Synthesis of Osteonectin by Human Retinal Pigment Epithelial Cells is Modulated by Cell Density

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PURPOSE. To determine whether human retinal pigment epithelial (HRPE) cells are able to synthesize the antiadhesive protein osteonectin, also known as secreted protein, acidic and rich in cysteine (SPARC). Additionally, because locally produced SPARC may modulate cellular behavior during tissue repair, to ascertain whether HRPE SPARC production and HRPE proliferation, migration, and/or differentiation are associated, in a simple HRPE wound-healing model.

METHODS. Immunohistochemical and Western blot analyses of SPARC protein expression by low- and high-density cultured HRPE cells were undertaken. Total RNA extracted from cultures was studied by reverse transcription-polymerase chain reaction (RT-PCR) and Northern blot analysis. Western and Northern blot analyses were evaluated by densitometry. Experiments were repeated with HRPE cells cultured in the presence of 1, 10, or 100 μM of the differentiating agents butyric acid (BA) and retinoic acid (RA).

RESULTS. HRPE cell cultures exhibited SPARC immunoreactivity. Western blot analysis of cell lysates and conditioned media showed a 43-kDa protein. RT-PCR and Northern blot analysis confirmed the presence of SPARC mRNA (with transcripts at 2.2 and 3.0 kb). Protein and mRNA transcript band densitometry revealed a higher proportion of SPARC protein and mRNA in high-density HRPE cell culture than in low-density culture. Neither BA nor RA (at the concentrations assessed) had a significant effect on SPARC production by HRPE cells in high- or low-density culture.

CONCLUSIONS. HRPE can synthesize SPARC. Although the findings do not support an invariable association between SPARC production by HRPE and HRPE proliferation, migration, or differentiation, they demonstrate that synthesis of SPARC by HRPE is modulated by cell density. (Invest Ophthalmol Vis Sci. 2000;41:2707–2711)

The 43-kDa glycoprotein, secreted protein acidic and rich in cysteine (SPARC), also known as osteonectin, is one of a group of secreted extracellular matrix proteins (matricellular proteins) that interact both with cell surfaces and a range of extracellular molecules (e.g., growth factors) but that generally have no structural role. Although the precise function of SPARC remains unclear, it possesses counter-adhesive properties that may permit changes in cell shape necessary for such cellular activities as proliferation and migration. Cell proliferation and migration are features of development and repair and, indeed, SPARC is produced in many developing organs and in healing wounds. Conversely, SPARC is also produced by cells in organs undergoing terminal differentiation, when cell proliferation and migration may be decreasing. Thus, for example, SPARC increases through differentiation in the chick retinal pigment epithelium and persists in the avian adult monolayer.4

We have noted an association between human retinal pigment epithelial (HRPE) cells and SPARC in the membranes of the anomalous wound-repair condition proliferative vitreoretinopathy (PVR), an observation that is consistent with the notion that HRPE have the ability to synthesize SPARC. Moreover, because PVR membranes contain migratory and proliferating cells on the one hand and on the other hand cells in various stages of transdifferentiation, it is possible that SPARC may be produced by HRPE cells during proliferation, migration, and/or differentiation. To investigate these possibilities, initially we determined whether HRPE cells have the ability to synthesize SPARC. Then we studied SPARC production in a simple in vitro model of wound repair using HRPE in low-density cultures, where cells are isolated, mitotic, and motile (i.e., in wound-repair phenotype), and in high-density cultures where cells are in contact and proliferation and migration are less marked. Finally, we investigated SPARC synthesis by HRPE cells treated with retinoic acid (RA) or butyric acid (BA), agents known to induce differentiation in these cells.5,6

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Supported by The Guide Dogs For The Blind Association; The Royal College of Surgeons of Edinburgh and The Blind Asylum; National Health Service Executive North West Regional Research and Development Directorate; The University of Liverpool Research Fund; Research into Eye Disease; and the Foundation for the Prevention of Blindness.

Submitted for publication November 17, 1999; revised March 20, 2000; accepted April 11, 2000.

Commercial relationships policy: N.

