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In vitro Transplantation of Fetal Human Retinal Pigment Epithelial Cells onto Human Cadaver Bruch's Membrane†

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Retinal pigment epithelium transplantation has been proposed as adjunctive treatment for age-related macular degeneration following surgical excision of choroidal neovascular membranes. The goal of this study was to develop a model to evaluate retinal pigment epithelium transplantation onto human Bruch's membrane in vitro. We investigated the ability of cultured fetal human retinal pigment epithelium to colonize human cadaver Bruch's membrane, determined the incubation time needed to form a monolayer and to exhibit apical microvilli and tight junctions, and assessed the production of basement membrane. Freshly enucleated (less than 48 hours old) human eyes were cut through the pars plana, and the anterior segment, vitreous, and retina were removed. The native retinal pigment epithelium was debrided with a surgical sponge. Bruch's membrane and choroid at the macula were trephined with a 7·0 mm diameter trephine and then incubated with 1/2 ml of Dulbecco's modified Eagle's medium + 15% fetal calf serum + basic fibroblast growth factor (1 ng ml^{-1}), and fetal human retinal pigment epithelium at a concentration of 242,000 cells ml^{-1}. Specimens were incubated for 1, 4, 6, 8, 12, or 24 hours. The specimens were fixed in half strength Karnovsky's fixative, processed, and analysed with scanning and transmission electron microscopy. The retinal pigment epithelium covered the debrided macular specimens to different degrees at different incubation times. After 1 hour, the cells started to attach and flatten (median percent coverage: 78%). The extent of Bruch's membrane coverage by fetal retinal pigment epithelium varied greatly between specimens. After 4–6 hours, the cells covered the entire debrided surface in a monolayer (median percent coverage: 97·2% at 4 hours, 99·8% at 6 hours). Tight junctions were observed, and the cells had few apical microvilli. The lateral cell borders were obliquely oriented with respect to Bruch's membrane, and the nuclei were elongated, exhibited prominent nucleoli, and were oriented parallel to Bruch's membrane. After 6–8 hours, cells started to become hexagonal (median percent coverage at 8 hours: 99·97%). Cells attached to the inner collagenous layer tended to be flatter than cells attached to residual native basement membrane. At 12 and 24 hours, expression of hexagonal shape, tight junctions, and apical microvilli were observed more frequently (median percent coverage: 99·87% at 12 and 100% at 24 hours). No newly formed basement membrane was observed at these time points. In separate experiments comparing attachment in the presence and absence of native RPE basement membrane, the presence of native retinal pigment epithelial basement membrane promoted the early attachment of the cells and more rapid expression of normal morphology. This in vitro system provides a reproducible way to study the adherence of retinal pigment epithelium to normal and diseased human Bruch's membrane.

Key words: retinal pigment epithelium; Bruch's membrane; retinal pigment epithelium transplantation; basement membrane; age-related macular degeneration.

1. Introduction

Surgically excised choroidal neovascular membranes (CNVs) from patients with age-related macular degeneration (AMD) uniformly include retinal pigment epithelial (RPE) cells and often include RPE basement membrane, portions of the inner collagenous layer of Bruch's membrane (BM), and occasionally, even portions of the elastic lamina and choriocapillaris (Lambert et al., 1992; Grossniklaus et al., 1994; Bynoe et al., 1994; Pollack et al., 1996, Rosa, Thomas and Green, 1996; Nasir, Sugino and Zarbin, 1997). Removal of the RPE may result in retinal and choriocapillaris degeneration (Korte, Reppucci and Henkind, 1984, Del Priore et al., 1995; Del Priore et al., 1996; Leonard et al., 1997), and RPE transplantation has been advocated as an adjunct to the surgical treatment of choroidal neovascularization in AMD (Algvere et al., 1994). Our goals were to establish an in vitro model to study the colonization of BM by RPE, to determine the incubation time needed for dispersed RPE cells to establish a monolayer on BM, and to determine the time needed for RPE cells to establish their in situ morphology after seeding onto BM explants.

