E-cadherin Mediates Aggregation-dependent Survival of Prostate and Mammary Epithelial Cells through the Retinoblastoma Cell Cycle Control Pathway*

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E-cadherin and the retinoblastoma tumor suppressor (Rb) are traditionally associated with diverse regulatory aspects of cell growth and differentiation. However, we have discovered new evidence, which suggests that these proteins are functionally linked in a physiologic pathway required for cell survival and programmed cell death. Pharmacological activation of protein kinase C (PKC) or inducible overexpression and activation of the α isozyme of PKC (PKCα) resulted in approximately 60% apoptosis of mammary and prostate epithelial cells. Interestingly, the surviving cells had undergone dramatic aggregation concurrent with increased E-cadherin expression. When aggregation was inhibited by the addition of an E-cadherin-blocking antibody, apoptosis increased synergistically. We hypothesized that survival of the aggregated population was associated with contact-inhibited growth and that apoptosis might result from aberrant growth regulatory signals in non-aggregated, cycling cells. This hypothesis was confirmed by experiments that demonstrated that E-cadherin-dependent aggregation resulted in Rh-mediated G1 arrest and survival. Immunoblot analysis and flow cytometry revealed that hypophosphorylated Rb was present in non-aggregated, S phase cultures concurrent with synergetic cell death. We have also determined that the loss of membrane E-cadherin and subsequent hypophosphorylation of Rb in luminal epithelial cells preceded apoptosis induced by castration. These findings provide compelling evidence that suggests that E-cadherin-mediated aggregation results in Rb activation and G1 arrest that is critical for survival of prostate and mammary epithelial cells. These data also indicate that Rb can initiate a fatal growth signal conflict in non-aggregated, cycling cells when the protein is hypophosphorylated as these epithelial cells enter S phase.

Tissue development and homeostasis are dependent on a complex microenvironment regulated by extracellular adhesion mechanisms as well as soluble growth regulatory molecules.

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The PKC1 family of serine/threonine protein kinases represents one of the most prominent signal transduction mechanisms activated by these environmental stimuli (1). Historically, PKC activity has been associated with the regulation of cell growth and differentiation; however, recent studies have demonstrated that specific isozymes of the PKC family may regulate apoptotic programs as well (2–4). Although PKC has become the focus of more extensive research in the apoptosis field, a specific mechanism has not been elucidated. A potential target of PKC action, with important ramifications in differentiation and development, is the cadherin family of transmembrane adhesion proteins. This hypothesis is supported by studies in which the PKC activator, 12-O-tetradecanoylphorbol-13-acetate (TPA) has been shown to induce redistribution of E-cadherin to membranes and premature compaction in the mouse embryo (5). Other studies using activators and inhibitors of PKC have also demonstrated a functional association between PKC and cadherins in a variety of cell types (6–9); however, the identity of PKC isozymes and their regulation of specific cadherin targets have not been reported.

In the prostate gland and mammary gland, inter-epithelial membrane adhesion is dependent on the homotypic interaction of E-cadherin (10, 11). Such homophilic cell-cell adhesion results in the formation of desmosomes and adherens junctions that are required for tissue morphogenesis and the maintenance of the differentiated phenotype (10). The intracellular domain of E-cadherin is linked to the actin cytoskeleton through its interaction with the cytoplasmic adapter proteins α-, β-, and γ-catenin/plakoglobin (12–14), which is essential for intercellular adhesion. Although central to the cell adhesion mechanism, E-cadherin has also been implicated in physiologic roles beyond the mere mechanical interconnection of cells. More recent evidence suggests that E-cadherin may also be associated with regulatory pathways involved in various aspects of cell fate including developmental decisions, cellular differentiation, and possibly cell survival (15, 16).

Homeostasis of the prostate and mammary glands is dependent upon androgenic and estrogenic steroids, respectively. Depletion of these hormones activates a series of poorly defined molecular events leading to involution and remodeling of the tissue resulting from apoptotic death of the luminal epithelium. Although critical for tissue homeostasis, steroid hormones may not be exclusive to the regulation of cell survival since suppression of epithelial apoptosis also depends on their homotypic placement with adjacent epithelial cells and their