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MATERIALS AND METHODS

HRPE and CRL2070 Cell Culture

HRPE cells were cultured in Ham’s F10 (Life Technologies, Paisley, UK) containing 20% fetal calf serum (FCS; Harlan Sera Laboratory, Crawley Down, UK), 1% each glutamine, fungizone (Life Technologies), and 1% each glucose, penicillin-streptomycin (Sigma, Gillingham, NY). Cultures were maintained in 5% CO2 at 37°C and grown to confluence in 150-cm2 flasks (Corning, Corning, NY) before passaging. Purity of cell cultures was examined with a microscope (Polyvar; Reichert-Jung, Germany). Cultures were treated with anti-rabbit or anti-mouse fluorescein isothiocyanate (FITC) conjugate (1:100; Sigma). RPE monolayers were previously characterized antiserum and monoclonal antibody to SPARC (6F-BON-1 and AON-1 respectively) were used. Cells were washed with phosphate-buffered saline (PBS) and fixed in methanol (5 minutes) and acetone (2 minutes) at −20°C. Nonspecific binding was blocked with normal goat serum before incubating the cells with primary antibodies at previously optimized dilutions of 1:1000 (LF-BON-1), 1:250 (AON-1) in PBS (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA). After a PBS wash, the RPE preparations were treated with anti-rabbit or anti-mouse fluorescein isothiocyanate (FITC) conjugate (1:100; Sigma). RPE monolayers were examined with a microscope (Polyvar; Reichert-Jung, Germany) equipped with epifluorescent optics.

SPARC Staining of RPE In Vitro

HRPE cells were seeded at a density of 7.5 × 103 cells per well on eight-chamber tissue culture slides (LabTek; Nunc, Naperville, IL) for immunofluorescence staining, as described. Preparations involving protein and mRNA analyses, HRPE cells were seeded on eight-chamber tissue culture slides (LabTek; Nunc, Naperville, IL) for immunofluorescence staining, as described. Preparations involving protein and mRNA analyses, HRPE cells were seeded on eight-chamber tissue culture slides (LabTek; Nunc, Naperville, IL) for immunofluorescence staining, as described.

Western Blot Analysis

Western blot experiments were conducted on cell lysates and conditioned media from HRPE cells. The tumor cell line CRL2070, together with pure SPARC protein (Hematologic Technologies, Essex Junction, VT), were used as positive controls. Electrophoresis was performed with 10% polyacrylamide gels. The bicinchoninic acid assay (Sigma) at 4°C overnight. Membranes were incubated for 1.5 hours at room temperature with maleic acid washing buffer (0.1 M maleic acid, 0.15 M sodium chloride, and 0.3% Tween 20) and 240 μL of each alkaline phosphatase- conjugated secondary antibody (Sigma), at a dilution of 1:2500 (containing 25% newborn calf serum) for 1 hour and subsequent chemiluminescent reaction using a commercial system (ECL, Amersham, Amersham, UK). Each experiment was conducted three times.

RNA Extraction and cDNA Synthesis

For each experiment, RNA was extracted from RPE monolayers using a kit (RNEasy; Qiagen, Crawley, UK). RNA (5 μg) was used as template for first-strand cDNA synthesis in a 25-μl reaction volume containing 5 μL 5× first-strand buffer (Life Technologies); 0.5 mM each of dATP, dCTP, dGTP, and dTTP; 1.25 μg oligo(dT) (Pharmacia, St. Albans, UK); 20 U RNase inhibitor; 10 mM dithiothreitol; and 2 μL reverse transcriptase (Superscript; Life Technologies). The reaction was incubated at 37°C for 1 hour and terminated by freezing.

Reverse Transcription–Polymerase Chain Reaction

Reverse transcription–polymerase chain reaction (RT-PCR) was performed in a 50-μl reaction mixture containing the following reagents: 0.25 μL DNA polymerase (Thermoprime; Advanced Biotechnologies, Epsom, Surrey, UK); 5 μL 10× PCR buffer; forward and reverse primers (0.5 μL of 1 μg/μL); 200 mM each of dATP, dCTP, dGTP and dTTP; 1.5 mM MgCl2; and 2 μL cDNA preparation. The cycling conditions for all primers were as follows: stage 1, 94°C for 2 minutes; stage 2, 35 cycles of 94°C for 30 seconds, 57°C for 30 seconds, 72°C for 60 seconds; and stage 3, 72°C for 5 minutes. The primers used for PCR were as follows: SPARC 5′-GATGGCGCTGACCACTTC-3′; 5′-CGATTATAGTCCCATTTT-3′; GAPDH 5′-GGTCAAGGA-GTCCITCCAGAT-3′; and 5′-GGTGAGGGTGGATGTCA- CG-3′. PCR products were separated by electrophoresis on a 1% agarose gel and the products visualized under ultraviolet illumination.

