

Apoptosis Induced by Inhibition of Intercellular Contact

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Abstract. The LIM 1863 colon carcinoma cell line grows as structural organoids of goblet and columnar cells around a central lumen and provides a model for the development of stem cells in the normal colon. The organoid structure can be disrupted by removal of calcium from the medium, resulting in a suspension of single cells. Upon readdition of calcium, the cells reform the organoid structure over a period of 24 h, and ultrastructural examination of the reforming cells reveals that this involves a complex process that we have termed clutching. To determine the adhesion molecules involved in organoid formation we attempted to block this process by single cell suspensions of LIM 1863 reseeded in the presence of monoclonal antibodies. An anti-integrin antibody directed against a conforma-

tional epitope on the αv subunit totally inhibited organoid reformation. As a consequence of this inhibition of cell contact the colon carcinoma cells rapidly underwent apoptosis. Investigations of the apoptotic pathway involved suggested an induction mechanism since the onset of apoptosis in the contact-inhibited cells showed specific increased synthesis of 68- and 72-kD proteins. In addition, immunoblotting of cytosolic and nuclear extracts of the cells revealed the rapid translocation of the tumor suppressor gene product, p53 to the cell nucleus upon induction of apoptosis. These results suggest that cell-cell adhesion may be a vital regulator of colon development overcome in tumor cells by loss of adhesion molecules or of functional p53 protein.

ONE of the problems in developing better strategies for the control of colon cancer is that little is known about the biology of the colonic mucosa, or the factors that regulate its proliferation and differentiation. Normal colonic crypts fail to grow long term in tissue culture after removal from the colon, and, in consequence, *in vitro* models are largely restricted to cell lines derived from colon carcinomas. An interesting feature of such cell lines is that a number of tumors have retained both phenotypic and morphologic markers of the mucosa, such as the expression of brush border enzymes and glandular formation (Namba et al., 1983; Pinto et al., 1983). The cell line used in this study, LIM 1863, shows a further degree of specialization, in retaining many of the structural and functional properties of the colonic crypt (Whitehead et al., 1987; Hayward and Whitehead, 1992).

In the small intestine a single layer of epithelial cells lines the crypts of Lieberkuhn and covers the villi (Lipkin, 1987). Several morphological cell types have been identified. The epithelium of the villus consists mainly of absorptive columnar cells (which express brush borders for an amplification of surface area of absorption) and of goblet cells which se-

crete mucus (Strand, 1983). The goblet cells respond to a variety of stimuli, such as specific neurotransmitters, by emptying their contents into the lumen (Hubel, 1989). Within the crypts, the most abundant cells are the proliferative undifferentiated crypt cells. Daughter cells migrate up the wall of the crypt where they begin differentiating until their structural features resemble those of the absorptive columnar cells on the sides of the villi. The undifferentiated cells at the crypt base, therefore, serve as precursors for the columnar cells, and there is convincing evidence that they also serve as precursors for other differentiated intestinal epithelial cells, including the goblet cells, caveolated and Paneth cells (Madara and Trier, 1987). In terms of tissue architecture, intestinal epithelia are polarized so that the basal lamina is on the side of the cell which faces the blood capillaries, while the secretory/absorptive surface and junctional complexes are at the apical or luminal end of the cell (Steward and Case, 1989).

The LIM 1863 cell line is derived from a colon carcinoma and grows as organized spheroids of cells ("organoids"), showing structural and functional specialization. The cells are arranged around a central lumen, with polarization of nuclei to the periphery. At least two morphological cell types are present—columnar cells complete with brush border, and goblet cells which secrete mucus into the lumen (Whitehead et al., 1987). In addition, non-viable single cells

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are continuously shed from the organoid, in a manner analogous to the shedding of terminally differentiated cells in the intestine. Hence, similarities can be observed with the normal colonic epithelium. However, while differentiated phenotypes become more prominent as cells move toward the apical surface in the normal crypts, Hayward and Whitehead (1992) have shown that the differentiated cell types in this tumor line can still be proliferative or in fact be precursor cells for further culture. The functional differentiation of LIM 1863 suggests the presence of precursor or stem cells, which can differentiate along the columnar or goblet pathways, and that the cell which gave rise to the LIM 1863 tumor was a committed progenitor cell (Hayward and Whitehead, 1992).

The influence of cell-cell and cell-matrix interactions upon proliferation and differentiation are clearly determining events in development, and this is likely to be the case in a dynamic adult organ like the colon where rapid proliferation and differentiation are coupled with cell migration. Escape from such controls of differentiation through loss of function and/or synthesis of cell adhesion molecules may contribute to the uncontrolled pattern of growth typical of malignant neoplasia (Pignatelli and Bodmer, 1990). Because of the highly structured organization of cells around a central lumen, the LIM 1863 cell line provides a unique opportunity to investigate cellular adhesion mechanisms involved in the maintenance of the novel organoid structure.

In the present study investigations were carried out to identify the molecular basis for this specialized arrangement of cells using antibody inhibition studies. Our results implicate a role for an intercellular adhesion molecule in the survival of this colon carcinoma line. We found that if we inhibited organoid formation with antibody, the cells underwent rapid apoptosis, or programmed cell death. This distinctive form of cell death—seen under a wide variety of both physiological and pathological conditions—differs markedly from necrosis on morphological, biochemical, and conceptual grounds (Cotter et al., 1990). Recent findings in thymocytes (Lowe et al., 1993; Clarke et al., 1993) have shown that there are at least two distinct apoptotic pathways, in one of which the tumor suppressor gene product p53 has been implicated as a mediator (for review see Lane, 1993), and our data implicate p53 in the contact-regulated apoptosis of the LIM 1863 cells.

Since the colon is rapidly dividing throughout life, it can be suggested that the LIM 1863 cells are representative of normal colonic crypt cells and that cell-cell attachment may be an important controlling factor in the regulation of apoptosis in these epithelial cells. In addition, these findings may have profound biological implications for an understanding of homeostatic and anti-tumor control mechanisms within the normal colonic mucosa.

Materials and Methods

Cell Culture

LIM 1863 cells (Whitehead et al., 1987) were routinely grown in RPMI 1640 (Cytosystems, Sydney, Australia) supplemented with 5% FCS (Cytosystems, Sydney, Australia) at 37°C in a 10% CO₂ atmosphere. Low calcium growth conditions were performed in a modified calcium-free RPMI 1640 medium (Cytosystems, Sydney, Australia) plus 1% FCS. This medium contains approximately 35 μM calcium derived from the FCS (Peek et al., 1989). Intact LIM 1863 organoids were disaggregated to a sin-

gle cell suspension by culturing in this calcium-depleted medium for at least 2 d, and then passage through a 19 gauge needle to completely separate loose clusters.

Organoid Reformation Assay

Cells were cultured under low calcium conditions until the organoid structure was lost and a single cell suspension produced as noted above. The cells were pelleted at 1,500 rpm for 5 min, and the supernatant discarded. The pellets were resuspended in complete RPMI 1640 plus 5% FCS before being aliquoted into a 96-well microtitre plate at a final density of 2×10^5 cells/well. The cells were cultured at 37°C in 10% CO₂. Antibodies were initially tested at a final concentration of 100 μg/ml, sodium azide used at a concentration of 0.2%.

Antibodies

The murine monoclonal antibodies PIF6 (anti-αvβ5) and R6G9 (anti-αvβ6) antibodies were kindly provided by Dr. Dean Sheppard (UCSF, San Francisco, CA). Dr. Michael Berndt (Baker Institute, Melbourne, Australia) provided the AK2 (anti-Ib) used as an isotype-matched control for 23C6. The antibodies 13C2 (anti-αv) and 23C6 (anti-αvβ3) were purified from ascites in our laboratories. The specificity of 23C6 has been reported and shown previously (Davies et al., 1989; Nesbitt et al., 1989; Bates et al., 1991). The anti-p53 monoclonal (PAb1801) was purchased from Oncogene Science Inc. (Uniondale, NY) and the monoclonal anti-α tubulin (clone DM1A) from Sigma Chem. Co. (St. Louis, MO). The anti-HMG-I/Y IgG was obtained from Dr. Raymond Reeves, Washington State University (Pullman, WA). Fab fragments of 23C6 were produced by papain digestion of purified antibody, isolation over protein A and visualized by SDS-PAGE and silver staining.

Confocal Microscopy

LIM 1863 organoids were fixed in 4% formaldehyde, and then incubated with the mAb 23C6 or an isotype-matched control (AK2) for 1 h, at a final concentration of 20 μg/ml. For the single cells, cells were reseeded into the reformation assay with either 23C6 mAb or AK2 for 2 h, and then fixed in 4% formaldehyde. A FITC-conjugated anti-mouse secondary antibody was added for 1 h, and the cells dried onto microscope slides. Images were obtained using a MRC-600 confocal system (Bio-Rad Labs., Richmond, CA) coupled to an Inverted microscope (Zeiss Axiovert, West Germany). Cells were observed through a coverslip with an oil immersion lens (magnification 40×, numerical aperture 1.3) and the confocal settings were maintained the same for all samples. Single optical sections were photographed.

