Regulation of Programmed Cell Death by Basement Membranes in Embryonic Development

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Abstract. The formation of the proamniotic cavity in the mammalian embryo is the earliest of many instances throughout development in which programmed cell death and the formation of epithelia play fundamental roles (Coucouvanis, E., and G.R. Martin. 1995. Cell. 83:279–287). To determine the role of the basement membrane (BM) in cavitation, we use embryoid bodies derived from mouse embryonic stem cells in which the LAMC1 genes have been inactivated to prevent BM deposition (Smyth, N., H.S. Vatansever, P. Murray, M. Meyer, C. Frie, M. Paulsson, and D. Edgar. 1999. J. Cell Biol. 144:151–610). We demonstrate here that LAMC1−/− embryoid bodies are unable to cavitate, and do not form an epiblast epithelium in the absence of a BM, although both embryonic ectodermal cells and extraembryonic endodermal cells do differentiate, as evidenced by the expression of cell-specific markers. Acceleration or rescue of BM deposition by exogenous laminin in wild-type or LAMC1−/− embryoid bodies, respectively, results in cavitation that is temporally and spatially associated with restoration of epiblast epithelial development. We conclude that the BM not only directly regulates development of epiblast epithelial cells, but also indirectly regulates the programmed cell death necessary for cavity formation.

Key words: organogenesis • extracellular matrix • laminin • apoptosis • stem cells

Introduction

The formation of cavities in solid blocks of cells is a widespread event in organogenesis throughout embryonic development. Over the last decade, it has become apparent that programmed cell death (PCD)1 plays a fundamental role in cavity formation in many tissues (Coles et al., 1993; Coucouvanis and Martin, 1995; Humphreys et al., 1996; Jacobson et al., 1997). Although it is known that basement membranes (BM) are necessary for the survival and differentiation of epithelial cells surrounding the cavities (Ekblom et al., 1980; Coucouvanis and Martin, 1995; Streuli, 1996), any involvement of BMs in the regulation of PCD and the mechanisms coordinating epithelialization with PCD during cavitation remain unknown.

Formation of the proamniotic cavity is the first instance of cavitation during mammalian development. Shortly before implantation, the inner cell mass (ICM) of the mouse embryo consists of a small group of cells separated from an outer layer of primitive endoderm by a BM (Salamat et al., 1995). Subsequently, the primitive endoderm cells remaining in contact with this BM differentiate to become visceral endoderm (VE), while the remaining ICM cells differentiate to become the epiblast, or primitive ectoderm (see Fig. 1). Initially, the differentiation of epiblast cells is reflected by an alteration in the profile of expressed genes, and is not accompanied by any obvious morphological differentiation (Kaufman, 1992; Rathjen et al., 1999). However, a few hours later, the epiblast cells in contact with the BM become polarized to form the columnar epiblast epithelium (CEE), while cells at the center of the ICM undergo PCD, thereby giving rise to the proamniotic cavity (Coucouvanis and Martin, 1995).