In this study we describe a culture system that permits evaluation of the behavior of fetal human RPE cells cultured on human cadaver BM explants. Our

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model is based on studies by Nicolaissen et al. who showed that such explants can be maintained in culture for at least 1 month (Nicolaissen et al., 1988; Vatne and Nicolaissen, 1988). These investigators seeded first passage human RPE onto the explants but did not focus on short incubation times, which is the purpose of the present study (Nicolaissen et al., 1988). In addition, the current study focuses on transplantation of RPE cells onto BM subjacent to the macular area only. Rapid attachment of RPE may reduce apoptosis of the transplanted cells (Tezel and Del Priore, 1997) and the possibility of complications such as proliferative vitreoretinopathy.

2. Materials and Methods

In preliminary experiments, we determined the number of cells needed to cover a 38·5 mm² area (surface area of a 7·0 mm diameter trephine). Our goal was to determine the number of cells that would form a monolayer with 100% coverage of the denuded BM specimen at an early time after seeding. Following the protocol described below, we initially seeded 242,000 cells (in 0·5 ml media) onto this surface and observed multilayers of cells. (This number of cells was derived from the in situ cell density.) We believe that the multilayers were due to the larger size of the cultured cells. We then seeded 121,000 cells in 0·5 ml media and obtained RPE monolayers over most of the explants although there were still foci of extranumerary cells even 24–48 hr after seeding. Debrided specimens without transplanted cells were also studied

TABLE I

Time course of RPE attachment and coverage on human Bruch's membrane explants

Incubation time	Specimen	% Coverage
1 hr	66M*	36·15 ± 15·43
	64F	33·70 ± 9·81
	84F	89·84 ± 7·69
	87F	86·42 ± 8·89
	75M	82·72 ± 7·60
4 hr	70M	99·21 ± ·0·80
	66M	93·99 ± 2·68
	93F	75·92 ± 5·53
	85F	93·15 ± 5·48
	64F	99·91 ± 0·18
6 hr	83M	89·70 ± 11·85
	65F	99·97 ± 0·07
	77M	98·41 ± 2·46
	68F	99·62 ± 0·48
8 hr	77M	99·35 ± 0·96
	83M	68·39 ± 27·63
	84F	99·99 ± 0·0001
	65F	99·80 ± 0·20
12 hr	93F	99·76 ± 0·25
	75M	97·31 ± 4·53
	66F	99·60 ± 0·31
	68F	99·88 ± 0·12
24 hr	87F	99·44 ± 0·70
	66F	99·88 ± 0·10
	85F	99·71 ± 1·00
	70M	100·0 ± 0·00

Data are reported as mean ± S.D. and represent the average of 8–10 measurements. See text for details.

* Abbreviation refers to age and sex of donor.

