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The fate of E- and P-cadherin during the early stages of apoptosis

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Abstract

Caspases are responsible for the proteolysis of many cytoskeletal proteins in apoptotic cells. It has been demonstrated here that during cisplatin-induced apoptosis of human embryo retinoblasts both E- and P-cadherin were degraded by caspases, giving initially major polypeptide products of apparent molecular weights 48 K and 104 K respectively. This proteolysis occurred over a similar time-scale to the observed degradation of PARP and to the onset of DNA fragmentation but appreciably later than p53 induction and cleavage of Mdm2 and p21. Addition of caspase inhibitors such as Z-VAD-FMK inhibited apoptosis and cadherin degradation. Co-immunoprecipitation studies carried out on viable cells confirmed previously observed complexes between cadherins and α and β catenin and between the catenins themselves. These interactions were sustained in apoptotic cells as long as the protein components remained intact. Using confocal microscopy it has been shown that cytoskeletal changes associated with apoptosis precede degradation of catenins and cadherins by several hours. In particular, after addition of cisplatin relatively rapid (within 3 h) re-localization of adherens junction proteins from the cell periphery to the cytoplasm was observed whereas little cadherin or catenin degradation occurred until 10 h. We conclude that neither caspase-mediated degradation of cytoskeletal components nor disruption of adherens junction protein-protein interactions is required for morphological change.

Keywords: E-cadherin; P-cadherin; apoptosis; caspases; cytoskeleton

Abbreviations: Ad2, adenovirus 2; ATM, mutated in ataxia telangectasia protein; E1A, early region 1A; HER, human embryo retinoblasts; PARP, poly (ADP-ribose) polymerase; Rb, retinoblastoma protein

Introduction

Apoptosis is a process of fundamental importance for the development and well-being of most, perhaps all, multicellular organisms. It plays a central role in morphological development, functioning of the immune system in vertebrates and homeostasis.[1–3] The apoptotic process is controlled by members of the Bcl-2 family.[4,5] These proteins, which are highly conserved having been identified in *Caenorhabditis elegans* (ced-9 gene product), viruses (for example adenovirus E1B 19K protein) and higher mammals, can serve as both positive (e.g. Bcl-2 and Bcl_{XL}) and negative (e.g. Bax and Bak) regulators of apoptosis. Just as the proteins which control apoptosis are highly conserved so are the mediators of the process. Over the last few years it has become apparent that a series of proteases, termed caspases, homologous to the product of the *Caernorhabditis elegans* ced-3 gene are responsible for putting apoptotic signals into practice.[6,7] Caspases can serve as initiators and effectors of apoptosis such that apoptotic signals result in activation of pro-caspases which can then cleave and activate other members of the family. These proteases can go on to degrade a wide variety of cellular proteins.

Caspase substrates are many and varied. Of particular importance for the progress of apoptosis are the caspases themselves,[6,7] I^{CAD}/DFF45 which serves as an inhibitor of the nuclease responsible for DNA fragmentation[8,9] and Bcl-2 homologues.[10,11] A number of important cellular regulators of the cell cycle and p53 activity such as pRb,[12] Mdm2,[13] p21[14] and ATM[15] are also degraded during apoptosis. A further broad class of proteins which appear to be a primary target for caspases are cytoskeletal components. Thus it has already been reported that keratins,[16,17] β catenin,[18] γ catenin,[19] lamins,[20,21] gelsolin[22] and vimentin[16] as well as cytoskeletal-associated kinases such as FAK[23] and PAK2[24] are substrates for caspases at various stages during apoptosis. It has been considered that caspase-mediated degradation of these and perhaps other cytoskeletal proteins may play a major role in inducing the morphological changes associated with apoptosis such as the commonly observed membrane blebbing, loss of cell-cell and cell-matrix contacts and cytoplasmic condensation. The most obvious candidates for involvement in the loss of cell-cell and cell-matrix contacts are components of the adherens junctions in which classical cadherins (such as E-, P- and N-cadherin) form extra cellular clusters and then tether these through their cytoplasmic domains, catenins, α actinin and vinculin to F actin filaments.[25–27] With this in mind we have investigated the stability of cadherins in adherent human cells undergoing apoptosis. In addition the temporal relationship of morphological changes occurring in apoptotic cells, caspase-mediated cadherin and catenin degradation, the maintenance of cadherin/catenin interactions and the degradation of other well-characterized caspase substrates have been compared.

Results

It has previously been shown that Ad2 E1A+N-*ras* HER 313A cells will undergo apoptosis in response to relatively low doses of cisplatin.[19] We reported rearrangement of adherens junctions together with degradation of β and γ catenin.[19] In the present study the fate of cadherins during the early stages of apoptosis has been examined.

Degradation of E- and P-cadherin during apoptosis

Preliminary Western blotting analysis showed that Ad2 E1A+N-*ras* HER313A cells expressed E- and P-cadherin at reasonably high levels as well as other cadherins of rather higher molecular weight which were detected with a pan-cadherin antibody.[28] (data not shown). Cells were induced to undergo apoptosis by the addition of cisplatin. Percentage of apoptotic cells detected by acridine orange staining at 3, 6, 10 and 16 h was 4, 21, 39 and 60% respectively (these values varied only marginally between experiments). Western blotting analysis (Figure 1) showed cleavage of E-cadherin beginning after about 6 h and P-cadherin at slightly later times. Degradation of the cadherins occurred over a similar time scale to that observed for β and γ catenin (Figure 1). No degradation of α catenin was observed as has been reported previously.[18,19] The major products of β and γ catenin proteolysis were about 75 K molecular weight as has been reported.[18,19,29]

It can be seen (Figure 1) that at 10 and 16 h a 48 K degradation product of E-cadherin was detected (using the antibody clone 36). This is consistent with proteolysis occurring at one of the potential caspase cleavage sites located towards the centre of the molecule. The most likely candidates are amino acids 476–479 (DVLD) or 549–552

