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REVIEW

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Basement membranes and artificial substrates in cell transplantation

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This article will concentrate largely on the current developments in the area of cell transplantations presented at the 1st Workshop for Cell Transplantation in Age-related Macular Degeneration. In particular, this brief review will address our current understanding of the role of cell–matrix interactions by covering the pathobiology of normal ageing Bruch's membrane; some of the problems faced at the time of surgery from a basement membrane prospective; the dedifferentiation and differentiation of RPE cells; and how the use of artificial substrates may address several of these issues. We will concentrate on problems related to age-related macular degeneration (AMD), the leading cause of irreversible blindness in Europe, America and other industrialized nations. AMD is likely to be a family of disorders, rather than a single biologic entity, characterized by the progressive loss of sight in the central portion of the visual field [6, 56, 71, 81].

Bruch's membrane

Evidence suggests that the pathological manifestations of AMD are the end stage of a lifelong continuum of change influenced by both genetic predisposition and environmental factors and focused at the level of Bruch's membrane. A number of age-related changes occur at the level of Bruch's membrane [26, 32]. Morphological and biochemical studies have demonstrated that the changes that occur to Bruch's membrane are typified by increased thickness and the progressive accumulation of deposits (such as drusen—see later) within the inner layers of the membrane.

Bruch's membrane is a basement membrane complex located between the retinal pigment epithelium (RPE) and the choroid. It is a pentalaminar structure with a central elastin layer bordered on either side by a collagenous zone. There is the basal lamina of the RPE at the innermost collagenous layer of Bruch's membrane and a second basal lamina, produced by the endothelial cells of the choriocapillaris, that is juxtaposed to the outer collagenous layer. Although much more remains to be learned, the basic ultrastructural organization and biochemical composition of Bruch's membrane are similar to basement membrane complexes in other tissues, such as the choroid plexus, kidney glomerulus, and airway alveoli, where an epithelial–endothelial juxtaposition occurs. All ionic exchange and metabolic traffic from the neural retina and RPE to the choroidal capillaries, and vice versa, must traverse Bruch's membrane, thus leaving the neural retina vulnerable to any disruptions of those processes [3]. It is well documented that Bruch's membrane undergoes a number of changes throughout life, including increased thickening, protein cross-linking and reduced permeability to nutrients as well as increased amounts of lipid deposition and the accumulation of basal laminar deposits and drusen (for more detailed reviews see [13, 22, 86].

Drusen are insoluble deposits that accumulate at the interface between Bruch's membrane and the RPE. Clinically, drusen are divided into two main morphologic phenotypes—hard and soft. Hard drusen are hemispherical structures with well-defined edges, whereas soft

drusen tend to be larger in diameter and more irregular in shape. The functional relationship between drusen and AMD is still not entirely clear, as the formation of hard drusen may be part of the normal ageing process with no profound pathogenic role in AMD. However, evidence suggests that the accumulation of large numbers of macular drusen is a direct precursor to geographic atrophy and the choroidal neovascularization (CNV) characteristic of late-stage AMD [14, 74]. Although a direct cause-and-effect relationship has not been specifically established, the appearance of numerous or large confluent drusen, particularly in the macula, is strongly correlated with the development and progression of the disease [3, 27, 33, 62].

Understanding the origin and molecular composition is likely to have important implications for AMD pathogenesis. Indeed, a recent study into the complete protein compositional profile of drusen from immunohistochemical screening of human donor tissues [20, 21, 51] has highlighted the fact that many of the newly identified drusen-associated molecules are also found in the pathologic deposits associated with other diseases, including Alzheimer's disease, atherosclerosis, elastosis, amyloidosis and glomerular basement membrane disease, [50], thus raising the intriguing possibility that common pathogenic pathways may be involved in their formation. (For a more detailed review on drusen, see [3, 22]).

