesion Complex Proteins Mediated by $\alpha_2\beta_1$ Integrin*

Received for publication, January 6, 2003, and in revised form, March 10, 2003
Published, JBC Papers in Press, April 3, 2003, DOI 10.1074/jbc.M300092200

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Previous studies have shown that collagen gel overlay induced selective proteolysis of focal adhesion complex proteins in Madin-Darby canine kidney (MDCK) cells. In this study, we examined whether morphological and biochemical changes were present in cells cultured on collagen gel. We found that focal adhesion complex proteins, including focal adhesion kinase (FAK), talin, paxillin, and p130cas, but not vinculin, were decreased within 1 h when MDCK cells were cultured on collagen gel. Gel-induced selective decrease of focal adhesion proteins was observed in all lines of cells examined, including epithelial, fibroblastic, and cancer cells. Matrigel also induced selective down-regulation of focal adhesion proteins. However, cells cultured on collagen gel- or matrigel-coated dishes did not show any changes of focal adhesion proteins. These data suggest that the physical nature of the gel, i.e. the rigidity, is involved in the expression of focal adhesion proteins. The collagen gel-induced down-regulation of focal adhesion complex proteins was caused by reduction of protein synthesis and activation of proteases such as calpain. Overexpression of a dominant negative mutant of discoidin domain receptor 1 (DDR1) or FAK-related non-kinase (FRNK) did not prevent collagen gel-induced down-regulation of the focal adhesion complex protein, whereas an anti-$\alpha_2\beta_1$ integrin-neutralizing antibody completely blocked it. Taken together, our results indicate that the rigidity of collagen gel controls the expression of focal adhesion complex proteins, which is mediated by $\alpha_2\beta_1$ integrin but not DDR1.

Adhesion of cells to the extracellular matrix (ECM) is a crucial event in multi-cellular organisms for the modulation of cellular processes such as cell growth, differentiation, and apoptosis (1–4). The integration of intracellular signaling and the structure of ECM elicited by ECM-integrin engagement may be mediated by focal adhesion complex proteins (5). Focal adhesion kinase (FAK) is a cytoplasmic non-receptor tyrosine kinase located close to focal adhesions and may play a central role in integrating signals from ECM-integrin and growth factors (6–8). FAK also plays important roles in the assembly of several signaling proteins to focal adhesions via interactions with a number of cellular proteins, including Src, Grb2, phosphatidyl-insitol-3 kinase, paxillin, Crk, talin, and p130cas (6, 9–13). Recent data have shown that FAK plays important roles in cell cycle progression (14), migration (8, 9, 15), adhesion (12), and the prevention of apoptosis (16, 17).

Three-dimensional collagen gel has been used as a cell culture vehicle for the study of morphogenesis by us and many laboratories (18–20). Collagen fibrils can transduce signals through integrins and the receptor tyrosine kinase discoidin domain receptor 1 (DDR1) (21). Interaction of ECM with its transmembrane receptor integrins causes subsequent cascades of protein-protein interaction and modification at the focal adhesion complex site and the recruitment of several cytoskeletal proteins to form the focal adhesion complex (22). Engagement of collagen with its receptor tyrosine kinase, DDR1, resulted in the tyrosine phosphorylation of DDR1 (23, 24). However, the signaling pathways triggered by activation of DDR1 have not been well understood.

Although collagen gel may exert both biochemical and biophysical impact on cells, its biophysical impact on cell biology has been largely unknown. Collagen gel is a flexible substrate with the rigidity of 30–100 pascals as measured by a dynamic mechanical analyzer. In contrast, a normal cell culture dish is non-flexible with a rigidity of more than 1 gigapascals. Therefore, the biophysical effects of collagen gel on cells could be due to its physical property, i.e. very low rigidity. To differentiate the biophysical effects of collagen gel from its biochemical impacts, we employed cells cultured on collagen gel and a collagen gel-coated dish for comparison. Our previous report showed that collagen gel overlay induced selective proteolysis of focal adhesion complex proteins in MDCK cells (25). In this study, we employed various cell lines to examine whether collagen gel affected the expression of focal adhesion complex proteins via its physical nature. We found that collagen gel, but not collagen gel coating, induced selective down-regulation of focal adhesion proteins in all lines of cells examined. Our data demonstrated that lowered substratum rigidity regulated the expression of focal adhesion proteins. The signaling mechanism whereby the collagen gel regulated expression of focal adhesion was explored further.

