Cellular Aggregation Facilitates Anoikis in MDCK Cells

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Abstract: Anoikis is a specific type of apoptosis caused by the detachment of anchorage-dependent cells from their supportive matrix. Aggregation of suspended cells is believed to suppress anoikis. Here we describe the effects of cellular aggregation on anoikis in Madin-Darby canine kidney (MDCK) cells. Suspension cultures of MDCK cells grown under conditions known to induce extensive cellular aggregation were less able to reattach to culture dishes, exhibited higher caspase-8 activity, and contained more sub-G1 cells than suspension cultures did with less cellular aggregation. When suspension cultures of MDCK cells were separated into aggregated cells and single cells, the aggregated cells had low caspase-8 activity regardless of suspension conditions, whereas the single cells had higher caspase-8 activity that increased with an increasing degree of aggregation. These results suggest that cell-cell interactions in cellular aggregates of suspended MDCK cells facilitate anoikis, causing more apoptosis in individual cells than when these interactions are absent.

Key words: apoptosis, anoikis, cellular aggregation, MDCK, caspase-8.

Normal epithelial cells require contact with the extracellular matrix (ECM) to survive [1, 2]. When detached from the ECM, they no longer receive survival signals from ECM receptors and undergo apoptosis by default [3]. This type of apoptosis is termed anoikis [2], and it plays important roles in the physiological induction of apoptosis during the development and maintenance of tissue homeostasis in the organism [4–6].

Cell-cell interactions, as well as cell-ECM interactions, affect a broad range of cellular activities by modulating the signaling pathways of each cell to regulate gene expression, cell cycle, and survival [7]. Epithelial cells have a propensity for cell-cell interactions involving epithelial cadherin [8]. When epithelial cells are detached from the ECM and maintained in suspension in vitro, many of them spontaneously interact with one another to form cellular aggregates. It is possible that cell-cell interactions between the cells in suspension affect certain aspects of anoikis. To elucidate the molecular mechanism of anoikis, we find it important to clarify whether the cell-cell interactions affect anoikis and, if so, to study the effects of cell-ECM detachment on anoikis separately from the effects of cell-cell interactions.

Several studies of anoikis have indicated that cell-cell interactions provide survival signals to cells that are maintained in suspension [5]. For example, in mouse proximal tubular cells [9] and in bronchial epithelial cells [10] the extent of anoikis in a suspension culture negatively correlates with the extent of cellular aggregation in it. Other studies have shown that anoikis is observed primarily in single cells in a suspension culture, rather than in aggregated cells [10–13]. Since the formation of cellular aggregates reflects cell-cell interactions, these results suggest that cell-cell interactions provide survival signals to the detached cells.

The role of cell-cell interactions in the anoikis of Madin-Darby canine kidney (MDCK) cells has not been reported, although MDCK cells were one of the first cell types in which anoikis was reported [2]. In fact, they remain a standard model system for studying anoikis in normal epithelial cells [4, 14]. In this work, we examined whether cell-cell interactions in suspension cultures of MDCK cells release survival signals. The extent of cellular aggregation in the suspension culture was controlled using multiple methods, and the relationship between cellular aggregation and anoikis was examined. In all experiments the indexes of cell death positively correlated, and those of cell viability negatively correlated, with the extent of cellular aggregation in suspension cultures of MDCK cells. These results suggest that anoikis is facilitated, rather than suppressed, by cell-cell interactions between MDCK cells in suspension.

METHODS

Cells. An MDCK cell line (RCB0995) was purchased from the Riken cell bank (Tsukuba, Japan). The cells were maintained in a 5% CO₂ atmosphere at 37°C in...
Eagle’s minimum essential medium (MEM) buffered with 1.3 g/l NaHCO₃ and supplemented with 0.292 g/l L-glutamine and 10% fetal bovine serum (FBS) (JRH Biosciences, USA). This medium is referred to as the standard culture medium.