In the field of transplantation a current problem we face at the time of grafting is an aged or damaged Bruch's membrane. How drusen or its composites may interfere with transplanted cell function is not yet understood, but some knowledge regarding how cells interact with aged and/or damaged Bruch's membrane is now complete (see next section). Interestingly recent work has shown that the complement regulatory protein (CD46), which can be found in drusen, also has a co-regulatory role with β1 integrin in RPE adhesion to Bruch's membrane [46]. However, there is still a gap in our knowledge as to the exact composition of Bruch's membrane and all of the receptors involved in RPE–Bruch's membrane interactions. This is due in part to the explosion of new information over the past decade or so regarding composition of basement membrane components elsewhere in the body and the number of potential receptors. Let us take laminin as an example of a protein known to be present in Bruch's membrane. Laminin is not a single protein but comprises multi-domain proteins built up from different modules. The diversity of the molecules is made greater in the occurrence of homologous but distinct α, β, and γ chains which combine into different trimeric isoforms and can be complicated further with different variants due to alternative splicing and proteolytic processing. At least 12 known forms of laminin trimer show a restricted expression in basement membranes [80], and although at least one member of the α- , β- , and γ-chain families appears to be present [48, 80] in every basement membrane studied, this does not preclude the presence of other laminins [80].

What will we be faced with at the time of surgery?

Initial work into pigment epithelial transplantation has followed surgical removal of CNV. Submacular surgery with CNV excision offers the possibility of removing larger CNVs whilst preserving the overlying retina, in the hope of preventing further photoreceptor damage and blindness associated with the subretinal bleeding and further scarring. However, visual recovery after CNV excision is usually poor in patients with AMD [47, 77, 79].

Clinical and histological studies have indicated that in AMD patients, CNV surgical removal is commonly associated with removal of adjacent native RPE and RPE basement membrane [4, 18, 40, 52] and with incomplete growth of RPE into the dissection bed [52, 55, 60, 61]. The resulting lack of functioning RPE cells contributes to the atrophy of the photoreceptors and of the underlying choriocapillaris [52, 58]. The environments in which the cells are transplanted clearly impact on the success of the operation. Obstacles to success of the transplant include immune rejection and graft failure.

The problem of immune rejection is covered in more depth elsewhere [72]. Although the subretinal space is an immunologically privileged site and is in part subjected to an immune response down-regulation that resembles anterior chamber-associated immune deviation (ACAID), it is known that RPE cells express HLA class I antigens and MHC II expression can be induced with growth factors such as IFN-γ [39, 59]. Indeed, it is well documented that an immune response can take place after RPE transplantation [2, 9], and long-term follow of cultured fetal RPE allografts in non-immunosuppressed patients with AMD has shown a high rate of rejection (75%) [2]. The stability of any immune privilege is also likely to be dependent on the maintenance of vascular integrity, since disruption of the blood–retinal barrier will expose the retina to immune surveillance [83]. This is likely to be a major factor in transplant rejection, with transplants appearing to fail more rapidly when placed over exudative areas [1, 2].

Attempts to limit rejection with immune suppression have had some success but are generally not well tolerated by elderly patients. It is hoped that rejection of transplants may be averted by transplanting autologous cells (RPE and IPE) removed in a previous biopsy from the patient. However, the transplanted cells may not survive in the subretinal space initially, irrespective of immune rejection. The major problem that cells face following transplantation is the need for adhesion and subsequent differentiation.

Cell–ECM adhesion

A number of donor cell types for transplantation have been studied, including fresh [36, 37, 38, 42], cryopreserved [11], cultured [41, 65] or immortalized RPE cells of animal or human origin [43], iris pigmented epithelial (IPE) cells [10, 59, 63, 78], stem cells [8, 64, 73], retina [49], photoreceptors [16] and Schwann cells [35, 44]. Irrespective of the cell type that is to be transplanted into the subretinal space in the hope of preventing further disease progress and functional deterioration, the cell's interactions with its surrounding substrate will be crucial to the success of any operation.

Cellular adhesion is key for the survival of any epithelium, and the RPE cell requires adhesion to a suitable substrate if it is to avoid undergoing death by anoikis or apoptosis. Indeed, work by Tezel and Del Priore showed that RPE cells die within 24 h if attachment to Bruch's membrane has not occurred [75]. Furthermore, even in successful RPE transplants in laboratory animals onto a normal Bruch's membrane or even onto in situ RPE cells there are considerable numbers of transplanted cells that do not adhere and subsequently die.