* This work was supported by National Health Research Institute Grant NHRI-EX91-9031SL and the Ministry of Education (MOE) program for promoting academic excellence of the University under grant number 91-B-FA09-1-4. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¶ The abbreviations used are: ECM, extracellular matrix; FAK, focal adhesion kinase; DDR1, discoidin domain receptor 1; MDCK, Madin-Darby canine kidney; DMEM, Dulbecco’s modified Eagle’s medium; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; Ab, antibody; TRITC, tetramethylrhodamine isothiocyanate; PYK2, proline-rich tyrosine kinase 2; FRNK, FAK-related non-kinase.

** C.-C. Wu, Y.-H. Wang, M.-J. Tang, and H.-C. Chang, unpublished observations.
treatment with the Klenow enzyme and ligase to generate the carboxyl-terminal truncated clone that encoded 524 amino acids. The truncated DDR1b clone was cloned into a pcDNA 3.1 plasmid. The plasmid containing DDR1b or truncated DDR1 was transfected into MDCK clone II B5 and selected by G418.

Immunofluorescence and Confocal Study—MDCK cells cultured on dish, collagen gel coated dish, or collagen gel for 4 h were washed three times with phosphate-buffered saline (PBS) and fixed using 4% paraformaldehyde prepared in PBS for 20 min at room temperature. Cells were then washed three times in PBS and permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature and then incubated with anti-FAK monoclonal Ab for 1 h. Cells were then washed and incubated with Alexa Fluor 488-conjugated goat anti-mouse Ab (Amersham-Pharmacia Biotech) and phalloidin-TRITC (from Sigma) for 1 h. The immunofluorescent images were taken by confocal microscopy (Leica, TCS-SP2).

RESULTS

Collagen Gel Induced Down-regulation of Focal Adhesion Complex Proteins—To understand the mechanism of collagen gel-induced changes in MDCK cells, we first examined the expression of focal adhesion complex proteins in MDCK cells cultured on dish, collagen gel-coated dish, or collagen gel. MDCK cells cultured on a collagen-coated dish had a slightly retracted morphology in comparison with cells cultured on a normal dish (Fig. 1A). Meanwhile, MDCK cells cultured on collagen gel exhibited round-up morphology 1 h after attachment and gradually developed more retractive and elongated morphology later on (Fig. 1A). We found that FAK was significantly decreased as early as 1 h in cells cultured on collagen gel, and the decrease persisted for 24 h (Fig. 1B). Meanwhile, FAK remained intact in cells cultured on dish or collagen gel-coated dish, suggesting that the physical property of collagen gel per se induced a decrease in FAK levels. Other focal adhesion complex proteins such as talin, paxillin, the FAK family protein PYK2, and the FAK downstream protein p130CAS were decreased with time course similar to that of FAK when the cells were cultured on collagen gel. However, vinculin, β-actin, the cytosolic protein c-Src, and the endoplasmic reticulum protein calnexin remained intact. These data suggested that collagen gel selectively regulated proteins located on focal adhesions, particularly in proteins contacting directly with integrin (e.g. FAK and talin) and located in integrin downstream signaling (e.g. PYK2, p130CAS, and paxillin), but not in the structural proteins or cytoskeletal associated protein in integrin downstream (e.g. vinculin, β-actin) or proteins localized other than focal adhesions (e.g. c-Src and calnexin).