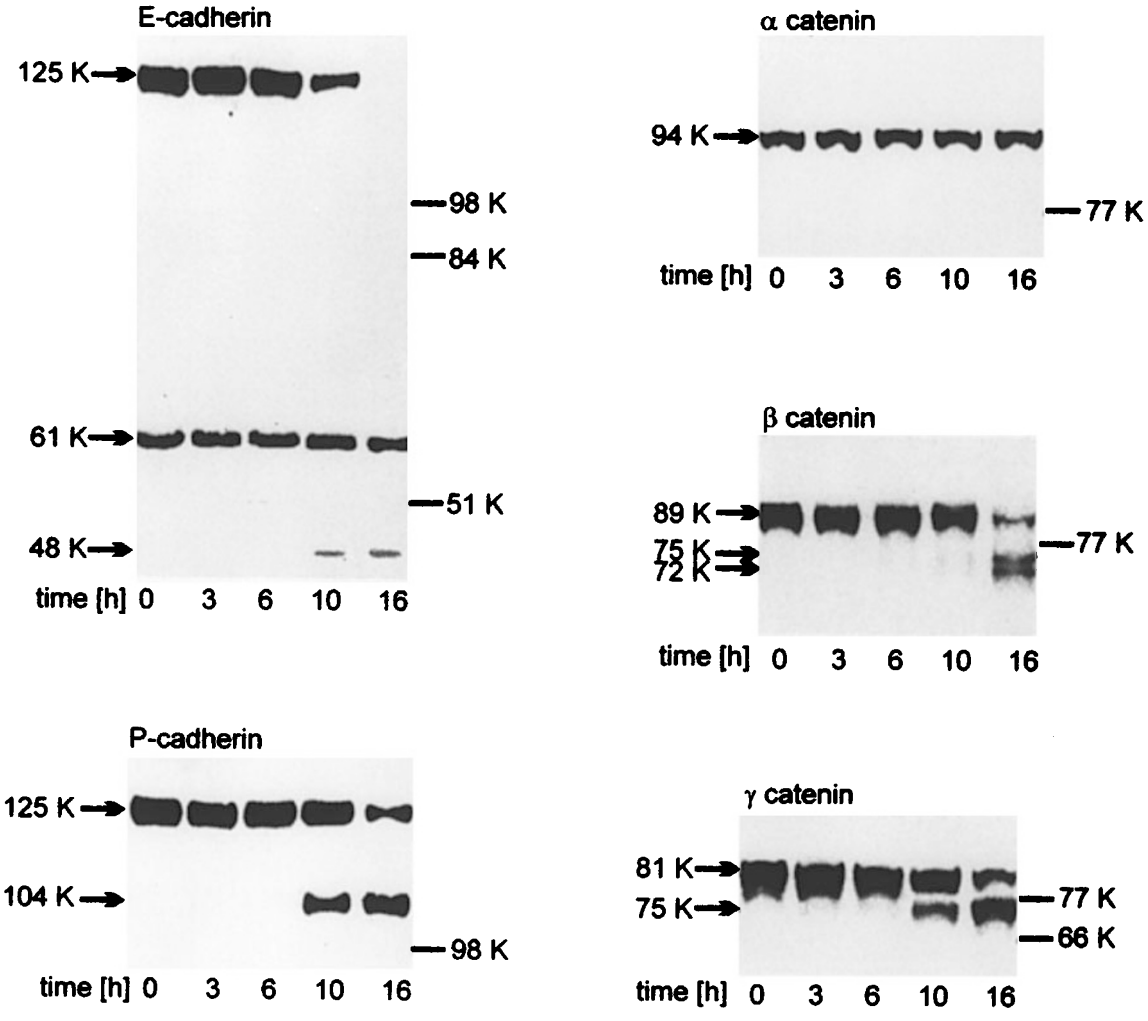


Figure 1 Integrity of cadherins and catenins during apoptosis. Ad2 E1A+N-*ras* HER313A cells were treated with cisplatin (16 μg/ml) and then harvested at the times indicated. Aliquots (containing 50 μg protein) were fractionated on polyacryamide gels and subjected to Western blotting using antibodies against E- and P-cadherin and α, β (mouse antibody) and γ catenin. The position of migration of marker proteins is shown on the right hand side of each panel, and the calculated molecular weights of the cadherins, catenins and their cleavage products on the left hand side. Percentage of apoptosis at each time point is given in the text

(DRED) (Table 1) which would give rise to C-terminal fragments of theoretical molecular weights 44.6 K or 36.2 K respectively (the epitope recognised by the antibody is located in the C-terminal domain). It is difficult, on the basis of SDS–PAGE, to predict the cleavage site more accurately due to glycosylation of cadherins. A 61 K, cross-reacting protein was recognised by the antibody against E-cadherin but was unaffected by apoptosis. A similar protein has been observed with other antibodies raised against E-cadherin (using clone 34 from Transduction Laboratories, clone ECCD-1 from Zymed and clone NCC-CAD-299 from Zymed) but its origins are, at present unclear. A major cleavage product of 104 K molecular weight resulting from P-cadherin degradation was seen (using antibody clone 56 from Transduction Laboratories) 10 h after induction of apoptosis. As the antibody epitope on P-cadherin is between amino acids 72–259 and the 104 K fragment is not co-immunoprecipitated with catenins (see below and Figure 4A) it is most probable that the site of cleavage of P-cadherin is close to the C-terminus of the protein possibly between amino acids 693–696 (Table 1). Again, protein glycosylation makes prediction of the site of cleavage on the basis of SDS–PAGE rather equivocal.

The sites of cleavage of β catenin must be close to the C-terminus. Western blotting with the rabbit antibody raised against the C-terminal 14 amino acids recognized neither of the two β catenin degradation products seen in Figure 1. We conclude therefore that both caspase sites must be present in the C-terminal region. The sequence ETAD (amino acids 692–695) perhaps provides one caspase recognition site close to the C-terminus.

To confirm that proteolysis of E- and P-cadherin was attributable to caspases, the effectors of apoptosis, Ad2 E1A+N-*ras* HER 313A cells were treated with cisplatin in the presence of caspase inhibitors. Results of Western blotting analysis after treatment with cisplatin and Z-VAD-FMK (40 μM) are shown in Figure 2. Addition of inhibitor appreciably delayed the onset of apoptosis (the percentage of apoptosis at each time determined by acridine orange staining is shown under the appropriate lane) such that over the time course shown here no degradation of P-cadherin was detected (Figure 2). Slight reduction in the level of E-cadherin was observed at 10–16 h although no