Adhesion to Bruch's membrane is therefore a crucial step in the success of the transplant procedure. However, the condition of Bruch's membrane will be difficult to predict, it will vary with each patient and it is likely to have an effect on adhesion. That deterioration of Bruch's membrane is problematic is supported by the work of Tezel et al. [76], who have shown that RPE cells have markedly different settlement rates on the different layers and components of Bruch's membrane. Furthermore, with both fetal and aged cells, the deeper the level of Bruch's membrane that is exposed the less adhesion is observed, with greatest adhesion occurring on the RPE basal lamina. Aged cells appear to be much less adept than fetal cells at repopulating Bruch's membrane. In addition, Shirigami et al. [70] have shown that embryonic RPE cells are unable to differentiate on severely damaged Bruch's membrane. It seems, on the other hand, that cultured RPE harvested from older donor eyes can attach and grow well and reach confluence in culture on substrates such as bovine ECM-coated culture dishes [28]. Work by Tsukahara and colleagues [79] has shown that freshly isolated autologous cells do not adhere as well to or survive on surfaces similar to that found in patients following removal of CNVs. Few if any cells were seen to survive on the submacular basement membrane or on the inner collagenous layer. This implies that attachment followed by proliferation would not repair such a defect. Indeed, it has recently been shown that RPE basement membrane supports RPE resurfacing of localized defects but the deeper portion of the inner collagenous layer impedes this [82]. However, these fundamental differences show that cells can be manipulated in culture prior to transplantation into the eye, and it is perhaps this point that bodes well for the future of transplantation.

Cell dedifferentiation and differentiation

Adhesion of the cells to an ideal substrate and in the correct environment is also crucial to the fate of the cells, with well-documented evidence that the nature of the substrate can alter the phenotype or differentiation state of RPE cells. A major problem for RPE or IPE injected as a suspension of isolated cells is that the transplanted RPE fail to regain a fully differentiated phenotype but instead form "quasi-monolayers", clumps of macrophage-like or fibroblastic cells: i.e. they are dedifferentiated. RPE differentiation and dedifferentiation are currently poorly understood. When RPE dedifferentiate, they lose their cuboidal shape, become fibroblastic in appearance, disperse, migrate and proliferate, resulting in contraction and distortion of the macula or even the whole retina [24]. This complication, a condition called proliferative vitreoretinopathy (PVR), can make RPE transplantation visually ineffectual [44]. PVR also complicates macular relocation surgery as an alternative surgical treatment for some AMD patients. It is known that when PVR does occur, profound visual loss may result. A major consideration in the strategy of RPE transplantation must be the avoidance of PVR [85].

Cell–matrix interactions are also key to the pathogenesis of PVR, and the membranes often contain numerous dedifferentiated RPE cells along with a number of extracellular proteins. The extracellular proteins include adhesive proteins such as collagen, laminin and fibronectin, as well as several matricellular proteins that have potential counter-adhesive functions. Two such matricellular proteins, thrombospondin 1 (TSP1) and osteonectin (or SPARC: secreted protein acidic and rich in cysteine), tend to be co-distributed with the dedifferentiated RPE cells in PVR membranes [69]. Their ability to modulate focal cell adhesions implies that TSP1 and SPARC may reduce RPE cell–matrix adhesion and so permit the key RPE cellular activities characterized in dedifferentiation, such as migration, shape change and proliferation [25]. Understanding dedifferentiation of RPE is fundamental, because RPE cells transplanted to non-immune privileged sites in the eye inevitably seem to adopt a fibroblast-like morphology [34]. If we are to obtain a functioning monolayer on Bruch's membrane by transplantation, establishment and maintenance of differentiated cells is imperative. There is a plethora of factors that can cause RPE cells to dedifferentiate, including ECM proteins and growth factors (such as HGF [17]) as well as the vitreous itself.