Northern Blot Analysis

Total cellular RNA was extracted using a kit (RNeasy; Qiagen). Total RNA (5 μg/lan) was denatured with formaldehyde (Sigma) and formamide (Sigma) and was size fractionated by electrophoresis through a 1% agarose gel (Life Technologies) containing formaldehyde. The RNA was then transferred to a nylon membrane (ICN, Irvine, CA) overnight and cross-linked with ultraviolet irradiation. A cocktail of four SPARC-specific, 3’-tailed, digoxigenin (DIG)-labeled oligonucleotides (Life Technologies) was used to probe the blotted membrane. Even loading of RNA samples was assessed through use of a 5′ and 3′ DIG-labeled 18S ribosomal RNA probe (Eurogentec, Oxford, UK). The blotted membrane was hybridized overnight at 42°C (Easy Hyb; Boehringer Mannheim, Indianapolis, IN). The membrane was successively washed at room temperature with 2× SSC (three times for 5 minutes; one time for 10 minutes), at 48°C with 0.1× SSC (two times for 15 minutes), and at room temperature with maleic acid washing buffer (0.1 M maleic acid, 0.15 M sodium chloride, and 0.3% Tween 20; one time for 3 minutes). Bands were visualized using a commercial system (CDP-Star; Boehringer Mannheim), and the blot was exposed to film (Hyperfilm; Amersham). The 3′-tailing of SPARC probes was performed using a DIG oligonucleotide 3′-tailing kit (Boehringer Mannheim). Each experiment was conducted three times.
Effect of BA and RA on SPARC mRNA Expression

For all experiments fresh BA and RA were used. Experiments were conducted under darkened conditions. Preconfluent and confluent HRPE cells were exposed to 1 μM, 10 μM, and 100 μM BA (sodium salt; Sigma) and 1 μM, 10 μM, and 100 μM RA (Sigma) in Ham’s F10 medium supplemented with 10% FCS for 24 hours and 72 hours, respectively. The preparations were incubated at 37°C and 5% CO₂. After each experiment, protein and total RNA were extracted from cells, subjected to Western and Northern blot analyses, respectively, followed by densitometric analysis. Experiments were conducted a minimum of three times.

RESULTS

SPARC Protein and HRPE Cells

RPE cells were immunoreactive for SPARC, and a similar pattern of staining for the protein was observed in both preconfluent and confluent HRPE cell cultures. The pattern of SPARC immunostaining in HRPE cells was of a granular perinuclear and a diffuse peripheral punctate nature (Fig. 1). Immunostaining associated with the extracellular matrix was also observed and appeared to consist of discrete foci of SPARC immunoreactivity arranged in threadlike or beadlike patterns (Fig. 1). Positive immunostaining was observed with both SPARC antibodies (LFBON-1 and AON-1). Controls in which the primary antibodies were omitted showed no immunoreactivity (Fig. 1).

Immunoblot of HRPE cell lysates and conditioned medium (standardized for protein concentration at 50 μg/lane) demonstrated the presence of a 43-kDa band (the expected molecular weight of SPARC) that displayed immunoreactivity with the monoclonal antibody AON-5031 (Fig. 2). A band of the same weight of SPARC was observed in CRL2070 cell lysates and with purified SPARC protein. Densitometric analysis of the bands representing SPARC from cultured RPE cells (conducted on standardized amounts of total protein and at the same time interval in culture) revealed significantly higher concentrations (14-fold increase; P < 0.05; t-test) of SPARC in confluent than in preconfluent cultures (Fig. 2).

SPARC mRNA and HRPE Cells

The primers designed to detect cDNA for SPARC produced a band of predicted size (514 bp), with the use of control cDNA from the CRL2070 SPARC-producing cell line (data not shown). When these primers were used on cDNA from cultured HRPE cells, SPARC mRNA was detected in preconfluent and confluent cells (Fig. 3). PCR using primers for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) demonstrated the integrity of the cDNA from the cultured HRPE cells.

Northern blot analysis of total RNA from cultured HRPE cells identified two mRNA transcripts migrating at 2.2 and 3.0 kb (Fig. 3) equivalent to that expressed by other human cells. The intensity of the 3.0-kb band appeared less than that of the 2.2-kb band. Only a single band of 2.2 kb was observed for the CRL2070 SPARC-producing cell line, which was consistent with its murine origin.² Densitometric analysis of the transcript bands from cultured RPE (conducted on standardized amounts of total RNA and at the same time interval in culture) revealed significantly higher levels of SPARC mRNA expression (2.5-fold increase; P < 0.01; t-test) in confluent than in preconfluent cultures (Fig. 3).

Effect of BA and RA on SPARC mRNA Expression

HRPE cells were exposed to RA and BA for times previously established to induce features associated with HRPE cell differentiation and to induce SPARC synthesis in other cell types.¹¹,¹² All three concentrations of RA induced features that have been described previously, including cellular flattening and polygonal shape. Similar changes were also observed in all the BA-treated cells, although these changes were not so pronounced as in the RA-treated cells. Treatment of preconfluent
ent and confluent HRPE cells with 1 \( \mu \text{M} \), 10 \( \mu \text{M} \), or 100 \( \mu \text{M} \) BA or 1 \( \mu \text{M} \), 10 \( \mu \text{M} \), and 100 \( \mu \text{M} \) RA for their respective incubation periods, induced no significant increase in SPARC protein or in mRNA expression (Fig. 4; data shown for BA only).

