Collagen gel overlay induces two phases of apoptosis in MDCK cells

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Wang, Yang-Kao, Hsi-Hui Lin, and Ming-Jer Tang. Collagen gel overlay induces two phases of apoptosis in MDCK cells. Am J Physiol Cell Physiol 280: C1440–C1448, 2001.—We previously demonstrated that collagen gel overlay induced cell remodeling to form lumen and apoptosis in Madin-Darby canine kidney cells. In the present study, we established that collagen gel overlay-induced apoptosis was initiated at areas exclusive of cell remodeling within 24 h (first phase) and extended into areas of cell remodeling within 48 h (second phase). Collagen gel overlay-induced apoptosis was accompanied by selective proteolysis of focal adhesion complex proteins, and the second phase on activation of caspases. Crouch et al. (7) showed that FAK serves as a target for proteases, either cysteine proteases or matrix metalloproteinases. Several pieces of evidence suggest that FAK could be a target for proteases, either cysteine proteases or caspases. Crouch et al. (7) showed that FAK serves as a specific, early and novel target for proteolysis during myc-induced apoptosis. Crouch et al. (7) showed that FAK serves as a specific, early and novel target for proteolysis during myc-induced apoptosis. Crouch et al. (7) showed that FAK serves as a specific, early and novel target for proteolysis during myc-induced apoptosis. Crouch et al. (7) showed that FAK serves as a specific, early and novel target for proteolysis during myc-induced apoptosis. Crouch et al. (7) showed that FAK serves as a specific, early and novel target for proteolysis during myc-induced apoptosis. Crouch et al. (7) showed that FAK serves as a specific, early and novel target for proteolysis during myc-induced apoptosis.

The focal adhesion complex has been recognized as an important site of signal transduction affecting cellular behavior such as migration, proliferation, differentiation, and survival. A central member of the focal adhesion complex is the tyrosine kinase pp125FAK (focal adhesion kinase, FAK) that plays a key role in the assembly of various signaling proteins recruited to focal adhesions. While FAK interacts with a number of cellular proteins, including Src, Grb2, phosphatidylinositol-3 kinase, paxillin, talin, and p130cas (5, 7, 29, 30, 38, 39). It has been established that FAK plays important roles in cell cycle progression (40), cell migration (2), cell adhesion (27), and prevention of apoptosis (9, 14). On the other hand, c-src, grb2, and p130cas have been implicated in FAK signaling in regulating these cellular functions. Talin is involved in FAK-mediated cytoskeletal organization (4). Other proteins, such as α-actinin, vinculin, and Crk, are also present in focal adhesion complex (24).

Several pieces of evidence suggest that FAK could be a target for proteases, either cysteine proteases or caspases. Crouch et al. (7) showed that FAK serves as a specific, early and novel target for proteolysis during c-src-induced cell death, the suppression of which was clearly linked to cell survival. Wen et al. (35) also demonstrated that FAK was sequentially cleaved into two different fragments early in Apo-2L-induced apoptosis possibly mediated by caspases-3/caspase-8. In addition to FAK, other focal adhesion complex proteins also play important roles in maintenance of cellular functions or in apoptosis. During platelet aggregation, activation of the cysteine protease calpain serves to degrade the focal adhesion complex proteins, such as c-src and talin, thereby mediating the postaggregation response (31). Chan et al. (3) showed that during ultraviolet irradiation-induced apoptosis, the focal adhesion complex proteins FAK, p130cas, and c-src were proteolyzed and that overexpression of FAK can partially rescue the ultraviolet-induced apoptosis. Recently, it was shown that overexpression of constitutive activated FAK rescued cells from apoptosis induced by disruption of cell-matrix interaction (9). On the other hand, fibroblasts underwent apoptosis when the inter-
action of FAK with the cytoplasmic domain of the \(\beta_1\)-integrin was inhibited by microinjection of peptide identical to the FAK binding site on the integrin or by microinjection of an antibody interfering with this binding (14). These data indicate that FAK plays an important role in maintaining cell survival.