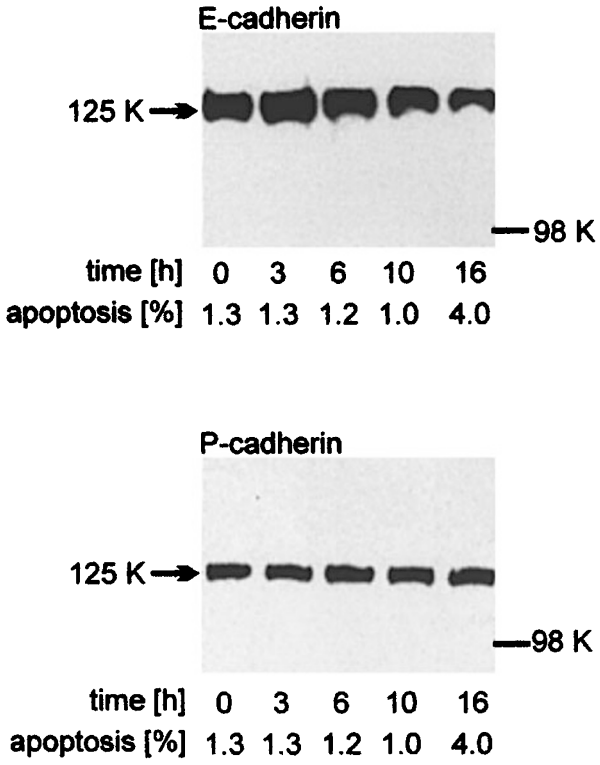


Figure 2 Inhibition of P-cadherin and E-cadherin cleavage and apoptosis with capase inhibitor. Ad2 E1A+N-*ras* HER313A cells were treated with Z-VAD-FMK (40 μM) and cisplatin (16 μg/ml). Cells were harvested at the times indicated and the percentage apoptosis determined by acridine orange staining. Aliquots (containing 50 μg protein) were subjected to Western blotting using the antibodies against E-cadherin and P-cadherin. The position of migration of marker proteins is shown on the right hand side of each panel, and the calculated molecular weights of the cadherins on the left hand side

Table 1

Cadherin predicted molecular weight (:observed mol weight)	Potential caspase cleavage site	Predicted products	Observed products
E-cadherin: 97.4 K (125 K)	aa 254–257 DQND	29.1 K+68.3 K	
	aa 288–291 DADD	30.0 K+67.4 K	
	aa 367–370 DTND	41.1 K+56.3 K	
	aa 476–479 DVLD	52.8 K+44.6 K	48 K
	aa 549–552 DRED	61.2 K+36.2 K	
	aa 587–590 DVND	65.1 K+32.3 K	
	aa 747–750 DTRD	82.7 K+14.7 K	
P-cadherin: 91.4 K (125 K)	aa 207–210 DQND	23.2 K+68.2 K	
	aa 241–244 DEDD	26.9 K+64.5 K	
	aa 264–267 DPHD	29.5 K+61.9 K	
	aa 320–323 DAND	35.5 K+55.9 K	
	aa 372–375 DDGD	41.1 K+50.3 K	104 K
	aa 540–543 DVND	59.6 K+31.8 K	
	aa 585–588 DDSD	64.6 K+26.8 K	
	aa 613–616 DTYD	67.3 K+24.1 K	
	aa 693–696 DTRD	76.2 K+15.2 K	

48 K degradation product was seen. This is consistent with the data shown in Figure 1, which indicates that E-cadherin is appreciably more sensitive to caspase-mediated degradation than P-cadherin. At late times (for example 48 h), even in the presence of the caspase inhibitor Z-VAD-FMK, apoptosis and consequent cadherin proteolysis occurred (data not shown). Inhibition of apoptosis and caspase-mediated degradation of cadherins was also observed (up to at least 24 h) with other caspase inhibitors Boc-Asp-FMK, Z-DEVD-FMK and Z-YVAD-FMK (all at a concentration of 40 μM, data not shown). The observation that all of these compounds inhibited cadherin degradation with approximately similar efficiencies means that it is difficult to predict which caspase is primarily responsible for the proteolysis. Resolution of this point will have to await *in vitro* studies using purified components. When the human epithelial cell line SW-13 was treated with cisplatin apoptosis was induced and degradation of E-cadherin, similar to that shown in Figure 1, was observed. In the presence of the caspase inhibitor Z-VAD-FMK no apoptosis was seen and E-cadherin remained intact (data not shown). Cleavage of E- and P-cadherin have also been observed in other cell-lines during apoptosis- for example adenovirus transformed rat kidney cells (data not shown).

