Summary. The aim of this study was to investigate the temporal and spatial expression of the matricellular protein SPARC (Secreted Protein, Acidic and Rich in Cysteine; also known as osteonectin) in human retinal pigment epithelial (RPE) cells, and compare the results with Bruch’s membrane thickness, employing immunohistochemistry.

Eyes from 36 human donors, 16 being ≤65 and 20 >65 years old, were included in the study. Intensity of SPARC immunoreactivity was evaluated using Aequitas Image Analysis software with two-way analysis of variance (ANOVA). Bruch’s membrane thickness was assessed by profile analysis and the association between RPE SPARC immunoreactivity and Bruch’s membrane thickness was investigated by fitting a linear mixed effects model to the data set.

Intensity of SPARC immunostaining in RPE cells was significantly lower in older donors (p<0.05 and p<0.001 for posterior and peripheral RPE cells, respectively). The anatomical localisation of the RPE cells also affected the intensity of SPARC staining, which was lower in posterior compared to peripheral cells (p<0.01). No correlation was observed between SPARC immunoreactivity in RPE cells and the thickness of the underlying Bruch’s membrane, in either posterior or peripheral regions. Our results suggest that RPE cell SPARC levels decline with age, a change that may play a role in the pathogenesis of age-related diseases such as age-related macular degeneration.

Key words: Ageing, Retinal pigment epithelium, Secreted protein, Acidic and rich in cysteine, SPARC, Osteonectin

Introduction

SPARC (Secreted Protein, Acidic and Rich in Cysteine), also known as osteonectin or BM-40, is a 43kDa matricellular protein with heat shock protein-like properties (Emerson et al., 2006). Alteration of SPARC tissue levels causes abnormal fibronectin fibre assembly at cell surfaces and modification of laminin-1 and collagen IV secretion (Barker et al., 2005; Yan et al., 2005). SPARC loss leads to morphological abnormalities of basement membrane structure with changes in adjacent cells. For example, SPARC loss causes anomalous synthesis and deposition of lens epithelial basement membrane components with dedifferentiation and degeneration of the cells (Yan et al., 2005).

Alteration in basement membrane (BM) structure is a feature of ageing in several human tissues, including the lens, cornea and retina of the eye. In the retina, the retinal pigment epithelium (RPE) basement membrane increases in thickness with age. This increase contributes to age-related thickening and irregularity of Bruch’s membrane (of which the RPE cell BM is a part) at the interface between choroid and retina (Sarks et al., 2007). The change in RPE cell BM is linked to age-related macular degeneration (AMD) and is due to abnormal aggregation of BM proteins between the basal surface of the cells and their BM (Marshall et al., 1994; Sarks et al., 2007). Given the role of SPARC in BM assembly, the observation that the protein is synthesized and secreted by RPE basally (Ratnayaka et al., 2007) and
Materials and methods

Donor tissue

Ethical approval for the study was granted by the local research ethics committee. Thirty six human donor eyes, each from a different donor, were obtained from the local eye bank (Royal Liverpool University Hospital, Liverpool, UK). The specimens had been fixed in 10% neutral buffered formalin within 48 hours of death and had no history of ocular disease and had been fixed in formalin and embedded in paraffin wax. Only eyes that had no history of ocular disease and had been fixed in 10% neutral buffered formalin within 48 hours of death were included in the study. Eyes were known only by their specimen number and thus they were age-masked throughout the investigation. The age range of the donors (18 male and 18 female) was 17-95 years, and donors were categorized as ≤65 or >65 years old, with 16 eyes in the ≤65 years category and 20 in the >65 group. Because of their archival nature, an immunoperoxidase method was used to investigate SPARC in the tissue.

Section preparation

The eyes were sectioned anteroposteriorly through the pupil. Serial sagittal sections (5 µm thick) of the wax-embedded tissues were deparaffinised, rehydrated and washed in PBS. In order to allow subsequent image analysis of immunohistochemical staining within the RPE cells, bleaching was performed on all tissue studied to remove melanin pigmentation from the cells (Alexander et al., 1986). Bleaching involved immersing tissue sections for 60 min in potassium permanganate, followed by 5 min immersion in oxalic acid, washing in distilled water and then incubating at 30°C for 10 min (to prevent tissue detachment from slides).

