

# Suppression of apoptosis by basement membrane requires three-dimensional tissue organization and withdrawal from the cell cycle

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Communicated by Melvin Calvin, University of California, Berkeley, CA, December 28, 1995 (received for review October 11, 1995)

**ABSTRACT** The basement membrane (BM) extracellular matrix induces differentiation and suppresses apoptosis in mammary epithelial cells, whereas cells lacking BM lose their differentiated phenotype and undergo apoptosis. Addition of purified BM components, which are known to induce  $\beta$ -casein expression, did not prevent apoptosis, indicating that a more complex BM was necessary. A comparison of culture conditions where apoptosis would or would not occur allowed us to relate inhibition of apoptosis to a complete withdrawal from the cell cycle, which was observed only when cells acquired a three-dimensional alveolar structure in response to BM. In the absence of this morphology, both the G<sub>1</sub> cyclin kinase inhibitor p21/WAF-1 and positive proliferative signals including c-myc and cyclin D1 were expressed and the retinoblastoma protein (Rb) continued to be hyperphosphorylated. When we overexpressed either c-myc in quiescent cells or p21 when cells were still cycling, apoptosis was induced. In the absence of three-dimensional alveolar structures, mammary epithelial cells secrete a number of factors including transforming growth factor  $\alpha$  and tenascin, which when added exogenously to quiescent cells induced expression of c-myc and interleukin- $\beta$ 1-converting enzyme (ICE) mRNA and led to apoptosis. These experiments demonstrate that a correct tissue architecture is crucial for long-range homeostasis, suppression of apoptosis, and maintenance of differentiated phenotype.

During development, acquisition of a differentiated phenotype generally requires that cells switch from a proliferative to a quiescent state. Recently, the cyclin kinase inhibitor p21 has been shown to play a central role in mediating the transition from a proliferative to a differentiated state via its ability to impair cyclin D1 activity and to prevent G<sub>1</sub>/S-phase progression (1, 2). Maintaining this differentiated state would also require cells to down-regulate the expression of factors that positively regulate proliferation, since such signals in combination with a block to cell cycle progression often result in apoptosis. For example, overexpression of c-myc or adenovirus protein E1A, which normally induce proliferation, will result in apoptosis when expressed in the absence of serum or in combination with cell cycle inhibitor p53 (3, 4). Moreover, premature activation of p34<sup>cdc2</sup> or unscheduled expression of cyclins can lead to apoptosis (5–8). Thus the ability to coordinate expression of cell cycle inducer and inhibitors is critical for maintenance of normal tissue homeostasis.

The basement membrane (BM) is well established as a potent mediator of differentiation (9, 10) and both cell cycle progression (11) and quiescence in normal tissues (12–14). Mammary epithelial cells, which actively proliferate during pregnancy, subsequently withdraw from the cell cycle and undergo functional differentiation as they deposit a laminin-

rich BM (10). This laboratory has demonstrated (15, 16) that the intact BM not only supports the differentiated phenotype but also suppresses apoptosis in these cells, whereas absence or degradation of the BM results in loss of differentiated function and apoptosis. Thus, these results suggest that the BM must play a central role in coordinating expression of both positive and negative regulators of the cell cycle and thus protect differentiated cells from undergoing apoptosis. In this study, we have directly assessed the expression of mediators of cell cycle progression and inhibition in mammary epithelial cells cultured in the presence or absence of BM. Furthermore, we investigated how BM components might be responsible for coordinating the expression of cell cycle regulatory genes and subsequently suppressing apoptosis. It appears that a three-dimensional organotypic structure that in culture arises only in the presence of a complete reconstituted BM is required for suppression of apoptosis.