**Low-calcium culture medium.** A serum-free low-calcium culture medium was prepared from S-MEM (GIBCO). It contained no calcium, but was supplemented with 0.292 g/l L-glutamine, ITS-A (Insulin-Transferrin-Selenium-A, GIBCO), 1 mg/ml bovine serum albumin (BSA), and 0.01 μg/ml epidermal growth factor (EGF). Low-calcium culture medium was prepared from S-MEM supplemented with 0.292 g/l L-glutamine and 10% FBS that had been dialyzed against phosphate-buffered saline (PBS) using cellulose tubing (MWCO 3500, Membrane Filtration Products, USA) to remove the free calcium present in the FBS. The concentration of contaminating calcium in the low-calcium media was less than 0.1 mM, as measured by Fura-2 fluorescence enhancement (data not shown). A high-calcium culture medium was prepared by adding 2 mM CaCl₂ to the low-calcium medium.

**Suspension culture.** Dishes and flasks for suspension culture were coated with poly-2-hydroxyethyl methacrylate (polyHEMA) as described [15]. Confluent MDCK cells were harvested from standard dishes with 0.25% trypsin containing 1 mM EDTA (Trypsin-EDTA, GIBCO) and seeded onto polyHEMA-coated dishes (or flasks). Assuming that all cells settled to the bottom, we determined that the surface cell density of the suspension culture, which was calculated by dividing the number of cells in the culture by the surface area of the bottom, was greater than 3 × 10⁴ cells/cm², unless otherwise indicated. For the experiments with sparse- and dense-surface cell density, the cells were suspended at 10⁴ cells/ml in the standard culture medium and seeded onto polyHEMA-coated flasks, either at 1.5 × 10⁵ cells/cm² (sparse-surface cell-density suspension, typically 11 ml of the cell suspension on a flask with a bottom area of 75 cm²) or at 1 × 10⁶ cells/cm² (dense-surface cell-density suspension, typically 270 ml of the cell suspension on a flask with a bottom area of 27 cm²).

**Cell viability measurements.** The cells were incubated in a standard culture medium supplemented with 5% alamar blue (BioSource International, USA) for 2 h at 37°C. Cell viability was calculated from the colorimetric change in the alamar blue in the culture medium, following the manufacturer’s instructions.

**Cell recovery measurements.** The cells were washed in PBS, trypsinized to yield a suspension of single cells, and counted under a microscope.

**Reattachment measurements.** The cells were collected in a standard culture medium, seeded onto cell culture dishes, and allowed to attach to the dishes for 1 h at 37°C. Then the culture medium, including unattached cells, was discarded, and a standard culture medium supplemented with 5% alamar blue was added and incubated for 2 h at 37°C. The amount of the attached cells was calculated from the colorimetric change in the alamar blue, following the manufacturer’s instructions.

**Caspase-8 activity measurements.** The cells were assayed with a FLICE/Caspase-8 colorimetric assay kit (BioVision, USA), following the manufacturer’s instructions. Briefly, they were lysed, and the resulting cell lysate was diluted to a total protein concentration of 0.5–2 mg/ml and assayed for the ability to cleave IETD-pNA, a specific substrate for caspase-8.

**Flow cytometry.** The cells were collected by gentle centrifugation and washed with PBS. The suspended cells, containing the cellular aggregates, were trypsinized to yield a suspension of single cells. The cells were fixed with 70% ethanol and stained with propidium iodide, and the histogram of the DNA content of the stained cells was analyzed using a FACScan flow cytometer equipped with Cell Quest software (Becton Dickinson, USA). In this method, apoptotic cells appear as a sub-G₁ cell population (cells with a fractional DNA content), since fragmented DNA leaks from the fixed cells prior to the measurement [16].

**Separation of single and aggregated cells.** The cells were applied to a 40 μm nylon cell strainer (Falcon); the cells that passed through the strainer were considered to be a population of single cells, and those that remained on the strainer were considered to be a population of aggregated cells, as described [13].

**RESULTS**

**MDCK cell death induced by detachment from ECM depends on cell density in suspension culture**

In several cell lines, the cell density of the suspension culture has been shown to affect the extent of anoikis. Smaller populations of cells have been shown to proceed to anoikis in suspension cultures at higher cell densities, rather than at lower ones [10, 17]. In these experiments, a certain population of the suspended cells had aggregated. The extent of cellular aggregation depended on the cell density in the suspension culture: the greater the cell density, the greater the extent of aggregation. These results support the idea that cellular aggregation in a suspension culture suppresses anoikis.

