Occasional articles

Review

Use of basement membrane markers in tumour diagnosis

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Introduction

Basement membranes are complex structures composed of a mixture of collagenous and non-collagenous glycoproteins and proteoglycans.[1-5] Their molecular composition and supramolecular arrangement differ in different sites according to functional requirements. Basement membranes of epithelia and endothelia can be visualised as continuous limiting plates or tubes separating different body compartments. In the vascular system basement membrane continuity is interrupted at only a few sites, notably the sinusoids of spleen and liver. Basement membrane components in splenic sinusoids are the structurally specialised ring fibres. In hepatic sinusoids only small amounts of basement membrane material are present in the space of Disse; no organised basement membrane is present. Elsewhere in the vascular system specialised filtering and diffusion properties are conferred by fusion of endothelial basement membranes with those of adjacent epithelia. This occurs in renal glomeruli and pulmonary alveoli. Similar fusion occurs in capillary basement membranes of the central nervous system.[1 3]

Basement membranes in mesenchymal tissues have another type of arrangement. In smooth muscle and adipose tissue individual cells are enveloped in basement membrane. Schwann cells in nerves are similarly wrapped, providing continuous tubes through which the nerve fibres course.[1 3] Individual fibres of striated muscle are likewise surrounded by a basement membrane sheath. Notably, fibroblasts, the potentially mobile cells of mesenchyme, do not have a basement membrane. The mobile cell populations of lymphoreticular and haemopoietic systems and cells in cartilage and bone also lack this structure.

Several studies have indicated the importance of basement membranes in orderly tissue regeneration

and repair.[1] For example, an intact basement membrane is necessary for regeneration of renal tubular epithelium following acute tubular necrosis and for repair of epidermis after skin damage. In the peripheral nervous system nerves can regenerate along the basement membrane tubes in which they are normally enveloped; neurons in the central nervous system do not have this capacity. Basement membranes are also important in embryogenesis for orderly cell migration.[5] Interactions between cells and basement membranes occur, at least in part, via specific cell surface receptors for different basement membrane constituents. These extracellular proteins influence cell morphology and differentiation as well as promoting cellular adhesion and movement.[5-11]

Ultrastructurally, basement membranes are characterised by the presence of basal lamina. This is further subdivided into lamina densa and lamina lucida. The lamina densa, sometimes referred to as the basement membrane proper, consists of tightly matted randomly orientated fibrils 3–4 nm in diameter, embedded in a dense matrix[1]; lamina lucida is an electron lucent zone between plasma membranes and lamina densa, sometimes traversed by small filaments (figure).[1] In sites where fusion of two basement membranes has occurred lamina lucida is present on both sides of lamina densa—for example, in the renal glomerulus that on the epithelial side is the "lamina rara externa", and that on the endothelial side is the "lamina rara interna".

Conventional methods of staining basement membranes, such as periodic acid Schiff (PAS) and reticulin, may stain not only basal lamina but also structures external to this. For example, many basement membranes have an outer fibrillar "extrinsic" layer known as lamina reticularis (figure).[5] By light microscopy it is not possible to resolve these different components. Immunohistological methods show that antibodies directed against constituents of
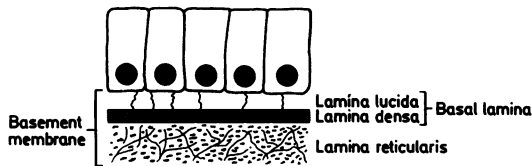
Figure *Diagrammatic representation of an epithelial basement membrane.*

any of these structures will give rise to light microscopic basement membrane delineation.