Following the discovery of a number of factors associated with RPE dedifferentiation in vivo, we further examined their role in more detail using an in vitro model

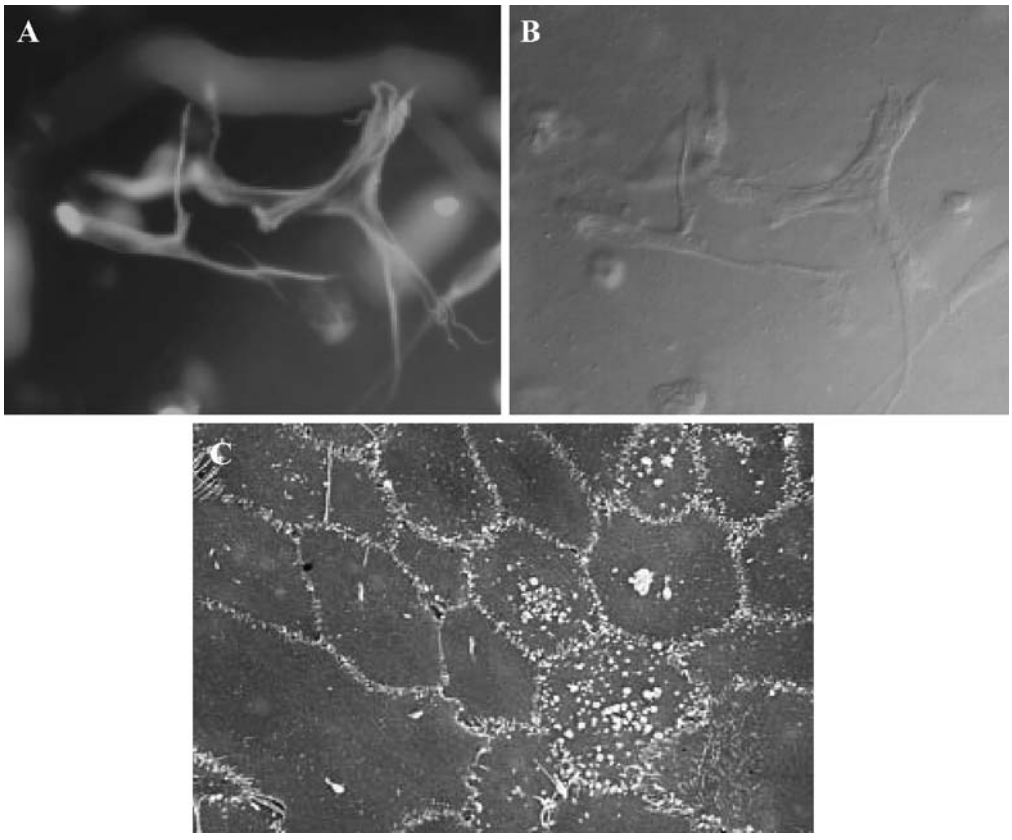

Fig. 1A–C Photomicrographs illustrating the different morphology of RPE cells in the presence of collagen type I. **A** Cytokeratin-positive RPE cells exhibit a spindle shape within a collagen type I matrix. **B** DIC picture of A. **C** A scanning electron micrograph illustrates that the RPE can adopt a typical epithelial phenotype when situated on collagen type I

of RPE dedifferentiation. We adapted an in vitro model [45] of contraction (i.e. the collagen matrix system) for this purpose.

The mechanisms by which RPE cells dedifferentiate and contract collagen matrices are beginning to be understood, and we have recently published data relating some of the different mechanisms involved in contraction [67]. These include cell-surface receptors such as integrins, cell–ECM interaction via glycoproteins (e.g. TSP1 and SPARC) [19, 25], lectins [29, 30] and enzymatic intervention (i.e. matrix metalloproteinases, MMPs) [68].

We have shown that TSP1 and SPARC are present during collagen contraction, but neutralizing these glycoproteins with monoclonal antibodies has not prevented RPE cells from dedifferentiating and contracting the surrounding collagen matrix [66]. However, antibodies directed against cell-surface receptor integrins $\alpha 2$ and $\beta 1$ subunits have significantly prevented RPE-mediated collagen matrix contraction [68].