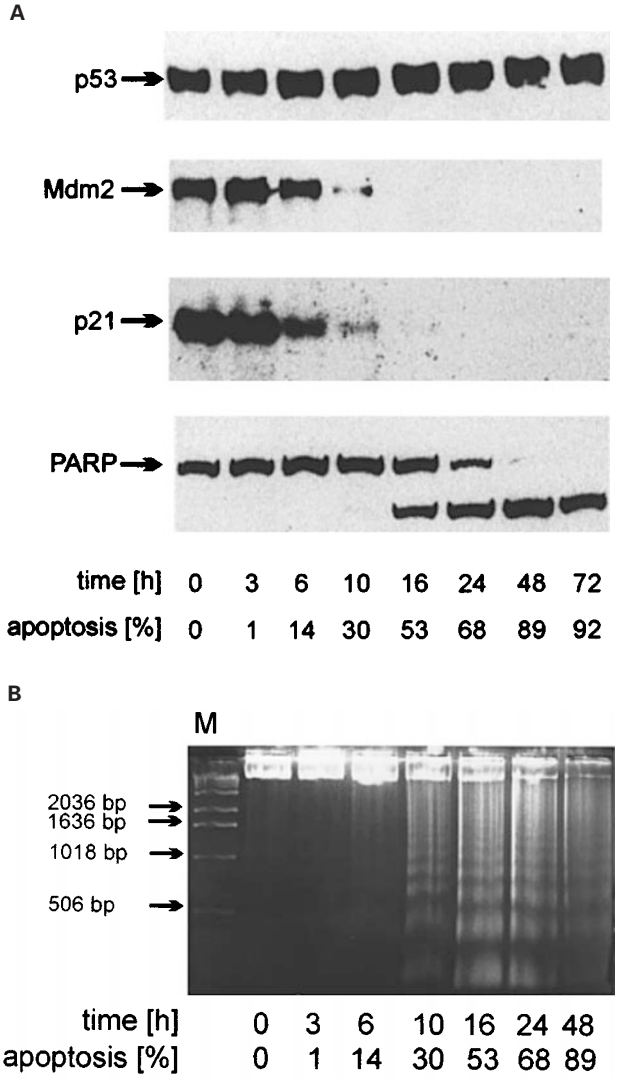


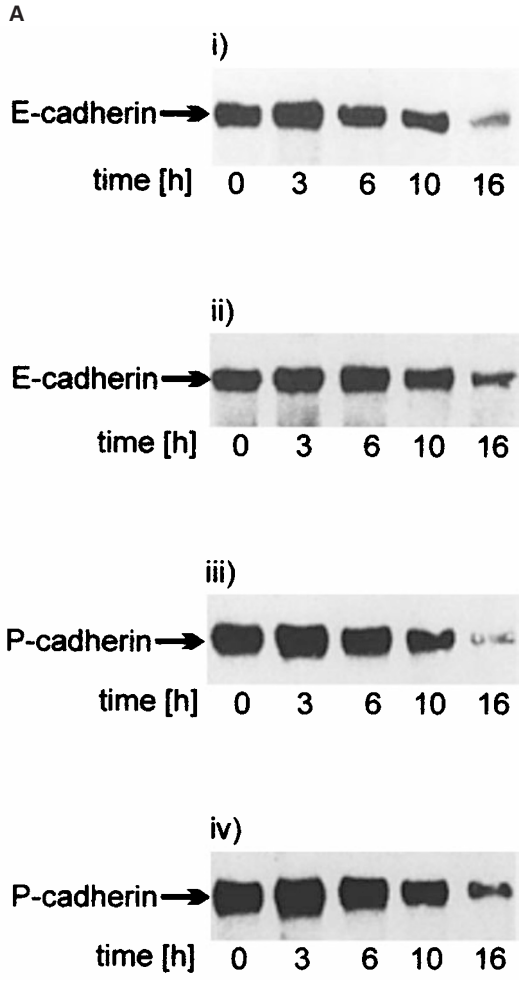
Figure 3 Protein expression and DNA fragmentation during cisplatin-induced apoptosis. Ad2 E1A+N-*ras* HER313A cells were treated with cisplatin and then harvested at the times indicated. (**A**) protein expression. Aliquots (containing 50 μg of protein) were fractionated by SDS–PAGE and subjected to Western blotting using antibodies against p53, Mdm2, p21 and PARP. Times at which samples were harvested are indicated under each track, together with the extent of apoptosis as determined by acridine orange staining. (**B**) DNA fragmentation. DNA was extracted from samples similar to these shown in (**A**) and fractionated on 2% agarose gels (see Materials and Methods). The size of molecular weight markers is indicated on the left-hand side

Early and late protein degradation during apoptosis

In order to understand better the significance of cadherin and catenin degradation for the apoptotic cell we considered that it was important to know whether cleavage occurred at the same time as other caspase-mediated proteolysis. Therefore Ad2 E1A+N-*ras* HER 313A cells were treated with cisplatin as described and subjected to Western blotting with a variety of antibodies (Figure 3A). p53 was induced by addition of cisplatin as would be expected.[30] It has previously been noted that the CDK inhibitor p21 serves as an early substrate for caspases during apoptosis.[14] Consistent with this suggestion it can be seen that the level of p21 decreased dramatically 3–6 h after addition of cisplatin, being virtually undetectable at 10 h. Similarly Mdm2 was lost over a similar timescale. On the other hand no cleavage of PARP could be detected until 10 h and appreciable quantities of the full-length protein could still be seen after 24 h. The degradation of E- and P-cadherin slightly preceded that of PARP but occurred appreciably later than p21 cleavage. The data presented in Figure 3B shows that DNA fragmentation coincided with E- and P-cadherin, β and γ catenin and PARP cleavage (Figures 1 and 3A).

Catenin-cadherin interactions during apoptosis

In adherens junctions in viable cells, a complex series of protein-protein interactions can be observed.[25] In the present study we set out to examine how these complexes were affected by the onset of apoptosis. Thus Ad2 E1A+N-*ras* HER313A cells were induced to apoptose by the addition of cisplatin as described. At times up to 16 h cells were harvested and aliquots containing equal amounts of protein were subjected to immunoprecipitation as described in Material and Methods. Figure 4A shows Western blots obtained after immunoprecipitation with antibodies raised against α catenin (Figure 4A, panels i and iii) or β catenin (rabbit antibody) (Figure 4A, panels ii and iv) and blotting for E-cadherin (Figure 4A, panels i and ii) and P-cadherin (Figure 4A, panels iii and iv). In viable cells protein complexes were seen as expected. For example P- and E-cadherin were co-immunoprecipitated using antibodies raised against α and β catenin. Similarly, when lysates were immunoprecipitated with the antibody which recognizes E-cadherin co-immunoprecipitated (i.e. bound) α and β catenin could be detected by Western blotting (data not shown). In cells where there was