**DISCUSSION**

Our results demonstrate that HRPE cells are capable of synthesizing SPARC. Moreover, some of the protein appears to be secreted by the cells and deposited in the extracellular matrix, consistent with a proposed role for SPARC in extracellular matrix assembly. In addition, we have shown that the proportion of SPARC to total protein in high-density cultures is greater than in low-density cultures and that this difference in SPARC expression is paralleled by higher SPARC mRNA levels in confluent HRPE cells. These observations suggest that the higher SPARC level in confluent cultures is, at least in part, due to increased production of the protein rather than decreased SPARC degradation at high cell density.

SPARC is produced in tissues undergoing development and repair. We have previously shown that SPARC is colocalized with HRPE cells in the anomalous reparative tissue of PVR membranes. Given possible roles for SPARC in repair-related cellular activities such as proliferation, migration, and differentiation, we questioned whether RPE-derived SPARC might be linked to HRPE cell proliferation, migration, and/or differentiation.

To investigate the possibility that SPARC production by HRPE cells is associated with differentiation of the cells, we used a range of concentrations of two established differentiating agents with both high- and low-density HRPE cell cultures. Although, as previously reported, the RA- and BA-treated HRPE cells adopted features similar to mature cells in vivo, and the difference in SPARC synthesis between low- and high-density culture was maintained irrespective of treatment, neither RA nor BA elicited any significant change in SPARC production by HRPE cells. BA and RA upregulate SPARC synthesis by some but not all cells. For example, RA increases SPARC expression by chick chondrocytes but has little effect on SPARC synthesis by human osteoblasts. Variance between cells in the effect of differentiating agents on SPARC production may reflect either species or cell type differences, or both. However, although BA and RA induce many of the features typical of differentiated HRPE cells (such as polygonal shape and arrested growth and migration), these agents do not appear to produce a mosaic of polarized HRPE cells (i.e., tertiary differentiation) in vitro. Thus, we cannot exclude the possibility that the cell density-dependent increase in HRPE SPARC synthesis is related to events at the end of retinal development.

A number of cell types exhibit increased SPARC synthesis when cell proliferation is in decline. Our observation that SPARC expression is upregulated in high-density culture (where HRPE cell proliferation is generally less abundant than in low-density culture) is consistent with the notion that in-

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**FIGURE 3.** (A) RT-PCR of human SPARC in HRPE cells from preconfluent (lane 1) and confluent (lane 2) cultures. A 514-bp band consistent with SPARC mRNA was identified in each reaction. The 500-bp band represents the GAPDH reaction control. (B) Northern blot analysis showing expression of SPARC mRNA in total RNA extracts from preconfluent HRPE cells (lane 2), confluent HRPE cells (lane 3), and the CRL2070 SPARC-producing cell line (lane 4). Lane 1: DIG-labeled molecular weight markers.

**FIGURE 4.** Northern blot analysis showing expression of SPARC mRNA in total RNA extracts from preconfluent and confluent HRPE cells before exposure of 1 \( \mu \text{M} \) BA (lanes 2 and 4, respectively) and after exposure to 1 \( \mu \text{M} \) BA (lanes 3 and 5, respectively). Lane 1 represents the DIG-labeled molecular markers.
creased SPARC synthesis by HRPE cells is associated with a decrease in HRPE cell proliferation. However, the finding that neither RA nor BA (which both inhibit RPE proliferation) increase HRPE SPARC synthesis suggests that termination of HRPE cell proliferation can occur without any need for increased SPARC production by these cells. Furthermore, because HRPE cell migration also is decreased in high-density cell culture, our findings do not support the concept that HRPE cell migration is related to increased SPARC synthesis by the cells, although further studies are required to establish the relationship between HRPE cell migration and SPARC synthesis.

The findings of our study suggest roles for HRPE-derived SPARC other than, or in addition to, HRPE cell proliferation, migration, and differentiation. SPARC has been implicated in the regulation of angiogenesis. It is well established that HRPE cells have an important function in the control of angiogenesis at the chorioretinal interface and that this function is mediated by a variety of HRPE-derived peptides and proteins (see, for example, recent review by Campochiaro14). Therefore, it is possible that production of SPARC by HRPE cells plays a role in the biology and pathobiology of vascularization at this site. If this is the case, we could expect an involvement of SPARC in the vascular abnormalities associated with age-related macular degeneration. We are currently investigating this concept.

Acknowledgment

The authors thank Larry Fisher who kindly supplied the LF-BON-1 antibody.

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