In the present study, we examined the effects of cell density in suspension culture on the viability of MDCK cells. The cells were maintained in suspension at either a density of 10⁴ cells/ml (low cell density) or 10⁵ cells/ml (high cell density). At indicated times, the cells were collected by gentle centrifugation, and the viability of the cells was assessed using the alamar blue reduction assay. The viability decreased during the suspension period (Fig. 1A). It was greater in suspension culture at the low cell density (Fig. 1A, open circles) than at the high den-
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Next, we assessed the viability of cells by counting the number of cells after suspension culture. More cells were recovered from the 24 h suspension culture at the low cell density than at the high density (Fig. 1D, panels “Cell, L” and “Cell, H”). These results indicate that more MDCK cells survive in suspension culture at low cell density than at high density.

MDCK cell death induced by detachment from ECM depends on calcium concentration in suspension culture

In several cell lines, the concentration of calcium in a suspension culture has been shown to affect the extent of anoikis. When such cells are maintained in suspension in the presence of EGTA [9] or EDTA [10], more cells proceed to anoikis. Because these metal chelators sequester free calcium in the suspension culture medium and suppress cellular aggregation via calcium-bound cadherin, these results have been interpreted to support the idea that unaggregated cells are more likely to undergo anoikis than aggregated cells in suspension culture.

In the present study, the effect of calcium concentration on cell viability was studied in a suspension culture of MDCK cells. The cells were maintained in suspension either in the low- or high-calcium medium (serum-free medium; see Methods). The viability of the suspended cells in the low-calcium medium (Fig. 1B, open triangles) was higher than in the high-calcium medium (Fig. 1B, filled triangles) during three days of suspension culture (Fig. 1B).

Next, the number of the cells remaining in the suspension culture was determined. More cells were recovered from the 24 h suspension culture in the low-calcium medium than in the high-calcium medium (Fig. 1D, panels “Ca, L” and “Ca, H”), with low or high calcium (“Ca, L” and “Ca, H”), with and without methylcellulose (“MC, +” and “MC, −”), were trypsinized to yield a suspension of single cells and counted under a microscope. Cell recovery was expressed as a percentage (normalized value relative to the cell number at 0 h suspension). Values represent mean ± SE from at least three independent experiments (*P < 0.05 by Student’s t-test).
The viability of suspended MDCK cells depends on cells that remain unaggregated in suspension culture

The viability of suspended MDCK cells depends on cells that remain unaggregated in suspension culture. The experiments described above indicate that the extent of cell death induced by detachment from ECM decreases as the density of the suspension culture decreases, as calcium concentration decreases, and when methylcellulose is added to the culture medium. All of these culture conditions are thought to decrease cellular aggregation and thus increase the proportion of unaggregated cells. In other words, then, our results suggest that more MDCK cells survive in suspension cultures under conditions that disfavor cellular aggregation.

Methylcellulose increases viability of MDCK cells in suspension culture

When epithelial cells (other than MDCK cells) are maintained in a suspension culture, more cells proceed to anoikis in the presence of methylcellulose [11] or collagen [12] than in the absence of these additives. Because methylcellulose and collagen increase the viscosity of the culture medium and thus suppress cellular aggregation, these results have been interpreted to support the idea that unaggregated cells are more likely to undergo anoikis than aggregated cells in a suspension culture.

Here, the effect of methylcellulose on cell viability was studied in a suspension culture of MDCK cells. The viability of these cells was higher in the presence of 2% methylcellulose (Fig. 1C, open squares) than in its absence (Fig. 1C, filled squares) during three days of suspension culture ($P < 0.05$ by Student’s t-test at 24 h and 48 h time points).