1 The abbreviations used are: PKC, protein kinase C; Rb, retinoblastoma; TPA, 12-O-tetradecanoylphorbol-13-acetate; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP biotin nick end labeling.
attachment to the extracellular matrix. The first visible stage of prostate and mammary involution is the disruption of intercellular adhesion and anchorage prior to the onset of apoptosis (17). Thus, disruption of basal and lateral adhesion is often associated with apoptosis in involuting tissues (17, 18). Previous studies have demonstrated that anchorage-dependent epithelium will undergo apoptosis following loss of integrin contact with the extracellular matrix (19, 20) or inhibition of integrin-mediated organoid formation (21). It is now believed that just as integrins function to mediate cell-extracellular matrix interactions in anchorage-dependent survival, cadherins may also act in such a capacity, possessing a functional role in the regulation of intercellular adhesion-dependent survival. Several studies have reported the association between N-cadherin-mediated intercellular adhesion and survival of gut and ovarian epithelium (22, 23), and recently an association between E-cadherin-mediated aggregation and survival of oral squamous carcinoma cells has been reported (24). These studies suggested that homophilic binding of cadherin molecules on adjacent cells could transduce apoptotic suppressive signals; however, the actual mechanism by which cadherins mediate these signals is not known.

We have previously demonstrated that integrin-mediated survival of prostate epithelial cells was uniquely regulated through the Rb cell cycle control pathway (25). We have also investigated the functional role of PKC in the regulation of epithelial apoptosis, and we have demonstrated that PKC signal transduction, like integrin-regulated mechanisms, is capable of recruiting a Rb-regulated apoptotic pathway in prostate epithelial cells (3). In many cell types, apoptosis is thought to be a default pathway occurring where opposing or conflicting cell proliferation signals arise. For example, in the absence of serum growth factors, overexpression of c-Myc can initiate such a conflict by promoting the aberrant entry of cells into cycle (26). We have found that such a conflict arises when Rb growth-suppressive activity opposes mitogenic signals in proliferating cells. In support of this possibility, we found that transient expression of a constitutively active form of Rb induced apoptosis and that the inhibition of Rb function, by transfection of the Rb inhibitory oncogene, E1a, suppressed apoptosis (3, 25). These findings are in stark contrast with studies in which homoygous deletion of the Rb1 gene resulted in increased apoptosis in mouse cells, suggesting an apoptotic-suppressive role for Rb (27–30). A possible explanation for this discrepancy is that Rb may influence both outcomes and participate in both cell survival and cell death.

The adhesion molecules that mediate intercellular and extracellular matrix contact (31) largely regulate the normal growth of cells. Thus, an inadequate cell adhesion system, in which cells can easily dissociate from the primary tumor, may be a key determinant in the metastatic progression of certain cancers. In tumors of epithelial origin, the disruption of cellular adhesion appears to arise through alterations in the E-cadherin/α,β-catenin/adenomatous polyposis coli system, and as such, the loss of growth control may not be due to growth-promoting mutations but by the escape from apoptotic mechanisms (31, 32). Accordingly, E-cadherin expression is often reduced in more advanced prostate tumors and lobular mammary tumors, supporting growing speculation about the role of E-cadherin as a metastasis-suppressor protein in these cancers (33–36).

Herein, we describe a novel cell survival mechanism initiated by PKC in prostate and mammary epithelial cells in which E-cadherin-mediated aggregation resulted in Rb activation and G1 arrest necessary for survival. However, non-aggregated cells, which do not arrest in G1, undergo apoptosis, which we believe results from a fatal cell cycle conflict that occurs in S phase cells which contain hypophosphorylated Rb. Additionally, these findings are supported by the observation that the loss of membrane E-cadherin and subsequent hypophosphorylation of Rb in luminal epithelial cells occur in the involuting prostate gland.

**MATERIALS AND METHODS**

**Cell Culture Reagents and Transfections**—The cell line LNCaP (ATCC) was propagated in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.). The cell line LNCaP was plated at 37 °C in a humidified atmosphere of 5% CO2 and subcultured weekly. The LNs17, a clone of LNCaP transfected with PKCo under control of the tetracycline-repressible promoter, was propagated as described (3). The human epithelial breast cancer cell line, SUM185 was grown in Ham's F-12 supplemented with 5% fetal bovine serum, 5 μg/ml insulin, 1 mg/ml hydrocortisone, gentamycin, and fungizone. SUM185 PE is one of 12 breast cancer cell lines developed in the laboratory of Dr. Stephen Ethier.2 Bryostatin 1, staurosporine, and TPA (LC Laboratories, catalog numbers B-6697, S-8451, and P-1680, respectively) were dissolved in 100% ethanol, aliquoted, and stored at −20 °C. Bryostatin 1 and TPA were used at a final concentration of 10 nM. Staurosporine was used at 50 nM concentration as a pretreatment 90 min prior to PKC activation.

