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Letter to the Editor

Changes in intercellular junctions during apoptosis precede nuclear condensation or phosphatidylserine exposure on the cell surface

Dear Editor,

An essential feature of apoptosis *in vivo* is rapid removal of the dying cell before it ruptures to invoke an inflammatory response. Histology of epithelia reveals apoptotic cells to be detached from their neighbours and engulfed by phagocytes.[1] This implies that following an apoptotic stimulus, detachment from neighbouring cells, substratum or extracellular matrix precedes or accompanies engulfment, and is likely to be a specific component of the apoptotic process.

Herein we report changes in cell-cell junctions during apoptosis in MDCK cells, a polarised epithelial line. We induced apoptosis by two different stimuli, a DNA damaging agent (etoposide) and a non-specific protein kinase inhibitor (staurosporine). Cells were cultured as confluent sheets and junctional changes examined in relation to characteristic apoptotic nuclear events and to phosphatidylserine exposure at the cell surface. The latter precedes nuclear condensation and is believed to be a signal for recognition of apoptotic cells by phagocytes. The concentrations of etoposide (50 μM) and staurosporine (400 nM) were chosen to allow sufficient time in which to examine changes in cell-cell interactions that might occur prior to the appearance of apoptotic morphology and cell detachment. Detached cells were shown to be apoptotic both by Hoechst 33528 staining for nuclear morphology and by DNA analysis for internucleosomal cleavage (DNA laddering). Apoptotic events induced by etoposide were also observed by time-lapse video microscopy of cell sheets.

By time-lapse microscopy apoptosis was first detectable as ruffling at the periphery of the dying cell which became slightly raised above the monolayer and then contracted.[2] Moreover, as the dying cell contracted its neighbours moved to fill the space vacated. The recruitment of viable neighbours and the detachment of the dying cell were continuous and simultaneous processes and at no point were gaps observed in the monolayer. Although the dying cell's immediate neighbours were most obviously involved, the migration included cells 2–3 distant from it. Thus, as the dying cell rose out of the monolayer and became phase-bright, confluence was maintained. The time between the first event at the cell periphery and rising out of the monolayer was 1–1.5 h. After its exit, the dying cell remained tethered to the monolayer by filamentous projections before final detachment.

MDCK cells have three types of intercellular junction, the zonula occludens (ZO) or tight junction which forms an apico-lateral seal between cell membranes, the zonula adherens (ZA), an adhesive ring just below the ZO, and the desmosome (DM) which gives punctate, sub-apical adhesion sites. We examined changes in these junctions during