In the present study, we established that collagen gel overlay-induced apoptosis could be classified into two phases. The first phase of apoptosis was observed at the areas exclusive of cell remodeling within 24 h. The second phase of apoptosis could be seen within areas of cell remodeling by 48 h. In addition, we also found that collagen gel overlay induced two phases of degradation of FAK. FAK was initially degraded into a 90-kDa product during the first 24 h and subsequently into a 80-kDa product during the second phase. These observations prompted the examination of whether proteolysis of FAK is the cause or result of collagen gel overlay-induced apoptosis. In this study, the association of FAK proteolysis with collagen gel overlay-induced apoptosis was explored.

**MATERIALS AND METHODS**

**Cell line.** Madin-Darby canine kidney (MDCK) cells (purchased from ATCC) were regularly maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37°C in humidified 5% CO\(_2\) atmosphere.

**Preparation of hydrated collagen gel.** Type I collagen was prepared from tail tendons of 1-mo-old rats according to the method of sonication. Type I collagen was diluted in 0.025 N acetic acid. For the preparation of agarose gel, a final concentration of type I collagen stock was 1% dissolved in chilled PBS and then lysed by lysis buffer containing 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5% Nonidet P-40 (on dish). Cells on gel and on dish were either pooled together or kept separated, depending on the purpose of the experiment. The lysate was further homogenized by the method of sonication.

**Western blot analysis.** We used the following antibodies to assess the protein levels in focal adhesion complex: anti-FAK kinase domain and anti-paxillin (Transduction Laborato-
ries); anti-FAK NH\(_2\)-terminal domain; anti-vinculin and anti-\(\alpha\)-actinin (Sigma); anti-talin (Biogenesia); and anti-e-src and anti-p130\(^{cas}\) (provided by Dr. H.-C. Chen, National Chung-Hsing University, Taichung, Taiwan). Protein concentration was measured by the method of Lowry et al. (21) using bovine serum albumin as the standard. For Western blot, 20 or 50 \(\mu\)g of cell homogenate from specific samples was resolved by 5% SDS-PAGE (for talin and vinculin) or 10% SDS-PAGE (for FAK, paxillin, \(\alpha\)-actinin, c-src, and p130\(^{cas}\)) and electrophoretically blotted onto nitrocellulose paper. The nitrocellulose paper was incubated with the specific antibody listed above, and then immunocomplexes were detected with horseradish peroxidase-conjugated IgG, and finally the immunocomplexes were made visible by fluorography with enhanced chemiluminescence detection kit (Amersham-Pharmacia International PLC).

**Determination of apoptosis.** The apoptosis was assessed by flow cytometry using propidium iodide as described previously (19, 33). MDCK cells were first treated with trypsin-EDTA (0.25%-2mM in PBS) to obtain a better cell suspension. The trypsin-digested cells were then washed with PBS-1 mM EDTA and fixed in 70% alcohol. After fixation, cells were treated with RNase (100 \(\mu\)g/ml in PBS) and stained with propidium iodide (40 \(\mu\)g/ml in PBS) and 4.3 vol of 1 M HEPES (1 vol), 0.17 M CaCl\(_2\) (0.1 vol), 1 N NaOH (0.1 vol), and 2.5% NaHCO\(_3\) (0.5 vol), 0.1 M HEPES (1 vol), 0.17 M CaCl\(_2\) (0.1 vol), 1 N NaOH (0.1 vol), and 2.5% NaHCO\(_3\) (0.5 vol), 0.1 M HEPES buffer to allow for the gelation. The agarose gel was prepared by the same protocol as described (33). After gelation, each culture was supplemented with 1.5 ml of culture medium containing DMEM and 20% FCS. The culture medium was replaced every other day.