Cell Labeling, Immunoprecipitation, and SDS-PAGE Analysis

Cells were labeled at the cell surface by lactoperoxidase-catalyzed iodination essentially as described previously (Bates et al., 1991) before lysis in an octylglucoside lysis buffer (100 mM I-O-n-octyl-b-D-glucopyranoside, 10 mM Tris, 150 mM sodium chloride, 2 mM phenylmethyl-sulfonyl fluoride, 20 mM imidazole, 50 mM iodoacetamide and 50 μg/ml soybean trypsin inhibitor) for 1 h. Lysates were centrifuged at 10,000 g for 10 min. For immunoprecipitations, the lysates were precleared with rabbit anti-mouse immunoglobulins (RAM)¹ (Dako, Glostrup, Denmark) coupled to Sepharose 4B beads (Pharmacia, Uppsala, Sweden) for a minimum of 2 h. Immunoprecipitations were carried out using antibodies directly coupled to Sepharose 4B, or indirectly using RAM-Sepharose 4B, and analyzed by 7.5% SDS-PAGE.

Biosynthetically labeled whole lysates were analyzed by 7.5% SDS-PAGE in one dimension. In addition, lysates were analyzed by 2-dimensional gel electrophoresis, with isoelectric focusing (IEF) in the first dimension and 7.5% SDS-PAGE in the second, as described (Celis and Bravo, 1984).

Nuclear Isolation and Extraction

After individual treatments, cells were washed in PBS and then lysed on ice in the presence of 1% NP-40 in run-on storage buffer (ROB; 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂). The lysate was layered

1. *Abbreviations used in this paper:* RAM, rabbit anti-mouse immunoglobulin.

over 30% sucrose in ROB, and centrifuged at 350 g for 5 min at room temperature. The nuclear pellet was resuspended in 100 μ l of ROB containing 200 U of DNase I (Bresatec, Adelaide, South Australia) and digested for 1 h at 37°C. After DNase treatment, 50 μ l of sample buffer containing 2.3% SDS was added to each sample, before SDS-PAGE.

Polymerase Chain Reaction

RNA was prepared by guanidinium thiocyanate extraction. Reverse transcription was performed in a 50- μ l reaction volume for 2 h at 42°C. Reverse transcription mixture consisted of 1 μ g RNA, 50 mM Tris-HCl pH 8.3, 75 mM potassium chloride, 10 mM dithiothreitol, 3 mM magnesium chloride, 500 μ M each dATP, dTTP, dCTP, dGTP (dNTPs) (Pharmacia, Uppsala, Sweden), 1 μ l oligo dT (0.2 μ g/ml) and 10 U of reverse transcriptase (Pharmacia, Uppsala, Sweden). Upon completion of the reaction the cDNA was phenol/chloroform extracted once, and 5- μ l aliquots used in the PCR reaction.

PCR was carried out in 100 μ l vol. The cycle protocol was as follows: cycles 1–3: 96°C for 2 min, 50°C for 1 min, and 74°C for 1 min; cycles 4–40: 96°C for 1 min, 50°C for 1 min and 74°C for 1 min. The reaction mixture consisted of 10 mM Tris-HCl pH 8.3, 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.01% gelatin, 5 μ l cDNA, 2 μ l of 10 mM dNTPs, 1 μ l of each oligonucleotide (concentration between 0.5–1.0 μ g/ml), and 0.5 μ l of Amplitaq polymerase (Cetus Corp., Emeryville, CA). On completion of the PCR reaction, products were examined on a 6.5% acrylamide gel.

The primers used were all 24-mers selected to encompass the integrin transmembrane and cytoplasmic domains to the stop codon. An actin probe was used as a positive control. The primers used were as follows:

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 $\alpha$ v 5' CACATTGGTTACCACTAATGTCAC 3'
      GACGTCAAAAATCAATACGATGT (antisense)
 $\beta$ 1 5' AATGGGAACAACGAGGTCATGGTT 3'
      CATAACGGGCACGTTTAGGGTGT (antisense)
 $\beta$ 3 5' TGCTCATTTGGCCTTGCCGCCCTGC 3'
      ACTATTCGTCAGTAGGAGTCTAGT (antisense)
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Western Blotting

For cytoplasmic proteins, cells were lysed in 100 mM octylglucoside lysis buffer, as described above. After classification by centrifugation (to remove nuclear material), whole cell lysates were resolved under 7.5% SDS-PAGE. The nuclear extracts were resolved under reducing SDS-PAGE on a 7.5–15% gradient gel. Proteins were then transferred to nitrocellulose (Gelman Sciences, Ann Arbor, MI) by electrophoresis for 3 h at 40 V in transfer buffer (20 mM Tris, 150 mM glycine, 20% methanol, 0.1% SDS). Residual protein sites were blocked overnight at 4°C in TTBS (25 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween) containing 5% skim milk. The nitrocellulose was incubated in TTBS with either the anti-p53 mAb PAb1801 (Oncogene Science, Inc. Uniondale, NY) at a final concentration of 1 μ g/ml, or an isotype-matched control mAb (AK2) at the same final concentration for 1 h. Blots were developed using enhanced chemical luminescence (ECL) (Amersham, UK).

Transmission Electron Microscopy

Intact organoids, single cells, and single cells allowed to reform in the presence or absence of 23C6 antibody were harvested and washed in PBS. After washing, the cells were fixed in Karnovsky's fixative overnight at 4°C. The cells were rinsed in PBS, postfixed with 1% osmium tetroxide for 4 h at room temperature, and then dehydrated in a sequential ethanol series. The cells were infiltrated and embedded in Spurr's resin and sectioned using a Reichert-Jung Ultracut E microtome (West Germany) to a final thickness of 80 nm. Sections were double stained with uranyl acetate and lead citrate, and examined with a Philips CM 10 transmission electron microscope (Philips Electronic Instruments, Mahwah, NJ) at an acceleration voltage of 80 kV.

Apoptosis and Analysis of DNA Degradation

To assess apoptosis under low power microscopy, HOECHST 33258 stain (Aldrich Chem. Co., Milwaukee, WI) was used. Briefly, cells from different treatments were cytospun onto glass slides, followed by a mild hypotonic treatment (1:1 ratio distilled water/growth medium) for 10 min. The cells were prefixed for 5 min with a 50% solution of fixative (3:1 methanol/acetic acid), and then fixed with neat fixative for 10 min. The preparations were

stained with HOECHST 33258 (0.1 μ g/ml) for 10 min, rinsed, and dried. Preparations were examined using immersion objectives, UG-1 excitation filter and a UV-absorbing barrier filter. Condensed and fragmented nuclei, typical of apoptosis, were easily distinguishable from intact nuclei and percentages were calculated by counting. Three random fields of view were observed at each time point, with a minimum number of 200 cells scored in each field.

For DNA degradation analysis, LIM 1863 cells were cultured under calcium-depleted conditions for 3 d before physically disaggregating to a single cell suspension. Cells (3×10^7) were harvested and split into three groups for short term culture. Control cells were resuspended in 3 ml of calcium-depleted medium in a 6-well tissue culture plate. One-third of the cells were resuspended in 3 ml of complete RPMI 1640 plus 5% FCS (with calcium) while the remaining 10^7 cells were resuspended in complete RPMI 1640 plus 5% FCS with the addition of the mAb 23C6 or Fab fragments at a final concentration of 100 μ g/ml. The cells were then cultured for 18 h at 37°C. The method employed for the analysis of DNA degradation was identical to that described by Neiman et al. (1991). Aliquots (40 μ l volume) of each of the three digests were analyzed by electrophoresis in a mixed 0.7% Seakem/1.1% NuSeive agarose gel (Edwards Scientific, Sydney, Australia). The DNA was resolved by ethidium bromide staining and observation under ultraviolet light.

Results

LIM 1863 Colon Carcinoma Cells Grow as Discrete, Structured Organoids

The colon carcinoma cell line LIM 1863 has been described (Whitehead et al., 1987) as exhibiting many of the structural and functional properties associated with the normal colonic mucosa. In culture, the cell line grows as discrete spheroids of cells or organoids in suspension (Fig. 1 A), and single cells shed from the organoids are clearly non-viable. Electron microscopy reveals a well differentiated structure (Fig. 1 B). The cells are arranged around a central lumen, and exhibit a characteristic brush border and tight junctional complexes. The nuclei are polarized to the basal surface and both goblet (secretory) and columnar (absorptive) cells can be identified. Whitehead et al. (1987) report that these morphological cell types are also functionally mature and express typical brush border enzymes.