Embryoid bodies (EBs), which are derived from differ-
Materials and Methods

ES Cell and EB Culture

The production of R1 mouse LAMC1+/− and LAMC1−/− ES cells has been described in detail previously (Smyth et al., 1999). The LAMC1−/− ES cells, used here as controls, were from the clone previously used to produce healthy heterozygous germine animals (Smyth et al., 1999). The absence of clonal artefacts in the LAMC1−/− cells used here was confirmed by rescue of the phenotype by adding laminin type-1 (Sigma Chemical Co.) to developing LAMC1−/− EBs (see Fig. 4). ES cells were cultured on mitomycin-treated STO feeder cells in gelatinized 3.5-cm tissue culture dishes. The culture medium was DMEM (GIBCO BRL) supplemented with 15% (vol/vol) ES grade FBS (GIBCO BRL), 0.1 mM β-mercaptoethanol, 1 mM µ-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 1,000 U/ml of LIF (ESGRO; GIBCO BRL). ES cells were subcultured every 2 d. Before EB formation, ES cells were passaged once on gelatinized tissue culture dishes and incubated in the above medium for 2 d to eliminate STO cells. To make EBs, ES cells were trypsinized, triturated, and split 1:10 by replating into bacterial petri dishes. For analysis of such events, cell differentiation in EBs and an equal number of LAMC1−/− EBs were selected using phase-contrast microscopy. α-Feto protein (AFP) primers were those used for riboprobe synthesis (see below), and BMP4 and FGF-5 primers were as described previously (Johanson and Wiles, 1995). GAPDH primers were as follows: forward (5′-GGTTGAAAGGGGACGTCAACGG-3′) and reverse primer (5′-GGTCATGAGTCTCCTCCAGAT-3′; product size, 520 bp). Semi-quantitative RT-PCR was performed as previously described to determine mRNA levels relative to that of GAPDH (Squitti et al., 1999).

Whole-mount In Situ Hybridization

A sequence containing nucleotides 309–770 of mouse AFP cDNA (Tilghman et al., 1979) was amplified by PCR with forward primer (5′-ACATCATGAATTGCTGGAG-3′) and reverse primer (5′-AGCGAGTTCTCCTTGGCAAACAC-3′), from cDNA reverse-transcribed from total RNA extracted from day 10 EBs. The PCR fragment was cloned into the T-Easy™ Vector (Promega) and transcribed with T7 or SP6 and digoxigenin-UTP for sense or antisense probes. Whole-mount in situ hybridization was performed as previously described (Leibl et al., 1999).

Results and Discussion

Fig. 1 shows a schematic diagram of the organization of cells and BM during the periimplantation stages of mouse development.

Cavitation Fails in LAMC1−/− EBs Despite the Presence of VE Cells

Histological analysis of LAMC1−/− control EBs, which are able to synthesize BMs and have a wild-type phenotype (Smyth et al., 1999), showed that they cavitated in suspension culture as expected (Fig. 2 a). In contrast, the...
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dermal cells in contact with the BM differentiate to become visceral endoderm (VE) cells, and the remaining ICM cells differentiate to become epiblast cells. (d) The epiblast cells in contact with the BM polarize to form the columnar epiblast epithelium (CEE), and the unpolarized epiblast cells in the center undergo programmed cell death, giving rise to the proamniotic cavity.

**Figure 1.** Schematic diagram showing the peri-implantation stages of mouse development. (a) Shortly after blastocyst formation, the cells on the surface of the ICM differentiate to become primitive endodermal cells. (b) The primitive endodermal cells deposit a basement membrane (BM). (c) After implantation, the primitive en-

**Figure 2.** VE cell differentiation and cavitation in EBs. (a and b) Toluidine blue-stained frozen sections of EBs after 7.5 d in suspension culture show that LAMC1+/- EBs had cavitated by this time (a), whereas the LAMC1-/- EBs failed to cavitate (b). (c and d) EM shows differentiation of cells with the morphological characteristics of VE in both LAMC1+/-(c) and LAMC1-/- (d) EBs. However, a BM is only present in the LAMC1+/+- control EBs (c, white arrow). (e and f) Whole-mount in situ hybridization for the VE marker AFP shows positive cells at the periphery of both LAMC1+/-(e) and LAMC1-/- (f) EBs. (g and i) RT-PCR analysis; GAPDH mRNA is shown as a loading control. (g) AFP mRNA is expressed late in both LAMC1+/+ and LAMC1-/- EBs; (h) Only a trace of FGF-5 mRNA is detectable in undifferentiated LAMC1+/- ES cells (U) and day 2 (d2) EBs, but FGF-5 is induced by day 10 (d10) in both LAMC1+/+ and control EBs; the double band results from splice variants (Johnsson and Wiles, 1995). (i) BMP4 mRNA is expressed in both LAMC1+/+ and control EBs at day 2; but, by day 10, levels are greatly reduced in the controls. VEs, visceral endoderm.
Additionally, whole-mount in situ hybridization showed that the VE marker AFP (Dziadek and Adamson, 1978) was expressed in some of the peripheral cells of both LAMC1−/− and LAMC1+/− control EBs (Fig. 2, e and f). Semi-quantitative RT-PCR was used to demonstrate that the relative levels of AFP mRNA were similar or even somewhat higher in the LAMC1−/− EBs than in controls while being absent in undifferentiated EBs (Fig. 2 g). Taken together, these results indicate that the BM, while having no apparent effect on VE cell differentiation, is necessary for the previously reported regulation of PCD by endodermal cells (Coucouvanis and Martin, 1995).

