Cell Anchorage Regulates Apoptosis through the Retinoblastoma Tumor Suppressor/E2F Pathway*

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Epithelial cells are dependent upon adhesion to extracellular matrix (ECM) for survival. We show that loss of β1 integrin receptor contact with extracellular matrix signals the inhibition of G1 cyclin-dependent kinase activity. Loss of cyclin-dependent kinase activity leads to accumulation of the hypophosphorylated (active) form of the retinoblastoma tumor suppressor protein (Rb). We present evidence that in epithelial cells deprived of matrix contact, the growth suppression signal elicited by hypophosphorylated Rb opposes stimulatory signals from serum growth factors, leading to a cell cycle conflict that triggers apoptosis. This apoptotic pathway is modulated by Bcl-2 through a novel mechanism that regulates Rb phosphorylation. We present evidence that the Rb-dependent apoptotic pathway functions in vivo in the apoptosis of the prostate glandular epithelium following castration.

Adhesion of epithelial and endothelial cells to an extracellular matrix (ECM) transmits signals that suppress apoptosis (for review, see Refs. 1 and 2). In response to decreases in hormone levels in the prostate following castration or in the mammary gland following weaning, proteases are released that disrupt levels in the prostate following castration or in the mammary gland following weaning, proteases are released that disrupt ECM not only provides an adhesive interaction, the cytoplasmic domain transmits signals that regulate growth and prevent apoptosis (5–7). In the absence of ECM contacts, adhesion-dependent cells arrest in the G1 phase of the cell cycle due, in part, to the loss of activity of G1-specific cyclin-dependent kinases (cdks), cyclin E-cdk2 and cyclin D-cdk4/6 (8, 9). Growth arrest also correlates with a dephosphorylation and activation of the retinoblastoma tumor suppressor protein (Rb), a transcriptional repressor that controls transition of cells from G1 to S phase by regulating expression of cell cycle genes (10). The activity of Rb is modulated by G1-specific cdks that phosphatase and inactivate Rb in late G1.

Here, we report that in epithelial cells, the disruption of β1 integrin contact with ECM triggers a loss of G1 kinase activity, resulting in the dephosphorylation (activation) of Rb. We demonstrate that this activation of Rb and its interaction with E2F are required for induction of apoptosis. Additionally, we present evidence that the apoptotic regulator, Bcl-2, can inhibit this apoptosis by regulating the activity of Rb. Finally, we provide evidence that apoptosis of prostate epithelium following castration occurs through this Rb-E2F-dependent pathway.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection Assays, and Western Blot Analysis—LNCaP cells, Bel-2-expressing LNCaP cells, and the DU145 cell line were cultured as described (11, 12). For suspension assays, cells were grown to 75% confluence and detached by trypsinization or by treatment with a blocking anti-β1 integrin antibody (LIA 1/2) followed by culture on polyHEMA (15 μg/ml) (5). Western blots were done as described (13) using anti-Bcl-2 (Dako M857), anti-Rb (Oncogene Science OP28-A; Pharmingen 14001A), anti-p16 (Calbiochem Ab-1), anti-p27 (Calbiochem Ab-2), anti-Bax (Pharmingen 13666E and 13686E), anti-E1a (M. Green, St. Louis University, St. Louis, MO), anti-p21 (Pharmingen 15091A), anti-E2F-1 (KI-20 from K. Helin, Danish Cancer Society, Copenhagen, Denmark), anti-Cyclin D1 (Pharmingen 14726E), and anti-Cyclin E (Santa Cruz C-19). For Rb overexpression assays, 2 μg each of the CD20 expression vector (pCMVCD20) (14) and the Rb or Rb-706 expression vector (15) was transfected into LNCaP cells. Cells were immunostained with rhodamine-conjugated anti-CD20 (Becton-Dickinson), and TUNEL (TdT-mediated dUTP-biotin nick end labeling) activity was detected using fluorescein isothiocyanate-conjugated avidin and biotinylated dUTP. At least 200 CD20(+) cells were examined for TUNEL activity in each assay in three independent experiments. Indicated amounts of pDN-E2F-1 were cotransfected with 1 μg of the pTA-E2F-CAT reporter (16), and CAT activity was determined as described (13). For stable clones, pDN-E2F-1, pCMV-E1a-12S, or pCMV-E1a-12S-928 was transfected into LNCaP cells, and clones were isolated in 500 μg/ml of G418.

Rat Prostate Studies—250-g male Sprague-Dawley rats were castrated, and at the indicated times the ventral prostate was excised, fixed in 10% formalin and embedded in paraffin. Immunohistochemical studies were performed on 5-micron sections overlaid with rabbit affinity-purified polyclonal Rb antibody (SC-50, Santa Cruz). The avidin-biotin-peroxidase method was performed (VectaStain ABC kit; Vector Laboratories), and slides were developed by immersion in 3,3-diaminobenzidine tetrachloride (0.5 mg/ml), containing 0.005% hydrogen peroxide followed by a hematoxylin counterstain. Nonimmune rabbit serum was used as a negative control. Northern blotting for Rb mRNA was done as described (17).