After Disruption the Organoid Structure Is Rapidly Reestablished by a Distinctive Clutching Process

The organoid structure of LIM 1863 is particularly resistant to disaggregation. However, this structure is lost after culture in low-calcium medium (Hayward and Whitehead, 1992) and the resulting loose clusters of cells can be mechanically disrupted so as to give rise to a viable single cell suspension, as outlined in Materials and Methods. When these single cells (Fig. 2 A) were subsequently reseeded into tissue culture medium containing calcium, a rapid intercellular adhesion event occurred, such that an organoid structure was reestablished within 24 h. Ultrastructural examination showed that this event was not merely random agglutination between adjacent cells. In the early stages of cell-cell contact, a previously unreported mechanism was observed, whereby one enterocyte would apparently engulf a neighboring cell with pseudopod-like cytoplasmic extensions. This novel form of attachment, termed "clutching," occurred in all cases when two single cells made contact, and did not affect the viability of the encapsulated cell, as evidenced by its well preserved nucleus, membrane, mitochondrial cristae, and other organelles (Fig. 2 B). These doublets of cells then

Cell-Cell Contact Is Required for Cell Survival

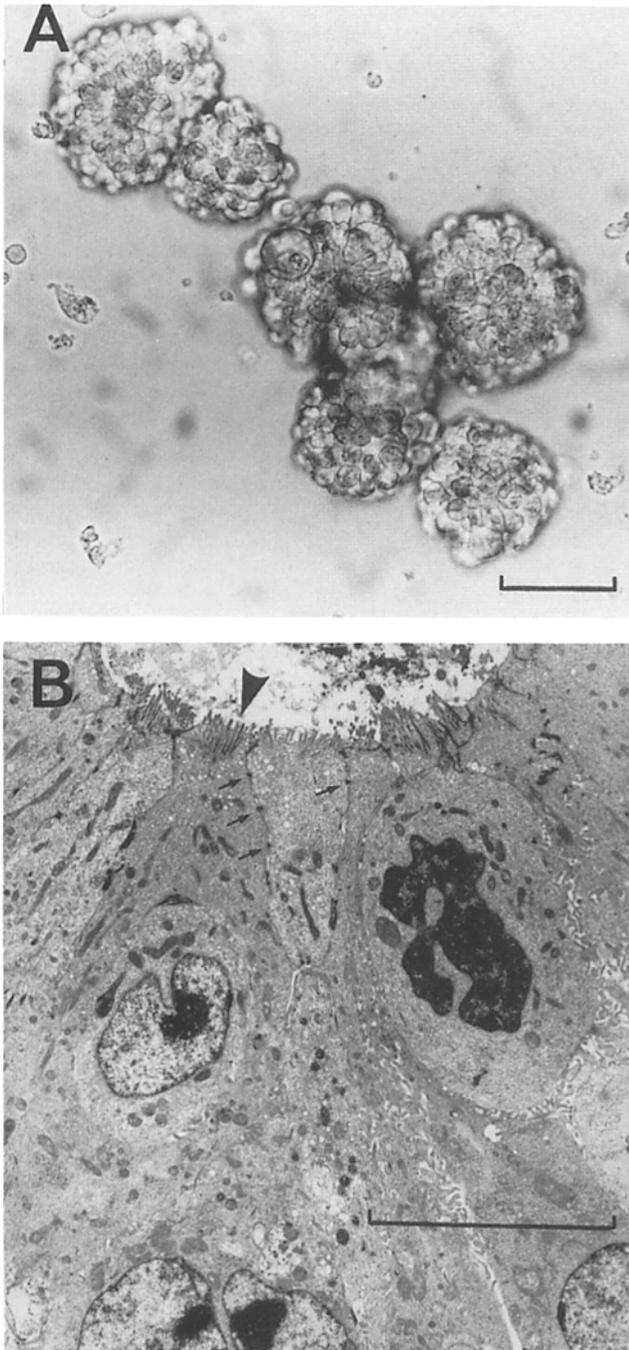


Figure 1. Morphology of LIM 1863 organoids. (A) Low magnification showing spheroid arrangement of cells in suspension. Bar, 250 μm . (B) Ultrastructural cross-sectional examination through organoid, displaying an arrangement of cells analogous to the intestinal epithelium. Note the microvilli of the luminal brush border (arrowhead) and desmosomes of the apical tight junctions (arrows). Bar, 10 μm .

provided foci for further cell attachment, and intercellular adhesion continued with a concentric appearance (Fig. 2 C). These data suggest that this form of reattachment is an active process, with a high energy expenditure for the cells involved, and indeed the addition of sodium azide (0.2%) inhibited organoid reformation.

The reformation event described above was adapted to microtiter plates to provide the basis of our assay system to analyze the molecules involved in the formation of the unique organoid structure. We observed that LIM 1863 cells failing to incorporate into the organoid structures did not survive. In addition, single cells reseeded at low density in the presence of calcium were unable to establish contacts and undergo organoid formation, and subsequently died.

In an attempt to identify the molecular basis of this mechanism, some 30 monoclonal and polyclonal antibodies were tested for their capacity to inhibit organoid formation. Of these, the antibody found consistently to inhibit reformation was the monoclonal 23C6, directed against a conformation-dependent epitope on the integrin αv subunit (Nesbitt et al., 1989), which totally inhibited cell-cell adhesion at a final concentration of 100 $\mu\text{g/ml}$ (Fig. 3), in either the presence or absence of FCS. The 23C6 antibody immunoprecipitates the $\alpha\text{v}\beta\text{3}$ complex from several cell types (Davies et al., 1989; Bates et al., 1991) although the 23C6 epitope on αv can be exposed when this subunit is in association with β subunits other than β3 , consequent upon ligand-induced conformational changes (Agrez et al., 1991). We failed to immunoprecipitate any specific bands from labeled LIM 1863 cells using the 23C6 antibody. Immunoprecipitation and PCR failed to detect expression of the integrin β3 subunit in these cells (Fig. 4 A) and the αv integrin was found to only associate with the β5 and β6 subunits (Fig. 4 B). From these results we postulate that αv integrins modulate cell adhesion-dependent organoid formation, but the avidity of 23C6 for αv associated with these other subunits is insufficient to enable immunoprecipitation.

To lessen the possibility that the binding of 23C6 was not sterically blocking the activity of another molecule involved in adhesion, Fab fragments were prepared and tested. The activity of the Fab fragments was identical to that of the intact antibody in showing total inhibition of organoid reformation at 100 $\mu\text{g/ml}$, indicating that the epitope recognized by 23C6 was intimately associated with the reformation event. In contrast to its inhibitory function in the assay, the addition of 23C6 had no effect on the structure of intact LIM 1863 organoids, nor another control colon cell line SW480 which grows as a monolayer (data not shown). Similarly, the addition of 23C6 to the microassay was ineffective in causing disruption after organoid reformation was well underway (see below).

To substantiate the role of the antigen bound by 23C6 in organoid reformation we performed immunofluorescent staining of LIM 1863 cells. In Fig. 5 A is shown the pattern displayed by intact organoids when stained by 23C6. The antigen appeared to be distributed primarily around the membrane of the cells, localizing to cell contacts. When single cells were allowed to bind 23C6 and organoid formation was blocked by the antibody, subsequent staining with a FITC-labeled secondary antibody revealed that the antigen was clustered into patches on the surface of the individual cells. This is illustrated in Fig. 5 B where single optical sections taken through an individual cell at 0.5- μm increments show the antigen to be patched on the cell membrane.

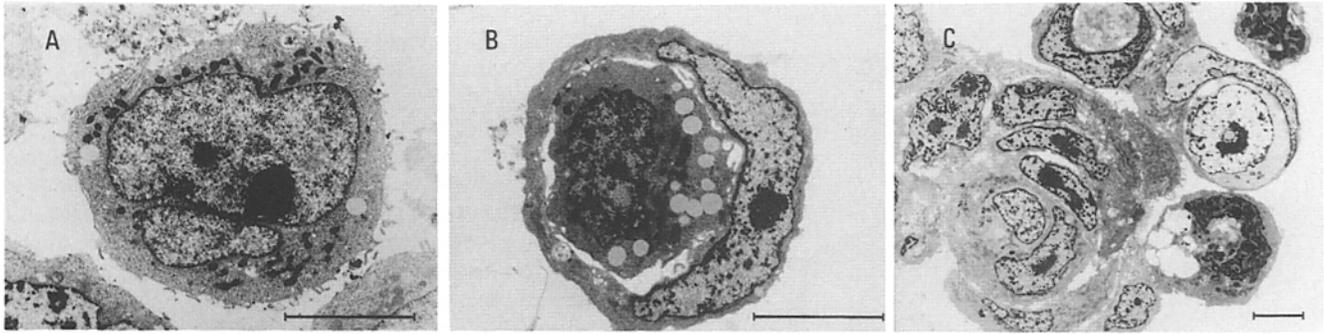


Figure 2. Reformation of organoids. Intact LIM 1863 organoids were disaggregated in low calcium (<math><35 \mu\text{M}</math>) medium to a single cell suspension (A) and allowed to reform organoid structures in RPMI 1640 (420 μM calcium) supplemented with 5% FCS. At various time intervals, samples were fixed and prepared for electron microscopy. (B) Clutching—initial adhesion between adjacent cells involved the engulfment of one cell by another—this form of attachment, whereby one cell appears to extend processes around its neighbor, we have termed clutching. This phenomenon was observed in all cases where two cells were found in contact. The cell being encapsulated remained viable, as evidenced by its well preserved nucleus, membrane, and organelles. (C) Successive cell attachment then appears to proceed in a concentric manner, where the clutching doublets of cells appeared to act as foci for subsequent cell adhesion. The concentric appearance results from the activity of the cells attaching to the doublets also extending processes to establish close proximal contacts. Bar represents 5 μm in all micrographs.