**Preparation of cell lysates.** MDCK cells cultured to 70–80% confluence were overlaid with type I collagen gel. At the appropriate time points, cells were harvested from both gel and dish. To release cells that adhered to the gel, we removed the collagen gel and incubated the gel with 3 mg/ml of type II collagenase (Worthington Biochemical) at 37°C for 10 min. After digestion, the sample was then centrifuged at 800 g, and cells in the pellet were collected (on gel). On the other hand, cells that remained on the dish were washed twice by chilled PBS and then lysed by lysis buffer containing 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5% Nonidet P-40 (on dish). Cells on gel and on dish were either pooled together or kept separated, depending on the purpose of the experiment. The lysate was further homogenized by the method of sonication.

**RESULTS**

Collagen gel overlay-induced apoptosis and cell remodeling could be separated. MDCK cells cultured to 70–80% confluence were overlaid with collagen gel, and the morphology of cells was observed under light microscope. Within 24 h of collagen gel overlay, MDCK cells underwent morphological changes and remodeled into lumen-containing structures (13, 32, 33). Mean-

Statistics. All data are expressed as means \(\pm\) SE of at least three independent experiments. One-way ANOVA was used to test for statistical differences. \(P < 0.05\) was taken to be statistically significant.
while, a small proportion of MDCK cells disintegrated into tiny vesicles that resembled apoptotic bodies in the area where cell remodeling was not present (Fig. 1A). Between 24 and 48 h of collagen gel overlay, apoptotic bodies increased in the area of cell remodeling (Fig. 1B). The remodeled and apoptotic cells could be separated by the removal of collagen gel. As shown in Fig. 1, C and D, apoptotic bodies remaining on dish were markedly reduced after the removal of collagen gel, indicating that apoptosis is associated with collagen gel. To quantify apoptosis in the population of cells adhering to gel or remaining on dish, we used FACS analysis. Our results showed that almost all cells adhering to gel were apoptotic (93.5 ± 2.7% at 24 h and 94.7 ± 1.5% at 48 h), whereas cells remaining on dish were mostly viable, with apoptosis ratio of 4.9 ± 1.5% at 24 h and 28.5 ± 2.7% at 48 h (Fig. 1E).

Collagen gel overlay induced selective proteolysis of focal adhesion complex proteins. We examined whether there were changes in the focal adhesion complex proteins in MDCK cells overlaid by collagen gel. After collagen gel overlay, cells adhering to both dish and gel were harvested at different time points, and the lysates were analyzed by Western blotting using a monoclonal antibody against kinase domain of FAK. We observed that FAK underwent distinct proteolytic changes with time in response to collagen gel overlay (Fig. 2A, top). A significant decrease in 125-kDa FAK was observed within 8 h of collagen gel overlay, along with the appearance of a major degraded product of 90 kDa. The intensity of FAK was gradually decreased 8–48 h after collagen gel overlay. The degraded fragment of FAK appeared to increase in intensity from 8 to 24 h (first phase), and another degraded product appeared between 24 and 48 h (second phase) with a molecular weight of 80 kDa. The pattern of FAK degradation was also analyzed by polyclonal antibody against NH2-terminal 2–17 amino acids of FAK. This antibody detected two degraded products of FAK that were similar to those recognized by anti-FAK kinase domain antibody (Fig. 2A, middle). The first degradation product of FAK (90 kDa) was increased from 8 to 24 h and decreased in 48 h, whereas the second degradation product (80 kDa) was increased 16–48 h after collagen gel overlay. In contrast, FAK remained intact even after agarose gel overlay for 72 h (Fig. 2B).

We investigated whether other focal adhesion complex proteins were also affected after collagen gel over-
The representative results of Western blots from 3 separate experiments showed that collagen gel overlay induces selective proteolysis of focal adhesion complex proteins. MDCK cells were overlaid with collagen gel for the indicated times, and total lysates collected from both dish and gel were analyzed by immunoblotting with the antibody against talin, p130<sup>cas</sup>, c-src, α-actinin, vinculin, or paxillin. A: collagen gel overlay induces proteolysis of talin, p130<sup>cas</sup>, and c-src; B: collagen gel overlay does not alter the level of α-actinin, vinculin, or paxillin. The immunoblots were stripped and reprobed with anti-β-actin antibody to demonstrate equal loading of protein. These figures are representative results of Western blots from 3 separate experiments.