RESULTS AND DISCUSSION

Loss of Matrix Contacts Induces Activation of Rb and Apoptosis—We found that primary cultures of human breast and tracheal epithelial cells, human umbilical vein endothelial cells, mink lung epithelial cells, and an Rb(+) human prostate epithelial cell line, LNCaP (11), all undergo apoptosis after they are detached from matrix, either by trypsinization or by the addition of a blocking anti-β1 integrin adhesion receptor

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1 The abbreviations used are: ECM, extracellular matrix; cdk, cyclin-dependent kinase; Rb, retinoblastoma tumor suppressor protein; TUNEL, TdT-mediated dUTP-biotin nick end labeling.

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antibody (Fig. 1A and results not shown). Apoptosis was confirmed by trypan blue exclusion assay, a positive TUNEL assay, labeling of chromosomal DNA, and nuclear condensation as determined by electron microscopy. These results are consistent with previous studies demonstrating that epithelial and endothelial cells undergo apoptosis when they lose contact with extracellular matrix (1, 5, 6).

Binding of β1 integrin receptors to extracellular matrix ligands not only provides an adhesive interaction, the β1 cytoplasmic tail interacts with cytoskeletal components and protein kinases and transmits signals that regulate cell cycle progression (7, 9). The transition from G1 to S phase is a key regulatory checkpoint in the cell cycle, which is controlled by G1 cyclins. Therefore, we examined expression of proteins that control G1 cdk activity. A rapid decrease in the level of cyclin D1 and cyclin E was observed (within 4 h) in LNCaP cells deprived of matrix contact (Fig. 1B). These cyclin regulatory subunits form active complexes with cdk4/6 and cdk2, respectively, that can hyperphosphorylate and inactivate Rb (10). We also found that the level of the cdk inhibitors p21 (SDI, WAF1, and CIP1) and p27 (KIP1), which block the activity of G1 cyclins (10), increased within 12 h following detachment from matrix (Fig. 1B). In contrast, there was little change in the level of the cdk inhibitor p16 (INK4B and MTS1) (Fig. 1B). The decrease in cyclins coupled with the increase in p21 and p27 suggested a loss of G1 cdk activity in cells deprived of matrix contact. Accordingly, we found that hypophosphorylated (activated) Rb accumulated when LNCaP cells (as well as the other epithelial and endothelial cells) were deprived of matrix contact (Fig. 1B and results not shown). This accumulation of hypophosphorylated Rb followed the rapid decrease in G1 cyclins and the increase in cdk inhibitors. The hypophosphorylated Rb accumulated, apoptotic cells were detected. When LNCaP cells were allowed to reattach, the changes in cyclin and cdk inhibitor expression and the accumulation of hypophosphorylated Rb were reversed (Fig. 1B and results not shown).

Rb Function Is Required for Apoptosis of Matrix-deprived Epithelial Cells—The importance of Rb in this process was suggested by the finding that epithelial cells with a mutated or inactive form of Rb, such as the DU145 prostate epithelial cell line, failed to undergo apoptosis when deprived of matrix contact (Fig. 1A and results not shown). As with Rb(+)-LNCaP cells, DU145 cells appeared to lose G1 cdk activity when detached from matrix (results not shown), suggesting that the signal to decrease cdk activity is sent but these cells do not undergo apoptosis, perhaps because they lack Rb.

Our finding that accumulation of hypophosphorylated Rb may be important for apoptosis in epithelial cells deprived of matrix contact was surprising given that hypophosphorylated Rb has been implicated in the inhibition of apoptosis (18, 19). To test whether Rb as a downstream target of integrin signaling (8, 9) promotes apoptosis, LNCaP cells were transfected with an expression vector for Rb or an inactive mutant, Rb-706. Cells were cotransfected with an expression vector for the B cell protein marker CD20, to identify transfected cells (red). A TUNEL assay (green) was then used to identify apoptotic cells. Arrows identify nuclei; in the Rb transfected cultures, the apoptotic nuclei are yellow due to the red-green overlap. The results are averages of three independent experiments, each in duplicate, and are present with standard deviations. B, TUNEL assay for apoptosis in wild type (wt) LNCaP and LNCaP transfectants following detachment from matrix. Cells were detached from matrix and cultured in suspension for 48 h as in Fig. 1A.
continued to proliferate in suspension (results not shown). Because E1a has cellular targets in addition to Rb, we derived LNCaP cell lines stably expressing an E1a construct containing a point mutation at nucleotide 928, which selectively blocks interaction with Rb without disrupting other E1a interactions or functions (20). E1a-928 did not block apoptosis, suggesting that the anti-apoptotic activity of E1a in epithelial cells is solely the result of inhibiting Rb function. These results are then a second way of demonstrating a role for Rb in inducing apoptosis of epithelial cells that lose matrix contact.