The Mechanism of Contact-inhibited Death Is Apoptosis

Based on our observations that LIM 1863 cells failing to reform organoid structures also failed to survive, we tested the possibility that these cells were undergoing apoptosis. Changes in nuclear morphology revealed by HOECHST staining provided a means to quantitate apoptosis occurring in the microassay. As can be seen in Fig. 6 A, control cells reseeded into calcium-depleted medium showed no increase

in the condensed or fragmented nuclei characteristic of apoptotic cells. This perhaps indicates that apoptosis in this system is dependent on calcium ions. Cells allowed to reform over the same time period were unsuitable for analysis by this technique because of the organoid structures but they do remain viable and continue to grow well in tissue culture. However, the inhibition of reformation by 23C6 lead to a time-dependent increase in the percentage of apoptotic cells. The kinetics of the process indicated a rapid induction from

Control

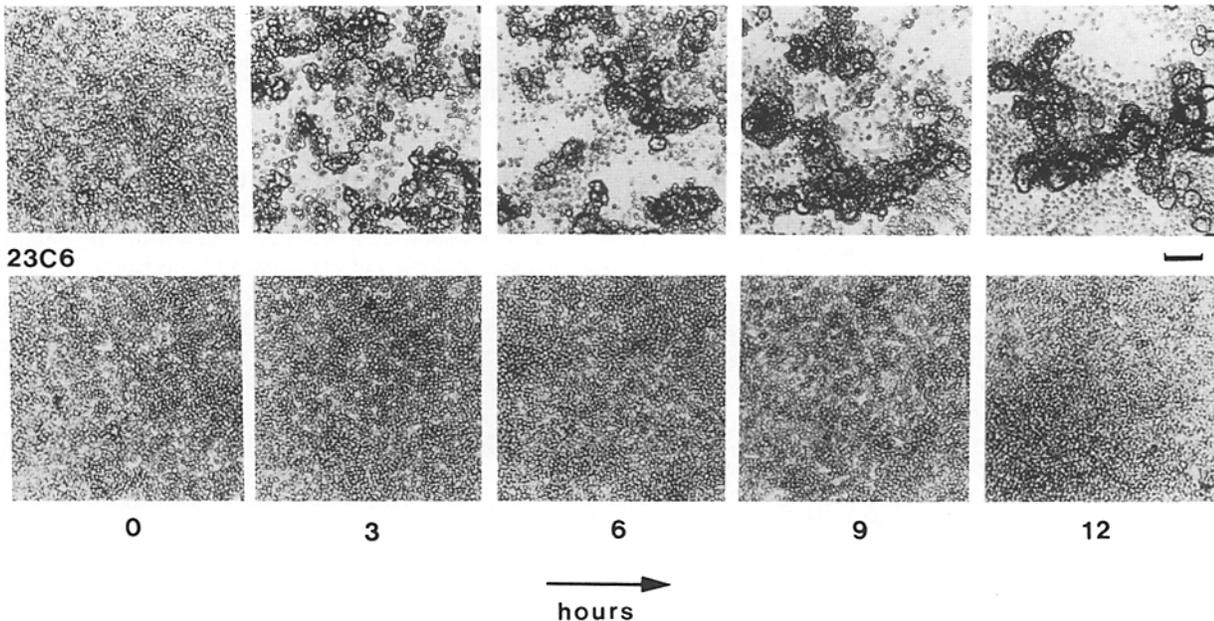


Figure 3. Antibody-mediated inhibition of organoid reformation. Single cell suspensions of LIM 1863 cells were reseeded in normal calcium medium in the absence (upper panel) or presence of the mAb 23C6 (lower panel) at a final concentration of 100 $\mu\text{g}/\text{ml}$. The addition of Fab fragments of this antibody under identical conditions yielded the same result. Experiments under serum-free conditions showed identical effects. Bar, 100 μm .

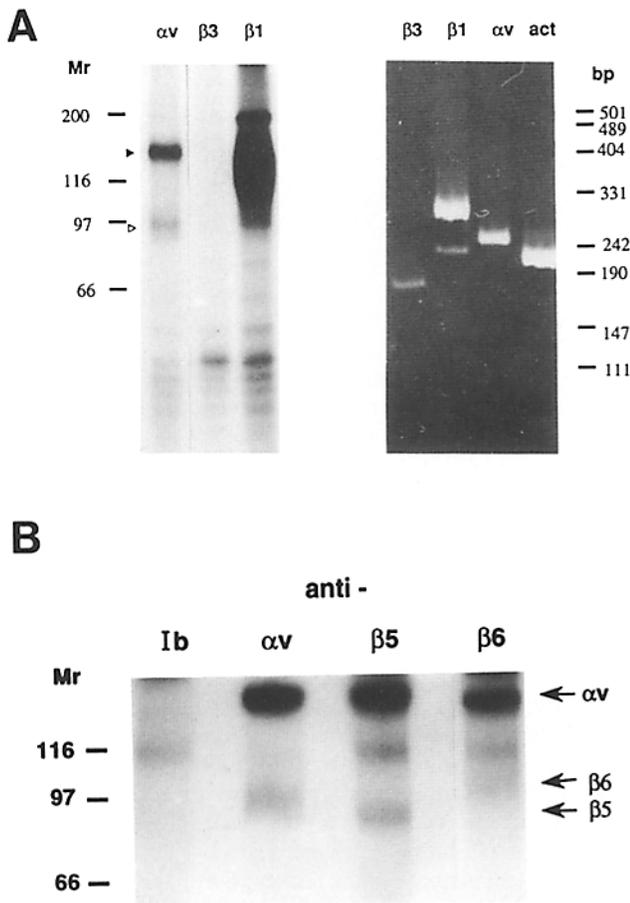


Figure 4. LIM 1863 cells do not express $\alpha v\beta 3$, but display the $\beta 5$ and $\beta 6$ integrin subunits in association with αv on the cell surface. (A) (Left) Autoradiography of materials immunoprecipitated with anti-integrin antibodies directed to the αv , $\beta 3$, and $\beta 1$ subunits from lysates of surface iodinated LIM 1863 cells, run under non-reducing 7.5% SDS-PAGE. LIM 1863 expressed the αv subunit (closed arrowhead) in association with a broad 95-kD β subunit band (open arrowhead). Immunoprecipitation with the anti- $\beta 3$ mAb failed to detect surface protein, while $\beta 1$ integrin was identified in association with at least one α subunit. Irrelevant control antibodies failed to detect surface proteins (not shown). Relative molecular masses (*Mr*) are shown to the left in kD. (Right) The PCR was used to amplify cDNA from mRNA extracted from LIM 1863 organoids. LIM 1863 organoids were positive for both αv and $\beta 1$ mRNA, and negative for $\beta 3$ —reflecting the surface expression of these glycoproteins. Actin (*act*) was included as a positive control. Molecular weight markers are shown to the right in base pairs (*bp*). The expected sizes of the mRNA amplified products are $\beta 3$, 200 bp; $\beta 1$, 300 bp; αv , 250 bp; and *actin*, 210 bp. (B) Organoids were immunoprecipitated with antibodies to αv , $\alpha v\beta 5$, $\alpha v\beta 6$, or platelet glycoprotein Ib (as a negative control) after surface iodination and lysis. Both $\alpha v\beta 5$ and $\alpha v\beta 6$ could be precipitated from the cell surface, indicating that the apparently singular β band resolved by the antibody to αv (13C2) actually represented a mixture of these two integrin subunits. The band at 116 kD is non-specific and most prominent in the indirect immunoprecipitates. Relative molecular masses are shown to the left in kD.

the four hour time point, resulting in a greater than 90% loss of viability after twelve hours.