Cells adhering to collagen gel exhibited proteolysis of focal adhesion complex proteins. We collected cells either adhering to gel or remaining on dish separately and analyzed the integrity of their focal adhesion complex proteins in these two different populations. Although the quantity of cells adhering to gel increased with time, the amount adhering to gel in a 60-mm dish was too low for the assessment of total protein content within 24 h of collagen gel overlay. We therefore had to load all the lysate of each sample into each well for Western blot analysis. In contrast, for the analysis of focal adhesion complex proteins in these two different populations, an equal amount of total protein was loaded for each sample. As shown in Fig. 4A, cells adhering to the gel exhibited only a degraded form of FAK, the intensity of which increased with time. In contrast, cells left on the dish showed intact FAK, talin, and p130<sup>cas</sup> with little sign of proteolysis (Fig. 4B). Taking these data together, we found that cells adhering to the gel were almost apoptotic and exhibited completely degraded FAK, p130<sup>cas</sup>, and talin throughout the time course, whereas cells left on the dish displayed little apoptosis or intact focal adhesion complex proteins.

First-phase apoptosis and FAK proteolysis could be blocked by protease inhibitor cocktail. We previously showed that cycloheximide prevented collagen gel-induced cell remodeling and apoptosis within 36 h (33). We examined whether cycloheximide might prevent collagen gel overlay-induced proteolysis of focal adhesion complex proteins. MDCK cells cultured to 70–80% confluence were pretreated with cycloheximide (1 μg/ml) for 1 h and overlaid with collagen gel in the presence of cycloheximide for another 36 h, and the biochemical changes of focal adhesion complex proteins were assessed. The results showed that collagen gel overlay-induced cleavage of FAK, talin, p130<sup>cas</sup>, and c-src was completely blocked by cycloheximide within 36 h of collagen gel overlay (Fig. 5).

To examine whether inhibition of proteolysis of focal adhesion complex proteins may prevent collagen gel overlay-induced apoptosis, we employed cell-permeable protease inhibitor PMSF (serine protease inhibitor), pepstatin A (aspartate protease inhibitor), and the protease inhibitor cocktail Complete, which inhibits a broad spectrum of serine, cysteine, and metalloproteases as well as calpain proteases. Cells were pretreated with each protease inhibitor for 1 h and then overlaid with collagen gel in the presence of the inhibitor for another 48 h. Among them, only the protease inhibitor cocktail attenuated collagen gel overlay-induced apoptosis. As shown in Fig. 6A, the protease...
inhibitor cocktail reduced collagen gel overlay-induced apoptosis from 11.4 ± 0.9% to 6.5 ± 0.5% (a, P < 0.05), which was approximately the basal apoptosis ratio for cells at the same confluence within 24 h. However, it could only partially inhibit collagen gel overlay-induced apoptosis at 48 h, from 41.0 ± 1.7% to 27.3 ± 0.2% (b, P < 0.05). In addition, protease inhibitor cocktail prevented collagen gel overlay-induced proteolysis of focal adhesion complex proteins in a dose-dependent manner (Fig. 6B), whereas pepstatin A and PMSF did not inhibit apoptosis and failed to inhibit degradation of FAK (data not shown). These data indicate that proteolysis of focal adhesion proteins is involved in the cause of collagen gel overlay-induced apoptosis within the first 24 h, especially in cells adhering to collagen gel.