To clarify whether collagen gel-induced selective decrease of focal adhesion complex proteins is a general phenomenon, we employed nine different cell lines representing normal epithelial cells (MDCK, LLCPK1, NRK52E), transformed/cancer cells (Change Liver, HeLa, TCCSUP, and U373MG), and fibroblasts (3T3 and 293). These cells were on a dish, collagen gel-coated dish, or collagen gel for 24 h, and their proteins in focal adhesion complex were analyzed by Western blot. We found that collagen gel induced a selective decrease in FAK, talin, PYK2, p130CAS, and paxillin in all of the cell lines examined (Fig. 2). These focal adhesion complex proteins remained unchanged when the cells were cultured on dish or collagen gel-coated dish, consistent with our notion that the collagen gel-induced decrease of focal adhesion complex proteins is regulated by the physical property of collagen gel.

Matrigel Also Induced Down-regulation of Focal Adhesion Complex Proteins—To understand whether the components of a basement membrane might exert similar effects, we employed matrigel. We cultured MDCK cells on a matrigel-coated dish or on matrigel for 4 h and analyzed the changes of focal adhesion complex proteins. MDCK cells cultured on a matrigel-coated dish showed relatively retracted cell shape, and on ma-
trigel they exhibited completely round-up morphology (Fig. 3A). The Western blot results showed that focal adhesion complex proteins were selectively decreased when MDCK cells were cultured on matrigel but remained unaltered when they were cultured on a matrigel-coated dish (Fig. 3B). These data indicate that down-regulation of focal adhesion complex proteins induced by collagen gel and matrigel is controlled by the physical property of gel, i.e. substratum rigidity.

In addition, we tested the effects of agarose gel (0.5–4%) on the expression of focal adhesion proteins. In our experience, the rigidity of agarose gel at a concentration as low as 0.5% is still higher than that of collagen gel of any concentration. For experiments of this purpose, agarose gel was coated with 0.3% collagen gel to ensure cell attachment. We found that cells cultured on agarose gel displayed spreading morphology regardless of the concentration of agarose gel (Fig. 3C). Meanwhile, focal adhesion complex proteins remained unchanged in cells cultured on agarose gel of any concentration (Fig. 3D). These data indicate that a threshold might be required for the substratum rigidity-regulated expression of focal adhesion complex proteins. However, the threshold of rigidity that causes down-regulation of focal adhesion complex proteins remains to be defined.

Collagen Gel-induced Down-regulation of Focal Adhesion Complex Proteins Is Mediated by Post-transcriptional Mechanism

To delineate whether the collagen gel-induced decrease in focal adhesion complex proteins is mediated by transcriptional regulation, we cultured MDCK cells on a dish, a collagen gel-coated dish, and collagen gel and analyzed the mRNA levels of FAK, talin, PYK2, p130cas, paxillin, c-Src, calnexin, or vinculin. β-actin was used as an internal control.
sis rates by assessing the ratio of $[^{35}S]$methionine incorporation into protein. As shown in Fig. 4B, collagen gel resulted in a significant decrease of total protein synthesis rates within 4 h. To examine whether the collagen gel-induced decrease in focal adhesion complex proteins is mediated by activation of proteases, we pretreated MDCK cells with different protease inhibitors for 1 h, then cultured the cells on collagen gel for 4 h in the presence of protease inhibitors, and analyzed their focal

Fig. 3. Matrigel and collagen gel exert similar effects on down-regulation of focal adhesion proteins. A, morphology of MDCK cells cultured on dish (C), collagen gel- or matrigel-coated dish (Co), and collagen gel or matrigel (G) taken under phase-contrast microscope at 4 h. B, MDCK cells were cultured on a dish (C), a collagen gel-coated dish (Co) or a matrigel (G), or a matrigel-coated dish (Co) or matrigel (G) for 4 h, and the cell lysates were harvested and analyzed by Western blot. C, morphology of MDCK cells cultured on dish (C), collagen gel-coated dish (Co), a collagen gel or an agarose gel (G) of various concentrations (0.5–4%). The pictures were taken under a phase-contrast microscope at 4 h. D, MDCK cells cultured on dish (C), collagen gel-coated dish (Co), or collagen gel (G) and agarose gel (0.5–4%) for 4 h, and the cell lysates were harvested and analyzed by Western blot by using specific Ab against FAK, talin, PYK2, p130cas, paxillin, and vinculin.