Immunohistochemistry

Tissue sections were washed with TBS, incubated with serum free protein block (DakoCytomation, Ely, UK) and immunostained with 5 µg/ml mouse anti-human/bovine SPARC antibody (Ab) (Haematological Technologies Inc., Cambridge, UK). Sections were washed with TBS and incubated with peroxidase-conjugated goat anti-rabbit/mouse secondary Ab (DakoCytomation, Ely, UK). After a final TBS wash, visualization was achieved with NovaRED (Vector Labs, Peterborough, UK). The preparations were dehydrated, transferred into xylene and mounted in DPX. RPE cells were photographed using a light microscope with a digital camera. Images were recorded under standardised conditions for analysis. Negative controls for this procedure included substitution of the primary Ab with anti-glucoseoxidase Ab [15 µg/ml] (Sigma, Gillingham, UK), an inappropriate Ab, and substitution of either the primary, or both primary and secondary Ab, with TBS. The presence of the SPARC-immunoreactive neuroretina and vascular endothelial cells served as internal positive controls (Kim et al., 1997; Sage et al., 1984).

Image analysis

Evaluation of the immunohistochemical results was undertaken using methods similar to those used by other investigators (Friedman et al., 2007; Sozo et al., 2007; Zafirellis et al., 2008). Specifically, images were analyzed using Aequitas™ Image Analysis software (Dynamic Data Links Ltd, Cambridge, UK), which generated data relating to the intensity of SPARC-positive staining in the RPE cells of each of the eyes in the investigation, allowing the relative intensity of staining to be directly compared between the eyes. The RPE cells in each image were delimited and all other areas were masked to exclude them from the analysis. Data from the analysis gave an over-all ‘gray-scale value’ of staining intensity for the RPE cells within each eye (the gray-scale is the computers linear measure of color intensity, running from ‘0’ black to ‘255’ white). Three randomly selected images were analyzed from the posterior RPE cells, and three from the peripheral RPE cells of each eye section investigated (posterior RPE cells were defined as those which lay temporal to the optic nerve head at the posterior pole of the eye, that is in the macular region; peripheral RPE cells were defined as those which lay immediately anterior to the equator of each globe). Since the immunohistochemistry was repeated three times for each of the eyes in the study, a total of 9 analyses for posterior RPE cells and 9 for peripheral RPE cells were performed for every donor eye. After the data from RPE staining was collected, staining in the choroid and neuroretina was evaluated from images of these structures by the same method.

Profile analysis of Bruch’s membrane

Bruch’s membrane thickness was assessed by profile analysis, in which tissue sections were stained with haematoxylin and eosin and photographed as described.
above. Images were analysed using an in-house computer program designed specifically to measure the thickness of biological structures. Bruch’s membrane thickness was deduced by determining the boundaries of the membrane and plotting 100 equidistant plumb lines, perpendicular to the lower boundary, along the length of the image. The distance between and height of each plumb line was used to construct a profile of the membrane. Data was used to find the average thickness along Bruch’s membrane (thickening of Bruch’s membrane with age is usually irregular in any one individual). Profile analysis was performed three times, on randomly selected images, for both regions of each eye in the study.

**Statistical analysis**

Data acquired from the three images for each eye section were averaged and the three repeated measurements obtained per region were analysed with two-way analysis of variance (ANOVA) to test whether SPARC concentration was significantly influenced by the age of the donor (where age is treated as a binary variable: ≤65 years and >65 years old: donor age information was obtained after data collection from image analysis from the linked coded database that held this record) and/or by the region of RPE cells (posterior or peripheral).

Linear association between SPARC concentration and thickness of Bruch’s membrane (where age and region of RPE cells were controlled for) was studied by fitting a linear mixed effects model to the data set.

**Results**

**The intensity of SPARC immunoreactivity in RPE cells is lower in older eyes**

Immunohistochemistry confirmed the presence of SPARC in the choroid, RPE cells and retina (Fig. 1). In the choroid and RPE cells, SPARC staining was present in a diffuse pattern throughout (Fig. 1). Comparing the two age groups revealed immunohistochemical staining intensity to be higher, indicating more SPARC protein was present, in the RPE cells of younger donors compared to older donors (Fig. 1). Two-way ANOVA revealed that age had a statistically significant influence upon SPARC protein expression by RPE cells (p<0.05 and p<0.001 for posterior and peripheral RPE cells, respectively; Fig. 2). The mean grey-scale value for the ≤65 years old group was 138.2 in posterior RPE cells and 129.1 in peripheral RPE cells, whilst the mean grey-scale value for the >65 years old group was 155.4 in posterior RPE cells and 150.5 in peripheral RPE cells (more intense labelling yields a lower grey-scale value; see above). The SPARC staining observed in the choroid and neuroretina however did not decrease with age (data not shown).

**Regional differences exist in SPARC immunoreactivity in RPE cells**

Two-way ANOVA also revealed that region of the RPE cells has a significant effect on SPARC protein concentration, with a higher concentration found in peripheral compared to posterior RPE cells (p<0.01). Immunohistochemical staining was observed to be more intense, thus relative SPARC protein concentration was higher in the peripheral RPE cells compared to the posterior RPE cells for 30/36 donors in this study (Fig. 3).