## METHODS

The CID-9 mammary epithelial strain (17) or a homogeneous scp2 epithelial subclone (18) were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium supplemented with insulin (5  $\mu$ g/ml), hydrocortisone (1  $\mu$ g/ml), and prolactin (3  $\mu$ g/ml). The latter cells were used only to confirm the findings with CID-9 cells (data not shown). In some experiments, medium was supplemented with 2% (vol/vol) fetal calf serum (Sigma) as indicated. Culture plates were coated with BM extracellular matrix derived from the mouse EHS tumor (referred to as exogenous BM; ref. 15) or with thick type I rat tail collagen gels, which were subsequently floated as described (19). Alternatively, a 1% soluble BM overlay was added to cultures as described (20).

For northern blot analysis, total RNA was isolated by the method of Chomczynski and Sacchi (21) and 20  $\mu$ g per lane was separated on 1% agarose/formaldehyde gels and transferred to nylon membranes. Blots were hybridized in Hybrisol (Oncor) solution at 42°C and probed with cDNA for c-myc (ref. 22; J. Campisi, Berkeley National Laboratory) mouse p21/WAF-1 (K. Huppi, NIH), cyclin D1 (A. MacAuley, UCSF), or mouse interleukin- $\beta$ 1-converting enzyme (ICE) (J. Yuan, Mass. Gen. Hospital). For western blot analysis, cells were lysed directly in 2 $\times$  Laemmli sample buffer, separated by SDS/PAGE on 5% gels, transferred to nylon membranes (Immobilon, Millipore), and probed with polyclonal rabbit anti-human retinoblastoma protein (Rb), C-15 (Santa Cruz Biotechnology). Antibody binding was visualized with enhanced chemiluminescence (Amersham). Nucleosomal DNA fragmentation, characteristic of apoptotic cell death, was

Abbreviations: BM, basement membrane; Rb, retinoblastoma protein; TGF- $\alpha$ , transforming growth factor  $\alpha$ ; TN, tenascin; ICE, interleukin- $\beta$ 1-converting enzyme.

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assessed after isolation of genomic DNA and electrophoresis through 1% agarose gels and visualization with ethidium bromide (16). Alternatively, DNA integrity was determined *in situ* by using the ApopTag kit (Oncor). DNA synthesis was determined after a 12-h incubation of cells with 10  $\mu$ M BrdUrd followed by fixation in 70% ethanol and staining with anti-BrdUrd kit (Boehringer Mannheim). A 2.4-kb cDNA corresponding to coding exons 2 and 3 of *c-myc* was inserted by blunt-end ligation into the *Not* I site of the op13RSV expression vector, which contains a *lac* repressor binding intron (Lacswitch, Stratagene). CID-9 cells were stably cotransfected with a p3SS vector encoding the *lac* operon repressor protein (Lacswitch, Stratagene) and the op13RSV-*myc* by using the calcium phosphate method (17). The p21 expression vector was constructed by inserting a blunted 0.85-kb cDNA coding region of mouse p21 into the *Not* I site of the op13RSV expression cassette described above and *scp2* cells were transiently transfected overnight by using the calcium phosphate method as described (17). Conditioned medium was collected from CID-9 cells cultured on plastic in serum-free medium and added daily to cells cultured on BM for a period of 72 h. Purified human tenascin (TN) C was purchased from Chemicon and purified transforming growth factor  $\alpha$  (TGF- $\alpha$ ) was a gift from Robert Coffey (Vanderbilt University). Fibronectin was purchased from Sigma.