We have also shown there is a role for MMPs during RPE cell-mediated contraction of collagen matrices [68]. Using a number of techniques (immunohistochemistry, ELISA, zymography) we were able to show expression of a number of MMPs during the contraction assay. Furthermore, the evaluation of a broad-spectrum MMP inhibitor, a hydroxamic derivative known as Galardin-MPI, which is known to inhibit the activity of all MMPs, has helped to determine that MMP production within the collagen gels was essential rather than incidental. We have shown that Galardin-MPI has no effect on cell viability, adhesion or proliferation and as such its anti-contractile effects appear to operate via inhibition of MMPs [68]. What was of particular interest, however, was that even when RPE cells are seeded on the same substrate (collagen type I) in otherwise identical conditions they still can adopt different morphologies, depending on whether the cells are seeded within or on the substrate [45, 68] (Fig. 1).

Underlying substrates

Since the key to healthy RPE cells is attachment to a healthy Bruch's membrane, our strategy for future transplantation into humans will follow one of two approaches. One route aims to create an ideal modified Bruch's membrane in situ prior to the addition of the cells. This may be take the form of carefully preparing the graft bed so that Bruch's membrane is stripped off host cells but remains fundamentally undamaged [57], or some further surface modification of the diseased Bruch's membrane

may take place in order to increase adhesion and differentiation. The second approach requires cellular transplants to be introduced under the macula as a functioning monolayer, intact from the outset. To achieve this it is necessary to identify the optimal substrates on which to grow the cells in culture prior to implantation. To date a number of substrates have been studied and cells have been grown on and transplanted on substrates such as cryoprecipitated membranes [12], anterior lens capsule [23, 31, 53], cadaver Bruch's membrane [7], Descemet's membrane [78], synthetic biodegradable polymer films [15], collagen type I [5] and as microspheres on cross-linked fibrinogen [54]. Most of this research concentrates on the use of either biological substrates or degradable substrates to sustain the RPE monolayer. However, the former are variable and not easy to handle, while we consider that degrading substrates have the potential to leave the cells without appropriate support and the potential to cause adverse tissue reactions at the site of the transplant as they degrade.

We decided to restrict our investigations to non-degradable polymer substrates after research and evaluation of numerous types of biomaterials used in the posterior segment of the eye. Certain characteristics are required for a material to be suitable in this application. The substrate needs to be sufficiently pliable for introduction into the eye at surgery (Fig. 2), as well as suitably robust in very thin films for handling during the operation. The substrate must also be able to be manufactured in a porous structure, which would allow the transport of both nutrients and waste from the underlying tissues to the transplanted RPE monolayer. In addition, the material should be both biostable and display excellent biocompatibility so that it can remain in the eye for the lifetime of the patient whilst maintaining a monolayer of functioning cells.

We presented some preliminary data on the potential of polyurethanes for use as substrates because considerable research has been performed on optimizing their structure to produce soft, elastomeric materials with enhanced biostability and excellent biocompatibility [87]. As such the mechanical properties of polyurethanes can be exploited for this application. Polyurethanes are a large family of polymers with a diverse range of properties dependent on the particular chemical structure. They can be cast into thin films which are robust and can be manufactured by electrostatic spinning to create a porous substrate. However, one of the disadvantages of polyurethanes is that they tend to have a hydrophobic surface and therefore are usually not well suited for growing a monolayer of well-adhered and -spread cells. To overcome this problem, we are researching into the effects of modifying the surface properties of the polyurethane without influencing the bulk mechanical properties of the film. It is hypothesized that if the surface properties of the membrane are optimized, the RPE cells will secrete and create their own basement membrane on the polymeric substrate to maintain their phenotype and produce a healthy functioning monolayer protected from the diseased Bruch's membrane.