Rb is a transcriptional repressor that is targeted to cell cycle genes through interaction with the E2F family of transcription factors (13, 16). Once Rb is concentrated at the promoter through interaction with E2F, it binds surrounding transcription factors, preventing their interaction with the basal transcription complex, thereby blocking transcription. Previously, it has been demonstrated that deletion of the E2F-1 gene, which prevents the recruitment of Rb to cell cycle genes, leads to a loss of cell cycle control and ultimately to tumor formation (21). LNCaP cell lines were created that stably express a dominant-negative form of E2F-1 (DN-E2F) that can bind DNA but is unable to recruit Rb to the promoter (22). DN-E2F blocked E2F function in transfection assays, and stable expression of DN-E2F prevented apoptosis in epithelial cells detached from matrix (Fig. 2B and results not shown). These experiments then provide a third method of demonstrating that Rb activity is required for inducing apoptosis in epithelial cells that are deprived of matrix contact. Further, the results indicate that a specific interaction between Rb and E2F is required to induce this apoptotic pathway.

Apoptotic Regulators of the Bcl-2 Family Can Determine Sensitivity to Apoptosis Induced by Matrix Deprivation—Our observations along with those of other laboratories suggest that accumulation of hypophosphorylated Rb is a general response of adhesion-dependent cells to the loss of matrix contact. Why do some cells respond to this growth suppression signal by undergoing apoptosis, whereas others do not? It has been shown that fibroblasts deprived of matrix contact accumulate hypophosphorylated Rb but in contrast to epithelial cells do not undergo apoptosis (8, 9). Apoptotic pathways can be regulated by a family of proteins that act as pro-apoptotic (e.g., Bax) and anti-apoptotic (e.g., Bcl-2) modulators (18). We observed an inverse correlation between Bcl-2 expression and sensitivity to apoptosis (Fig. 3, A–C). Little or no Bcl-2 was detected by Western blot in cultured LNCaP cells that undergo apoptosis when deprived of matrix contact (Fig. 3B). Likewise, Bcl-2 is not evident in the glandular prostate epithelium, which undergoes apoptosis following castration (23). However, Bcl-2 levels are high in the castration-resistant basal epithelium and stromal fibroblasts of the prostate gland (stromal fibroblasts from other tissues also express Bcl-2) (23). Additionally, primary cultures of prostate stromal fibroblasts also expressed high levels of Bcl-2 (Fig. 3C).

The ratio of pro- and anti-death Bcl-2 family members is thought to determine whether cells are susceptible to apoptosis (18). In contrast to Bcl-2, the pro-apoptotic protein Bax was expressed in LNCaP cells, and its level did not change when cells were detached from matrix (Fig. 3B). Therefore, the high Bax to Bcl-2 ratio that we observe in the epithelial cells is consistent with susceptibility to apoptosis. We decreased this ratio by using LNCaP clones that stably express Bcl-2 (Fig. 3C) and found that apoptosis was inhibited when Bcl-2-expressing cells were deprived of matrix contact (Fig. 3A). The ratio of pro- and anti-apoptotic proteins may then be one mechanism that determines whether cells will respond to the loss of matrix contact by growth suppression or apoptosis.

How might Bcl-2 inhibit apoptosis in epithelial cells that lose matrix contact? Surprisingly, we found that expression of Bcl-2 in LNCaP cells prevented the accumulation of hypophosphorylated Rb that occurs when epithelial cells are deprived of matrix contact (Fig. 3D). Although Bcl-2 did not affect expression of cdk inhibitors in adherent cells, the levels of p21 and p27 failed to increase when the Bcl-2-expressing cells were detached from matrix (Fig. 3E and results not shown), providing an explanation for why Rb continued to be phosphorylated. We also examined the ability of Bcl-2 to induce hyperphosphorylation of Rb in another set of experiments. When the concentration of serum growth factors is decreased, hypophosphorylated Rb normally accumulates, and the cell growth arrest (10). We found that when parental LNCaP cells were cultured in reduced growth factor medium for 24 h, hypophosphorylated Rb did indeed accumulate and cell growth was arrested; however, under the same conditions, Bcl-2 continued to be hyperphosphorylated in the Bcl-2-expressing LNCaP cells, and the cells proliferated (results not shown). These experiments are then a second method of demonstrating that Bcl-2 promotes hyperphosphorylation of Rb in the epithelial cells. In support of our findings, it has been reported that Bcl-2 can also promote accumulation of hyperphosphorylated Rb in B cells (24).