## RESULTS

**BM Is Required for Cell Cycle Withdrawal and Suppression of Apoptosis in Cultured Mammary Epithelial Cells.** In previous studies, we showed (16) that mammary cells cultured on BM in the absence of serum differentiate and will not undergo apoptosis. We also showed (16) that the ability of BM to suppress apoptosis was not related to factors contained within the BM preparations, as growth-factor-depleted preparations or endogenously deposited BM were also capable of supporting survival of mammary epithelial cells. When cultured in the presence of this exogenous BM, CID-9 cells adopt a three-dimensional alveolar morphology, while cells cultured on tissue culture plastic assume a flattened cobblestone morphology (Fig. 1A). When CID-9 cells are placed on the exogenous BM, they down-regulate expression of *c-myc* and cyclin D1 mRNA, with levels remaining low for at least 120 h after culturing under these conditions (Fig. 1B and C). Consistent with a quiescent state, no hyperphosphorylated Rb was detected in cells cultured on BM (Fig. 1D). Under serum-free conditions, CID-9 cells also express high levels of the cell cycle inhibitor p21. Expression of p21, however, was not affected by the presence or absence of BM (Fig. 1E). In contrast, in the absence of BM, CID-9 cells continued to express high levels of *c-myc* and cyclin D1 mRNA and hyperphosphorylated Rb for at least 120 h, analogous to actively proliferating cells maintained in 2% serum (Fig. 1B-D).

Subsequent labeling with BrdUrd revealed that, despite the persistent expression of markers of G<sub>1</sub> progression and phosphorylated Rb, less than 6% of CID-9 cells cultured without BM were entering S phase 72 h after removing serum (Fig. 2). Moreover, in contrast to actively proliferating cells or cells cultured on BM, between 10 and 15% of the CID-9 cells cultured in the absence of a BM began to apoptose within 96 h after removing serum (Fig. 2). Thus, a combination of high levels of *c-myc* and cyclin D1 arising in the absence of BM, and the expression of p21 observed after removal of serum accompanied apoptosis in these cultures.

To directly determine whether the presence of these conflicting signals for proliferation (*c-myc* and cyclin D1) and growth arrest (p21) contribute to the apoptosis observed in mammary epithelial cells, we ectopically expressed *c-myc* in CID-9 cells by using an inducible RSV-*myc* expression plasmid. CID-9 cells were allowed to become quiescent by cultur-

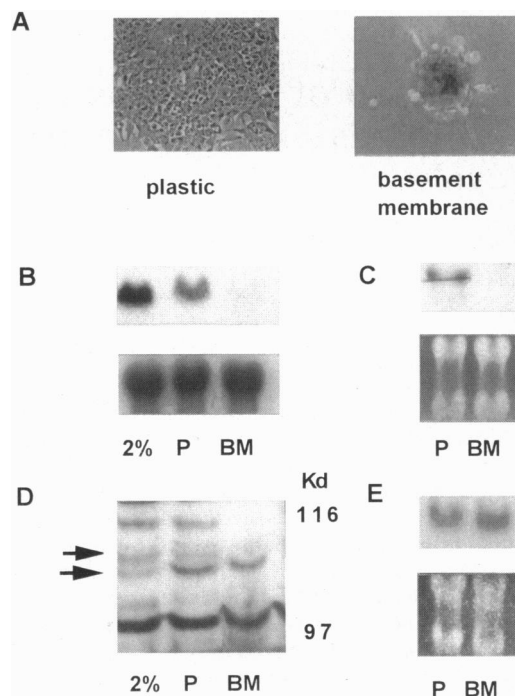


FIG. 1. Expression of cell cycle mediators in the presence and absence of BM. (A) Morphology of CID-9 cells cultured on plastic or on reconstituted BM after 4 days in serum-free medium. (B) Northern blot analysis of *c-myc* expression (Upper) from 20  $\mu$ g of total RNA from CID-9 cells in the presence of 2% serum (2%) or after 100 h in serum-free medium in the presence of exogenous BM or on tissue culture plastic (P). (Lower) Relative expression of 28S rRNA in each of the corresponding lanes. (C) Northern blot analysis of cyclin D1 expression (Upper) in CID-9 cells cultured on BM or tissue culture plastic (P) after 120 h in serum-free medium. (Lower) Ethidium bromide staining of total RNA loaded for each sample in the corresponding gel. (D) Western blot for Rb protein in CID-9 cells cultured in the presence of 2% serum (2%) or after 120 h in serum-free medium on BM or plastic (P). One-fifth of the total cell lysate from  $5 \times 10^5$  cells was separated by SDS/PAGE on 5% gels, transferred to nylon membranes, and incubated with a 1:100 dilution of rabbit polyclonal anti-human Rb antibody. The upper arrow indicates the position corresponding to hyperphosphorylated Rb, and the lower arrow indicates the position of the hypophosphorylated protein. (E) (Upper) Northern blot analysis of p21 mRNA expression in CID-9 cells cultured on BM or plastic (P) after 120 h in serum-free medium. (Lower) Ethidium bromide staining of total RNA loaded for each sample in the corresponding gel.