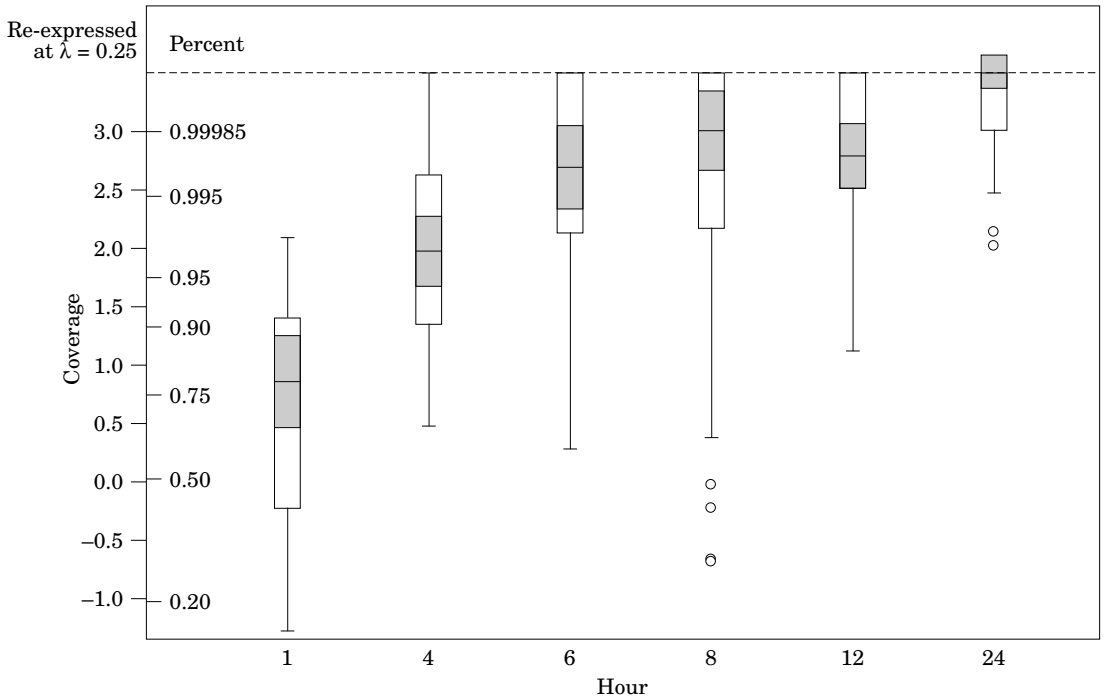

FIG. 1. Boxplot of the re-expressed data to illustrate the change in percent coverage over time. The horizontal bar within the box is the median. The shaded box represents the central 50% of the data, and the upper and lower lines represent the tails of the distribution. Values that fall outside the two tails are deemed 'outside values' and are plotted as ○. □ (McGill, Tukey and Larsen, 1978) define a 95% confidence interval around each median.

to assess the amount of RPE basement membrane remaining.

Isolation of Specimens

Human eyes were obtained 24–48 hr after death from the Arizona Lion's Tissue Bank. Donor age was between 64 and 93 years (mean 79 years) with death to enucleation times ranging from 1 hr 50 minutes to 8 hr 55 minutes. No eye had clinically evident macular degeneration. The eyes were incised through the pars plana. The corpus vitreous was removed, and the retina was gently peeled off and severed from the optic nerve. Under stereo microscopic observation, the RPE was removed hydraulically by Balanced Salt Solution (BSS-Sodium Chloride 0·64%, Potassium Chloride 0·075%, Calcium Chloride Dihydrate 0·048%, Magnesium Chloride Hexahydrate 0·03%, Sodium Acetate Trihydrate 0·39%, Sodium Citrate Dihydrate 0·17%, Sodium Hydroxide, Hydrochloric Acid and Water) through a 30 gauge needle attached to a 5 ml syringe. The RPE (identified by their shape and pigment content) still attached to BM were debrided with a wet surgical sponge (Alcon) and then washed off. The choroid-sclera in the macular area was trephined with a 7·0 mm diameter trephine to produce explants of BM attached to the underlying choroid and sclera. Two BM explants were generated from each donor, and the explants were used to assay RPE attachment at different time points (Table I).

RPE Cell Culture

Cultures of fetal human RPE cells from a single cell line from a 20 week old fetus were maintained in 35 mm diameter bovine corneal endothelial cell extracellular matrix (BCE-ECM)-coated plates (Song and Lui, 1990) with Dulbecco's modified Eagle's medium (DME-H16) supplemented with 15% fetal calf serum (FCS), 300 μg ml^{-1} glutamine, 50 μg ml^{-1} gentamicin, 100 U ml^{-1} Pen-Strep, 2·5 μg ml^{-1} fungizone, and 1 ng ml^{-1} basic fibroblast growth factor (bFGF). The plates were incubated in a humid atmosphere of 91% air, 9% CO_2 at 37°C. Five days before the experiment, RPE cells either in second, third, or fourth passage were trypsinized and seeded on new 35 mm diameter BCE-ECM-coated plates. At the time of the experiment, when the plates were confluent, the RPE were harvested by trypsinization (Song and Lui, 1990). After washing the cells with Ca^{2+} free-PBS, the dish was incubated at 37°C for 1 to 2 minutes with 0·05% Trypsin-EDTA (GIBCO BRL), until cells became rounded. Trypsin-EDTA was then removed, and the cells collected, by gently flushing, and then resuspended in regular media. Cells were counted before being seeded onto BM. For each experiment, cell viability was tested by plating a portion of the RPE suspension onto BCE-ECM-coated culture dishes and incubating the cells for the same time as was being tested on the BM explants. Viability under these circumstances was 100% at all times.