In contrast to the Bcl-2-expressing LNCaP cells, expression of Bcl-2 in fibroblasts does not appear to prevent accumulation of hypophosphorylated Rb when cells are detached from matrix (Refs. 8 and 9 and results not shown). Bcl-2 can act at multiple points in the apoptotic pathway; in addition to inducing phosphorylation of Rb, Bcl-2 can regulate the activity of transcription factors (i.e., NF-κB and NFAT), regulate an antioxidant pathway, and inhibit interleukin 1β-converting enzyme-like proenzymes (caspases) that ultimately execute the apoptotic process in many cells (8). Therefore, Bcl-2 may block apoptosis of fibroblasts that are deprived of matrix contact through one of these other mechanisms. Alternatively, the intracellular environment in fibroblasts may be such that growth suppression in cells deprived of matrix contact does not initiate a conflicting signal that triggers apoptosis.
Activate of Rb Precedes Apoptosis of Prostate Epithelium—To determine whether an Rb-dependent apoptotic pathway may be responsible for apoptosis of epithelial cells in vivo, we examined expression and activation of Rb in the rat ventral prostate following castration. The glanular epithelium constitutes approximately 85% of the cells in the ventral prostate, and nearly 80% of these cells undergo apoptosis within 7 days following castration (25). In response to decreasing levels of androgen, proteases that digest the epithelial cell extracellular matrix are released (4). These proteases are thought to trigger apoptosis at least in part by digesting the extracellular matrix substrate for glandular epithelial cells (1). We found an increase in apoptotic cells 2 days after castration, and a peak of apoptotic cells was seen by day 4 (Fig. 4 and results not shown). The apoptotic act was essentially complete by day 7. No evidence of apoptosis was seen in stromal fibroblasts or basal epithelial cells. Expression of Rb in the glandular epithelium preceded this apoptosis. Approximately 2% of the prostate glandular epithelium showed expression of Rb in the nucleus before castration. The number of Rb(+) cells increased to 10% at day 1 following castration, 60% at day 2, and 78% at day 4. This increase in Rb was paralleled by an increase in Rb mRNA (results not shown). By day 7 following castration when apoptosis had diminished, Rb was only evident in approximately 2% of the remaining glandular epithelial cells (similar to the pattern before castration), and the expression of Rb mRNA had returned to base line (results not shown). Similar to our results in the LNCaP prostate epithelial cell line that had been deprived of matrix contact (Fig. 1B), it has been reported that the levels of cyclin D1 and cyclin E decrease in the prostate following castration (26), and we found that the level of p27 increases (results not shown). Together, these results suggest that G1 cell activity diminishes following castration and that the Rb that accumulates in the glandular epithelium following castration is likely to be in the hypophosphorylated form. A Western blot of prostate tissue 2 days after castration revealed that Rb was indeed exclusively in the hypophosphorylated form (results not shown). The correlation between expression of hypophosphorylated Rb and apoptosis following castration together with our results in cultured cells suggests that accumulation of hypophosphorylated Rb may be important for signaling a common apoptotic pathway in epithelial cells in vitro and in vivo.

Apoptosis is thought to be a default pathway in cells where opposing or conflicting cell proliferation signals arise. For example in c-myc-induced apoptosis, overexpression of c-myc can initiate such a conflict by promoting entry of cells into the cell cycle when other essential cell cycle growth factors are not present (19), and Rb-mediated growth arrest would be expected to alleviate such a conflict. In contrast, Rb-mediated growth suppression appears to precipitate a conflict in epithelial and endothelial cells that lose matrix contact. This conflict could arise when the Rb growth suppression signal opposes mitogenic signals from serum growth factors that promote progression through the cell cycle. In support of this possibility we found that epithelial cells that are no longer growth factor-responsive due to either serum starvation or growth arrest by contact inhibition are unable to undergo Rb-dependent apoptosis (Fig. 1A and results not shown).

Although Rb has been shown to inhibit multiple p53-dependent apoptotic pathways (18, 19), accumulation of hypophosphorylated Rb and growth arrest in G1 occurs in multiple p53-independent pathways (24, 28). One such p53-independent pathway is induced by release of the lipid second messenger ceramide, which causes accumulation of hypophosphorylated Rb, G1 arrest, and apoptosis (29, 30). Apoptosis occurs normally in the prostate luminal epithelium of p53(−/−) mice following castration, indicating that this process is p53-independent (27).

Therefore, accumulation of hypophosphorylated Rb and G1 arrest appear to have opposing roles in signaling different apoptotic pathways (which appears to be determined at least in part by whether there is a role of p53 in the process). It is likely that the role of Rb is to simply arrest the growth of cells in G1, and it is the intracellular and extracellular environment that dictates how a cell responds to this growth suppressive signal.

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