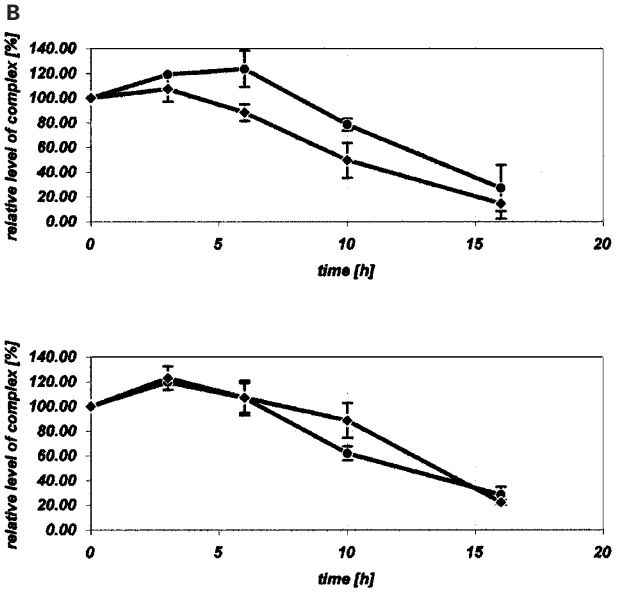


Figure 4 Co-immunoprecipitations of E- and P-cadherin with α and β catenin. Ad2 E1A+N-*ras* HER313A cells were treated with cisplatin (16 μg/ml) for the times indicated. Cells were harvested and solubilized in immunoprecipitation buffer as described in Materials and Methods. Using equal amounts of protein, immunoprecipitation was carried out with antibodies against α catenin or β catenin (mouse antibody). Immunoprecipitated proteins were fractionated by SDS–PAGE and electrophoretically transferred to nitro-cellulose membranes which were incubated with antibodies raised against E-cadherin or P-cadherin. Western blots are shown in (**A**); panels i and iii, immunoprecipitation with an antibody raised against α catenin, panels ii and iv immunoprecipitation with and antibody raised against β catenin (rabbit antibody); panels i and ii, Western blotted with an antibody raised against E-cadherin; panels iii and iv Western blotted with an antibody raised against P-cadherin. (**B**) shows results of quantitation of Western blots similar to these shown in **A**. Blots obtained from three separate experiments were scanned using a Bio Rad GS 690 Imaging Densitometer. All values were normalized to the amount of co-immunoprecipitated (i.e interacting or bound) protein from viable cells (time 0) and this was taken as 100%, (●) immunoprecipitated with and antibody against α catenin; (◆) immunoprecipitated with an antibody against β catenin (rabbit antibody); upper panel, blotted for E-cadherin; lower panel blotted for P-cadherin

appreciable apoptosis (10 and 16 h) a reduction in co-immunoprecipitated protein was observed. Densitometric scanning of Western blots obtained from three separate experiments similar to that shown (Figure 4A) allowed a quantitative assessment of the data. It can be seen (Figure 4B) that there was relatively little difference in the stability of complexes between P-cadherin and α and β catenin (Figure 4B lower panel). There was somewhat less E-cadherin/α

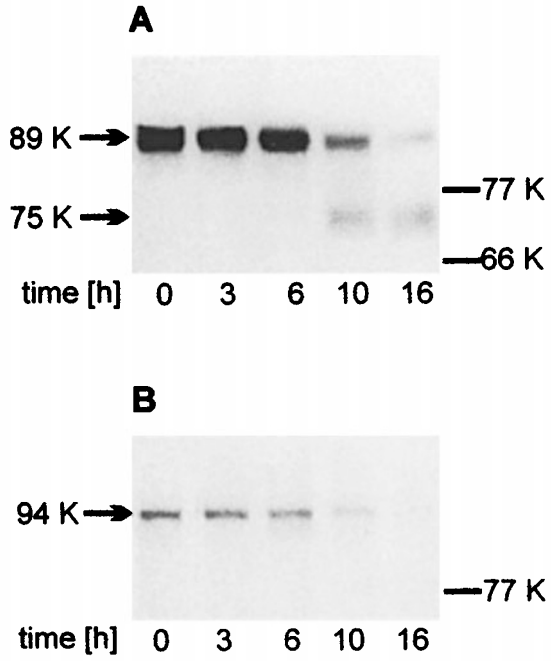


Figure 5 Co-immunoprecipitation of α and β catenin. Ad2 E1A+N-*ras* HER313A cells were treated with cisplatin, harvested, solubilized and subjected to immunoprecipitation as described in the legend to Figure 3. (**A**) Proteins were immunoprecipitated with an antibody against α catenin and Western blotted for β catenin (mouse antibody). (**B**) Proteins were immunoprecipitated with an antibody against β catenin (mouse antibody) and Western blotted for α catenin. The position of migration of marker proteins is shown on the right hand side of each blot, and the molecular weights of the co-immunoprecipitated catenins and proteolytic fragments is shown on the left hand side