There have been reports of antibodies that directly induce apoptosis by binding to a cell surface antigen that triggers

an irreversible apoptotic response (Trauth et al., 1989; Itoh et al., 1991). It was important to determine whether the 23C6 mAb was acting directly in this way, or whether it functioned through its capacity to inhibit close cellular contact and subsequent organoid formation.

This was addressed in two ways. In the first series of experiments intact 23C6 mAb or Fab fragments were added to the reformation assay at different time intervals after reseeding. By morphologic examination it was clear that once intercellular contacts were formed, the antibody had no effect on the cells involved, although it was still able to prevent single cells from joining these clusters. LIM 1863 cells from the clusters were viable and continued to grow as organoids in subsequent culture, while the single cells underwent apoptosis, as determined by HOECHST staining (data not shown). Thus it appears that 23C6 is not delivering an apoptotic signal. To substantiate this, 23C6 Fab fragments were added to single cells at the time of calcium addition, and then washed out at different time periods, resulting in varying exposure times to the antibody. After a 4-h incubation, the numbers of clusters of reforming cells were counted under light microscopy, and a relative degree of reformation was determined (Fig. 6 B). Brief exposure (30 min) to the antibody had no effect on reformation, while there was a corresponding decrease correlating with the length of exposure. Similarly, even after a 60-min exposure before washout, the residual clusters were perfectly viable and continued to grow when returned to culture. Based on these physiological data, it is clear that 23C6 is only effective in inducing apoptosis by preventing cellular adhesion, and that it does not trigger the response directly.

To further confirm that the morphology we were observing was apoptotic, DNA degradation analysis was performed on the cells illustrated in the light micrographs of Fig. 7 A: single cells reseeded into low calcium medium; reformed organoids after an 18-h incubation period in calcium-rich medium (where there is a proportion of cells that have failed to incorporate into the structure); and cells inhibited from reforming with 23C6 mAb.

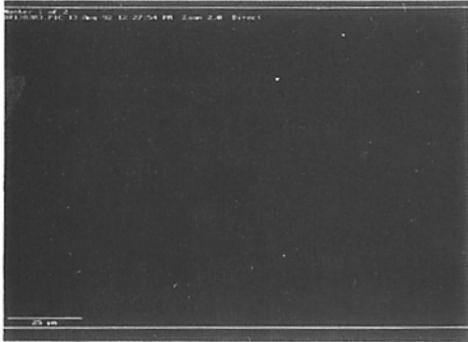
The DNA degradation results show that the 23C6-treated cells displayed a pattern of degradation characteristic of extensive intranucleosomal cleavage. In contrast, DNA from the parallel cultures in low-calcium medium or in the absence of antibody showed little DNA breakdown. The minor breakdown seen in lane *b* of Fig. 7 A can be attributed to the cells which failed to incorporate into the organoids.

Ultrastructural analysis confirmed the occurrence of apoptosis in antibody-treated cells. A representative cell from normal calcium conditions in the continued presence of mAb 23C6 (Fig. 7 B) shows a classical apoptotic morphology. Both the nucleus and cytoplasm are highly condensed, and vacuolation of the cell has commenced. Also typical of apoptotic cells, a number of organelles are still well preserved even at this stage, as demonstrated by the presence of intact mucin granules, rough endoplasmic reticulum, and intact lysosomes.

These results demonstrate that LIM 1863 cells undergo self-destruction by apoptosis within a few hours when the cells are inhibited from reforming organoid structures by the antibody 23C6. In addition, cells seeded at low density exhibited a similar time course and evidence of apoptosis as seen in these antibody-inhibited cells.

A

Control



23C6

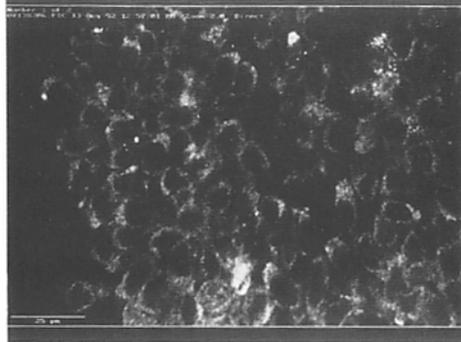
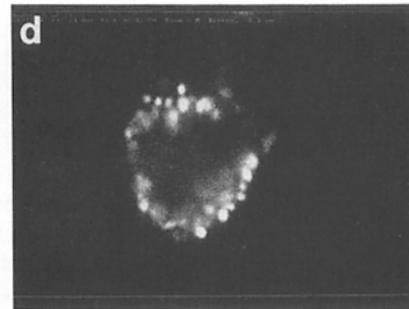
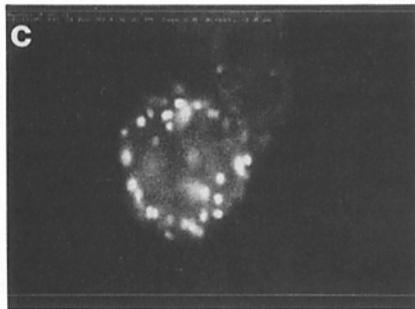
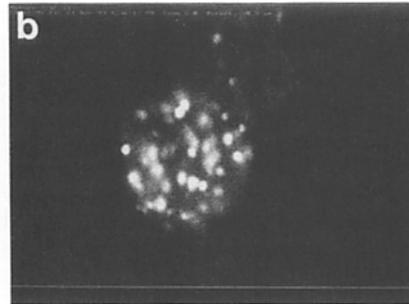
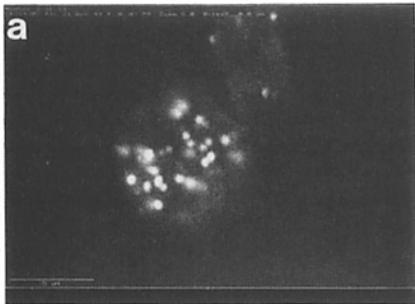
**B**

Figure 5. Immunofluorescent detection of the antigen recognized by 23C6 on LIM 1863 organoids and single cells undergoing adhesion inhibition. (A) Intact LIM 1863 organoids were fixed in 4% formaldehyde, and then stained with either 23C6 (right) or an isotype-matched control mAb (AK2) at a final concentration of 20 $\mu\text{g/ml}$ for 1 h at room temperature. A FITC-conjugated secondary antibody was used, and the cells observed under confocal microscopy. A single optical section is presented from the middle of the organoid. Bar, 25 μm . (B) Single cell suspensions were reseeded in the reformation assay in the presence of 23C6 for 2 h, fixed in 4% formaldehyde, and stained with a FITC-conjugated secondary antibody. Single optical sections were taken at 5- μm increments starting at the top of an individual cell (a-d), revealing a patching distribution of the antigen, which is restricted to the cell surface membrane. Bar, 5 μm .

Apoptosis in LIM 1863 Is Accompanied by Protein Synthesis and Nuclear Translocation of p53

The molecular events that drive the apoptotic process are not entirely resolved, and, for example, reports vary as to a requirement for protein synthesis when different cell types have been used (see Discussion). To begin to dissect this process in the organoid system we examined the expression of proteins synthesized during the initiation of apoptosis.

To determine the synthetic requirements of the colon cells preparing to undergo apoptosis, ^{35}S -labeling and two-dimensional gel electrophoresis were carried out. Cells were allowed to either reform cellular contacts for 4 h, or were inhibited from doing so by the antibody 23C6 over the same period. This time period represents an early point in the apoptotic event (Fig. 6 A). The results (Fig. 8) show that, overall, macromolecular biosynthesis was lower in the cells

undergoing apoptosis, as compared to those cells reforming organoid structures. However, careful examination of the two profiles revealed specific molecular differences. Whereas the synthesis of most proteins was decreased in the inhibited cells, the expression of another group of proteins was consistently increased. The apoptotic cells showed a marked increase in the turnover of a molecule of ~ 72 kD. Also, a 68-kD molecule appeared only in the apoptotic cells, possibly as a result of de novo synthesis. The identity of these proteins is currently under investigation, but their appearance at this time strongly suggests a role in the apoptotic cascade.