**Experiments**—For the E-cadherin blocking antibody studies, LNCaP cells were plated on 5 μg/ml fibronectin-coated tissue culture plates, and the SUM185 cells were plated on poly-lysine-coated tissue culture plates (Collaborative Biomedical Products), and all cells were allowed to attach for 24–48 h prior to experimentation. LNCaP and SUM185 cells were identically plated, and the cultures were 50% confluent prior to treatment. For both cell lines, the E-cadherin blocking antibody (Zymed Laboratories Inc., catalog number 13-5700) and the IgG2a antibody isotype control (PharMingen, catalog number 03020D) were added to the cultures 1 h prior to the addition of TPA. Both antibodies were used at a concentration of 60 μg/ml in culture. Cell viability was followed by trypsin blue exclusion (Life Technologies, Inc.) or by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy méthoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt assay (Promega).

**Experimental Cell Culture Plating and Density**—LNCaP, LNs17, and SUM185 log phase cells were plated and allowed to attach to the tissue culture flask for 48 h, at which time the cells were at 50% confluence (subconfluent) prior to PKC activation for all experiments unless otherwise described. For confluent cultures and subconfluent plus E-cadherin-blocking antibody cultures (Fig. 6), log phase cells were plated out at a higher density so that 48 h post-plating the cells were at 70% confluence. At this point one culture was given the blocking antibody and the other allowed to grow to 100% confluence. Both cultures were then treated with 10 nM TPA and harvested for viability and protein extraction 48 h later.

**Western Blot and Protein Analysis**—Expression of E-cadherin and Rb proteins was determined by Western blot analysis using the following antibodies: anti-human E-cadherin (Zymed Laboratories Inc., catalog number 13-1700), anti-rat E-cadherin (Transduction Laboratories, catalog number C20820), anti-Rb (PharMingen, catalog number 14001A), and anti-human E1a (Calbiochem, catalog number DP11). The donkey anti-mouse peroxidase-conjugated IgG (Amresco, catalog number E574) was used as a secondary antibody for all monoclonal antibodies described here. For protein extraction, tissue culture cells were lysed in 50 mM Tris at pH 7.5, 120 mM NaCl, 0.5% Nonidet P-40 with the following protease inhibitors: 1μM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 200 μg/ml orthovanadate. The cells were allowed to lyse on ice for 1 h; the resulting lysates were centrifuged, and the supernatants were collected and quantitated. Rat prostate lysates were prepared from frozen tissue as described below in a buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS in phosphate-buffered saline with the following protease inhibitors: 40 μM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 200 μg/ml orthovanadate. Protein lysates were quantitated using a Bradford assay, separated by 6% Tris/glycine precast Novex gels, and analyzed under denaturing antibody conditions using the Nordic and Eta (Amersham Pharmacia Biotech) detection systems as described previously (3).

On-line address is as follows: http://www.cancer.med.umich.edu/umbnymdh.html.
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Flow Cytometry Analysis—LNCaP cells (log phase cells) were grown and PKC-activated as previously reported (3). Cell cycle analysis of asynchronous LNCaP cultures treated with TPA over a 48-h time course was accomplished utilizing propidium iodide staining and multicycle analysis. Cultures were harvested, fixed with 70% ethanol, stained in a solution containing 5 μg/ml propidium iodide, 50 μg/ml of Range A, phosphate-buffered saline, and analyzed by flow cytometry with multicycle (see below). For experiments involving growth arrest through high density contact inhibition, LNCaP cells were plated at higher density and retained in culture for 5 days. For experiments manipulating late G1/S cell cycle arrest, subconfluent LNCaP cultures (2 days after plating) were treated for 3 days with 2 μM hydroxyurea (Sigma). Hydroxyurea cultures were released from the block with hydroxyurea-free medium 5 h prior to PKC activation. LNCaP cells were prepared at the times indicated and stained with propidium iodide and Hoechst for flow cytometry as described (37, 38). Flow cytometry was done at the University of Michigan flow cytometry facility using a Coulter ELITE cell sorter (Beckman-Coulter, Miami, FL) equipped with a 5-watt UV-capable laser. Analysis regions were set to include viable, dead, and apoptotic cell populations, based on untreated control samples. Data, acquired to 105 events per sample, were analyzed using Coulter’s ELITE software. DNA histograms resulting from the H342 staining were analyzed using algorithms available in MultiCycle software (Phoenix Flow Systems, San Diego, CA) to estimate G1, S, and G2/M compartment percentages.