Tissue Culture Conditions

The isolated BM-choroidal explants were kept in the trephine and placed in tissue culture plates. Fetal human RPE cells were harvested and resuspended in medium at a density of $2{\cdot}42 \times 10^5$ cells ml^{-1}; 0·5 ml of this suspension was pipetted into the trephine onto the explants. The explants were incubated at 37°C for 1 ($n = 5$, mean age $\pm$ s.d. $= 75{\cdot}2 \pm 10{\cdot}3$), 4 ($n = 5$, mean age $\pm$ s.d. $= 75{\cdot}6 \pm 12{\cdot}7$), 6 ($n = 4$, mean age $\pm$ s.d. $= 71{\cdot}4 \pm 8{\cdot}2$), 8 ($n = 4$, mean age $\pm$ s.d. $= 77{\cdot}2 \pm 8{\cdot}7$), 12 ($n = 4$, mean age $\pm$ s.d. $= 75{\cdot}5 \pm 12{\cdot}2$), or 24 ($n = 4$, mean age $\pm$ s.d. $= 77 \pm 10{\cdot}5$) hr.

Preparation of the Specimen

At different incubation times, samples were placed in 1/2 strength Karnovsky's fixative and postfixed in osmium tetroxide. Samples were bisected at this time. Half of the sample was processed for transmission electron microscopy (TEM) and half for scanning electron microscopy (SEM). Specimens were processed with care, maintaining the cell surface side up to keep the attached cells from being dislodged. Specimens for TEM were en bloc stained in 2% uranyl acetate. After ethanol dehydration, tissue was infiltrated and embedded in epon. Blocks were sectioned at 0·5 μm with a MT-5000 microtome and stained with toluidine blue for light microscopic analysis. Selected blocks were thin sectioned for TEM examination and examined on a Zeiss 10C. Specimens for SEM were dehydrated, critical point dried, and sputter coated with 20 nm gold.

Area Analysis

Eight to 10 non overlapping areas were photographed from the center of the specimen at 250 X with an ISI DS130 SEM for area analysis. Micrographs were taped onto a Kurta IS/AD8 digitizing board connected to a Quadra 800 computer. Areas to be measured were outlined using a free hand cursor, and the size of each area was quantified using NIH Image software, version 1·49. Comparison with the total measurable area of every picture gave the percentage of coverage for each micrograph. In some cases, the measurable area did not include areas with folds moving out of the plane of focus since these could not be measured accurately. The percent coverage of these 8–10 non overlapping areas was measured for each specimen. The mean of percent coverage was calculated for each single standard picture (Table I). To quantify the change in percent coverage over time, notched boxplots (McGill, Tukey and Larsen, 1978) of the original estimates of the percent coverage of each standard area were generated (Fig. 1). Since these plots show the data to be skewed considerably, with non homogeneous

variance and several outside values at each time period, they were re-expressed using Tukey's Lambda transformation (Tukey, 1986, Valleman, 1995). This transformation allowed us to stretch the tails of the hourly distributions so that those values close to 100% could be viewed more closely. To determine whether the increase in percent coverage was linear over the first 8 hours, a regression analysis, Lowess and cubic spline smoothers (Valleman, 1995) were applied to the re-expressed data. Welch's ANOVA and a Tukey-Kramer multiple range test (Tukey, 1986) were also used to specify real differences between data collected at early and later hours. Data analysis utilized Data Desk (v. 5·0) statistical software from Data Description, Inc., Ithaca, NY, U.S.A. and JMP (v. 3·2) statistical software from SAS Institute, Carey, NC, U.S.A.

TABLE II

Mean and median† of the percent coverage over time*

Percent coverage			
Hour	Mean*	Re-expressed value (at $\lambda = 0{\cdot}25$)	Median†
1	66%	0·884	78·0%
4	92%	1·95	97·2%
6	97%	2·6	99·8%
8	92%	2·887	99·97%
12	99%	2·7	99·87%
24	99·76%	3·31	100%

* As stated in the manuscript, calculated from raw data.

† Calculated from the re-expressed value $\left(\frac{p^{\lambda}-(1-p)^{\lambda}}{\lambda}\frac{1}{2^{\lambda}}\right)$.