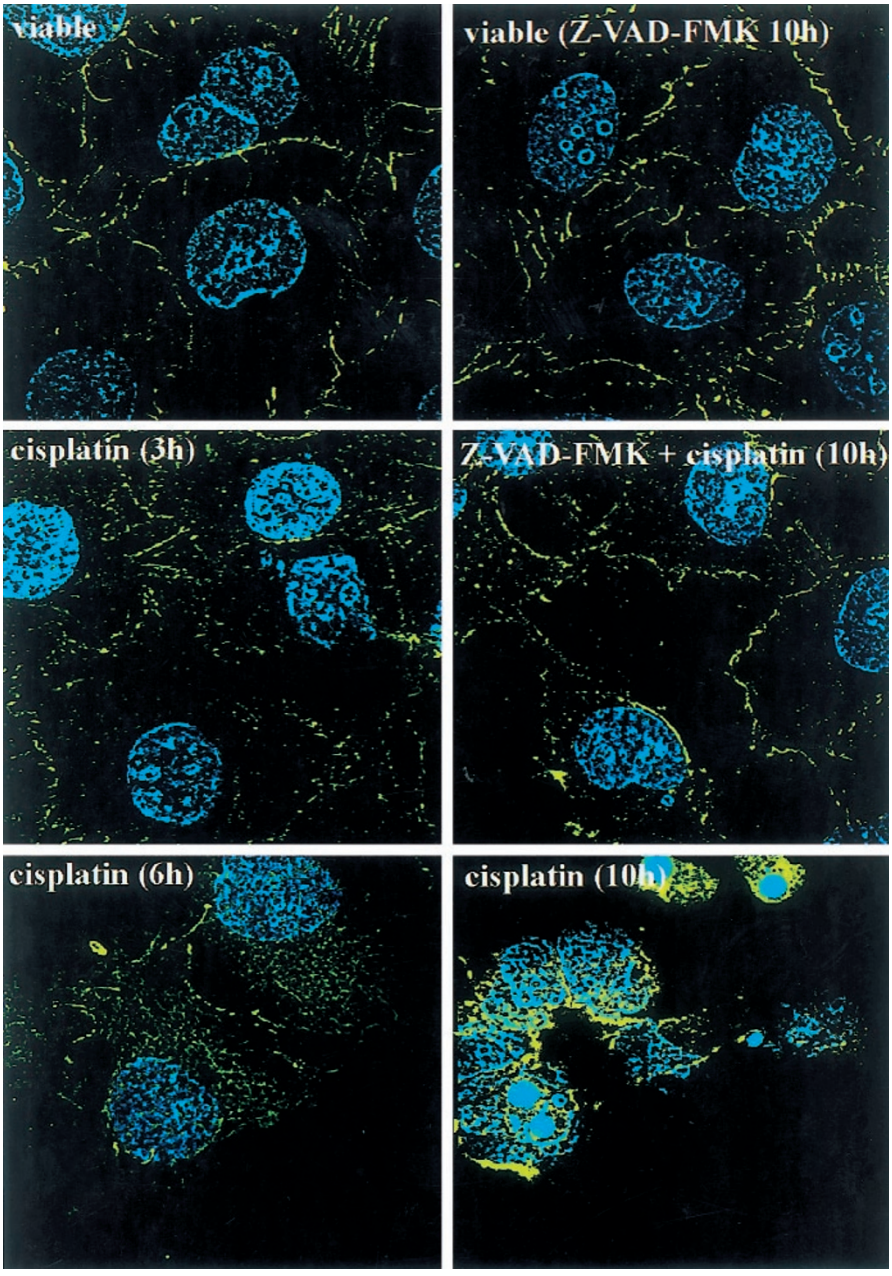


Figure 6 Subcelluar distribution of E-cadherin in viable and apoptotic cells. Ad2 E1A+N-*ras* HER313A cells were grown on glass multiwell slides. Cells were either fixed immediately (upper left and panel), or treated with cisplatin for the times shown (central or lower panels). Slides were also incubated with the capase inhibitor Z-VAD-FMK (upper and central right hand panels as indicated). Cells were fixed with paraformaldehyde and stained for E-cadherin (green) and for DNA DAPI (blue). Cells were viewed by confocal microscopy (see Materials and Methods). (Magnification objective $\times 63$)

catenin complex present during the relatively early stages of apoptosis (6 and 10 h time points) than of the E-cadherin/ β catenin complex (Figure 4B upper panel). Whether this difference is significant is not clear at present. Similar result to that for E-cadherin was obtained for the major cadherin recognized by the pan-cadherin antibody (data not shown). It is interesting to note that the proteolytic fragment of apparent molecular weight 104 K seen after degradation of P-cadherin (Figure 1) was not co-immunoprecipitated with catenins (Figure 4A). This suggests that the catenin binding site has been lost and that the initial site of cleavage is located towards the C-terminus of the protein (where the catenin binding site is located).

Whilst there are some slight differences in the stability of the complexes during apoptosis (Figure 4B), it is clear that the primary factor governing the ability to co-immunoprecipitate partner proteins is the presence of intact protein. Similar co-immunoprecipitation analysis was performed to

examine the stability of α catenin/β catenin complexes during the early stages of apoptosis (Figure 5). Slight reduction in co-immunoprecipitated (i.e. bound) species was observed over the first 6 h with a dramatic reduction at 10 and 16 h. It is presumed that this is attributable to caspase-mediated degradation of β catenin since there is relatively little loss of α catenin over a 48-h time course.[19]

It is interesting to note that two fragments produced by caspase-mediated degradation of β catenin were co-immunoprecipitated with α catenin 10 and 16 h after induction of apoptosis (Figures 1 and 5). Both polypeptides were recognized by the mouse monoclonal antibody used in this study but not by the rabbit antibody raised against a C-terminal peptide of β catenin. These observations confirm the presence of two caspase cleavage sites close to the C-terminus of β catenin, since the α catenin binding site is located close to the N-terminus. It is interesting to note, however, that there was a reduction in intensity of β-catenin cleavage products, compared to the full-length protein, suggesting that appreciable further cleavage occurred at later times resulting in the loss of β-catenin bound to α-catenin.

Re-localization of adherens junctions prior to caspase-mediated degradation

It is now well-established that there are marked changes in the cytoskeleton during apoptosis. It was our aim to see to what extent changes in localization of the adherens junctions might result from catenin-cadherin degradation or complex disruption. Ad2 E1A+N-*ras* HER 313A cells were grown on multiwell glass microscope slides and induced to apoptose with cisplatin. Cells were fixed, stained with appropriate antibodies raised against E- and P-cadherin and viewed by confocal microscopy. In viable cells E-cadherin can be seen primarily in adherens junctions around the cell periphery (Figure 6, upper left panel). By 3 h appreciable punctate staining was visible within the cytoplasm with disruption of staining around the plasma membrane (Figure 6 centre left-hand panel). Six hours after addition of cisplatin most of the E-cadherin (and presumably the adherens junctions) was present in the cytoplasm and had re-located away from the cell periphery. When cisplatin was added in the presence of the caspase inhibitor Z-VAD-FMK apoptosis was inhibited and there was little re-localization of E-cadherin from the cell membrane even after 10 h (Figure 6, centre right hand panel). At 10 h in the absence of caspase inhibitor, all of the E-cadherin was concentrated in the perinuclear region in the cells which remained reasonably intact and around the condensed chromatin in the apoptotic bodies (Figure 6, lower right hand panel). Similar micrographs to those shown in Figure 6 were obtained when cells were stained with the antibody against P-cadherin and the pan-cadherin antibody (data not shown). Staining with antibodies against α and β catenin showed similar re-localization of these proteins during apoptosis, therefore it seems reasonable to suppose that the pattern seen represents changes in intact adherens junctions rather than isolated P- and E-cadherin. By comparison of the data presented in Figure 6 with those shown in Figures 1, 4 and 5, it can be seen that re-localization of the adherens junctions in apoptotic cells precedes caspase-mediated cadherin and catenin degradation and disruption of cadherin/catenin interactions.

Discussion

It is now becoming clear that caspase-mediated degradation of proteins during apoptosis is commonplace. Substrates for caspases form a very heterogeneous group ranging from caspases themselves to cell cycle regulators such as pRb[12] and p21[14] to protein kinases as diverse as DNA PK[31] and FAK.[23] A further major target for caspases is proteins of the cytoskeleton. It has long been known that proteolysis of lamins[20,21] occurs during apoptosis leading to collapse of the nuclear lamina and probably contributing to condensation of the chromatin. In addition caspases cleave β catenin,[18] γ catenin,[19] keratins,[16,17] vimentin[16] and fodrin[32] as well as regulatory components such as gelsolin.[22]