Rat Castration and Histology—250-g male Sprague-Dawley rats were castrated at the indicated times, and the ventral prostate was excised and immediately frozen in liquid nitrogen. Immunohistochemical studies were performed on 3-μm serial frozen sections fixed in 100% methanol and incubated with the rat anti-E-cadherin antibody (Transduction Laboratories, catalog number C20820) diluted 1:40,000. The secondary antibody was a biotinylated horse anti-mouse (Vector Laboratories, catalog number BA-2001) conjugated with peroxidase and was used at a 1:200 dilution. The sections were counterstained with hematoxylin, dehydrated, and mounted. For direct comparison of E-cadherin expression and TUNEL analysis, the TUNEL assay was performed as described by the manufacturer (Apoptag kit, Oncor) in the subsequent section. In this fashion, alternating sections were used for immunohistochemistry and TUNEL analysis. The sections were examined at ×1000 magnification.

RESULTS

**Activation of PKC Results in Apoptosis and Aggregation-associated Survival of Prostate and Breast Epithelial Cells**—We have previously shown that prolonged activation of PKC recruited a novel apoptotic pathway in prostate epithelial cells (2, 39). We examined PKC isozyme specificity of this mechanism in detail using a tetracycline-regulated expression system in which the α member of the PKC family (PKCα) was expressed 40-fold over basal levels in the parental prostate epithelial line, LNCaP (3). In this earlier study, we demonstrated that activation of exogenous PKCα in the transfected clones resulted in dramatic morphological changes and subsequent apoptosis. Identical results were obtained in this study by using the PKC activator, 12-O-tetradecanoylphorbol-13-acetate (TPA), not only in the parental LNCaP cells but also in the human breast epithelial cell line, SUM185. Treatment of LNCaP and SUM185 cells with TPA resulted in approximately half of the cells undergoing apoptosis. Interestingly, the extent of cell survival correlated with increasing cell density such that high density cultures were more resistant to PKC-induced apoptosis (Fig. 1). This density-associated survival could possibly be explained by the observation that all surviving cells had undergone dramatic aggregation (Fig. 2, a, b, and d, e). The association between survival with high density cultures suggested that cells, which were in closer proximity at the time of PKC activation, were more likely to aggregate and survive.

**E-cadherin Mediates Aggregation-dependent Survival of Prostate and Mammary Epithelial Cells**—The observation that cell survival was closely associated with increased aggregation suggested a regulatory role for E-cadherin in survival of prostate and mammary epithelial cells. To begin to evaluate the functional role of E-cadherin in epithelial aggregation and survival, we examined the expression of E-cadherin protein in aggregated and non-aggregated populations of both prostate and mammary epithelial cells following activation of PKC. Protein extracts from the aggregated population of LNCaP cells and SUM185 cells were analyzed for E-cadherin expression using an E-cadherin-specific monoclonal antibody (Fig. 3). Immunoblotting revealed strong, yet transient induction of E-cadherin protein post-PKC activation in both cell lines. Induction of E-cadherin was first detected by 6 h, which coincided precisely with the onset of cellular aggregation. This finding suggested that E-cadherin-mediated aggregation is necessary for survival following apoptotic stimuli. We postulated, however, that a perturbation to E-cadherin-mediated adhesion must occur in the non-aggregated population as dissociated or individual cells were very rarely observed in treated cultures. Therefore, we examined the expression of E-cadherin in the non-aggregated population. Due to decreased basal adhesion, the non-aggregated cells were separated from aggregated cells as suspended cells. In this non-aggregated population, we found very little expression of E-cadherin at the 48-h time point, which correlated with a dramatic reduction in cell viability (Fig. 3). In comparison, E-cadherin expression continued to increase in the aggregated populations through 48 h without loss of viability.

Increased aggregation concurrent with E-cadherin induction strongly suggested that E-cadherin was mediating the survival of prostate and breast epithelial cells. To establish a functional role for E-cadherin in intercellular adhesion and cell survival, we attempted to disrupt PKC-induced aggregation in prostate and mammary epithelial cells with an E-cadherin-blocking antibody. In the presence of the blocking antibody, aggregation was completely inhibited, and the aggregated cells underwent synergistic cell death with more than a 3-fold increase in apoptosis as compared with minus antibody and isotype controls (Fig. 4, A and B). Although more diffuse and somewhat rounded by treatment with the blocking antibody, the LNCaP and SUM185 cells remained viable and continued to proliferate.