Recent data indicate that degraded collagen gel could induce morphological changes of vascular smooth cells and degradation of focal adhesion complex proteins via activation of the calcium-sensitive protease calpain (1). To clarify whether calpain is involved in collagen gel overlay-induced degradation of focal adhesion complex proteins and apoptosis in MDCK cells, we used ALLN, which is a specific inhibitor of calpain.
However, ALLN could neither prevent collagen gel overlay-induced remodeling nor block collagen gel overlay-induced apoptosis (data not shown).

**Second-phase apoptosis and FAK proteolysis could be blocked by ZVAD-fmk.** To elucidate whether caspases were involved in collagen gel overlay-induced apoptosis, we employed the following tetrapeptide caspase inhibitors: YVAD-CHO, cell-permeable DEVD-fmk, and ZVAD-fmk. Cells were preincubated with either inhibitor alone or inhibitors in combination for 1 h and then overlaid with collagen gel in the presence of the same inhibitor for 48 h. None of the caspase inhibitors affected collagen gel overlay-induced cell remodeling. Among these caspase inhibitors, only ZVAD-fmk attenuated collagen gel overlay-induced apoptosis in a dose-dependent manner (Fig. 7A). Nevertheless, it did not completely block collagen gel overlay-induced apoptosis. Interestingly, we found that DEVD-fmk and ZVAD-fmk but not YVAD-CHO partially blocked proteolysis of FAK induced by collagen gel overlay (Fig. 7B). These inhibitors selectively blocked the formation of degraded product 80 kDa during the second phase. The pattern of FAK degradation resulting from the presence of these caspase inhibitors within 48 h of collagen gel overlay is reminiscent of that observed at 24 h in the absence of any inhibitor. Taken together, protease inhibitor cocktail and ZVAD-fmk might inhibit the first and second phase of apoptosis, respectively. To test this possibility, we employed a combination of protease inhibitor cocktail and ZVAD-fmk. As shown in Fig. 8A, both protease inhibitor cocktail and ZVAD-fmk only partially inhibited collagen gel-induced apoptosis within 48 h. However, a combination of them completely block collagen gel overlay-induced apoptosis from 39.1 ± 1.7% to 5.1 ± 0.2%. Interestingly, the proteolysis of focal adhesion complex proteins was significantly inhibited by the combination of protease inhibitor cocktail and ZVAD-fmk (Fig. 8B). These results suggest that proteolysis of focal adhesion complex proteins is partially mediated by caspases.

![Fig. 7. ZVAD-fmk blocks collagen gel overlay-induced apoptosis and proteolysis of focal adhesion complex proteins of the second phase. MDCK cells were incubated with YVAD-CHO (200 µM), DEVD-fmk (200 µM), ZVAD-fmk (100 or 200 µM), or a combination of these tetrapeptides (Y + D + Z, 200 µM each) 1 h before and also during the collagen gel overlay. After 48 h incubation, total cells were harvested. The apoptosis ratio was assessed by FACSScan analysis (A), and the changes of focal adhesion complex proteins were analyzed by Western blot (B). The immunoblots were stripped and reprobed with β-actin antibody to demonstrate equal loading of protein. Data represent means ± SE of 3 independent experiments in duplicate (a, P < 0.05 vs. DMSO control; b, P < 0.01 vs. ZVAD-fmk 200 µM). NSB, nonspecific binding.](http://ajpcell.physiology.org/)

![Fig. 8. Combination of protease inhibitor cocktail and ZVAD-fmk completely blocks collagen gel overlay-induced apoptosis as well as proteolysis of focal adhesion complex proteins. MDCK cells were incubated with PI (1 mg/ml), cell-permeable ZVAD-fmk (200 µM), or combination of these inhibitors 1 h before and also during the collagen gel overlay. After 48 h, total cells were harvested. The apoptosis ratio was assessed by FACSScan analysis (A), and the changes of focal adhesion complex proteins were analyzed by Western blot (B). The immunoblots were stripped and reprobed with β-actin antibody to demonstrate equal loading of protein. Data represent means ± SE of 3 independent experiments in duplicate (a, P < 0.05 vs. DMSO control; b, P < 0.01 vs. ZVAD-fmk 200 µM).](http://ajpcell.physiology.org/)
teins, including FAK, talin, and p130cas, was also completely blocked by a combination of protease inhibitor and ZVAD-fmk (Fig. 8B).