3. Results

Light and electron microscopic examination of debrided specimens showed variable removal of RPE basement membrane. The percentage of the basement membrane present ranged from 60% to 80%. In some cases, superficial damage to the inner collagenous layer was observed. In these experiments, the elastic lamina was never damaged. No specimens showed intact RPE cells. On the light microscopic level, intact (native) RPE (Fig. 2) can be distinguished from the seeded RPE on the basis of several morphological characteristics (e.g., cell shape, apical-basal polarization, and basal infoldings). Intact RPE can be identified on the SEM level by the large number of microvilli on their surface (Nicolaissen, Davanger and Arnesen, 1982).

By 1 hr after seeding, dissociated RPE started to attach and flatten on the denuded BM. The percentage of coverage at this time varied widely and ranged from 34% to 90% (mean 66%, median 78%) (Tables I, II and Fig. 1). As seen with SEM, the cells showed considerable variation in shape with some being very rounded, others flattened and well attached, and some cells showing elongate shape [Fig. 3(A)]. High magnification of BM areas that were not covered by cells showed bare collagen without RPE basement membrane [Fig. 3(B)]. The seeded cells were usually larger than cells in situ. Rare multilayered cell clusters were observed. The density of surface projections was variable. By TEM most RPE appeared well attached in

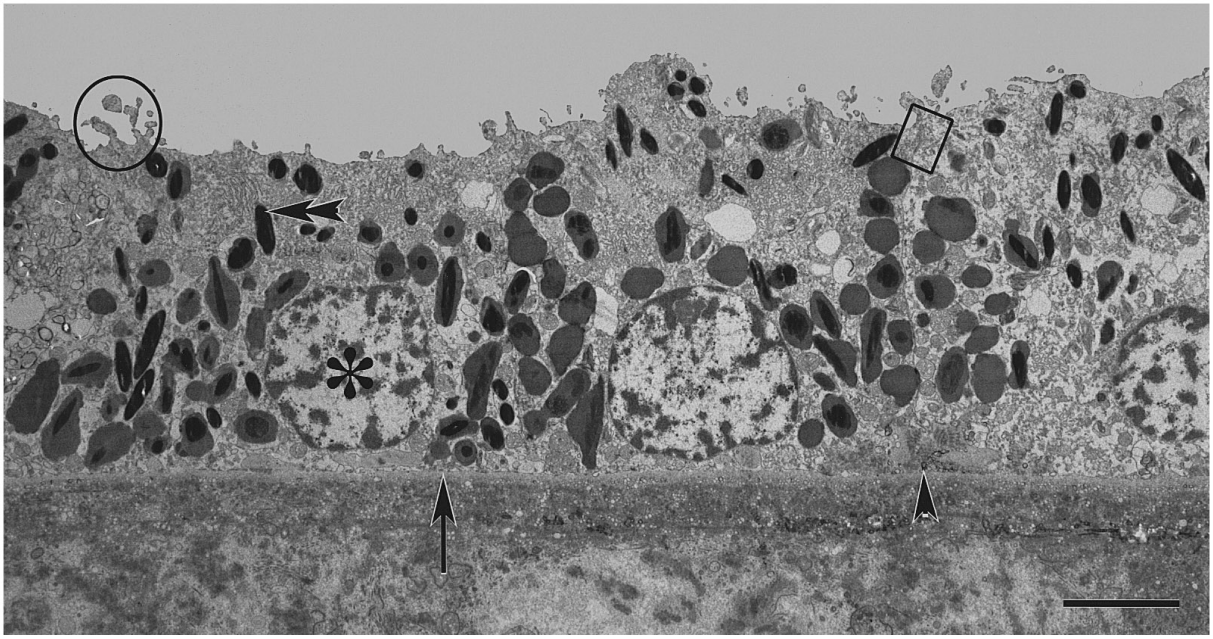

FIG. 2. TEM of undebrided human Bruch's membrane specimen. Apical villous processes (circle), basement membrane (arrow), basally situated nuclei with dispersed chromatin (∗), and numerous pigment granules (double arrowhead) are present. Some areas of basal laminar deposit are evident (arrowhead). Tight junctions (square) are present at the apical lateral borders of the cells. Bar = 2·0 μm.