The tumor suppressor gene product p53 has been implicated in a number of experimental apoptotic systems (Yonish-Rouach et al., 1991; Shaw et al., 1992; Lowe et al., 1993; Clarke et al., 1993). Hence to determine whether p53 might also be involved in the apoptosis of contact-inhibited colon carcinoma cells we used Western blotting of both cyto-

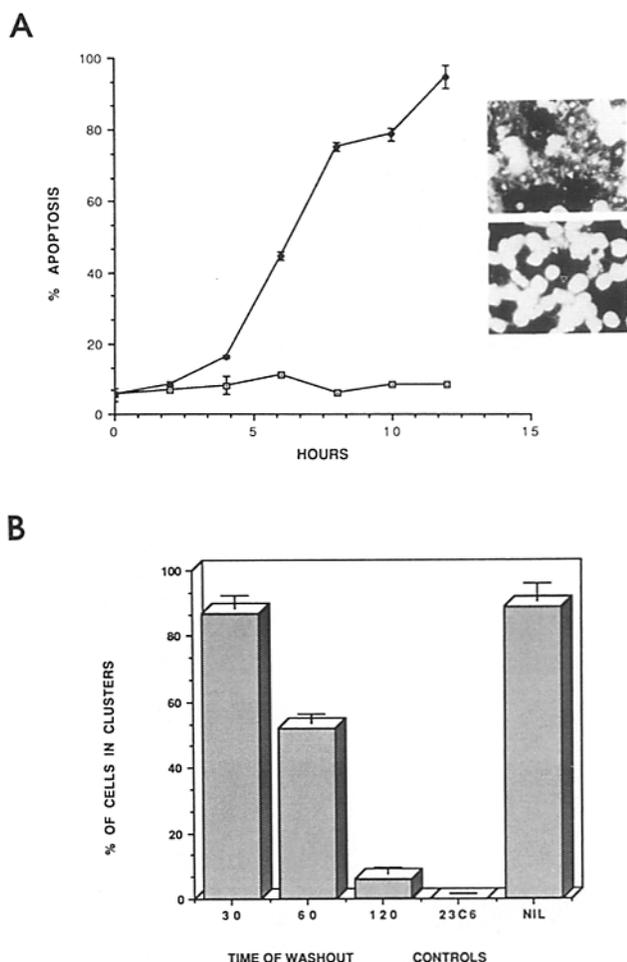


Figure 6. Kinetics of apoptosis induction by 23C6. (A) Single LIM 1863 cells were reseeded in calcium-free medium (open boxes), or in calcium-rich medium and the continued presence of 23C6 antibody (closed circles) for 12 h. The degree of apoptosis was determined by HOECHST staining at 2-h intervals. (Inset). Appearance of apoptotic nuclei (upper panel) showing condensation and fragmentation, or normal nuclei (lower panel). Photographs taken after 10 h culture. (B) In the control wells, single cells were reseeded in the reformation assay in the continuous presence (23C6) or absence (NIL) of 23C6 Fab fragments for 4 h. For the washout experiments, cells were reseeded in the presence of 23C6, which was subsequently washed out after 30, 60, or 120 min. These cells were then cultured for 4 h. The degree of reformation was determined by counting the numbers of cells forming intercellular contacts. A cluster was defined as two or more cells in contact.

solic and nuclear lysates from intact organoids, single cells, and cells undergoing either organoid reformation with time (O₂, O₄, O₆) or (Fab) antibody-induced apoptosis over the same time course.

Fig. 9 A shows high levels of p53 expression in the cytosol of both intact organoids and single cells. In cells allowed to reform over a six hour time period, the level of this p53 expression remained constant. By contrast, cells inhibited from reforming contacts through addition of 23C6 showed a marked decrease in cytosolic p53 levels by two hours. No p53 was detectable by 4 h and remained absent at the final time point. These time points correlate with the induction time of apoptosis in this line (compare with Fig. 6 A). Also,

in the single cells, reforming cells and organoids, a higher molecular weight band (around 140 kD) could be detected in addition to the native p53, and this too was absent from the cells undergoing apoptosis. To ensure that this loss of p53 from the cytosol in the contact-inhibited cells was not due to general protein degradation, a control anti-tubulin antibody was used and the expression of this protein did not change under the same conditions (Fig. 9 A).

Nuclear extracts from cells undergoing the same experimental conditions were prepared, and immunoblotted for p53. As is shown in Fig. 9 B, there was a concomitant increase in the levels of p53 in the nuclei of the apoptosing cells detectable by 2 h, and increasing at the four and six hour time points—corresponding to the loss from the cytosol. A polyclonal antibody against the high mobility group proteins, found exclusively in the nucleus, acted as a protein control. These non-histone proteins were selected because they are expressed at elevated levels in actively proliferating cells, and the observable reduction in high mobility group protein levels in the apoptotic cells is consistent with their role as structural components affecting the conformation and structure of chromatin (Nissen et al., 1991). Taken together, these data indicate that p53 translocates from the cytoplasm to the nucleus, during the initiation of apoptosis in these colon carcinoma cells.

Discussion

The results presented in this paper demonstrate a role for intercellular contact as a regulator of apoptosis. By blocking the cellular reattachment and morphogenetic rearrangement of colon carcinoma cells in a unique assay system, we were able to induce apoptosis in the cell line LIM 1863.

Intercellular adhesion is clearly a determining event in the formation and maintenance of the organoid structure of the LIM 1863 cell line. One of the aims of the study was to characterize molecules involved in this process. Of a panel of antibodies tested, the 23C6 antibody to the integrin $\alpha\beta 3$ complex inhibited intercellular adhesion of the LIM 1863 cells, and, even as Fab fragments, caused complete inhibition of organoid formation. That the antigen being bound by 23C6 was implicated in intercellular contacts was suggested by immunofluorescent staining of intact organoids and (inhibited) single cells analyzed by confocal microscopy.

The LIM 1863 cells, however, do not express detectable levels of the $\beta 3$ integrin subunit, and we could not identify the 23C6 antigen by immunoprecipitation analysis. In our hands the 23C6 antibody immunoprecipitates the $\alpha\beta 3$ integrin complex from a number of cell types, including osteoblasts, macrophages, and embryonic fibroblasts (Davies et al., 1989; Bates et al., 1991), and the mAb has been shown to bind a complex-specific epitope on the $\alpha\beta$ subunit (Nesbitt et al., 1989). However, we have also presented evidence that this cryptic epitope on $\alpha\beta$ can be exposed when $\alpha\beta$ is in association with β subunits other than $\beta 3$, consequent upon the conformational change induced by the tripeptide arginine-glycine-aspartic acid (Agrez et al., 1991). We show here that in LIM 1863 cells the subunits associated with $\alpha\beta$ are $\beta 5$ and $\beta 6$.

It is reasonable to suggest therefore that 23C6 is able to bind these $\alpha\beta$ integrins with low avidity, sufficient to block intercellular adhesions but not to enable immunoprecipita-

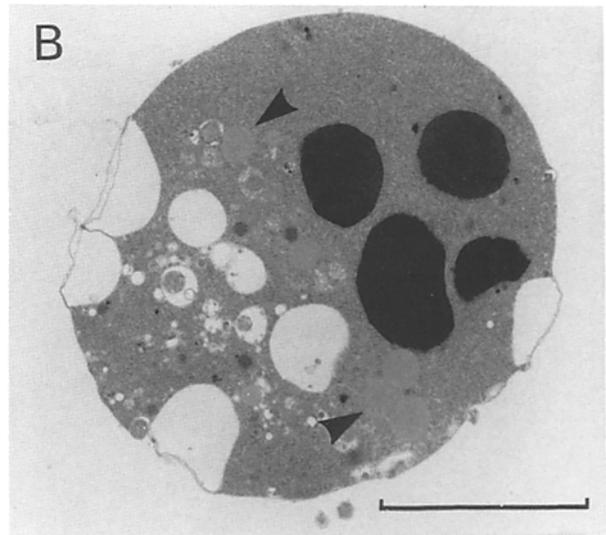
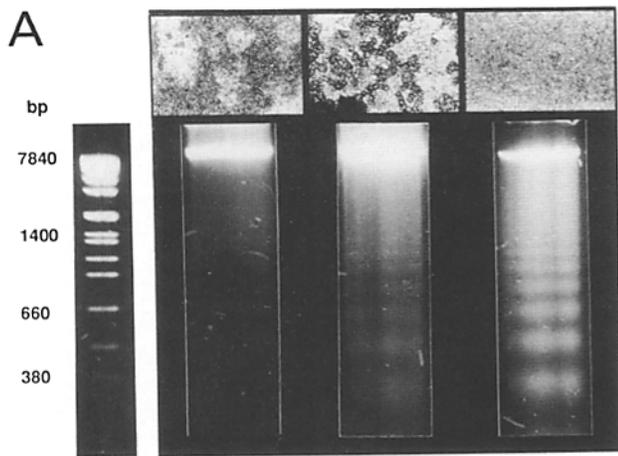


Figure 7. Apoptosis is characterized by DNA cleavage and ultrastructural changes in morphology. (A) Single LIM 1863 cells maintained in low calcium conditions for an 18-h incubation period (lane *a*) formed loose clusters (micrograph) and exhibited no DNA degradation. Cells reseeded in the presence of calcium and allowed to reform for 18 h (lane *b*) formed organoid structures, as seen in the micrograph. However, a proportion of cells failed to incorporate into the organoids—these cells are likely to be responsible for the minor DNA breakdown observed. The addition of 23C6 mAb (100 $\mu\text{g}/\text{ml}$) to cells in the presence of calcium (lane *c*) totally inhibited reformation, and the pattern of DNA degradation was characteristic of extensive intranucleosomal cleavage, typical of apoptosis. DNA markers are shown to the left in base pairs. (B) A typical cell from culture under normal calcium conditions in the presence of 23C6 exhibited many morphological features characteristic of apoptosis. The nucleus is highly condensed and vacuolation of the cell has commenced. No viable mitochondria could be observed at this point, although some organelles, including lysosomes and mucin granules (arrowheads), were still well preserved. Bar, 5 μm .