Next, the number of cells remaining in the suspension culture was determined. More cells were recovered from the 24 h suspension culture containing methylcellulose than from the culture lacking methylcellulose (Fig. 1D, panels “MC,” +” and “MC,” −”). These results suggest that more MDCK cells survive in a suspension culture with methylcellulose than in one without methylcellulose.

Caspase-8 activity in MDCK cells

Caspase-8, which is an initiating caspase in the death-receptor-mediated apoptosis pathway [18], has also been proposed as an initiating caspase in anoikis of MDCK cells [19, 20]. Therefore we measured caspase-8 activity as an index of anoikis in MDCK cells.

The extent of cellular aggregation in suspension culture was again varied, either by changing the cell density of the suspension culture (Fig. 2B, circles) or by adding methylcellulose to the culture (Fig. 2B, squares). In both instances, caspase-8 activity negatively correlated with
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The proportion of single cells in the suspension culture (Fig. 2B). These results indicate that more caspase-8 is activated in suspension cultures of MDCK cells under conditions that favor cellular aggregation.

Taken together, these findings suggest that anoikis is facilitated, rather than suppressed, by cellular aggregation in suspension cultures of MDCK cells. We therefore sought to study the effects of cellular aggregation on anoikis in MDCK cells in more detail.

Anoikis progression in cells maintained in suspension with low or high calcium

MDCK cells were prepared in low- or high-calcium media containing dialyzed FBS (see Methods). MDCK cells maintained in suspension at low calcium for 24 h appeared separated from one another (Fig. 3A, LCa). When they were collected and allowed to reattach to standard culture dishes in a standard culture medium, most of the cells reattached to the dishes (Fig. 3B, LCa). In contrast, MDCK cells maintained in suspension at high calcium formed many cellular aggregates (Fig. 3A, HCa). When they were collected and allowed to reattach to standard culture dishes in a standard culture medium, only a third of the cells reattached to the dishes (Fig. 3B, HCa). These results show that when MDCK cells are maintained in suspension in low calcium, the cells do not aggregate, and they retain their ability to attach to the ECM.

As an index of anoikis, prelytic DNA fragmentation, a hallmark of apoptosis [16, 21], was quantified as sub-G1 cell content. Suspension cultures with high calcium contained more sub-G1 cells than cultures with low calcium (Fig. 3C). The cells suspended in high calcium also had
We also measured the activity of caspase-8 in each cell population. After maintaining cells in suspension for 24 h, we found that cell lysate from the aggregated cell populations showed slightly higher (<2-fold) caspase-8 activity (Fig. 3G, filled bar) than before suspension (Fig. 3G, att). Single cells collected from suspension cultures in low calcium (Fig. 3G, LCa, open bar) exhibited a level of caspase-8 activity similar to that of aggregated cells (Fig. 3G, filled bar). In contrast, single cells collected from suspension cultures in high calcium had >3-fold greater caspase-8 activity (Fig. 3G, HCa, open bar) than before suspension (Fig. 3G, att).

We assessed how consistent the results would be before and after the strainer separation by comparing the results obtained with and without separation. The caspase-8 activity of a cell suspension in low calcium was estimated as the sum of the single cell number (Fig. 3F, LCa) multiplied by the caspase-8 activity of the single cells (Fig. 3G, LCa, open bar), and the aggregated cell number (Fig. 3F, [100 – the value in panel “LCa”] / 100) multiplied by caspase-8 activity of the aggregated cells (Fig. 3G, LCa, filled bar). The resulting value is shown in Fig. 3H, LCa. Likewise, caspase-8 activity of a cell suspension in high calcium was estimated from Fig. 3F (HCa) and G (HCa), and is shown in Fig. 3H (HCa). The calculated values of the activity (Fig. 3H) agree well with the results obtained in the independent experiments without the strainer separation (Fig. 3D), verifying that the higher caspase-8 activity than those suspended in low calcium (Fig. 3D). These results indicate that induction of anoikis is greater in high-calcium suspension cultures than in low-calcium suspension cultures.