Extraction and identification of basal lamina proteins was initially hampered by the difficulty of obtaining pure basal lamina. The unique basement membrane collagen (type IV) was first extracted from glomerular basement membranes that lack an outer lamina reticularis.[12] Purification of other basal lamina constituents was facilitated by the culture of tumour cell lines producing basal lamina in excess, notably the Engelbreth-Holm-Swarm (EHS) sarcoma[13] and teratocarcinoma cells.[14]

Principal constituents of basal lamina include type IV collagen,[15] the high molecular weight glycoprotein laminin,[16] and proteoglycans,[17 18] principally heparan sulphate proteoglycan. Entactin[14] and nidogen[19] are further basal lamina proteins. These are probably related if not identical molecules.[20] The adhesive glycoprotein fibronectin is present in lamina reticularis as well as in some, if not all basal laminae, in association with cell surfaces.[21-24]

The principal fibrillar components of lamina reticularis are interstitial collagens types I and III.[5 20 25 26] Additional collagenous molecules associated with some but not all basement membranes are collagens types V, VI, and VII.[25-31] Collagen type VII is probably a principal constituent of anchoring fibrils which link the basal lamina of skin and other stratified epithelia to underlying connective tissue.[32]

The heterogeneous composition of basement membranes has been shown by production of monoclonal antibodies with selective reactivity for basement membranes in different sites.[33 34] In many cases the underlying biochemical basis for this heterogeneity is not known. It may be partly attributable to differing extrinsic components. Further evidence for heterogeneity is provided by diseases such as Goodpasture's disease and pemphigoid, in which anti-basement membrane autoantibodies are restricted to specific locations.[35 36]

BASEMENT MEMBRANES IN TUMOURS

A property fundamental to malignant disease is invasiveness. It might seem that the first barrier a carcinoma must transgress before infiltrating surrounding tissues is the basement membrane.[37] Consequently many studies have investigated this possibility. Early investigations used staining techniques such as PAS and reticulin.[38] The advent of the electron microscope permitted detailed ultrastructural examination of basal lamina in tumours and inflammatory conditions.[39-43] More recently, biochemical characterisation and production of antibodies against different basement membrane constituents has meant that application of immunohistological techniques could be applied to the problem. This has the advantage over ultrastructural analysis that much larger volumes of tissue can be rapidly sampled and examined.

Both ultrastructural and immunohistological studies have shown that in general there is loss and fragmentation of basal lamina in malignant tumours of both epithelial and mesenchymal origin.[39-61] Reduplications may also be seen. Interpretation of these phenomena has differed. It is clear that they reflect abnormal turnover of basement membranes due either to increased breakdown or decreased synthesis of their constituents.[62-66] Less clear is the importance of these changes to tumour behaviour. They might represent the primary event in invasion.[63] Alternatively, they may simply be an epiphenomenon reflecting abnormal cellular differentiation.[67-69] The distinction has an important bearing on the use of basement membrane markers in tumour diagnosis.

Yet another interpretation of the importance of basement membrane constituents in neoplasia is that substances such as laminin are actually necessary to promote invasion and metastasis.[70] This concept originated from experiments on transplantable murine tumours[71]: the ability of some tumours to metastasise is related to their ability to secrete or bind to laminin.[71-74] An important factor may be whether they possess unoccupied laminin receptors.[74-76] These might help tumour cells to bind to basement membranes of vessels and those at distant sites, as well as to laminin produced by the tumour cells themselves.

The purpose of this review is to discuss whether basement membrane immunohistological analysis can be used to determine (*a*) if a tumour is invasive, (*b*) to distinguish malignant tumours from benign "lookalikes", (*c*) to determine tumour prognosis and (*d*) to determine tumour histogenesis.

CHOICE OF MARKER

For immunohistological study of basal lamina it is preferable to use an antibody directed against a protein which is ubiquitous in this structure and which is confined to this structure. It should also be possible to show that immunohistological delineation of basal lamina at light microscopy correlates with distribution of basal lamina observed ultrastructurally. Antibodies

against type IV collagen and against laminin for the most part seem to fulfil both these criteria. In tumours, however, it is sometimes possible to detect small amounts of extracellular immunoreactive laminin where no basal lamina can be detected ultrastructurally.[77] This may represent secreted protein which has not become organised into a macromolecular structure. Alternatively, it may simply reflect the sampling problems inherent in ultrastructural examination of tissue. It is also sometimes possible to show the presence of intracellular laminin, presumably reflecting synthetic activity of a cell.[44 78]

Immunohistological demonstration of fibronectin is less suitable for investigating basal lamina changes per se as this protein is not confined to this structure. Observation of changed distributions of fibronectin and other basement membrane and stromal proteins may nevertheless prove of interest in their own right.[79 80]