tion analysis. In support of this, by chemically cross-linking the 23C6 mAb bound to surface labeled LIM 1863 cells before lysis and immunoprecipitation analysis, we were able to detect a faint band in the position of αv , although the associated β subunits were not detectable (Bates, R. C., unpublished data). Thus, while the nature of the cell surface molecule bound by the 23C6 mAb has not been unequiv-

ally determined, our data implicate a role for an αv integrin complex as the cell-cell adhesion molecules involved in establishing the substantial contacts outlined above. The integrins have been characterized as the primary mediators of cell-extracellular matrix adhesion (Ruoslahti and Pierschbacher, 1987), and they serve as one of the many families active in cell-cell adhesion (Kaufman et al., 1989; Lampu-

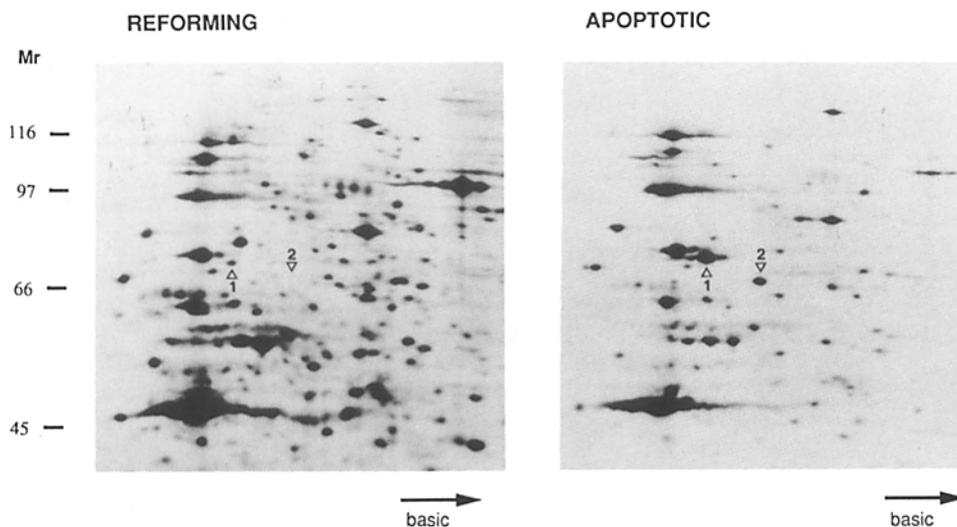


Figure 8. The induction of apoptosis is accompanied by protein synthesis. Single LIM 1863 cells were biosynthetically labeled with ^{35}S as described in Materials and Methods. Cells were reseeded into the reformation assay, in the absence (left) or presence (right) of 23C6 mAb, for 4 h. Intercellular contacts were reestablished in this time period in the absence of antibody, and apoptosis was induced in the presence of antibody (see Fig. 3). Total cell lysates were analyzed by two-dimensional gel electrophoresis with isoelectric focusing in the first dimension, and 7.5% SDS-PAGE in the second. Of

the total volume of lysates, 5% of the reforming and 7.5% of the apoptotic were loaded to each gel, which were subjected to fluorography overnight. There is a general reduction in the level of biosynthesis in the apoptotic cells, however, there is a marked increase in synthesis of a 72-kD protein (arrowhead labeled 1) compared to the reforming, as well as apparent de novo synthesis of a 68-kD protein (arrowhead labeled 2) not seen from the reforming cells. These same increases were noted in five of five experiments.

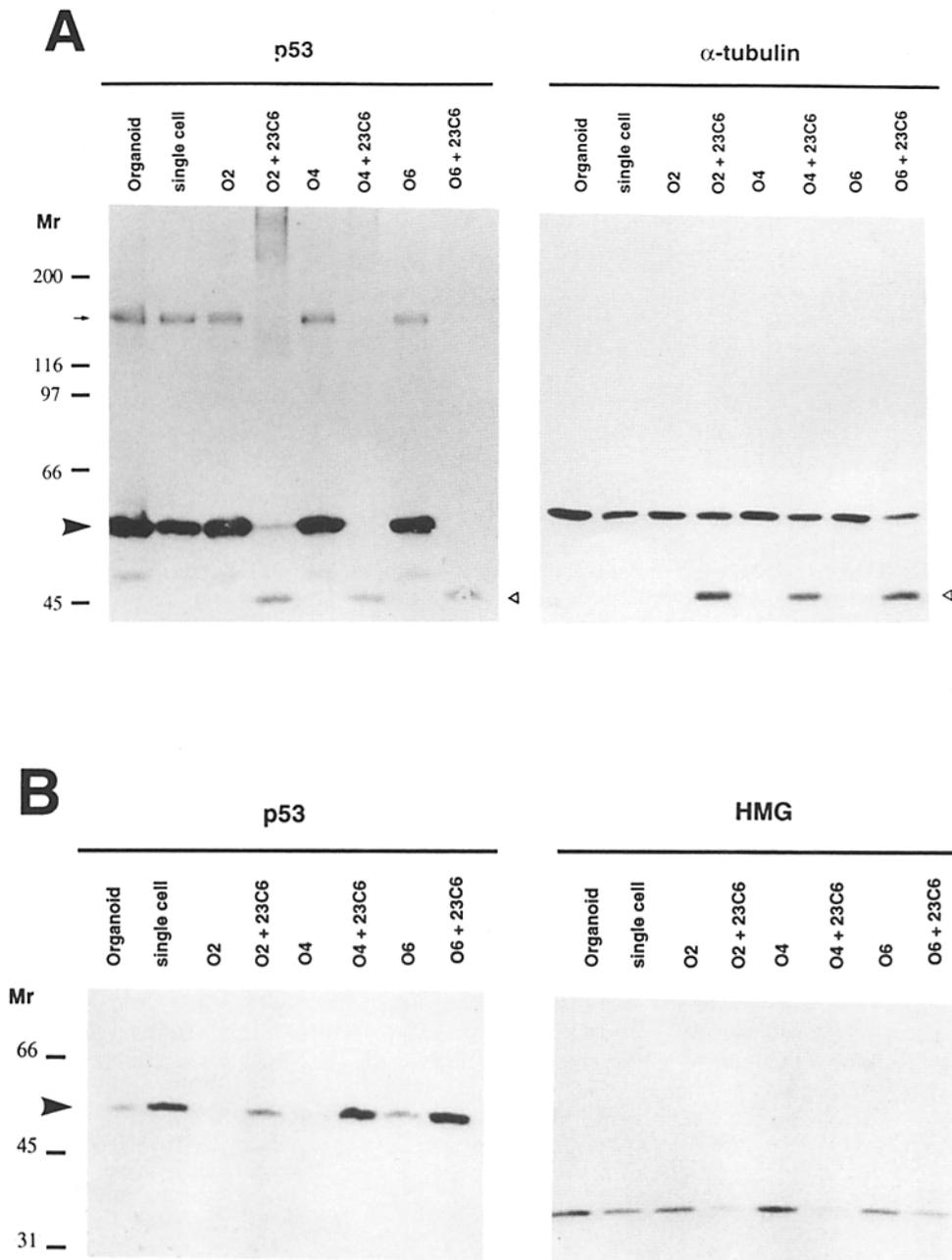


Figure 9. Translocation of p53 protein from the cytosol to the nucleus of apoptosing cells. (A) (Left) Equal volumes of cytoplasmic lysate from intact organoids, single cells, or cells seeded in the reformation assay taken at 2, 4, and 6 h time periods in the absence (O2, O4, O6) or presence of 23C6 Fab (+23C6), were subjected to 7.5% SDS-PAGE, and (after transfer to nitrocellulose) blotted with an anti-p53 antibody (PAb1801). (Right) Control immunoblot using an anti-tubulin antibody (Clone DM 1A). The immunoblots were developed using enhanced chemical luminescence (ECL) and were exposed for 1 min. Similar data were obtained in 3 of 3 experiments. Relative molecular masses (Mr) are shown to the left in kD. The position of p53 (closed arrowhead), a higher molecular weight band (arrow), and artefactual cross-reactivity of Fab in the 23C6 treatments (open arrowheads) are marked. (B) (Left) Nuclear extracts of cells under identical experimental conditions as described above, subjected to gradient 7.5–15% SDS-PAGE, and then immunoblotted with the anti-p53 antibody (PAb1801). (Right) Control immunoblot of nuclear lysates using an anti-HMG-1/Y antibody to the high mobility group proteins. Relative molecular masses are shown to the left in kD. The position of p53 is marked by the closed arrowhead.

gnani et al., 1991). These molecules are also intimately involved in the delivery of signals into the cell in response to extracellular stimuli (Hynes, 1992; Kapron-Bras et al., 1993). Confirmation of their involvement in the present system will provide another facet to the functional role of this family of receptors.