**DISCUSSION**

In this study, we first defined the model in which collagen gel overlay could induce two different phases of apoptosis. Collagen gel overlay-induced apoptosis of the first phase was observed in cells that lost their intact focal adhesion complex proteins. These cells were excluded from those that formed lumen but remained adhering to collagen gel even when they developed apoptosis during 24-h overlay. The second phase of apoptosis occurred to the cells that had remodeled to form lumen and contacted to collagen gel directly during 24–48 h overlay. The induction of two-phase apoptosis by collagen gel overlay has not been reported before. Interestingly, the cellular mechanism involved in apoptosis of these two phases is also different. In the first phase of apoptosis, activation of protease(s) other than caspases thatcleaves focal adhesion complex proteins plays important roles. On the other hand, caspases are required for execution of apoptosis in the second phase. Because MDCK cells are heterogeneous in their phenotypic property, it is possible that the two collagen gel overlay-induced phases of apoptosis in MDCK cells may reflect the heterogeneity of the cell line. To rule out this possibility, we employed a single clone of MDCK, clone II 3B5 (17). 3B5 cells exhibited apoptosis and cell remodeling as well as FAK proteolysis in response to collagen gel overlay (data not shown). Therefore, we tend to believe that cell heterogeneity is not the cause.

In the present study, we demonstrated that FAK, the central component of focal adhesion, was cleaved into 90-kDa fragments as early as 8 h during the phase of proteolysis; a further degradation product of 80 kDa appeared from 24- to 48-h overlay. Compared with the previous reports that demonstrated proteolysis of FAK induced by activation of caspases, the degradation pattern of FAK in our study may not be similar. Levkau et al. (20) demonstrated that during growth factor deprivation-induced apoptosis in vascular endothelial cells, FAK was cleaved by caspase activation within 2 h. Wen et al. (35) also demonstrated in Apo-2- and Fas-induced Jurkat or H460 cell apoptosis, respectively, that FAK was degraded at a similar time course to Levkau's finding. In these studies, they found several putative caspase cutting sites in the COOH terminus of FAK, which generated 33- or 41-kDa COOH-terminal degraded fragments, and the predicted size was similar to their experiments (20, 35). These studies showed that the different degraded products of FAK were caused by the activation of different caspases such as caspase-3 or -6. In our study, using anti-NH2-terminal antibody of FAK, we detected a 90-kDa (first phase) and an 80-kDa (second phase) degraded product after collagen gel overlay of 24 and 48 h, respectively. Most importantly, we found that caspase inhibitors did not prevent the collagen gel overlay-induced first phase of apoptosis. These results rule out the involvement of caspases in collagen gel overlay-induced first phase of apoptosis or FAK proteolysis.

Recent studies have demonstrated that proteases other than caspases, such as calpain and cathepsin D, may contribute to the induction of apoptosis (16, 25, 26, 28, 36, 37). In these studies, only calpain has been reported to cleave FAK and thereby contribute to the induction of apoptosis (37). On the other hand, it has also been shown that activation of calpain may induce FAK proteolysis, the result of which is associated with platelet activation and adhesion, but not cell death (15). We have tried to delineate whether a specific protease is involved in collagen gel overlay-induced apoptosis by testing the effects of different protease inhibitors. However, none of the inhibitors of serine protease, aspartate protease, or calpain can prevent collagen gel overlay-induced proteolysis of FAK or apoptosis. Nevertheless, a protease inhibitor cocktail can completely block collagen gel overlay-induced apoptosis and degradation of FAK in the first phase. Proteolysis of FAK is therefore responsible for collagen gel-induced apoptosis during the first phase.