ing on BM in serum-free medium for 100 h prior to induction of *c-myc*. Forty-eight hours after induction, we observed a marked increase in *c-myc* and cyclin D1 levels (Fig. 3A) that was accompanied by a marked induction of apoptosis in 7–10% of the cells (Fig. 3B). Similarly, we induced p21 expression in proliferating mammary epithelial cells by transfection with an RSV-p21 expression plasmid. A marked increase in apoptotic cells (up to 7%) was observed in the RSV-p21, but not the control, RSV-CAT-transfected cells (Fig. 3C and D).

**Three-Dimensional Tissue Morphology Conferred by the BM Is Required for Cell Cycle Withdrawal and Suppression of Apoptosis.** To determine which BM components may be responsible for suppressing the signals that result in apoptosis, we used a drip technique where a purified solution of a BM component in serum-free medium is added to monolayers of cultures on plastic (20). Despite the fact that this soluble overlay of BM components is sufficient to elicit expression of the  $\beta$ -casein milk protein gene (20), we observed that the cells still underwent apoptosis and continued to express high levels of *c-myc* (Fig. 4A and B). Unlike cells cultured on the reconstituted BM, this method does not support the formation

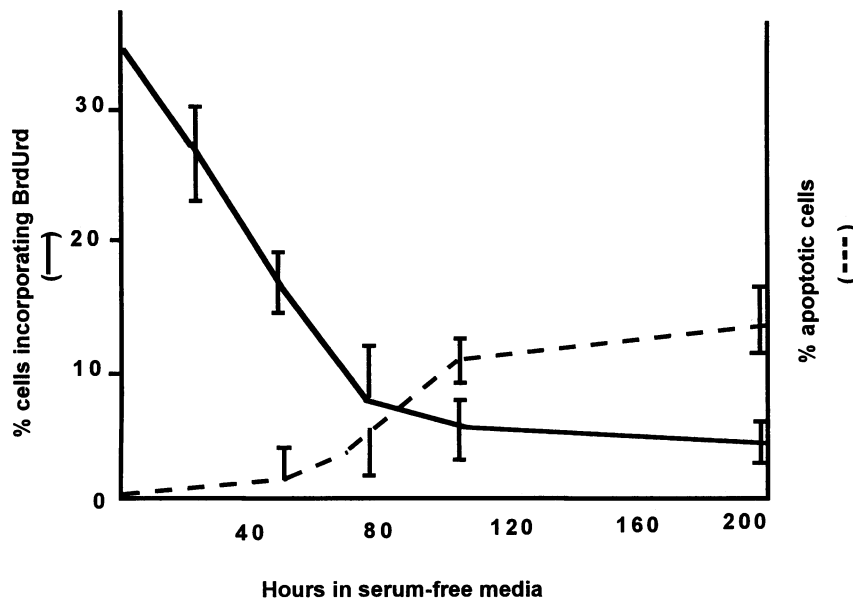


FIG. 2. DNA synthesis and apoptosis in cells lacking BM. Percentage of CID-9 cells incorporating BrdUrd (solid line) or undergoing apoptosis (dashed line) when cultured in the absence of BM at various times after removal of serum. BrdUrd incorporation was assessed after incubation for 12 h with 10  $\mu$ M BrdUrd. The number of apoptotic cells was determined in parallel samples after staining with the ApopTag kit (Oncor). Each point represents the mean  $\pm$  SD for a minimum of four samples.