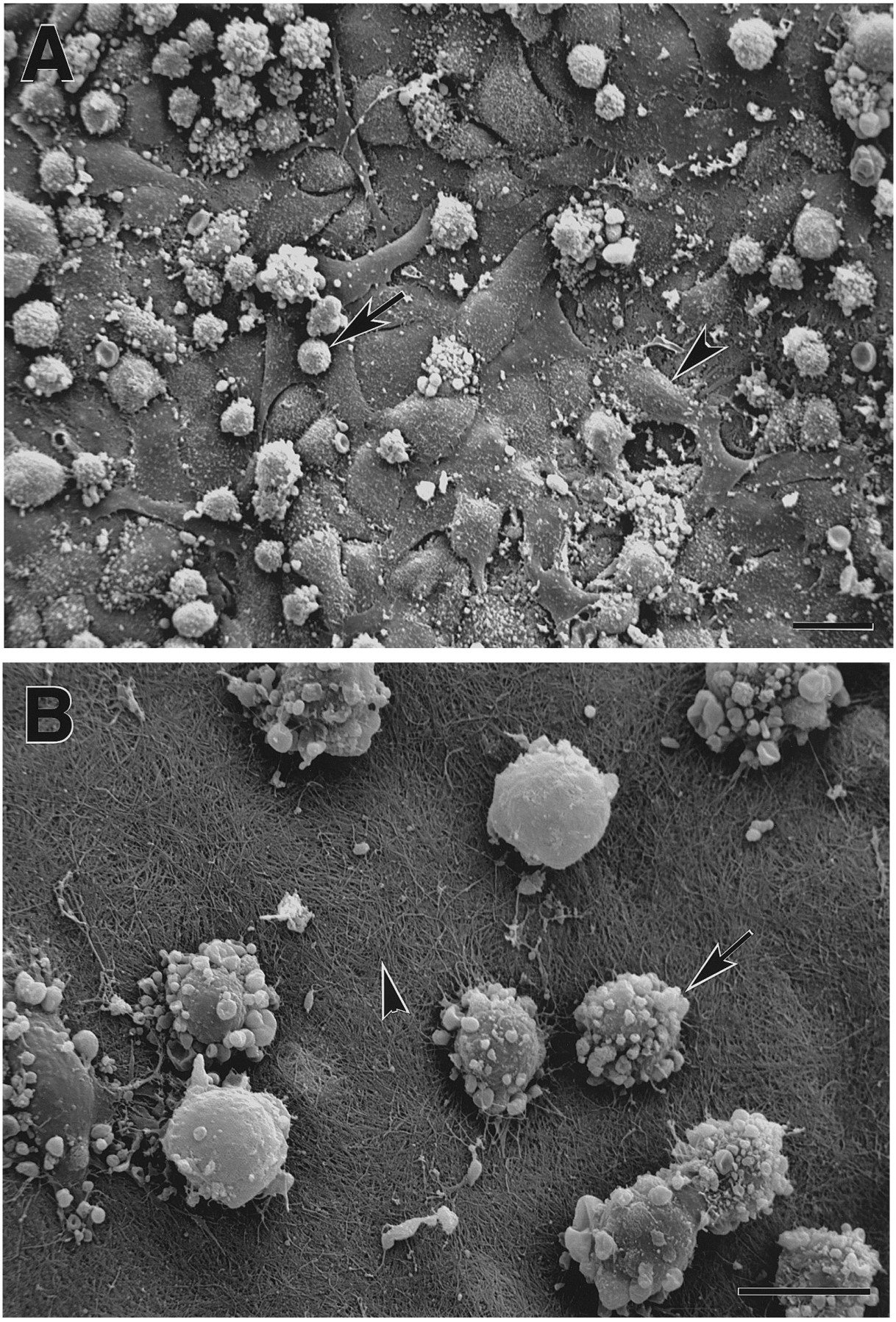

FIG. 3. For legend see p. 54.