It is of interest to know whether focal adhesion complex proteins other than FAK were degraded after collagen gel overlay. We found that only proteins present upstream of the focal adhesion complex such as FAK, talin, c-src, and p130cas were affected. Con-
versely, proteins participating in the downstream of the integrin signal or associated with cytoskeletal proteins, such as paxillin, vinculin, and α-actinin, remained intact. The selective proteolysis of focal adhesion complex proteins induced by collagen gel overlay may result in a blockade of FAK downstream signaling that regulates cell viability (3, 11, 12, 14). Chen and Guan (5) demonstrated that FAK could form a stable complex with its potential substrate phosphatidylinositol 3-kinase. The latter was known to be involved in antiapoptotic signal (18, 23). In addition, Carragher et al. (1) demonstrated that the association of FAK with other focal adhesion complex proteins such as paxillin and p130cas was markedly diminished by degradation of FAK. Therefore, the degradation of focal adhesion complex proteins may trigger apoptosis via the loss of their integrity of focal adhesions as well as antiapoptotic signals. On the other hand, cleavage of focal adhesion complex proteins prevents the cells from adhering to the dish. It is therefore not surprising that all the apoptotic cells at the first phase were found to stick to collagen gel, instead of remaining on the dish.

Furthermore, during 24–48-h collagen gel overlay, massive apoptosis was observed. The apoptosis of this phase was observed in cells that formed lumen and directly contacted with collagen gel. To examine whether direct contact with collagen gel may trigger apoptosis, we cultured cells in or on collagen gel. In our previous report, we discovered that MDCK cells developed apoptosis when cultured in collagen gel (19). In addition, our current study has shown that epithelial cells, but not fibroblasts or tumor cells, developed apoptosis when seeded on collagen gel (6). It is therefore likely that epithelial cells may develop apoptosis on direct contact with collagen gel, regardless of membrane site. Although those with intact focal adhesion complex proteins may form lumen with each other and escape the first phase of apoptosis, they may undergo apoptosis during the second phase due to direct contact with collagen gel. During collagen gel overlay-induced apoptosis of the second phase, ZVAD-fmk, but not YVAD-CHO or DEVD-fmk, significantly attenuated collagen gel overlay-induced apoptosis and proteolysis of FAK. It is thus concluded that activation of caspase(s) other than caspase-1, -3, or -7 plays an important role in triggering apoptosis of the second phase. The underlying mechanisms involved in collagen gel overlay-induced caspase activation remain to be elucidated.

The study reported here demonstrates that collagen gel overlay-induced proteolysis of focal adhesion complex proteins is not the cause of cell remodeling but is associated with collagen gel overlay-induced apoptosis. The temporal and spatial changes of collagen gel overlay-induced cell remodeling and the two phases of apoptosis are summarized in Fig. 9. Collagen gel overlay initiates a series of proteolysis that selectively degrades the focal adhesion complex proteins in a small proportion of cells, the results of which may trigger drastic morphological events. Cells that are depleted of intact focal adhesion complex proteins can neither form lumen structures nor adhere to the culture dish. However, they tend to stick to collagen gel and develop apoptosis due to loss of survival signals from focal adhesion complex proteins during the first phase of apoptosis. In contrast, cells exhibiting intact focal adhesion complex proteins tend to develop lumen structure within 24 h of collagen gel overlay. Between 24 and 48 h, cells that form lumen start to develop massive apoptosis where they directly contact collagen gel. The apoptosis initiated during the first phase is dependent on the activation of protease(s) other than caspases, whereas the apoptosis observed during the second phase results from the activation of caspases. The nature of protease(s) that selectively degrade the focal adhesion complex proteins in collagen gel overlay-induced apoptosis of the first phase is still unknown, as is the identity of the caspase(s) involved in apoptosis of the second phase. It should be of interest to explore further the mechanisms involved in collagen gel overlay-induced activation of protease(s) as well as caspases.

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