Fig. 4. Collagen gel-induced down-regulation of focal adhesion complex proteins is mediated by post-transcriptional mechanisms. A, RT-PCR results of FAK, talin, and c-Src mRNA expression in MDCK cells cultured on a dish (C), a collagen gel-coated dish (Co), or a collagen gel (G) for the indicated times. The total RNA was extracted by a Trizol commercial kit. 0.2–1 μg of total RNA was used for RT-PCR as described under “Experimental Procedures.” GAPDH was used as an internal control. B, protein synthesis rates in MDCK cells cultured on a dish (C), a collagen gel-coated dish (Co), or collagen gel (G) for the indicated times. Cells were serum starved for 1 h followed by 1 h of treatment with $[^{35}S]$methionine. Cellular proteins were precipitated by 10% trichloroacetic acid, and the precipitates were then solved by 1 N NaOH. The radioactivity was measured by β-counter. C, Western blot analysis of FAK, talin, and p130cas in MDCK cells on a dish (C), a collagen gel-coated dish (Co), or a collagen gel (G) with the treatment of protease inhibitors, i.e. Me2SO (DMSO, 0.3%), ALLN (10 μM), calpeptin (50 μM), or protease inhibitor mixture (PI) (1 mg/ml) for 4 h. β-actin was used as an internal control.
adhesion complex proteins. The results showed that the calpain inhibitors, ALLN and calpeptin, partially prevented the collagen gel-induced decrease in FAK, talin, and p130cas (Fig. 4C). Taken together, these data indicate that the collagen gel-induced lower expression of focal adhesion complex proteins is mediated by the reduction of protein synthesis rates and the activation of proteases.

To visualize whether focal adhesion complex proteins were actually diminished by collagen gel, we employed immunofluorescence studies. MDCK cells were cultured on a dish, a collagen gel-coated dish, and collagen gel for 4 h, fixed by paraformaldehyde, and stained with phalloidin-TRITC and anti-FAK Ab and subsequently goat-anti-mouse IgG conjugated with Alexa Fluor 488 (green) and phalloidin-TRITC (red). The immunofluorescence images were taken by confocal microscopy (Leica TCS SP2).

Collagen Gel-induced Down-regulation Is Mediated by \( \alpha_\beta_1 \) Integrin but Not DDR1—Collagen fibrils may exert their effects via two separate receptor systems, integrin and DDR1 (21, 23, 24). To delineate how the physical property of collagen gel transduces signals into cells, we performed stable transfection of DDR1 and its dominant negative mutant in MDCK clone 3B5 cells. We established several stable transfectants that overexpressed the control plasmid, wild type DDR1, or the C-terminal truncated mutant of DDR1. As shown in Fig. 6A, both 3B5 cells and the control transfectant (PC11) exhibited endogenous DDR 1, D-1 and D-9 overexpressed DDR1, whereas N-36 and N-38 overexpressed truncated DDR1 as detected by the anti-Myc antibody. There was a basal level of DDR1 phosphorylation in both PC-11 and D-9 cells even in the absence of collagen. Upon stimulation of soluble type I collagen (200 \( \mu \)g/ml) for 2 h, the PC-11 cell did not show further activation of DDR1 phosphorylation, but D-9 cells exhibited marked activation of DDR1. Both N-36 and N-38 clones exhibited little phosphorylation of DDR1 regardless of the presence of soluble collagen, indicating that the truncated DDR1 can be used as a dominant negative mutant (Fig. 6B). To determine whether the \( \alpha_\beta_1 \) integrin or DDR1 is involved in collagen gel-induced down-regulation of focal adhesion complex proteins, we cultured these DDR1 transfectants on collagen gel with or without the anti-\( \alpha_\beta_1 \) integrin antibody (5E8) (26) for 4 h. As shown in Fig. 6C, D-9 cells showed a more round-up morphology when cultured on a collagen gel-coated dish or a collagen gel than the PC-11 control cells. In contrast, N-38 cells were more flattened when cultured on a collagen gel-coated dish or a collagen gel than PC-12 and D-9 cells. On the other hand, 5E8 markedly reduced the expression of either PC-11 or N-38 cells cultured on a collagen gel. These morphological data suggest that the functions of \( \alpha_\beta_1 \) integrin and DDR1 are opposite, with the former causing cell extension and the latter triggering retraction. As shown in Fig. 6D, overexpression of wild type or dominant negative DDR1 did not alter collagen gel-induced down-regulation of FAK, PYK2, and p130cas. However, 5E8 could alleviate a collagen gel-induced decrease in levels of FAK, PYK2, and p130cas in all of these transfectants. These data indicate that collagen gel-induced down-regulation of focal adhesion complex proteins is mediated by \( \alpha_\beta_1 \) integrin, but not DDR1.