of three-dimensional tissue-type structures (Fig. 4C). Similarly, when we cultured CID-9 cells on floating collagen gels, another condition where BM components are present but mammary epithelial cells fail to organize into three-dimensional structures, the cells also underwent apoptosis and expressed high levels of c-myc (Fig. 4). Only cells that were cultured on recon-

stituted BM and capable of acquiring a three-dimensional tissue morphology (Fig. 4C) were protected from apoptosis and were capable of down-regulating c-myc expression (Fig. 4A and B).

When a three-dimensional tissue organization is lacking, mammary epithelial cells secrete factors, including TN (23) and TGF- $\alpha$  (24), which in turn, interfere with the expression of milk proteins. To determine whether factors secreted by CID-9 cells lacking a three-dimensional organization contributed to apoptosis, we treated cells cultured on BM with

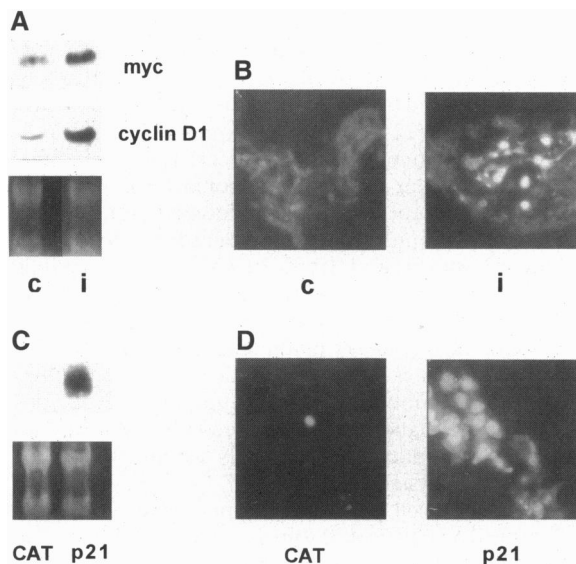


FIG. 3. Disrupting the balance of positive and negative mediators of the cell cycle induces apoptosis. (A) Northern blot analysis of c-myc or cyclin D1 expression in uninduced control (c) or induced (i) CID-9 cells cultured on BM under serum-free conditions. (Bottom) Ethidium bromide staining of total RNA loaded in each of the corresponding lanes. (B) *In situ* detection of fragmented DNA in uninduced control (c) and induced (i) CID-9 cells stably transfected with inducible RSV-myc expression plasmids. (C) (Upper) Northern blot analysis of p21 mRNA expression in scp2 cells cultured in the absence of BM, 48 h after transient transfection with RSV-CAT or RSV-p21 expression plasmids. (Lower) Ethidium bromide staining of total RNA loaded into each of the corresponding lanes. (D) *In situ* detection of fragmented DNA in scp2 cells, 48 h after transfection with RSV-CAT or RSV-p21 expression plasmids.