The observation that PKC activation resulted in morphologic
changes, in addition to E-cadherin accumulation, suggested that the surviving, aggregated cells had undergone dramatic phenotypic alterations. However, the fact that PKC activation also resulted in apoptosis of non-aggregated cells was supportive evidence that PKC recruits a pathway, which leads to either survival in an aggregated state or leads to apoptosis in non-aggregated cells. To confirm that these events were regulated through a common PKC mechanism, we examined the ability of staurosporine, a potent catalytic inhibitor of serine/threonine kinases, including PKC, to inhibit these changes. Not only did staurosporine prevent the changes in cell morphology and aggregation (Fig. 2, c and f), it also inhibited E-cadherin induction (Fig. 3B) and apoptosis (Figs. 1 and 2, c and f).

**E-cadherin-mediated Aggregation Results in G<sub>1</sub> Growth Arrest and Survival**—We had previously demonstrated that the Rb cell cycle control mechanism plays a crucial role in regulating apoptosis induced by anchorage disruption (25). In the current study, we have shown that the survival of mammary and prostate epithelial cells following PKC activation appears to be dependent on cellular aggregation. In the presence of the E-cadherin-blocking antibody, aggregation of prostate and mammary epithelial cells was inhibited, and the cells were hypersensitive to PKC-induced apoptosis. We postulated that E-cadherin-dependent aggregation, similar to integrin-mediated anchorage, might mediate survival by influencing Rb activity and cell cycle regulation as well. To determine if a functional link existed between E-cadherin and Rb, we first examined the cell cycle profile of LNCaP cells at the onset of PKC activation (0 h) as well as cell viability and Rb phosphorylation in four TPA-treated populations during a 48-h TPA time course (Fig. 5). TPA treatment of subconfluent, log phase LNCaP cells resulted in complete Rb hypophosphorylation within 24 h and 53% apoptosis by 48 h. However, TPA treatment of log phase cultures results in two distinct populations as follows: aggregated surviving cells and non-aggregated apoptotic cells, both of which contained hypophosphorylated Rb. How could such divergent effects, survival of aggregated cells and apoptosis of dissociated cells, be explained by Rb activation? When we specifically examined the aggregated population at the 48-h time point, we found that these cells expressed only hypophosphorylated Rb and had accumulated in G<sub>1</sub> but were completely viable, suggesting that contact-induced G<sub>1</sub> arrest facilitated survival. This was confirmed by the finding that when a subconfluent culture was grown to confluence and

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**Fig. 2.** PKC activation results in cellular aggregation of prostate and breast epithelial cells. Photomicrographs were taken of LNCaP prostate cells (a–c) and of SUM185 breast cells (d–f) as follows: untreated (a and d), treated with 10 nM TPA for 6 h (b and e), or pretreated for 90 min with 50 nM staurosporine (c and f) prior to TPA treatment, and viability was obtained 48 h later by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay. The data were presented as an average and S.D. of quadruplicate counts expressed as a percentage of untreated control. Magnification × 200.

**Fig. 3.** Accumulation of E-cadherin is associated with aggregation of prostate and breast epithelial cells. Protein extracts were analyzed by immunoblot analysis employing an E-cadherin-specific monoclonal antibody. A, protein extracts were obtained at the indicated times from aggregated or non-aggregated PKCa-expressing LNCaP prostate cells treated with 10 nM bryostatin 1 and from SUM185 breast cells treated with 10 nM TPA (collectively labeled as PKC). B, identical cultures were also pretreated for 90 min with 50 nM staurosporine prior to PKC activation (STS/PKC). The percent viable cell number was measured by trypan blue exclusion at 24 and 48 h post-PKC activation and is presented as an average of triplicate cell counts.
arrested in G1 prior to PKC activation, the culture exhibited only a 3% reduction in viability following 48 h of TPA exposure (Fig. 5). In support of this observation, we found that cells cultured at subconfluent densities and arrested in G1 by serum starvation also contained the hypophosphorylated form of Rb prior to PKC activation and were resistant to apoptosis (Fig. 5). The finding that aggregated cells were growth-arrested suggested that aggregation-mediated survival may depend on the ability of cells to initiate Rb-dependent G1 arrest. To help confirm the role of E-cadherin-mediated adhesion in Rb and cell cycle regulation, we examined cell cycle profile at the time of PKC activation (0 h). We also determined cell viability and Rb phosphorylation in cultures plated at the same density as the confluent cultures but were pretreated with the E-cadherin-blocking antibody (Fig. 5). The blocking antibody inhibited PKC-induced aggregation when added to subconfluent cultures. Immunoblot and flow cytometric analysis revealed that these dissociated cells contained the inactive, hyperphosphorylated form of Rb and were in log phase growth prior to TPA treatment (0 h). Addition of TPA to non-aggregated cells resulted in the conversion of Rb to the hypophosphorylated form within 24 h and resulted in 87% apoptosis at 48 h. This result was in complete contrast to the slight apoptosis (3%) observed in the aggregated population at the same time.