METHODS
The most reliable methods of showing the presence of basement membrane proteins in tissue sections entail the use of fresh frozen tissue or tissue which has been fixed in ethanol before processing in paraffin wax (Sainte Marie method).[81] Type IV collagen, laminin, and fibronectin, however, can usually be effectively shown by immunohistological techniques in enzymatically digested formalin fixed, paraffin wax embedded tissue.[82–86] This is an obvious advantage if fresh or specially prepared tissue is unavailable. Digestion is essential and choice of enzyme is important. For reasons that remain obscure, trypsin and some brands of commercial protease are ineffective for use with these extracellular proteins. Digestion with pepsin or protease type VII or XXIV (Sigma) can produce optimal results.[86] Use of fixatives other than formalin can also affect staining. Whereas most polyclonal antisera against these proteins should allow them to be shown in routinely processed tissue, some monoclonal antibodies are unreactive in these conditions. This may be explained by loss of immunoreactivity of some but not all epitopes of a protein during the fixation or embedding procedure.

Is it invasive?

Use of basement membrane immunohistological techniques to distinguish invasive from in situ neoplasia presupposes that basement membrane disruption is essential for invasion to occur. The studies carried out by Barsky *et al* suggested that this, indeed, was the case.[51] The authors examined neoplasms from various different tissues including breast, skin, pancreas and prostate and reported that benign and in situ lesions have intact basement membranes with linear staining for type IV collagen and laminin; most invasive carcinomas lack immunoreactivity for both these proteins. They reported that cases of in situ carcinoma with microinvasion show thinning, fragmentation, and disruption of the basement membrane in the foci of microinvasion but not elsewhere. Other studies have been less absolute in their conclusions. Many invasive tumours show variable retention of basal lamina proteins at their periphery.[50 58 67 69 87] Furthermore, dysplastic and in situ lesions in a variety of sites may have discontinuous or interrupted basement membrane staining.[49 53 54 57 59 68 78] Consequently, loss of basement membrane continuity cannot be used as a simple criterion of invasion.

Several studies of invasive breast carcinoma have described variation in laminin immunoreactivity both within and between tumours.[44–46 55 56] This ranges from discontinuous and linear in the better differentiated, invasive ductal carcinomas to complete absence in moderately differentiated and undifferentiated tumours. Variation in quantity of laminin shown in different studies may reflect technical variations, in particular, fixation. It may also reflect variation in interpretation of the results as laminin in tumours may be visualised as a much thinner and finer line than that seen around normal glands.[77] Notably, continuous linear staining for type IV collagen and laminin, such as that found around normal breast acini, has not been described at the margins of invasive ductal and lobular breast carcinoma. Fragmentation of basement membrane, however, may be seen around intraductal carcinoma of breast.[44 78]

In contrast to breast carcinomas, linear type IV collagen and laminin may be found at the margins of well differentiated squamous cell carcinomas of the larynx, oropharynx, skin and cervix.[50 58 67 68 88] Invasive carcinomas, however, generally show at least focal discontinuities of basement membrane and it may be totally lost. Basal cell carcinomas are usually completely enveloped by basal lamina, although this is not invariable and it tends to be fragmented in deeply infiltrative tumours.[87 89 90]

It has been suggested that the differing amount of basal lamina seen around different tumour types is a reflection of their cell of origin and degree of differentiation.[47 67 68] Relative paucity of basal lamina in invasive breast carcinoma might be a reflection of relative paucity of myoepithelial differentiation in these tumours. In contrast, squamous and basal cell carcinomas might be expected to produce larger amounts. Other neoplasms producing large quantities of basal lamina are adenoid cystic carcinomas and some pleomorphic adenomas.[23 78] This would be consistent with their putative myoepithelial origin.

Analogous to the breast, dysplastic and in situ lesions of squamous epithelia of skin, oropharynx, and

larynx may be associated with either continuous or interrupted basement membrane staining. Interruptions seem to be less common in dysplasia of the cervix, where linear immunoreactivity for the principal basal lamina constituents has been reported.[49] It was suggested that this represents the longer time course of progression from in situ to invasive carcinoma in this site and the possibility of regression[49]. In a series of cases of cervical intraepithelial neoplasia, however, Richards and Furness described basement membrane interruptions that increased in number with increasing severity of dysplasia.[91] Foci of microinvasive carcinoma had a completely different pattern of immunoreactivity, being absent or completely fragmented, and it was suggested that this could be of potential diagnostic use.