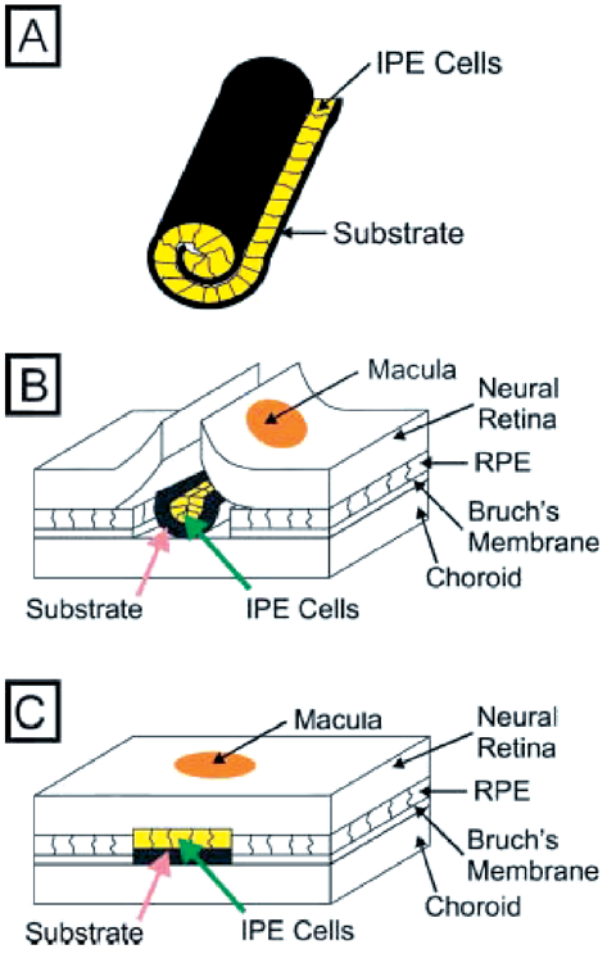

Fig. 2A–C Schematic diagram of the procedure for IPE transplantation. **A** IPE cells isolated from an iridectomy are cultured on a substrate and rolled into a cylinder prior to surgery. **B** The IPE roll is placed by syringe beneath the retina. **C** The IPE cylinder is unrolled in place beneath the photoreceptors of the macula and the retina is replaced

We found that surface modification using gas plasmas was a useful and practical way to increase the hydrophilicity of polyurethanes [84]. Cell culture experiments with an immortalized RPE cell line (ARPE-19) and wild-type human RPE examined the extent of cellular adhesion and proliferation on various substrates as well as potential detrimental effects such as apoptosis or toxicity. We found that both the untreated polyurethane films studied did not support RPE adhesion and proliferation, whereas on the gas plasma-treated surfaces cell cultures reached confluence within 3 days (Fig. 3).

To conclude, until we understand more of the cell–ECM interactions involved in both health and disease, cel-

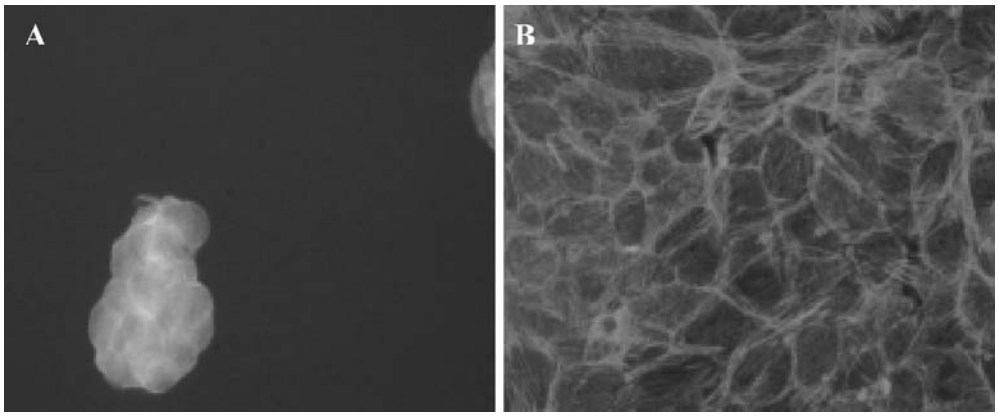

Fig. 3A,B Photomicrographs of RPE cells stained with phalloidin. The untreated polyurethane prevents cells from attaching and spreading (**A**), whereas the gas plasma treated substrate enables formation of a confluent monolayer (**B**)

lular transplantation will always represent a massive challenge both in the laboratory and theatre alike. Furthermore, obtaining animal models more akin to AMD in which one could evaluate how well transplants would fare with a defective Bruch's membrane would be of immense use in the field of transplant biology. Finally, although tissue engineering could offer hope and involve the use of polymeric templates to replace diseased or lost RPE, there is still much to be done to define the most suitable and reliable transplantation method for use in humans.

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