Apoptosis Results from a Fatal Growth Signal Conflict in S Phase Cells—Our results demonstrated that aggregated, G1-arrested cells were resistant to TPA-induced apoptosis, whereas cycling cells remained highly sensitive. We next attempted to determine what phase of the cell cycle that TPA-treated cells were susceptible to apoptosis. Propidium iodide staining of asynchronous LNCaP cells treated with TPA resulted in a 3-fold reduction in S phase (Fig. 6A). To confirm that apoptosis was actually occurring in S phase, we examined the susceptibility of synchronized S phase cultures to TPA-induced cell death utilizing Hoechst and propidium iodide staining and flow cytometry (Fig. 6B). When PKC was activated in cells synchronized at the G1/S border and released into S phase for 5 h, we observed an extremely rapid decline in cell viability that was reduced to 41% by only 8 h and 13 and 6% at 24 and 48 h, respectively (Fig. 6B and data not shown). We have previously postulated that PKC-induced apoptosis resulted from a conflict between Rb growth-suppressive signals in opposition to growth-promoting signals in proliferating epithelial cells (3). However, mechanistic details of this conflict were not elucidated. Because of the dramatic apoptosis that is induced in S phase cultures, we speculated that the hypophosphorylation of Rb as cells enter S phase might manifest such an apoptotic conflict. Surprisingly, TPA treatment not only resulted in Rb hypophosphorylation in the S phase cells, but it occurred with kinetics correlating precisely with cell death. Following PKC
activation, approximately 50% of Rb protein was dephosphorylated by 8 h, which correlated with 59% apoptosis at this time. Total conversion to the active conformation was complete by 24 h which correlated to 87% reduction in viability, which was 2-fold greater than the levels of apoptosis seen in asynchronous cells (Fig. 5).

To determine whether functional inhibition of endogenous Rb could suppress S phase apoptosis, we overexpressed the Rb inhibitory oncogene, E1a, in LNCaP cells. As shown in Fig. 6C, E1a-expressing LNCaP cells did not undergo apoptosis after addition of TPA. Because E1a has cellular targets in addition to Rb, we used a control E1a expression vector with a mutation at nucleotide 928 that blocks interaction with Rb without disrupting growth to 100% confluence (Contact-inhibited) or treated with 10 nM TPA following 5 days in serum-free medium (Serum-starved) or pre-treated as a subconfluent culture with the E-cadherin-blocking antibody prior to treatment with 10 nM TPA (α-E-cadherin). Whole cell protein extracts were prepared at the indicated times following PKC activation and were analyzed by immunoblotting employing an Rb-specific monoclonal antibody. Viable cell number was measured by trypan blue exclusion expressed as a percentage of untreated control.

**DISCUSSION**

E-cadherin is essential for the formation of intercellular junctional complexes and the establishment of cell polarity, and its role in epithelial differentiation and maintenance of tissue integrity is well established (10, 43). In the current study, we have demonstrated that E-cadherin functions in a novel adhesion-dependent survival pathway that can suppress apoptosis of prostate and mammary epithelial cells. Our observation that PKC activation resulted in the rapid accumulation of E-cadherin and aggregation suggested that PKC initiated a phenotypic program in surviving prostate and mammary epithelial cells. Regardless of the phenotypic outcome, the observation that staurosporine inhibited all morphologic and molecular changes induced by TPA suggested that the fate of these cells, whether survival in an aggregated state or death of non-aggregated cells, had vastly divergent outcomes originating from a common PKC signal.