Although it is apparent that loss of basement membrane integrity cannot in general be used as a simple diagnostic criterion of invasion, the abnormalities of this structure associated with dysplasia and neoplasia demand further investigation. Diagnostic applications depend on a better understanding of changes observed.

PROBABILITY OF INVASION

A particularly important question is whether loss of basement membrane integrity in dysplasia and in situ carcinoma indicates a greater probability of progression to invasive malignancy. If this were proved to be the case it might be a useful adjunct to grading dysplasias and identifying the potentially most aggressive changes. This possibility has been explored in the urinary bladder.[59] In a retrospective study of 69 superficial and 15 invasive urothelial carcinomas it was found that although superficial "non-invasive" tumours may have either intact or interrupted basement membranes, the incidence of progression of superficial tumours with patchy or absent basement membranes was significantly greater than those with complete basement membranes. There was some association between basement membrane staining and histological grade but this did not reach significance.

A complicating factor is that basement membrane interruptions may be found in association with inflammation. It has been reported that breaks in basement membrane at sites of inflammation are small and sharply defined in contrast to the irregular discontinuities found in in situ carcinoma.[54] Others have suggested that the gaps in basement membrane seen in dysplasia may actually be due to enzymes produced by inflammatory cells and that this might promote invasion.[59] Further studies are obviously required in this area.

LIMITED TISSUE

Another situation in which it has been suggested that immunostaining of the basal lamina may have a diagnostic role is in the analysis of endometrial curettings. In a study of benign, premalignant, and malignant endometrium Furness and Lam reported that in normal endometrium and benign cystic hyperplasia, glandular basement membranes are nearly continuous even in the menstrual phase.[57] Small numbers of breaks are found in atypical hyperplasia, with increasing numbers the greater the abnormality. Invasive tumours were said to have a strikingly different pattern with many breaks in basement membrane, even when well differentiated. It was thought that the differences between benign and atypical hyperplasia were insufficient to be a useful diagnostic tool. The differences between atypical hyperplasia and invasive carcinoma, however, were thought to be sufficiently distinctive to have a potential role in the analysis of endometrial curettings where lack of tissue makes invasion hard to assess.

In other studies of endometrium it has been noted that stromal cells are enveloped by laminin but only at certain stages of the menstrual cycle.[92 93] Stromal laminin was observed in secretory endometrium and in 69% of cases of cystic hyperplasia. In contrast, it was not present in proliferative endometrium or in atypical hyperplasia or carcinoma. This was also suggested to be of potential diagnostic value.

TYPE VII COLLAGEN

A basement membrane antibody produced more recently is the monoclonal antibody LH7·2 which reacts with type VII collagen.[94] This is only reactive on fresh, frozen tissue sections. Unlike the basal lamina antigens laminin and type IV collagen, type VII collagen is restricted to basement membranes of stratified epithelia and is not present around naevus cells. In malignant melanomas it was found that loss of an intact LH7·2 positive basement membrane beneath an intraepithelial proliferation of malignant melanocytes correlated with increased tumour thickness.[95] It was suggested that the relatively good prognosis of thin malignant melanomas may be associated with confinement of the tumour above intact epidermal basement membrane. The pattern of laminin and type IV collagen deposition in malignant melanomas is analogous to that in carcinomas with variable deposition around tumour nests or individual tumour cells and sometimes total absence.[96 97]

The antibody to collagen type VII has also been applied to basal cell carcinomas. Its pattern of reactivity was similar to that of laminin and type IV collagen, but in general it was diminished in amount relative to these two proteins and relative to strong staining of epidermal basement membrane. In two deeply infiltrative basal cell carcinomas fragmentation of basal lamina staining was accompanied by total loss

of type VII collagen immunoreactivity (d'Ardenne *et al*, unpublished observations). The presence or absence of type VII collagen may have an influence on the locally infiltrative capacity of these tumours.