Integrins play important roles in transducing mechanosignals in various cell types (27). The mechanostress applied through integrin-specific adhesion sites may trigger tyrosine phosphorylation of integrin downstream signaling proteins and result in cytoskeletal stiffening (28, 29). In addition, Wang et al. (30) found that focal adhesion kinase is involved in mechanosensing during fibroblast migration. To understand whether FAK is involved in the collagen gel-induced decrease of focal adhesion complex proteins, we employed 3B5 cells transfected wild type FAK and the endogenous FAK inhibitor, FAK-related non-kinase (FRNK) (31). These cells were cultured on a dish, a collagen gel-coated dish, and a collagen gel for 4 h, and the focal adhesion proteins were assessed by Western blot. We found that overexpression of either FAK or FRNK could alter the collagen gel-induced decrease of focal adhesion complex proteins, suggesting that FAK is not involved in this process (Fig. 7). Taken together, these results indicate that the collagen gel-induced decrease expression of focal adhesion complex proteins is mediated by \( \alpha_\beta_1 \) integrin without the involvement of FAK.

**DISCUSSION**

In this study, we demonstrate that the physical property of the collagen fibrils causes selective down-regulation of focal adhesion complex proteins in all types of the cells examined. Collagen gel-induced down-regulation of focal adhesion complex proteins results in the disappearance of functional focal adhesions and stress fibers, which may contribute to a decrease in intracellular tension. Previous data showed that increased mechanical force to integrin-mediated adhesions increased the cytoskeletal anchorage and stiffness of the cell (28, 29). We showed, in contrast, that collagen gel resulted in a marked decrease in focal adhesion complex proteins via physical property, i.e. lowered substratum rigidity. It is likely that cells losing expression of focal adhesion complex proteins may exhibit even lower cell strain, which is consistent with the cell response to the reduced mechanical stress of the local environment.

We showed that cells cultured on collagen gels did not spread well and displayed reduced stress fibers as well as down-regulation of focal adhesion proteins. It should be of interest to ask whether the observed changes could be due to a lack of spread-
ing rather than the gel per se. In this study, collagen gel induced down-regulation of focal adhesion proteins in all of the cell lines examined. However, collagen gel-induced reduction on cell spreading was not observed in all lines. For example, collagen gel did not block cell spreading in 293 or TCCSUP cells. On the other hand, cells cultured on a matrigel-coated dish and cells overexpressing DDR1 both showed limited cell extension, but their focal adhesion proteins remained intact, and the levels of these proteins were unaltered (Fig. 3, A–D). In Fig. 6, C and D, D-9 cells show reduced spreading on a normal culture dish as well as a collagen gel-coated dish, but they display intact focal adhesion complex proteins. In addition, we also examined whether reduced cell extension by deprivation of cell-matrix interactions affected expression of the focal adhesion complex. The result showed that cells cultured on albumin- or poly(2-hydroxyethyl methacrylate)-coated dishes exhibited intact FAK, despite the fact that they could not adhere, and they spread and gradually underwent apoptosis (data not shown). Taken together, collagen gel-induced down-regulation of focal adhesion proteins may not be mediated through the inhibition of cell spreading.