**Relative SPARC protein concentration in the RPE cells does not correlate with Bruch’s membrane thickness**

Although we found, consistent with the literature, that Bruch’s membrane thickness increases with age, no correlation was observed between the relative SPARC protein concentration in the RPE cells and the thickness of the underlying Bruch’s membrane, in either of the two regions investigated (Fig. 4A,B). Analysis based on a linear mixed-effects model failed to reveal a statistically significant relationship between these two variables in either posterior or peripheral regions of RPE cells, with p>0.05 for each region, respectively.

**Discussion**

Our findings confirm that SPARC is present in choroid and retina, and suggest that in the RPE (but not the rest of the retina or choroid) levels of the protein decline with age. We also observed that SPARC expression was lower in posterior RPE cells, when compared to peripheral RPE of the same donor, at all ages investigated. However, we could not find any correlation between the intensity of SPARC immunoreactivity in the RPE cells and the thickness of Bruch’s membrane.

Several studies have shown that human RPE manufacture SPARC for both secretion and retention by the cells and that it is degraded after secretion (Magee et al., 2000; Hiscott et al., 2002; Ratnayaka et al., 2007). Hence it seems likely that SPARC in the RPE cells is synthesised by the cells, rather than being taken up from the surrounding milieu. Thus the question arises as to why RPE cell SPARC levels decline with age. SPARC shares many similarities with heat shock proteins (HSPs). SPARC can be induced by heat shock and other stresses (Sauk et al., 1991; Neri et al., 1992; Kudo et al., 1994) and the promoter region of mouse and bovine SPARC genes have been found to include a ‘heat shock element-like’ sequence (Kudo et al., 1994). In addition, SPARC has been shown to act as a chaperone, protecting against unfolding and aggregation of other proteins in response to stress (Martinek et al., 2007), SPARC was...
Fig. 1. SPARC staining in the retina and choroid. Sections through retina and choroid immunohistochemically stained for SPARC (A to D) and, as a control, for glucose oxidase enzyme (E and F). F was counterstained with haematoxylin. Melanin in choroid and RPE has been bleached in all the sections. The detachment of the neuroretina in A-C is an artefact (due to differential shrinkage of the neuroretina during processing) and it is normally located where the star is placed. In D, the star is placed over the neuroretina. A to D. Staining for SPARC protein is seen in choroid (c) and RPE (arrow). In D, the photoreceptors (P) and outer nuclear layer (O) of the neuroretina can also be seen to be immunoreactive for SPARC. A. Posterior RPE cells from a young donor (35 years old). B. Posterior RPE cells from an older donor (78 years old). C. Peripheral RPE cells from a young donor (35 years old). D. Peripheral RPE cells from an older donor (78 years old). The RPE cells of younger donors demonstrate more intense staining than older donors, and intensity is also greater in peripheral than in posterior RPE cells. Controls employing the inappropriate primary antibody, E and F, demonstrate no reaction product and confirm the effect of the bleaching process on the removal of melanin from choroid and RPE cells (the inner nuclear layer (I) of the neuroretina can also been seen in these sections together with the photoreceptors (P) and outer nuclear layer (O)). The location of Bruch’s Membrane (B) is marked for orientation purposes. Scale bars: 50 µm.
Fig. 2. Relationship between SPARC protein concentration in the RPE and age of the donor. SPARC protein concentration is lower in the RPE cells of older donors in both posterior and peripheral regions of the eye. Y-axis values are inverted for ease of interpretation; lower gray-scale values indicate a higher protein concentration. Analysis based on two-way ANOVA found the association between age and SPARC protein concentration significant at p<0.05 and p<0.001 for posterior and peripheral RPE cells, respectively. In addition the region of RPE cells investigated exerts an influence, with relatively higher concentration found in peripheral rather than posterior RPE cells (p<0.01), in both younger and older donors.

Fig. 3. Regional differences in SPARC concentration within the RPE. For 30/36 of the donors investigated, relative SPARC protein concentration was found to be higher in the peripheral, rather than posterior, RPE cells. (For ease of interpretation this graph depicts data for only nine randomly selected donors included in the study.) Donors are coded by their specimen number (“EOU number”) to ensure an age-masked study.