**Epiblast Cell Differentiation in LAMC1−/− EBs**

Several lines of evidence have been presented suggesting that VE regulates epiblast cell differentiation. For example, disruption of the VE-specific gene Evx1 inhibits epiblast cell differentiation (Spyropoulos and Capecchi, 1994), and factors expressed by an endodermal cell line can induce the differentiation of ES cells to an epiblast-like cell population in vitro (Rathjen et al., 1999). Given the need for a BM to obtain cavitation of the epiblast, we wished to determine whether the BM was necessary for all aspects of epiblast differentiation, or, alternatively, if the role of the BM was more restricted to being a requirement for polarization of CEE cells. Therefore, RT-PCR was used to determine the relative mRNA levels of FGF-5, a gene that is not expressed in the undifferentiated ICM cells before implantation, but subsequently is turned on in epiblast cells just before they become polarized to form the CEE (Haub and Goldfarb, 1991). The results show that the profile of FGF-5 expression in EBs mimics that seen in vivo; only trace amounts of FGF-5 mRNA were present in undifferentiated LAMC1+/− ES cells and at early time points during EB differentiation, whereas the levels increased at later time points (Fig. 2 h). BMP4 mRNA levels were also investigated, as this signaling molecule is normally expressed in early epiblast cells before cavitation, but not in the CEE, and has been implicated in the PCD observed in EC cell-derived EBs (Coucouvanis and Martin, 1999). We found that while BMP4 mRNA levels were initially similar in LAMC1−/− and control LAMC1+/− EBs, the levels were maintained in the LAMC1−/− EBs, whereas they were markedly reduced in the control EBs that had cavitated by this time (Fig. 2 i). The maintenance of BMP4 mRNA expression in the LAMC1−/− EBs, together with the FGF-5 data, indicates...
that the BM has no apparent effect on initial epiblast cell differentiation. The development of epiblast cells is considered to be an obligatory intermediate step in the differentiation of ES cells into embryonic cell lineages (Rathjen et al., 1999). Thus, the fact that myoblasts, endothelial and neuronal cells can differentiate from LAMC1−/− ES cells (Smyth et al., 1999) also indicates that the BM is not required for at least some aspects of epiblast cell differentiation, although it is necessary for the polarization of cells to form the CEE.

**Relationship between PCD, Epiblast Cell Polarization and BMs**

In control LAMC1+/− EBs, we found that the first stage of cavitation involved a loss of cell–cell contact between the polarized CEE cells and the cells positioned at their apical surface (Fig. 3 b). Subsequently, small pockets of cell debris could be identified at the apical surface of the CEE (Fig. 3 c), and, finally, a cavity became evident as the debris was phagocytosed by the cells of the CEE (Fig. 3 d). During cavitation, cell debris was restricted to the apical surface of the CEE, and was never observed in the vicinity of the unpolarized epiblast cells (Fig. 3 a). TUNEL analysis of control and age-matched LAMC1−/− EBs showed that whereas only randomly scattered TUNEL-positive cells were present in the LAMC1−/− EBs (Fig. 3 h), clusters of TUNEL-positive cells were observed exclusively at the apical surface of the CEE in control EBs (Fig. 3 f). Thus, there is a precise correlation between the development of the CEE and PCD.