### Anoikis progresses differently in single and aggregated cells

In suspension cultures of several cell lines, anoikis has shown to be more prevalent in single cells than in aggregated cells [10–13] (summarized in Table 1). To see whether the extent of anoikis differs between the single and the aggregated cells in suspension cultures of MDCK cells, the cells that had been maintained in suspension for 24 h, either in low or high calcium, were collected and separated into single and aggregated cell populations by passage through a 40 μm cell strainer (Fig. 3, E–H).

After maintaining the cells in suspension in low calcium, we found that most of the cells passed through the strainer and were thus collected as a single cell population (Fig. 3, E and F, LCa). The size and shape of most of the cells in the population of single cells appeared to be normal and exhibited no apoptotic characteristics (Fig. 3E, LCa). In contrast, after maintaining the cells in suspension in high calcium, we noted that only a third of the cells passed through the strainer (Fig. 3, E and F, HCa). It is interesting that most of the cells in this latter population of single cells appeared to shrink (Fig. 3E, HCa), suggesting that apoptosis was occurring in the population of single cells.

### Table 1. Cellular aggregation and the extent of anoikis in various cell types.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Single cell dominant mixture</th>
<th>Aggregated cell dominant mixture</th>
<th>Anoikis examined separatelya</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse proximal tubular</td>
<td>+++</td>
<td>+</td>
<td>nd</td>
<td>Bergin et al., 2000 [9]</td>
</tr>
<tr>
<td>Bronchial epithelial</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>Aoshiba et al., 1997 [10]</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>nd</td>
<td>nd</td>
<td>+++</td>
<td>Zhang et al., 2004 [13]</td>
</tr>
<tr>
<td>Ras-transformed fibroblast</td>
<td>+++</td>
<td>+</td>
<td>nd</td>
<td>McGill et al., 1997 [17]</td>
</tr>
<tr>
<td>Mouse mammary epithelium</td>
<td>nd</td>
<td>nd</td>
<td>+++d</td>
<td>Pullan et al., 1996 [12]</td>
</tr>
<tr>
<td>MDCK</td>
<td>+</td>
<td>+++</td>
<td>+ / +++a</td>
<td>This study</td>
</tr>
</tbody>
</table>

The extent of apoptosis: +++, high; +, low; –, not detectable; nd, not determined. aExtent of cellular aggregation in suspension cultures was deliberately controlled. Suspended cell mixtures containing single and aggregated cells were studied. bExtent of cellular aggregation in suspension cultures was not specified, except in the case of MDCK cells. cThe cells were cultured in semisolid media containing methylcellulose to prevent cellular aggregation. d They were cultured in gels of collagen I or Engelbreth-Holm-Swarm matrix to control cellular aggregation. e The extent of cellular aggregation in suspension cultures was deliberately controlled. Suspended cells were separated into single and aggregated cell populations and studied. The extent of anoikis in populations of single cells depended on the extent of cellular aggregation in the suspension culture.
measurements were consistent.

The difference in the extent of anoikis between low- and high-calcium medium can be attributed to the difference in the extent of cellular aggregation in the suspension cultures. Alternatively, this difference may be a direct effect of calcium ions acting on the regulatory pathways of apoptosis, since calcium receptors are shown to be involved in apoptosis [22] and MDCK cells have calcium receptors on the surface [23].

To see whether the extent of anoikis is varied by changing the extent of cellular aggregation without varying the calcium concentration in the culture medium, we maintained MDCK cells in suspension either at a sparse- or dense-surface cell density, and the relationship between cellular aggregation and the extent of anoikis was studied as follows.

**Progression of anoikis in cells maintained in suspension either at a sparse- or dense-surface cell density**

After suspension at a sparse-surface cell density (see Methods) for 24 h, most of the cells separated from one another, and nearly no cellular aggregates were formed (Fig. 4A, sps). In contrast, cells maintained in suspension at a dense-surface cell density formed many cellular aggregates (Fig. 4A, dns). After the suspension was cultured for 24 h at a sparse-surface cell density, most of the cells reattached to the normal culture dishes (Fig. 4B, sps). After culturing at a dense-surface cell density, however, less than half of the