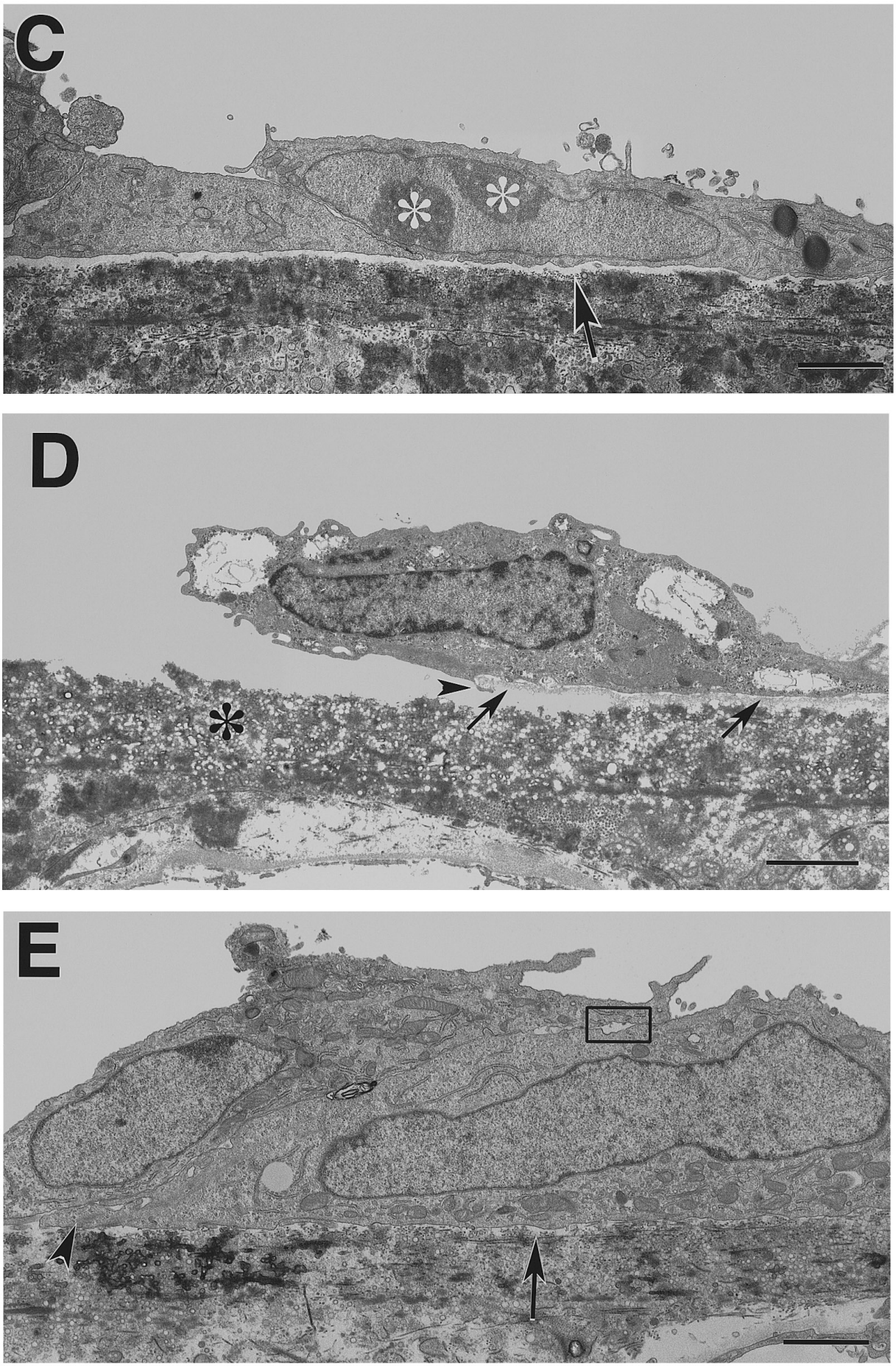

FIG. 3. For legend see p. 54.

areas where the basement membrane was present [Fig. 3(C), (D)] i.e., the plasma membrane was closely apposed against subjacent BM. RPE were also identified in areas lacking basement membrane [Fig. 3(E)], although this was rarely observed at this time point. In general, lateral cell borders were obliquely oriented with respect to BM, and the nuclei were elongated, exhibited prominent nucleoli, and were oriented parallel to BM. At 1 hr, cells that were attached to bare collagen without basement membrane appeared more rounded [compare Fig. 3(C) with 3(E)]. In one specimen, basal laminar deposit (Green and Enger, 1993) was present on one edge of the specimen. RPE cells appeared to attach well to basal laminar deposit although their profiles were rounded, not unlike profiles observed in cells attached to the inner collagenous layer (see below). Although most RPE cells did not exhibit the features of polarization seen in situ (Fig. 2), tight junctions were present between adjacent cells. Newly formed basement membrane did not appear to be present.