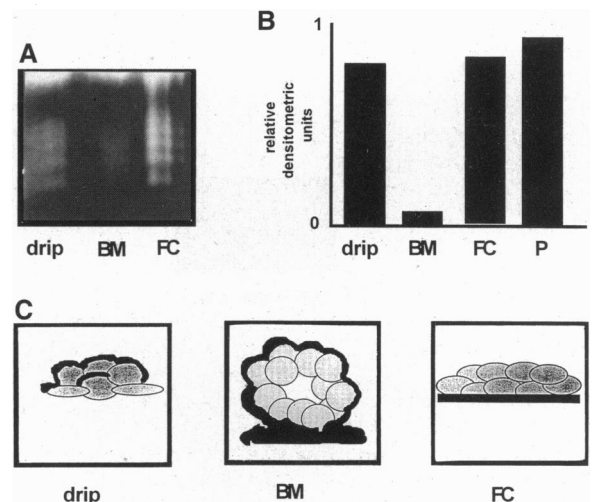


FIG. 4. Correlation between lack of three-dimensional morphology, elevated c-myc expression, and apoptosis in CID-9 cells. (A) Analysis of DNA integrity in CID-9 cells cultured on plastic with a 1% BM solution dripped (drip) or cultured directly on BM or on floating collagen gels (FC). (B) Densitometric analysis of c-myc mRNA levels in CID-9 cells cultured on plastic with a 1% BM solution dripped (drip) or cultured directly on reconstituted BM, floating collagen gels (FC), or plastic alone (P). Levels of c-myc mRNA were normalized to levels of 28S rRNA expression and values are expressed relative to normalized c-myc expression in cells cultured on plastic. (C) Diagrammatic representation of cellular morphology adopted by CID-9 cells when cultured on plastic with 1% BM solution dripped (drip) or cultured directly on reconstituted BM or floating collagen gels (FC).

conditioned medium derived from cells cultured on plastic in serum-free medium or directly with TGF- $\alpha$  (20 ng/ml) or TN (5  $\mu$ g/ml). Each of these treatments resulted in an induction of DNA fragmentation characteristic of apoptosis (Fig. 5A). TGF- $\alpha$  has previously been shown to induce c-myc and cyclin D1 mRNA in quiescent mammary epithelial cells (25) and like TGF- $\alpha$ , addition of conditioned medium or purified TN also resulted in a significant upregulation of c-myc expression (Fig. 5B). Each of these treatments was also accompanied by the induction of ICE mRNA expression (Fig. 5C; ref. 15). In contrast to TN and TGF- $\alpha$ , addition of purified fibronectin (5  $\mu$ g/ml), which is also produced at high levels by cells lacking BM (19), did not induce expression of c-myc, ICE mRNA, or apoptosis in cells cultured on BM (data not shown).

## DISCUSSION

Deregulation of genes involved in cell cycle progression, including aberrant cyclin expression or forced overexpression of c-myc in the absence of serum, results in apoptosis in a variety of cultured cell types including fibroblasts (4), neurons (6), and lymphocytes (8). These findings have suggested that signals that promote cell proliferation, in combination with conditions that favor growth arrest, create a conflict resulting in programmed cell death. Our data show that in mammary epithelial cells, the BM is necessary to down-regulate expression of c-myc and cyclin D1 and to suppress apoptosis under conditions that promote differentiation. Although previous

studies have demonstrated that the BM is necessary to suppress expression of positive proliferative signals (13, 14), the impact of this process on cell survival was not addressed. Furthermore, we show that the formation of three-dimensional tissue-type structures induced in the presence of BM is necessary to suppress apoptosis and also to suppress expression of factors such as TGF- $\alpha$  and TN, which can act to induce expression of c-myc and cyclin D1.

Although our studies have only addressed the role of cell cycle regulation and its relationship to apoptosis in cultured cells, it is likely that a similar phenomenon also occurs *in vivo* during involution of the mammary gland. Involution, which is initiated by proteolytic degradation of the existing BM (26) and culminates in large-scale apoptosis, is accompanied by up-regulation of both positive and negative regulators of cell cycle including c-myc, p53, and TGF- $\beta$  (27). Furthermore, dissolution of alveolar structures during normal involution or through premature expression of stromelysin 1 in transgenic mice is also accompanied by up-regulation of TN (ref. 23, and N. Thomasset, Z.W., and M.J.B., unpublished results). We now show that TN, like TGF- $\alpha$  (25), can also induce expression of c-myc in quiescent cultured mammary epithelial cells.