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**Fig. 4.** Anoikis of MDCK cells is suppressed in a suspension culture at sparse-surface cell density. MDCK cells (1 × 10⁴ cells/ml in standard culture medium) were maintained in suspension for 24 h either at 1.5 × 10³ cells/cm² (sps: sparse-surface cell density) or at 1 × 10⁵ cells/cm² (dns: dense-surface cell density). (A) Phase contrast images. Bar: 100 µm. (B) Reattachment of the cells was quantified as in Fig. 3B. (C) Sub-G₁ cell population of the cells was quantified as in Fig. 3C. (D) Caspase-8 activity of the cells was quantified as in Fig. 3D. (E–H) After the cells were maintained in suspension, they were separated into populations of single and aggregated cells, as in Fig. 3. (E) Phase contrast images of the population of single cells. Bar: 100 µm. (F) The percentage of the single-cell population was determined as in Fig. 3F. (G) The caspase-8 activity of each cell population was determined as in Fig. 3G. (H) Estimated caspase-8 activity in cultures before separation by strainer, as calculated from the cell number (F) and the caspase-8 activity (G) of each cell population. See Results for details. Values are the mean ± SD of three independent experiments (*P < 0.05 by Student’s t-test).
cells reattached to the dishes (Fig. 4B, dns). These results suggest that MDCK cells suspended at a sparse-surface cell density do not aggregate, and they retain their ability to attach to the ECM.

The sub-G₁ contents of cells suspended at a dense-surface cell density were twofold higher than before the suspension, whereas the sub-G₁ content of cells suspended at a sparse-surface cell density was comparable to that of the cells before suspension (Fig. 4C). Unlike in the sub-G₁ content measurement, no appreciable difference was detected in the caspase-8 activity between the two suspension conditions, partly because of large statistical errors (Fig. 4D).

These results suggest that more anoikis occurred in the suspension culture at a dense-surface cell density than in the suspension culture at a sparse-surface cell density, implying that anoikis is facilitated in suspension cultures under conditions favoring cellular aggregation.

Progression of anoikis in single and aggregated cells

To see whether the extent of anoikis differs between the single and the aggregated cell populations, we used a cell strainer to separate cells maintained in suspension for 24 h at either sparse- or dense-surface cell densities into single and aggregated cell populations (Fig. 4, E–H). After maintaining cells in suspension at a sparse-surface cell density, most of the cells passed through the strainer and were thus collected as a population of single cells (Fig. 4, E and F, sps). The sizes and shapes of these single cells appeared to be normal (Fig. 4E, sps). Only a fourth of the cells suspended at a dense-surface cell density was collected as a population of single cells (Fig. 4, E and F, dns); these single cells appeared to shrink (Fig. 4E, dns), suggesting that apoptosis was occurring in them.

The results show that when MDCK cells are suspended under conditions that facilitate aggregation, single cells in the suspension culture exhibit more apoptotic characteristics than those suspended under conditions that suppress it.

After maintaining cells in suspension for 24 h at a sparse- or dense-surface cell density, the caspase-8 activity of the aggregated cells was found to be comparable to that of cells before suspension (Fig. 4G, filled bar). Single cells collected from the sparse-surface cell density suspension exhibited an approximately two-fold greater caspase-8 activity (Fig. 4G, sps, open bar) than before suspension (Fig. 4G, att). In contrast, single cells collected from the suspension at a dense-surface cell density exhibited 6-fold greater caspase-8 activity (Fig. 4G, dns, open bar) than before suspension (Fig. 4G, att).

To determine the consistency between the results obtained before and after the strainer separation, we compared the results obtained with and without the separation. The caspase-8 activity of the suspension mixture before separation was calculated from the caspase-8 activity (Fig. 4G) and the cell number (Fig. 4F) of each population obtained after the separation. The calculated values of the activity (Fig. 4H) are in agreement with the results obtained from the independent experiments without the strainer separation (Fig. 4D).