We showed that collagen gel-induced down-regulation of focal adhesion proteins was mediated by α5β1 integrin but not DDR1. Western blot analysis of DDR-1 in MDCK cells harboring plasmid control (PC-11), DDR1b (D-1 and D-9) or C-terminal truncated clones (N-36 and N-38) using anti-DDR1 Ab (top panel) or anti-Myc tagged Ab (middle panel). B, Western blot analysis of immunoprecipitated (IP) DDR-1 and DDR-1 tyrosine phosphorylation in various DDR1 transfectants treated with soluble type I collagen (200 μg/ml). C, morphological changes of PC-11, D-9, and N-38 cells cultured on dish, collagen gel-coated dish, and collagen gel for 4 h with or without anti-α5β1 integrin Ab (5E8). D, Western blot analysis of FAK, PYK2, and p130Cas in various DDR1 transfectants cultured on dish (C), collagen gel-coated dish (Co), or collagen gel (G) for 4 h with or without anti-α5β1 integrin Ab.

Fig. 7. Collagen gel-induced down-regulation of focal adhesion complex proteins is mediated by FAK. Western blot analysis of FAK, talin, PYK2, p130Cas, paxillin, c-Src, and vinculin in MDCK transfectants overexpressing FAK or FRNK cultured on dish (C), collagen gel-coated dish (Co), or collagen gel (G) for 4 h. β-actin was used as an internal control.

The biomechanical regulatory mechanism exertsed by collagen gel in MDCK cells is mediated by α5β1 integrin but not...
DDR1. Previous studies showed the focal adhesion might be a potential mechano-sensor (34). In endothelial cells, shear stress-dependent signals are mediated by $\alpha_{\beta_3}$ and $\alpha_{\beta_1}$ integrins (27). In addition, stretching of the substratum promotes the assembly of focal adhesions (30, 35) and triggers integrin-dependent signaling (36). These data indicate that integrins play important roles in mediating biochemical as well as biomechanical stimuli. In this study, we demonstrate that FAK is not the downstream mediator of integrin for rigidity-regulated focal adhesion complex expression. Recent studies suggest that, besides FAK, integrin-linked kinase and caveolin-1 also play roles in integrin downstream signaling (37). In addition, mechanosensitive protein kinases have been proposed to reside in the caveolae located in the plasma membrane of the endothelium (38). These possibilities remain to be elucidated.

Because focal adhesion complex proteins play very important roles in cell migration and survival as well as proliferation, it is of interest to speculate why the mature kidney does not require the expression of intact focal adhesion complexes. Once the nephrogenesis is completed, there is little sign of cell proliferation or migration in kidney. Thus, the requirement of focal adhesion complex proteins in mature kidney is dampened. Besides, although focal adhesion complex proteins can provide anti-apoptosis functions (16, 39), mature kidney epithelial cells may have other survival machinery. Several lines of evidences showed that collagen fibril is associated with inhibition of cell proliferation. It was found that fibril collagen inhibited cell proliferation by increasing p27 expression as well as inhibition of the expression of cyclin D1 and cyclin E-associated kinase proliferation. It was found that fibril collagen inhibited cell proliferation by increasing p27 expression as well as inhibition of the expression of cyclin D1 and cyclin E-associated kinase proliferation. It was found that fibril collagen inhibited cell proliferation by increasing p27 expression as well as inhibition of the expression of cyclin D1 and cyclin E-associated kinase proliferation.

Acknowledgments—The authors thank Ms. Tsu-Ling Chen and Yan-Shou Ger for their excellent technical assistance and Dr. Hong-Chen Chen for the gift of MDCK 3B5 cells harboring FAK and FRNK.

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J. Biol. Chem. 2003, 278:21886-21892.
doi: 10.1074/jbc.M300092200 originally published online April 3, 2003

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