Fig. 4. A. Relationship between SPARC protein concentration in posterior RPE and thickness of Bruch’s membrane. No significant correlation between SPARC protein concentration in the RPE and thickness of Bruch’s membrane was observed in the posterior region of the eye, p>0.05, as determined by analysis based on a linear mixed-effects model. B. Relationship between SPARC protein concentration in peripheral RPE and thickness of Bruch’s membrane. No significant correlation between SPARC protein concentration in the RPE and thickness of Bruch’s membrane was observed in the peripheral region of the eye, p>0.05, as determined by analysis based on a linear mixed-effects model.
SPARC in human RPE cells

observed to inhibit the thermal aggregation of alcohol dehydrogenase to an extent greater than that of the well characterised chaperone protein αβ-crystallin (Emerson et al., 2006). Therefore, it is plausible that SPARC has a HSP role. Age has a well documented adverse effect upon the ability of cells to mount a heat shock response. Hence the same mechanisms that are proposed to attenuate transcription of other HSPs, such as the interference with protein-DNA interactions and levels of the transcription factors necessary for activation of heat shock genes (Jurivich et al., 2005), may be responsible for lower SPARC levels in the aged RPE cells. Indeed, several other RPE cell HSPs are reduced with age (Nordgaard et al., 2006). Thus the decline in RPE cell SPARC production may reflect a general decline of HSPs with age.

A postulated HSP function of SPARC in RPE cells may also help to explain the regional differences in the relative concentration of SPARC protein within posterior and peripheral RPE cells. The level of SPARC protein was generally found to be lower in the posterior RPE cells, when compared to the peripheral RPE of the same donor, at all ages investigated. Although HSPs are generally induced as a response to oxidative stress (Strunnikova et al., 2001; Kaarniranta et al., 2005; Decanini et al., 2007), the evidence of an early age-related decrease of RPE cell HSPs (Nordgaard et al., 2006) suggests that the greater photo-oxidative stress that posterior rather than peripheral RPE cells are subject to throughout life could lead to the regional differences in RPE cell SPARC levels. Differential expression has previously been reported for several genes and proteins between macular and peripheral regions of RPE cells, Bruch’s membrane and choroid including insulin-like growth factor and catalase (Ishibashi et al., 2004; Kociok and Joussen 2007; Mullins et al., 2007).

SPARC has multiple and complex roles in cell biology (Bornstein and Sage, 2002). SPARC has been shown to inhibit cell proliferation in a concentration-dependent manner, by arresting progression from G1 to S phase of the cell cycle (Funk and Sage, 1991). Similarly, SPARC influences cell adhesion so that higher levels of the protein abrogate focal adhesions to promote an intermediate or weak state of cellular adhesion, thus inducing cell rounding with prolonged exposure to the protein (Sage et al., 1989). Additional functions of SPARC include interactions with a number of proteins that influence cell adhesion and matrix assembly, including several other proteins expressed by RPE cells such as laminin and members of the matrix metalloproteinases (MMPs), metallo-disintegrins (ADAMs) and the ADAM with thrombospondin repeats (ADAMTS) families (Webster et al., 1999; Sheridan et al., 2001; Bevitt et al., 2003). As a consequence of these and other roles, SPARC is thought to play an important part in regulating cell shape and differentiation. Since loss of RPE cell uniformity is a feature of ageing and of early AMD (Sarks et al., 2007), it is possible that age-related reduction of SPARC levels in the RPE cells plays some role in this change. Further evidence that changes in RPE cell SPARC levels might play a role in AMD comes from a recent in vitro study, in which levels of SPARC protein produced by RPE cells were down regulated by 2-fold in cells cultured from the macular of AMD patients compared to RPE cells cultured from the macular of age-matched controls (An et al., 2006). Finally, the abnormal BM aggregates deposited in the thickened RPE cell BM in age and AMD contain laminin-1, fibronectin and collagen type IV (BM proteins that are modulated by SPARC), while functional abnormalities of other matrix-regulating proteins that are highly expressed in RPE, such as cystatin C, are also associated with AMD (Marshall et al., 1994; Yan et al., 2005; Paraoan et al., 2009).

Given SPARC’s roles in matrix assembly and cell-matrix interactions, we speculate that alterations in RPE cell SPARC concentration might relate to morphological changes in Bruch’s membrane. However, changes in Bruch’s membrane thickness did not appear to correlate with RPE cell SPARC levels when age was controlled for. Although with increasing age the relative SPARC protein concentration was found to decrease whilst the thickness of Bruch’s membrane increases, we found that one variable could not be used to predict the other. Therefore it seems that age-related variations in RPE cell SPARC concentration are independent of Bruch’s membrane thickness. Nevertheless, given the multiple functions of the protein, decline in RPE cell SPARC protein could play a role in the pathogenesis of AMD - perhaps with regard to the attachment and behavior of the RPE cells, or the decrease in HSP activity offered by SPARC.

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References