DISCUSSION

The main findings of this study are as follows: (a) anoikis progresses more extensively in suspension cultures of MDCK cells containing more cellular aggregates than in those with fewer cellular aggregates (Figs. 2–4), (b) apoptotic cells in suspension cultures are mostly single cells (rather than aggregated cells) (Figs. 3 and 4), and (c) the extent of apoptosis in populations of single cells is related to the extent of cellular aggregation in the suspension culture. High caspase-8 activity was detected only in populations of single cells derived from suspension cultures that contained many cellular aggregates, whereas low caspase-8 activity was detected in populations of single cells derived from suspension cultures with few cellular aggregates (Figs. 3 and 4).

Thus a suspension culture of MDCK cells with many cellular aggregates contains a mixture of a great many aggregated cells with low apoptotic characteristics and a small number of single cells with high apoptotic characteristics. An MDCK cell suspension culture with few cellular aggregates, however, contains a mixture of a small amount of aggregated cells with low apoptotic characteristics and a great many single cells with low apoptotic characteristics.

In other cell types, cellular aggregation in suspension cultures has been shown to suppress anoikis [5]. For example, the observation that suspension cultures with an extensive cellular aggregation contain fewer apoptotic cells has been reported with mouse proximal tubular cells [9], bronchial epithelial cells [10], and ras-transformed fibroblasts [17]. In MDCK cells, in contrast, suspended cell cultures with more cellular aggregates contain more apoptotic cells (Fig. 2). Thus the effect of aggregate formation on anoikis in suspension cultures appears to differ between MDCK cells and other cell types, as summarized in Table 1.

When populations of single cells and populations of aggregated cells are separately examined after culturing them in suspension, the former is more apoptotic than the latter, as reported for bronchial epithelial cells [10], squamous cell carcinoma [11, 13], and mouse mammary epithelial cells [12]. This was also seen in the present study with MDCK cells maintained in suspension under conditions favoring aggregation (Figs. 3G, HCa and 4G, dns). When a significant amount of cellular aggregates are present in suspension culture, MDCK cells behave similarly to other cell types because aggregated cells are
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When MDCK cells were suspended under conditions suppressing aggregation, the progression of apoptosis in populations of single cells was suppressed to a level similar to that in aggregated cell populations (Figs. 3G, LCA and 4G, sps; summarized in Table 1). Therefore in the absence of cellular aggregates, anoikis proceeds more slowly in populations of single MDCK cells in suspension than in the presence of aggregates.

These results may be explained by the following hypotheses: (I) anoikis of a single MDCK cell alone is slow; and (II) when cellular aggregates are formed in MDCK cell suspension cultures, apoptotic processes are activated in some cells of a cellular aggregate, and these apoptotic cells immediately leave the aggregate, yielding single apoptotic cells and aggregated nonapoptotic cells (Fig. 5).

The idea that cellular aggregation can facilitate anoikis is feasible if the interaction between cell surface molecules on neighboring cells, such as death ligands and death receptors, plays a role in the induction of anoikis. The role of death receptors in the anoikis of MDCK cells is not yet clearly understood, though it has been studied in other cell types [24–26]. Nevertheless, a death-receptor-associated death domain has been shown to be involved in anoikis of MDCK cells [19, 20]. Even if the type II death receptor pathway plays a role in anoikis of MDCK cells, as has been suggested [14], the involvement of the death receptor may be difficult to detect because only a small fraction of the death receptors needs to be activated to initiate apoptosis [18].

Facilitation of anoikis by cellular aggregation may also be feasible if cell-cell interactions affect cell cycle progression [27]. Sensitivity to apoptosis may depend on the phase of the cell cycle [28, 29]. In a situation of this kind, cells that proceed through the cell cycle may be more sensitive to apoptosis than cells that have been arrested [28]. Although noncancerous adherent cell types require cell-ECM interactions to complete the cell cycle [17, 30, 31], if cell-cell interactions support some phases of cell cycle progression in suspended cells, cellular aggregation may increase sensitivity to apoptosis in suspended cells and thus facilitate anoikis.

In conclusion, the effects of cell-cell interactions on anoikis in suspension cultures of MDCK cells have been studied by means of several methods. The progression of anoikis was found to positively correlate with cell-cell interactions. The next step to do would be to begin figuring out the molecular players in this facilitation of anoikis.

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REFERENCES

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