Following PKC activation we found that the surviving population of aggregated cells contained hypophosphorylated Rb, were growth-arrested, and were resistant to apoptosis. These results strongly suggested that E-cadherin-mediated survival is achieved through Rb-mediated G1 arrest and precluded apoptosis. By inhibiting aggregation with an E-cadherin-blocking antibody, we found that the cells contained hyperphosphorylated Rb and remained in logarithmic growth at the time of PKC activation. Following activation of PKC, these non-aggregated cells rapidly converted Rb to the active, hypophosphorylated form that preceded a 3.5-fold increase in apoptosis. This result suggested that Rb activity was involved in signaling apoptosis in cycling cells. If aggregated, G1-arrested cells are able to suppress apoptosis, then in what phase of the cycle are cells susceptible to apoptotic signals? We determined that PKC-induced apoptosis occurred in the S phase of the cell cycle. This was demonstrated in two experiments in which we observed a dramatic reduction in viable S phase cells from asynchronous cultures and almost complete loss of cell viability in synchronized S phase cultures.

The functional role of Rb is traditionally associated with cell cycle regulation; however, recent studies have accumulated significant data linking Rb activity with programmed cell death (44-46). We have previously demonstrated a functional role for Rb in signaling apoptosis of epithelial cells induced by anchorage disruption or inducible overexpression and activation of PKCα (3, 25). In the latter study, we demonstrated that Rb-signalated apoptosis was executed through the caspase family of cysteine proteases (3); however, the exact mechanism by
which Rb initiates this apoptotic response was unclear. Since a variety of regulatory signals from the extracellular environment are critically important in determining cell fate, we and others (26) have hypothesized that opposing signals can arise in some cells, such as forced c-Myc expression in fibroblasts in the absence of serum growth factors, resulting in dysfunctional cell cycle and apoptosis. A similar conflict occurs in our model, one in which Rb activation provides a growth inhibitory signal in direct opposition to growth-promoting signals in proliferating cells. In support of this possibility we found previously that when this conflict was prevented by eliminating Rb function in Rb\(^{2/2}\) cells or by transfection with the Rb inhibitory oncogene, E1a, apoptosis did not occur (see Ref. 25 and Fig. 6C). Although many aspects of PKC regulation of cellular differentiation and development are known, the mechanism by which PKC induces apoptosis in epithelial cells is not completely understood. The theory that defective developmental programs may precipitate some forms of apoptosis substantiates our observations that the rapid hypophosphorylation of Rb in S phase cells does not result in G\(_1\) arrest but leads to a fatal conflict of growth regulatory signals. These findings may begin to address inconsistencies surrounding the functional role of Rb in the regulation of programmed cell death. One view, strongly supported by RB knock-out studies, suggests that Rb may play a protective role in developing tissue by suppressing apoptotic programs (27–30). We believe that Rb also has the capacity to initiate an apoptotic program, which results from conflicting growth regulatory signals in the cell. The results of this study may provide an explanation as to how Rb can regulate cell survival through the induction of G\(_1\) arrest. Alternatively, our results also suggest that Rb can initiate a cell death program when it is hypophosphorylated as cells enter S phase.

Short term PKC activity is necessary for the regulation of cell growth and differentiation (1). However, sustained activation of PKC is thought to have oncogenic activity in various types of adenocarcinomas (47). E-cadherin, whose function is critical for epithelial differentiation, has also been implicated in tumorigenesis, specifically as a metastasis suppressor protein (48). Supporting this hypothesis is the observation that the loss of E-cadherin is a common event in advanced adenocarcinoma of the prostate (34). In the mammary gland the same trend is prevalent; however, late stage or metastatic tumors exhibit some dependence on intercellular adhesion suggesting that the loss or gain of cell-cell adhesion during tumor progression is a regulated and dynamic process (20, 36). Some of the genetic events that occur in prostate tumorigenesis involve
alterations in cell death programs present in the normal, androgen-dependent prostate epithelium. One of the most common genetic alterations associated with prostate cancer is allelic loss at the RB locus as reported in 27–67% of prostate tumors examined (49–51); however, the pathobiological role of Rb in this disease has not been elucidated.