In the study presented here we have shown that both E- and P-cadherin can be added to the list of cytoskeletal caspase substrates. Cleavage of these proteins occurs at about the same time as degradation of β and γ catenin (Figure 1), vimenin (data not shown) and PARP (Figure 3). Although there appears to be only slight reduction in level of either P- or E-cadherin up to six hours after addition of cisplatin, complete proteolysis occurs by 24 h with E-cadherin being degraded rather more rapidly (Figure 1). Because of the high levels of glycosylation of cadherins it is difficult to determine accurately by SDS–PAGE the size of the proteolytic fragments resulting from caspase activity. However, it seems likely that the observed site of cleavage of E-cadherin is at residue 479 which would give rise to a C-terminal fragment of theoretical molecular weight of 44.6 K, which would be recognized by the clone 36 antibody (raised against an immunogen comprising the C terminal intracellular domain). The 61 K band detected detected by the E-cadherin clone 36 antibody in Figure 1 (as well as other antibodies) is a cross-reacting, probably irrelevant, protein seen in many cell types even when E-cadherin is not expressed. The major P-cadherin cleavage product, of observed molecular weight 104 K, probably arises through loss of a C-terminal peptide. This suggestion is based on the observation that the cleavage product is not co-immunoprecipitated with catenins (Figure 5). Since the binding site on P- and E-cadherin for β and γ catenin is located very close to the C-terminus it seems reasonable to propose that cleavage at the site DTRD (amino acid 693–693, Table 1) is an initial step in degradation of P-cadherin at the same time disrupting binding to catenins. In addition to the degradation of P- and E-cadherin shown in Figure 1 other high molecular weight (135 K) cadherin components are expressed in Ad2 E1A+N-*ras* HER313A cells and have been shown to be degraded during apoptosis using a pan-cadherin antibody raised against the highly conserved C terminal domain of chicken N-cadherin.[28] A previous study has shown degradation of VE-cadherin during apoptosis of endothelial cells but this was attributed to the action of metalloproteinases rather than caspases.[29]

As confirmed by the Western blots presented in Figure 3, not all proteins are degraded by caspases at the same

rate after initiation of apoptosis. Thus, following addition of cisplatin there is an increase in p53 level (this is rather less marked in Ad2 E1A+N-*ras* HER313A cells than in many other cell lines we have studied [data not shown]). This is accompanied by an increase in level of the p53-regulated protein Mdm2 as has been noted previously.[33] There is no increase in p21 expression however as might be expected.[34] Reasons for this are not clear at present but could conceivably be due to the presence of the viral oncogene (AdE1A) or mutant *ras* gene. Levels of p21 are low in Ad2 E1A+N-*ras* HER313A cells (the protein shown in Figure 3A was only detected after prolonged exposure of the autoradiograph). This may be a contributory cause to the ease of induction of apoptosis in these cells since it has been suggested that very low levels of p21 favour an apoptotic response rather than cell cycle arrest.[35,36] It appears that if there is to be an apoptotic response by the cell (rather than p53-mediated cell-cycle arrest) degradation of cell cycle inhibitors, such as p21, occurs rapidly as observed here (Figure 3). At much later times degradation of the majority of cellular proteins by caspases occurs. The cadherins and β and γ (but not α) catenins are degraded at this time. Laddering of DNA is co-incident with this process at least under conditions of apoptosis induction described here.

The assocation of cadherins with catenins appears to be critical for cell-cell adhesion and cell-matrix interaction. With this in mind the ability of E- and P-cadherin to interact with α and β catenin during apoptosis was examined. It was shown that complexes were stable up to about 6 h with most remaining intact at 10 h (Figure 4A and B). By 16 h however little cadherin/catenin complex could be detected. From a comparison of the data in Figures 1 and 4 it seems likely that there is no particular targeting/disruption of the complexes prior to general degradation of the protein components. This might be considered somewhat surprising in view of the rapid changes in localization of the adherens junctions seen very soon after the onset of apoptosis and long before cadherin or catenin degradation is appreciable (compare Figures 1 and 6). Densitometric scanning of the Western blots from the co-immunoprecipitation studies suggests that the complexes between E-cadherin and β catenin were rather more stable than those containing α catenin (Figure 4). Whether this slight but reproducible difference has any significance will have to wait future investigations. Co-immunoprecipitation of cadherin with the antibody raised against α catenin depends on the presence of the ternary complexes α catenin/β catenin/cadherin or α catenin /γ catenin/cadherin whilst β catenin binds directly to cadherin. Despite the minor differences in Figure 4B it appears that catenins and P- and E-cadherin and the majority of the cadherin/catenin complexes are stable during the early stages of apoptosis in Ad2 E1A+N-*ras* HER313A cells.

Previous studies have suggested that the proteolytic fragment formed following caspase-mediated degradation of β catenin is unable to bind α catenin.[18] However, the data presented here indicate that β catenin is cleaved at two sites giving rise to large fragments which encompass the N-terminus and which bind α catenin. It seems likely that the difference between our observations (Figures 1 and 5) and those to Brancolini *et al*.[18] may be attributed to different cell type and/or different method of inducing apoptosis.

The photomicrographs shown in Figure 6 clearly demonstrate that the initial changes in localization of E-cadherin occur several hours before protein degradation is apparent. Based on staining of α, β and γ catenin[19] it is clear that distribution of E-cadherin shown in Figure 6 serves as an accurate marker for intact adherens junctions. Therefore it is likely that changes in their distribution during the onset of apoptosis (for example at 3 and 6 h time points shown in Figure 6) are not attributable to caspase-mediated protein degradation or disruption of catenin/cadherin complexes. Presumably other mechanisms triggered by apoptotic stimuli but not requiring widespread caspase activity directed against the adherens junction proteins may be responsible for changes in the cytoskeleton. Indeed evidence to support this contention has been published.[37] In that study it was shown that caspase 3 activity is not required for membrane blebbing in Rat-1 fibroblasts.[37] However, in the system described here very few morphological changes were seen in the presence of caspase inhibitor Z-VAD-FMK (Figure 6). This is consistent with data obtained from studies of apoptotic endothelial cells where caspase inhibitors effectively inhibited membrane blebbing and protein degradation.[38] Interestingly Ad2 E1A+N-*ras* HER313A cells treated with cisplatin for 3 h were essentially indistinguishable from viable cells by phase contrast microscopy (data not shown). This implies that the initial re-localization of the adherens junction proteins (seen at 3 h) has relatively few effects on the overall morphology of the cell. However, at 6 h there are gross changes in localization of the adherens junction proteins (Figure 6) and cell shape (data not shown). Even so it is not until 10 h that cadherins and catenins begin to be cleaved.

We conclude, therefore that the observed cytoskeletal changes are not due to adherens junction degradation but to the action of caspases directed against other substrates. Significantly it has recently been shown that caspase-mediated cleavage of FAK precedes cell detachment and substantial membrane blebbing during apoptosis in endothelial cells.[38] Degradation of FAK resulted in loss of focal adhesions, loss of binding to paxillin and probably loss of enzymic activity.[38] Whether degradation of focal adhesion proteins is a first step to cytoskeletal disassembly in many cell types will have to wait further investigation, but in view of the evidence presented up to now it appears a likely candidate.