Marti *et al.* (28) have reported a marked induction of AP-1, the transcriptional activator composed of the products of the immediate early response genes *jun* and *fos*, during mammary gland involution. Preliminary observations in our laboratory have noted that AP-1 levels are directly suppressed in the presence of BM in culture (C. Roskelley and M.J.B., unpublished results). Whether high levels of AP-1 contribute to the expression of genes that directly or indirectly activate the apoptotic machinery is not known. However, both in culture and *in vivo*, the presence of the BM and subsequent three-dimensional organization of cells appear necessary to coordinately down-regulate the expression of genes contributing to proliferation and apoptosis and simultaneously induce expression of genes associated with the differentiated phenotype.

The ability of the BM to suppress expression of positive proliferative stimuli and to maintain the differentiated phenotype has profound implications in the BM's role as a putative tumor suppressor (12). Direct overexpression of cyclin D1 in mouse mammary tumor virus-cyclin D1 transgenic mice results in development of adenocarcinoma (29). We have observed a high incidence of adenocarcinoma in transgenic mice where mammary epithelial BM is degraded through overexpression of stromelysin 1 (refs. 30 and 31 and unpublished results). Thus, since precise regulation of cell cycle genes including cyclin D1 (32) is essential for proliferation and development of mammary epithelium, the ability of BM to regulate these genes would be critical in maintaining the differentiated phenotype and preventing hyperplastic growth in the mammary gland. We have indeed observed that malignant human mammary epithelial cells are unable to perceive or respond to BM signals (12, 33).

How a normal cell perceives inappropriate growth signals during culture on plastic or during involution and subsequently activates the genes that make up the apoptotic machinery, and how the BM keeps such machinery in check remain intriguing questions and are under investigation.

We thank Peter Jones and Connie Myers for many helpful discussions and preparation of the figures. This work was supported by the Office of Health and Environmental Research, U.S. Department of Energy (Contracts DE-AC03-76-SF00098 and DE-AC03-76-SF01012) and by grants from the National Cancer Institute (CA 57621), the National Institutes of Health (8744), and a fellowship to N.B. from the Medical Research Council of Canada.

**Note.** While this manuscript was under review, Sheikh *et al.* (34) reported that overexpression of p21 induces apoptosis of human breast carcinoma cell lines.

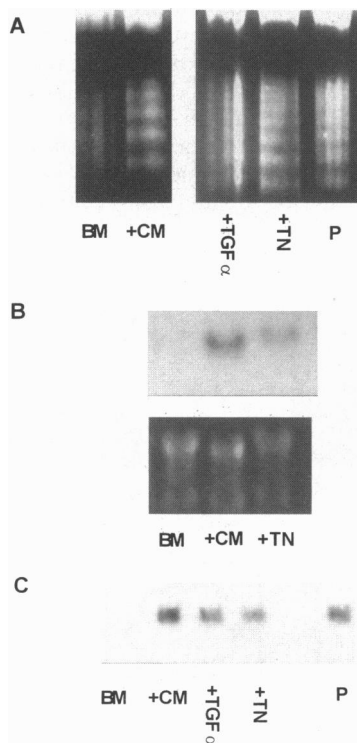


FIG. 5. Factors secreted in the absence of three-dimensional tissue-like morphology induce apoptosis. (A) Analysis of DNA integrity in cells cultured on BM alone (BM) or on BM after addition of conditioned medium from cells cultured on plastic (CM) or after addition of TGF- $\alpha$  (20 ng/ml) or TN (5  $\mu$ g/ml) or on plastic alone (P). (B Upper) Northern blot analysis of c-myc RNA expression in CID-9 cells cultured on BM alone (BM) or on BM after addition of conditioned medium (CM) or TN (5  $\mu$ g/ml) for 72 h. (B Lower) Ethidium bromide staining of total RNA loaded into each of the corresponding lanes. (C) Northern blot analysis of ICE mRNA expression in cell cultured in BM alone (BM) or on BM after the addition of conditioned medium (CM), TGF- $\alpha$  (20 ng/ml) or TN (5  $\mu$ g/ml) or cultured on plastic alone (P).

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