Benign or malignant?

A slightly different diagnostic problem is the distinction of an overtly invasive malignancy from a completely benign, non-neoplastic "look-alike". Two examples are the distinction of tubular carcinoma of the breast from sclerosing adenosis and distinction of pancreatic adenocarcinoma from chronic pancreatitis. The possibility of using basement membrane immunohistological techniques as a diagnostic aid has been explored in both these situations. Complete absence of basement membrane around tubular carcinomas has been noted both ultrastructurally and by immunohistology.[56 98] This is in contrast to continuous and linear staining of basement membrane in benign lesions. This may be of diagnostic value provided the possibility of false negative results is recognised (see below).

The value of laminin staining for distinguishing between chronic pancreatitis and adenocarcinoma of the pancreas has been investigated by Haglund *et al*.[99] They reported mostly irregular and discontinuous laminin around invasive ductal carcinoma, although it was almost continuous around some well differentiated glandular structures. They concluded that immunohistochemical analysis for laminin might be a useful diagnostic aid provided enough tissue is available for examination. In contrast, it was not found to be useful for distinguishing between benign and malignant mucinous cystic neoplasms or islet cell tumours, all of which possessed a nearly intact basement membrane.

Its use has also been investigated in the thyroid to determine if basement membrane staining might help in the sometimes difficult distinction of encapsulated, well differentiated follicular carcinoma from follicular adenoma.[100-102] It was found that basement membranes were only consistently deficient in the widely invasive tumours—that is, those that do not present any diagnostic difficulty. Encapsulated microinvasive tumours may have intact basement membrane around trabeculae and follicles and this can be seen even in areas of vascular invasion. In some microinvasive tumours, however, there is widespread loss of immunoreactive laminin and fibronectin.

A difficulty with the use of basal lamina markers to distinguish benign from malignant neoplasms is that the positive diagnosis (malignancy), is supported by the negative observation (lack of basal lamina immunoreactivity). Extreme caution must therefore be exercised to ensure that the immunostains have actually "worked" if basal lamina markers are to be used in this manner. Positive staining of endothelial basement membranes is not always a reliable indicator of staining efficacy as antigen accessibility may differ in different types of basement membrane. For example, excessive deposition of interstitial collagen in epithelial basement membranes may mask basal lamina antigens.[77] Positive staining of non-neoplastic epithelial basement membranes in the test section is nevertheless the best internal positive control available. In practice, this type of staining can only be used to support diagnoses for which there is already strong suspicion on other grounds.

Prognosis

Quantity of laminin around invasive carcinoma has been reported to correlate with its degree of differentiation, but few formal studies of its relation to prognosis have yet been made. It might be predicted that the better differentiated carcinomas would have a greater amount of laminin and a better prognosis. This may prove a general rule but it may only apply to some types of neoplasm. Tubular and mucinous carcinomas of the breast are recognised to have a relatively good prognosis and yet both are completely devoid of basal lamina. As noted above, it also seems that the amount of basal lamina a tumour is likely to have depends partly on its origin. Squamous and transitional cell carcinomas tend to have more than adenocarcinomas, and adenocarcinomas of stomach and colon, for example, tend to have more than adenocarcinomas of the breast. Each type of tumour therefore requires separate consideration.

In general, tumour prognosis is related to stage and grade. Although there is a tendency for basal lamina quantity to be related to histological grade,[69 103 104] there seems to be a poor correlation between patterns of basal lamina and stage or extent of spread of a tumour. If basal lamina immunostaining is to be of value as a prognostic variable, it must provide further information to that already provided by conventional staging and grading methods. Two studies have suggested that this is indeed the case. In a study of 75 bladder carcinomas, 27 superficial and 48 invasive, Daher *et al* reported that the invasive tumours could be divided into two groups: those with conserved or only partially fragmented basal lamina; and those with widely fragmented or absent basal lamina.[105] The latter had significantly lower short term survival, independent of stage or histological grade.