Tumor suppression is achieved not only by cell cycle arrest but by the initiation of cell death programs as well. The role of the p53 tumor suppressor gene product in apoptosis has been extensively documented in cells that are responding to DNA-damaging agents, chemotherapeutic agents, or in cells that have a deregulated cell cycle (reviewed in Ref. 1). Although Rb has been shown to inhibit multiple p53-dependent apoptotic pathways (1, 23), accumulation of hypophosphorylated Rb leading to G1 arrest and apoptosis occurs in multiple p53-independent pathways as well (36, 37). Two such p53-independent pathways are induced by release of the lipid second messenger ceramide (38, 39) or by DNA-damaging agents (40), both of which result in the accumulation of hypophosphorylated Rb, G1 arrest, and apoptosis. We have examined the contribution of p53 to apoptosis in the LNCaP model and have found that although p53 protein is rapidly induced 30–40-fold over basal expression following UV irradiation, there is absolutely no induction of p53 following PKC activation.3 Coupled to the observation that apoptosis occurs normally in androgen-dependent prostate epithelium of p53(−/−) mice following castration (41) indicated that apoptosis in prostate epithelium is a p53-independent process. The ability of epithelial cells to survive and proliferate in the absence of extracellular contact is critical for tumor progression. We postulate that E-cadherin functions to monitor extracellular contact, which during differentiation or tumorigenesis may control contact-independent growth of autonomous cells by inducing apoptosis. Thus, the inactivation of the E-cadherin/Rb apoptotic pathway may be a critical regulatory control that is lost during the metastatic progression of epithelial tumors.

Hormone ablation, by surgical or pharmacological castration, induces rapid involution of the prostate gland. This process involves extensive remodeling of the ductal architecture that regresses to an atrophic or underdeveloped state. The predominance of apoptotic cells observed in the luminal compartment 3–4 days following castration indicates that this regression results from programmed cell death of the luminal epithelium. The first visible stage of individual cell death is the loss of intercellular adhesion as the desmosomal contacts between the epithelial cells are lost prior to cytoplasmic and nuclear condensation (17). We investigated whether an E-cad-

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**Fig. 7.** Loss of membrane E-cadherin correlates with Rb hypophosphorylation and apoptosis in the luminal epithelium of the prostate following castration. A, whole prostate protein extracts were prepared from an intact animal and from castrates at the indicated times and analyzed by immunoblot employing a rat E-cadherin-specific monoclonal antibody (E-cad) or by immunoprecipitation and immunoblotting using Rb-specific antibodies (Rb). The immunoprecipitation control distinguishes the hyperphosphorylated (110 kDa) and the hypophosphorylated (105 kDa) species from cycling LNCaP cells for molecular weight comparison. B, 3 μm serial sections of rat ventral prostate gland were obtained from intact animals (a and b) and 72-h castrates (c and d). Immunohistochemical analysis was performed on a single section from the intact (a) and 72-h castrate (c) using a rat E-cadherin-specific monoclonal antibody. The succeeding 3-μm sections from the intact animal (b) and from the 72-h castrate (d) were subjected to TUNEL analysis. Arrows indicate individual apoptotic cells (d), and the corresponding E-cadherin membrane staining from the previous 3-μm section (c). Magnification × 1000.

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**Fig. 8.** Schematic representation of aggregation-mediated survival and apoptosis during TPA-induced aggregation and apoptosis. Prostate and mammary epithelial cells undergo dramatic aggregation following activation of PKC. All cells appear to hypophosphorylate Rb in response to PKC activation. Cells that undergo aggregation will arrest in G1 and survive; however, cells that are in S phase when Rb is activated undergo apoptosis.

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3 M. L. Day, X. Zhao, C. J. Vallorosi, C. T. Powell, C. Lin, and K. C. Day, unpublished observations.
herin/Rb-regulated pathway was activated in the prostate gland following castration. Examination of E-cadherin expression and its cellular distribution following castration revealed a dramatic reduction in the junctional membranes prior to apoptosis. Previous studies have reported that the levels of prostatic cyclin D1 and E are reduced following castration (40), suggesting that G1 cyclin-dependent kinase activity is diminished and that Rb is likely in the hypophosphorylated form. This observation that Rb was hypophosphorylated subsequent to the loss of E-cadherin and coincident to the onset of apoptosis during involution of the prostate gland suggested that androgen ablation signals the same apoptotic pathway in epithelial cells as was observed in vitro.

Considering the coordinated regulation between proliferation and cell death that is required for normal growth, it is not surprising that apoptosis is a fundamental component of numerous developmental programs (52). Therefore, it is feasible that cell survival and cell death could be regulated through extracellular and intracellular environment dictate whether an epithelial cell will survive in a quiescent or differentiated state or die in response to this Rb signal.

In conclusion, our data suggest that E-cadherin may play a role in the regulation of homeostasis through a pathway that coordinates cell survival and cell death. Many extracellular growth regulatory pathways terminate with the modulation of Rb activity and the regulation of G1 transit. Although we do maintain that the primary role of Rb is to signal G1 arrest, we also suggest that coordinated communications between the extracellular and intracellular environment dictate whether an epithelial cell will survive in a quiescent or differentiated state or die in response to this Rb signal.

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