Materials and Methods

Cell lines

Ad2 E1A+N-*ras* HER313A cells were produced by transfection of Ad2E1A together with mutant N-*ras* DNA into human embroyo retinoblasts (HER).[39] Cells were grown in Hepes-buffered Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% foetal calf

serum (FCS) and 2 mM glutamine. SW-13 cells derive from a human adrenal cortex carcinoma[40] and were grown in Leibovitz medium supplemented with 8% FCS and 2 mM glutamine.

Apoptosis was induced by the addition of cisplatin (16 μg/ml in the tissue culture medium). Apoptosis was inhibited by the addition of caspase inhibitors to tissue culture medium, added to a final concentration of 40 μM immediately prior to the addition of cisplatin. Caspase inhibitors (Z-DEVD-FMK, Z-VAD-FMK, Z-YVAD-FMK and Boc-Asp-FMK) were purchased from Enzyme Systems Products and dissolved in DMSO prior to use.

Detection of apoptosis

Acridine orange staining To determine the rate of apoptosis, cell suspensions were mixed 1 : 1 with acridine orange (10 μg/ml) and then viewed by fluorescence microscopy after 5 min. The number of apoptotic cells in a sample of at least 200 cells was counted.

DNA fragmentation Ad2 E1A+N-*ras* HER 313A cells were treated with cisplatin (16 μg/ml), harvested by aspiration and pelleted by centrifugation (3000 r.p.m. at 4°C for 5 min). 10^6 cells were resuspended in 10 mM EDTA, 50 mM Tris, 0.5% N-lauroylsarcosine (Sigma), 0.5 mg/ml proteinase-K by vortex mixing and incubated for 1 h at 50°C. After adding RNase, samples were incubated again for 1 h at 50°C and then fractionated on a 2% agarose gel. Gels were stained with ethidium bromide (0.5 μg/ml) for 15 min, destained with H_2O for 30 min and viewed under UV light.

Western blotting and immunoprecipitation

For Western blotting studies cells were harvested, washed in cold saline and solubilized in 9 M urea, 50 mM Tris HCl pH 7.4, 0.15 M β-mercaptoethanol. Proteins were fractionated by polyacrylamide gel electrophoresis (PAGE) in the presence of SDS and then electrophoretically transferred to nitro-cellulose membranes. Antigens were detected using appropriate antibodies (see below) and visualised by ECL (Amersham).

Immunoprecipitation was essentially as described.[41] Briefly, cells were harvested, washed in cold saline and solubilized in 0.825 M NaCl, 50 mM Tris HCl pH 7.4, 1% NP40. Lysates were clarified by ultra-centrifugation at 35 K for 30 min and insoluble material discarded. Solutions were precleared by incubation with Sepharose 4B on a rotator for 1 h. Appropriate antibodies (see below) were added for a further 1 h and antibody-antigen complexes collected on protein G-agarose (Sigma) for 1 h on a rotator. After washing, immunoprecipitated proteins were fractionated by SDS–PAGE and then subjected to Western blotting as described above.

Immunofluorescence studies

Ad2 E1A+N-*ras* HER 313A were grown on glass multiwell slides and were induced to apoptosis by the addition of cisplatin (16 μg/ml). At the appropriate times cells were fixed in paraformaldehyde (4% in PBS) and permeabilized with acetone at −20°C for 10 min. Fixed cells were stored at −20°C. For staining, cells were thawed to room temperature and non-specific binding sites were blocked in blocking buffer (20% v/v heat inactivated goat serum, 0.1% BSA w/v and 0.1% sodium azide in PBS) for 30 min. Cells were immunostained by diluting the appropriate antibody in the blocking buffer and incubating for 90 min in a humid box at 37°C. The antibody was removed by washing the slides twice in PBS for 15 min. FITC-labelled anti-species antibody was diluted into blocking buffer before incubating for 60 min at 37°C. Again the antibody was removed by washing the slides twice in PBS for 15 min. Nuclei were visualized by DAPI (0.1 μM) staining. The slides were mounted in the presence of DABCO (2%, 1,4 diazobicyclo-[2.2.2] octane in 80% glycerol/PBS). Cells were viewed by confocal microscopy using a Zeiss Axiphot fluorescence microscope. Images were recorded with the Biovision software package (Impro Vision) and a low-light-level video camera. The two channels were recorded independently and pseudo-colour images generated and superimposed with Adobe Photoshop 3.0 software.

Antibodies

Mouse monoclonal antibodies against E- and P-cadherin were from Transduction Laboratories and were diluted 1/1000 and 1/200 respectively in Western blotting studies. The same antibodies were diluted 1/20 for immunofluorescence. The antibodies raised against E-cadherin (clones 34 and 36) recognize an epitope in the C-terminal cytoplasmic domain (amino acids 735–883). The antibody against P-cadherin (clone 56) recognizes an epitope towards the N-terminus of the proteins (between amino acids 72 and 259). A pan-cadherin antibody was from Sigma and was raised against a highly conserved C-terminal region of chicken N-cadherin.[28] It was diluted 1/20000 for Western blotting studies and 1/50 for immunofluorescence. Additional antibodies against E-cadherin were obtained from Zymed (clone ECCD-1 and clone SHE78-7) and against P-cadherin from Zymed (clone NCC-CAD-299). Antibodies against α, β and γ catenin were from Sigma and were diluted 1/4000 for Western blotting studies. A rabbit antibody which recognises α catenin was raised against a synthetic peptide equivalent to amino acids 890–901 of the human protein (the C-terminal 11 residues). Antibodies against β catenin were raised either in rabbit or mice. The rabbit antibody was against a synthetic peptide equivalent to amino acids 768–781 of the human protein (the C-terminal 11 residues). The mouse monoclonal (clone 15B8) was raised against chicken β catenin.[42] A mouse monoclonal (clone 15F11) raised against chicken γ catenin was also used.[42] Five μl of each antibody was used for each immunoprecipitation reaction. p53 was identified using a rabbit polyclonal antibody (CM1, a generous gift from Professor David Lane, University of Dundee), Mdm2 using a mouse monoclonal (2A10, a generous gift from Professor Arnold Levine, Princeton), p21 using a rabbit polyclonal antibody (Santa Cruz) and PARP using a rabbit polyclonal antibody (Boehringer Mannheim). FITC-labelled anti-mouse IgG was from Sigma and was diluted 1/30 for immunofluorescence studies.

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