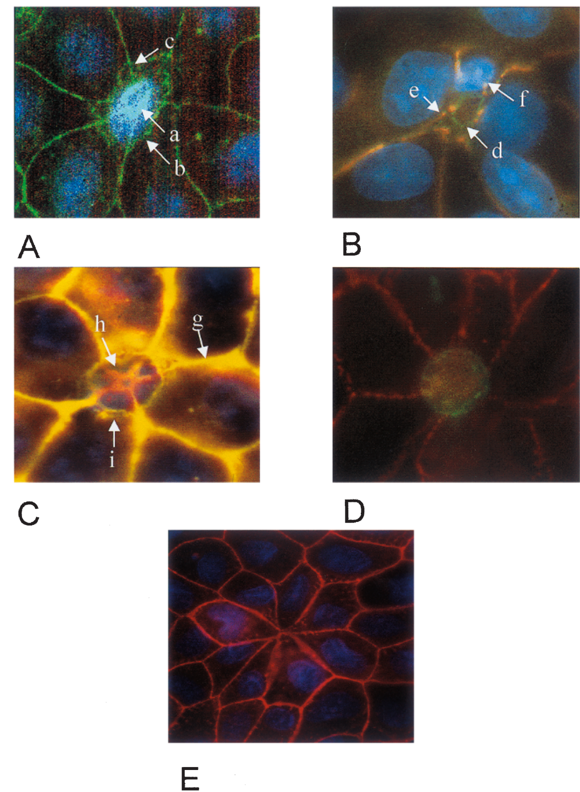

Figure 1 For all panels: cells were fixed in ice-cold methanol as previously described.[6] Monoclonal antibodies used were: mouse anti-desmoplakin 11-5F;[6] rat anti-occludin MOC37;[7] rat anti-E-cadherin ECCD1 (R&D Systems, Oxford, UK). FITC and Cy3 conjugated secondary anti-rat and anti-mouse antibodies (Jackson Immunoresearch, PA, USA) were used as appropriate. Annexin V-FITC (Brandt) was used to stain cells prior to fixation. (**A**) Cells were treated with staurosporine for 4 h, fixed and stained for occludin. The central cell has two rings of occludin staining (a and b) the inner ring has radiating spokes, c, which are colinear with the junctions between adjacent cells. (**B**) Cells treated as (**A**) and stained for occludin (green) and desmoplakin (red). Coincident staining appears orange-yellow. The central cell has a ring structure as (**A**), but this stains for occludin alone. The edge of the cell stains for both desmoplakin and occludin. The nucleus of this cell is displaced from the occludin ring, indicating that the latter is not a perinuclear structure. (**C**) Cells treated as (**A**) and stained for E-cadherin (red) and desmoplakin (green). Coincident staining appears orange-yellow. The pattern of E-cadherin staining in early apoptotic cells matches that of occludin, with a central ring, h, radiating arms colinear with the junctions between adjacent cells. The periphery of the cell still stains for both E-cadherin and desmoplakin. (**D**) Treated monolayers were stained with annexinV-FITC, fixed then stained for desmoplakin. AnnexinV positive staining was only observed in cells which had rounded and raised out of the monolayer. (**E**) Cells treated with etoposide for 6 h. The rosette at the centre of the cell is characteristic of post-detachment where a circle of cells have invaded substratum. Desmosomes have been formed along the extended borders between these cells

apoptosis by immunofluorescence. Staurosporine and etoposide produced identical changes. Normal cells within the monolayer show a single peripheral ring of staining for the ZO protein occludin, but cells undergoing apoptosis show two rings, one peripheral (Figure 1A, arrow b) and one towards the centre (arrow a). 'Spokes' of occludin which are co-linear with the junctions between the surrounding cells radiate from the central ring (arrow c). In Figure 1B double staining of occludin (green, FITC) and of DMs for desmoplakin (red, Cy3) shows that ZO and DM are coincident at the cell periphery (yellow; arrow e) but that only occludin is present in the central ring (green, arrow d). Critically, the nucleus of this cell is displaced from the occludin ring, indicating that this structure is not perinuclear. In contrast to DMs, the ZA component E-cadherin shows a similar distribution to that of occludin (Figure 1C), being co-distributed with desmoplakin at the cell periphery (arrow i) and present alone in the central ring and 'spokes' (arrow h). This suggests that occludin and E-cadherin are co-distributed, but dual staining was not possible since both antibodies were from rat.

We interpret these observations to indicate that as an apoptotic cell is excluded from the monolayer, the surrounding cells that move to occupy its space form new junctions in order to maintain the epithelial barrier. Since apoptotic cells are extruded from the monolayer the new junctions must be formed beneath the apoptotic cell. In the 'ring-spokes' structure we believe that the spokes represent new junctions between the encroaching viable cells and the ring represents a junction between the encroaching cells and the apoptotic cell. Both ring and spokes consist of a ZO accompanied by a ZA. The peripheral ring of the apoptotic cell represents the pre-existing junctions between the cell and its neighbours.

These observations differ in two respects from those of Peralta Soler *et al.*[3,4] who observed restructuring of ZO and ZA in association with epithelial cell apoptosis. Firstly our observations show apoptotic cells being extruded from the epithelial sheet whereas Peralta Soler *et al.* observed them being engulfed by adjacent epithelial cells. Secondly we show that ZO and ZA are formed in the absence of DM. Further observations show that ZO and ZA formation precede DM formation.

Nuclear staining of cells with ring-spoke structures revealed slight condensation but not the condensed and fragmented nuclei of late apoptotic cells. We therefore used another marker of apoptosis, annexin V staining, to indicate membrane phosphatidylserine exposure. No annexin V-positive cells were seen within the monolayer. Only when cells had contracted and begun to detach was annexin V staining detected (Figure 1D). This indicates that the adhesive changes leading to cell extrusion and detachment precede annexin V positivity. Figure 1E shows a rosette of encroaching cells shortly after the apoptotic cell has become detached from the monolayer. Here the apoptotic, PS-exposed cell has detached from the monolayer and new DMs have formed along the junctions between cell, demonstrating the later formation of DMs in the sequence of events.

We propose that the apoptotic epithelial cell detaches from the cell sheet as part of a continuous process which involves occupation of the vacated space by neighbouring viable cells. As part of this process new intercellular junctions are formed so that the epithelial barrier is not compromised. A recent study observed internalisation of E-cadherin and subsequent caspase-dependent cleavage.[5] This would seem inconsistent with a requirement to maintain the epithelial barrier, unless there are two pools of E-cadherin, one being broken down and another involved in new junction assembly.

In order to detach, the apoptotic cell must pass through its own apical junctional ring and thus it must lose polarity. This presumably involves early loss of cell-substratum contact. What emerges is a complex process involving the early remodelling of junctional contacts and the early recognition of the apoptotic process by neighbouring cells. Study of the cellular signals involved in these early events seems imperative.

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