We obtained unequivocal morphological evidence of apoptosis in cells prevented from reestablishing cellular contacts by the mAb 23C6. Apoptosis is a process of active cellular self-destruction in which a series of events are initiated such that the cell participates in its own death (Wyllie et al., 1980). In many systems this process is dependent on cations, since the endonuclease required for the intranucleosomal cleavage is calcium and magnesium dependent (Duvall and Wyllie, 1986; Cotter et al., 1990); this also appears to be the case in our system since the cells in calcium-depleted

medium did not undergo apoptosis (Figs. 2 A, 6 A and 7) even with the loss of tight cellular contacts (Fig. 2 A). Despite the ubiquity of the process, relatively little is known of the triggers or mechanisms that mediate the process (Schwartz et al., 1990). A growing body of evidence has identified many possible regulators of programmed cell death in different tissues. Cytokines, such as TGF- β (Oberhammer et al., 1992) or TNF- α (Robaye et al., 1991), have been identified as exogenous factors involved in apoptotic control. Other growth factors, such as nerve growth factor, can suppress the process (Kannan et al., 1992). A cell surface antigen, known as APO-1 (Oehm et al., 1992) or Fas (Itoh et al., 1991), which displays significant structural similarity to the members of the tumor necrosis factor/nerve growth factor receptor superfamily, can directly induce apoptosis when engaged by specific antibodies. We addressed the possibility that the

23C6 mAb may have been acting in a similar way, but found that the effect of the mAb was reversible until such time as reformation had begun in the control cells. Further, if cell-cell contact and clutching had already begun, the mAb was no longer inhibitory and did not induce apoptosis. Taken together, these data strongly suggest that the 23C6 mAb is triggering apoptosis through its capacity to inhibit close cellular contacts and subsequent organoid formation. Since this form of cell death is dependent on active cell participation, it can also be potentially suppressed (Williams, 1991). Our data suggest that cellular contact in these tumor cells is a means of suppression of a death signal, a similar conclusion to that of Kataoka et al. (1993) who demonstrated that intercellular contact between T lymphoma cells and 'normal' stromal cells could inhibit apoptosis of the lymphoma cells.

Because LIM 1863 is an epithelial carcinoma line, our findings may also be of some general importance towards an understanding of epithelial morphogenesis. The mechanisms which determine how various epithelia acquire their morphologies are not well understood. While there is evidence of mesenchymal and soluble factors exerting an influence (e.g., Montesano et al., 1991) the process of cell rearrangement is a less widely appreciated determinant of tissue morphogenesis (Gumbiner, 1992). Our findings also lend support to data implicating the important role of cell-cell contact in establishing an identity or fate for groups of equivalent cells as espoused by Greenwald and Rubin (1992). The single cells may be viewed as an equivalence group, with the same set of potential fates when reseeded into the assay. Intercellular contact provides a signal for reformation and subsequent survival. By inhibiting this contact, the cells do not receive the same signals and express a default option—in this case, death by apoptosis. Cells that establish successful intercellular contacts undergo the truly remarkable process that we have described as clutching in Fig. 2 B. Successive ultrastructural examinations indicate that after the initial clutching event, the resulting doublets of cells act as cores for further adhesion, such that development of the organoid structure continues in a concentric manner (Fig. 2 C). These observations underscore the importance of intercellular contacts for the survival and growth of this tumor cell line.

Our results may also have important implications for the biology of tumor growth. In the circulation, metastasizing tumor cells have been reported to adhere to other circulating tumor and host cells to form tumor cell emboli (Raz, 1988). It was proposed that this protected the tumor cells from the mechanical stresses of the blood flow, or helped their arrest in the fine capillaries. Some twenty years ago it was reported that lung colonization of disaggregated cell suspensions of single tumor cells was never as good as preformed aggregates (Liotta et al., 1974, 1976). More recently, Kobayashi et al. (1993) reported that mouse carcinoma cell lines selected for drug resistance *in vivo* failed to express this resistance *in vitro* unless grown as multicellular spheroids. Also, the monodispersed cells were found to be more sensitive to alkylating agents—which often induce apoptosis—than the aggregates. Our data suggest a possible mechanism for these observations in that the survival of cells detaching from a primary tumor mass may be seriously compromised unless they can form cell aggregates and thereby suppress apoptosis.

Investigations were made into the possible molecular events which drive the apoptotic cascade. Current understanding of the genetic regulation of apoptosis centers on the three models of activation, determined by the cell lineage being investigated, as proposed by Cohen (1993). Induction mechanisms depend on new gene expression; in other systems it would appear that the suicide program is being constitutively inhibited by factors with short half-lives, and is referred to as release; and in transduction systems inhibitors of protein synthesis have no effect, possibly indicating that the cells already possess the necessary apoptotic molecules at any given time. Our two-dimensional analysis (Fig. 8) revealed the consistent synthesis of two proteins by the apoptotic cells. One protein showing increased synthesis had a molecular weight of 72 kD. We are currently investigating the possibility that this protein is one of the heat shock proteins. If so, the increased expression is likely to be a consequence of apoptosis, rather than part of the initiation of the process. A 68-kD protein appeared, restricted to the apoptotic cells, and while the identity of this protein is also unknown at this point, its late appearance and consistent expression only in apoptotic cells suggests that it may play a significant role in the apoptotic cascade.

A potentially significant finding was that of the translocation of p53 from the cytosol to the nucleus in the apoptotic cells. The p53 protein is a tumor suppressor gene product, with the wild-type able to negatively regulate cell proliferation by growth arrest in G1, to inhibit *in vitro* transformation, and also to suppress tumorigenesis (for review see Lane, 1992; Vogelstein and Kinzler, 1992). It is the most frequently altered gene in the development of many human cancers (Hollstein et al., 1991) and several distinct mutational "hot spots" have been identified (for review see Ullrich et al., 1992). Such mutation, or the inactivation of tumor-suppressor activity by complexing with other proteins such as the DNA tumor virus, SV40 T antigen, or the oncogene product MDM2 (Oliner et al., 1992), are considered to be universal steps in most human malignancies. It is well established that the wild-type p53 binds strongly to double stranded DNA and can activate transcription (Farmer et al., 1992) hence movement to the nucleus is paramount for the protein to exert its profound biological effects. Moll et al. (1992) recently reported that p53 could be found in the cytoplasm of both normal and malignant breast tissue, and that nuclear exclusion of this protein, by sequestering it in the cytoplasm away from its site of action, effectively resulted in the inactivation of its tumor-suppressor ability in some breast carcinomas.

In addition, it has been shown that wild-type p53 is capable of inducing apoptosis when transfected into cell lines deficient in this form of the protein (Yonish-Rouach et al., 1991; Shaw et al., 1992); however, by transfecting previously immortalized cells and expressing p53 in non-physiological states, the role of p53 needs to be interpreted with some caution. More recently, the existence of p53-dependent apoptotic pathways have been described for thymocytes (Lowe et al., 1993; Clarke et al., 1993) using gene knock-out experiments. Our results (Fig. 8) clearly demonstrate a rapid translocation of p53 from the cytosol to the nucleus at a time when the cells are undergoing apoptosis, between 2 and 4 h. The kinetics of apoptosis induction (Fig. 6 A) correlate with the disappearance of the cytoplasmic p53. In the apoptosing

cells, increased nuclear levels were apparent by 2 h, and all cytosolic p53 was absent by 4 h, the same time point at which the proportion of cells undergoing apoptosis begins dramatically to increase. It is reasonable to suggest, therefore, that translocation of p53 to the nucleus in our model might be involved in the subsequent induction of the observed apoptosis, and this hypothesis is currently under investigation. The p53 expressed by LIM 1863 cells is intrinsically highly phosphorylated and appears to be a mutant form of the protein since it can be precipitated with the PAb240 monoclonal antibody (Oncogene Science Inc.) (Bates, R. C., unpublished data). If this result can be confirmed by sequencing, the LIM 1863 cell line will provide a valuable model for apoptotic regulation by an endogenous, mutant p53.

Cellular interactions and cell-cell adhesion are important for normal development and loss of such adhesions are associated with neoplastic transformation. The colonic mucosa is one of the most rapidly proliferating epithelial tissues in the body and stem cells which escape positional controls might well be expected to contribute towards oncogenesis. Apoptosis, triggered by the loss of cell contacts, could serve as a physiological tumor control mechanism by deleting these aberrant cells. Evasion from this control would thereby represent progressive steps in the development of malignancy.

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