To demonstrate that the BM was responsible for both the PCD and polarization of the CEE cells, the mutant phenotype of the LAMC1−/− EBs was rescued by the addition of exogenous laminin type-1. This resulted in the deposition of a BM-like sheet defined by anti-laminin type-1 and antiperlecan immunoreactivity between the outer endoderm and inner core cells of LAMC1−/− EBs (Fig. 4, a–d). In addition, CEE cells were found aligned on this sheet, and cells at the apical surface of the CEE cells had either detached or had undergone PCD, thereby forming a cavity (Fig. 4 e). The indirect effect of the BM on the PCD in the epiblast indicates either that the CEE is responsible for inducing PCD of those cells positioned at its apical surface, or, alternatively, the PCD was induced by a VE cell-derived molecule with restricted diffusion and whose synthesis was dependent upon contact of VE cells with the BM. To decide between these alternative hy-
hypotheses, we made use of the observation that a complete BM is not observed in wild-type LAMC1+/− EBs until after day 4 of differentiation (results not shown). However, by adding exogenous laminin at the start of differentiation, the rate of BM deposition was accelerated so that a BM was evident by day 2 of differentiation (Fig. 5, a–d). This observation supports our previous conclusion that laminin expression is the rate-limiting step in BM deposition (Smyth et al., 1999). In addition, histological analysis of these EBs showed that the early deposition of a BM-like sheet was accompanied by premature CEE formation and the initiation of cavitation (Fig. 5 f). However, the expression of AFP was unaffected by the absence of a BM (Fig. 2 f) and did not appear prematurely in laminin-treated EBs (results not shown). Thus, although the differentiation of the mature VE phenotype occurs independently of the BM, the PCD of epiblast cells is closely linked to the differentiation of CEE cells, which in turn is dependent upon a BM.

Conclusions

Our results demonstrate a novel BM-dependent mechanism for the coordination of cellular events leading to cavitation. First, extra-embryonic endodermal cells deposit a BM and also induce undifferentiated ICM cells to become epiblast cells (Spyropoulos and Capel, 1994; Rathjen et al., 1999). This induction is independent of the BM as it also occurs in LAMC1−/− EBs. Second, the epiblast cells in contact with the BM become polarized to form the CEE, the survival of which is then dependent upon contact with the BM (Cavoukianis and Martin, 1995). Finally, unpolarized epiblast cells that lie at the apical surface of the CEE undergo PCD, thereby forming the cavity. Our data show for the first time that laminin (and consequently the BM) could be both a death signal (acting indirectly) and a survival signal (acting directly). While the mechanism responsible for this indirect BM-dependent PCD remains to be determined, the fact that it is observed directly at the apical surfaces of newly polarized CEE cells is consistent with the involvement of a CEE-derived factor whose diffusion is highly restricted or, alternatively, a cell–cell contact phenomenon within the epiblast.

During organogenesis in later development, cavity formation occurs in many tissues including the exocrine glands (Hieda and Nakanishi, 1997), lungs (Schuger et al., 1995), mammary glands (Humphreys et al., 1996) and kidneys (Coles et al., 1993). In the submandibular gland and lung, both initially derived from solid masses of cells, there is a strong association between development of a continuous BM, epithelialization, and cavity formation (Schuger et al., 1995; Hieda and Nakanishi, 1997). Although the mechanism of cavity formation has not been investigated in most cases, PCD followed by phagocytosis of cell debris by the epithelial cells recently has been demonstrated in mammary gland (Humphreys et al., 1996) and kidney development (Coles et al., 1993). Therefore, it is likely that the ability of BMs to coordinate both epithelialization and cell death is used throughout development whenever a lumen or cavity is to be created from a solid structure.

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