Forster *et al* described the results of laminin immunostaining in a series of 158 rectal adenocarcinomas.[106] Sixty two per cent had well defined basement membrane laminin, and the corrected five year survivals for laminin positive and laminin negative tumours were 65% and 23%, respectively. There was partial correlation of laminin positivity with tumour grade, but multivariate analysis indicated it is a better

indicator of prognosis than conventionally assessed histological grade. Duke's staging remained the best independent prognostic variable.

Tumour histogenesis

Basement membrane markers cannot be used as "markers" of tumour cell histogenesis in the conventional sense as they are common to such a wide range of cell types. As described in the introduction, however, their organisation differs in different tissues and a similar type of organisation may be found in their malignant counterparts. When using basement membrane markers in this context, it is important to remember that malignant tumours may lose their basement membranes altogether. A negative result is therefore non-contributory.

In attempting to differentiate a sarcoma from a carcinoma, the presence of basal lamina around individual cells as opposed to groups of cells points to a sarcoma. This can occasionally be of value—for example, in the diagnosis of spindle cell tumours of the kidney (d'Ardenne, unpublished observations). This is analogous to use of a reticulin stain but the results are more specific and easier to interpret. Presence of basal lamina around individual tumour cells also indicates that they are not of fibroblastic origin as fibroblasts lack this structure. Positive laminin staining in a sarcoma excludes a diagnosis of fibrosarcoma or a malignant fibrous histiocytoma.[61 107 108] It may also be helpful in the diagnosis of vascular tumours to elucidate the relation of the tumour cells to basement membranes, again in a manner analogous to a reticulin stain.[61 109 110]

A tumour which has a very distinctive pattern of basal lamina deposition is adenoid cystic carcinoma.[42 78 111] In this neoplasm abundant basal lamina may be found either among individual tumour cells or lining the characteristic cystic spaces. This may be of value in distinguishing adenoid cystic carcinomas from cribriform adenocarcinomas, especially in the breast. In cribriform adenocarcinomas the cystic spaces represent true glandular lumina and are consequently not lined by basal lamina.

Demonstration of basal lamina is not of assistance in distinguishing adenoid cystic carcinomas from pleomorphic adenomas as both can have a very similar pattern of reactivity.[23] In general, however, pleomorphic adenomas have variable and irregular amounts of basal lamina at the margins of tumour islands.[23 112]

Identification of basal lamina has previously been recommended by ultrastructural pathologists as a useful landmark when attempting to solve the type of problem described above.[78 111 113] The major advantage of using basal lamina immunohistological techniques is the facility of looking at much larger volumes of tissue.

Conclusion

The relation of basal lamina to tumour invasiveness is a complex subject, many aspects of which have yet to be clarified. Although it is apparent that invasion cannot be regarded simply as penetration of neoplastic cells through a basement membrane barrier, the few studies done indicate that abnormalities of this structure are found in most invasive tumours and in many severe dysplasias. The importance of this phenomenon is indicated by the few studies in which basal lamina abnormalities have been related to subsequent tumour behaviour. It is not possible with this type of investigation to determine whether basal lamina disruption is simply a manifestation of abnormal tumour differentiation. Nevertheless, as basal lamina is essential for the maintenance of normal tissue architecture it seems likely that abnormalities in basal lamina contribute to the architectural disruption of neoplastic tissue. This may in turn be important for the occurrence of overt invasion.

From the diagnostic point of view it is clear that in most situations delineation of basal lamina cannot be used as an absolute criterion to distinguish between invasive and non-invasive malignancy. Possible exceptions to this generalisation are a few situations in which the distinction to be made is between a totally benign and an overtly malignant invasive tumour.

An important question remaining to be fully answered is whether basal lamina status may provide additional or possibly even more relevant information about invasive potential of dysplasias than commonly applied methods. Similarly, the prognostic importance of basal lamina and its cell surface receptor molecules in invasive neoplasms requires further investigation.

A totally separate question is the value of basal lamina markers in determining tumour histogenesis. In this situation their function is to supply architectural information as well as to distinguish between basal lamina-producing and non-basal lamina-producing cells. The advent of monoclonal antibodies with tissue restricted basement membrane reactivity may enhance the specificity of this approach. At present, basement membrane immunohistology can be regarded as an occasionally helpful adjunct to other diagnostic methods.

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