At 4 hr incubation, a monolayer of RPE cells covered denuded BM. The percentage coverage of the BM button ranged from 76% to ~ 100% (Tables I, II and Fig. 1) with a mean of 92%, median 97·2%. Compared with 1 hr incubation, the cells appeared flatter, and their shape was more uniform [Fig. 4(A)]. In all specimens at this time point, the RPE cells still were larger and, except in a few areas, lacked the hexagonal shape seen in confluent cultures and in undebrided RPE. At this time we also noted irregularly shaped cells with numerous surface projections sitting on top of the monolayer. By TEM, the seeded cells appeared well attached throughout most of the specimens in areas with basement membrane [Fig. 4(B)]. In areas where the inner collagenous layer of Bruch's membrane was exposed, cell attachment was variable with some cells exhibiting close apposition of the plasma membrane and subjacent BM, and others exhibiting gaps between the plasma membrane and subjacent BM. Cells had a very flattened appearance independent of the presence of RPE basement membrane. The specimen which showed the most regularly shaped cells (No. 70M, 99·2% coverage, see Table I) had the most basement membrane present under the cells. Interestingly, this specimen also showed a large amount of basal linear deposit (Green and Enger, 1993; Sarks, 1976) compared to other specimens at this time point. In some cells apical microvilli were present, and apical-lateral tight junctions between cells were present. No newly formed basement membrane was noted at this time.

At 6 hr incubation, we observed an almost complete RPE monolayer with 90 to ~ 100% (mean 97%, median 99·8%) coverage (Tables I, II and Fig. 1). In 1 specimen (No. 83M) we found a high standard deviation (11·05%). We suspect this specimen showed patchy coverage due to specimen handling. (SEM showed lifted edges indicating that perhaps some of the RPE had lifted off the surface.) At this time we began to see the expression of hexagonal shape in some areas [Fig. 5(A)]. Foci of cell multilayers were observed with the top layer of cells exhibiting elongate, irregular shape. By TEM we saw gaps between the basal RPE plasma membrane and the subjacent inner collagenous layer in some areas [Fig. 5(B)]. These cells were more flattened and spindle shaped than cells attached to the RPE basement membrane, and their cell borders were still obliquely oriented with respect to underlying BM. In general, where RPE were attached to basement membrane, they appeared to be more regular in shape, less flattened, and more tightly apposed to each other. Their cell borders had a more vertical orientation. Cells that were attached to basal laminar deposit appeared similar to those attached to basement membrane, i.e., they were well attached and not as spindle-shaped [Fig. 5(C)]. We saw no difference in the attachment of cells in areas rich in basal linear deposit compared to specimens with very little basal linear deposit. Apical microvilli were variably present, and apical lateral tight junctions were present frequently.

At 8 hr incubation, we saw a layer of cells with > 99% coverage (mean 92%, median 99·97%) in all of the specimens except for one, which showed $68 \pm 28\%$ coverage (Tables I, II and Fig. 1). This specimen had a large quantity of red blood cells on its surface, possibly from breaks in the specimen due to handling. In areas adjacent to the red blood cells, there were no attached cells. Other areas away from the red blood cells showed complete coverage by RPE. In general, RPE were still larger than in situ, and more cells showed

FIG. 3(A) SEM of RPE cells one hour after plating on debrided Bruch's membrane. The cells exhibit variable shapes from rounded (arrow) to flattened (arrowhead). Bar = 20 μm. (B) High magnification SEM of RPE cells one hour after plating onto debrided Bruch's membrane without RPE basement membrane. Most cells have a rounded appearance (arrow) when seeded onto the inner collagenous layer. Pseudopods are present on the majority of the cells. Collagen fibers of the inner collagenous layer are evident (arrowhead). Bar = 10 μm. (C) TEM of RPE cells 1 hr after plating onto debrided Bruch's membrane. Cells show close adherence to RPE basement membrane (arrow). Cells are flattened, and nuclei are elongate with prominent nucleoli (asterisks) and are oriented parallel to Bruch's membrane. Bar = 2·0 μm. (D) TEM of RPE cells 1 hr after plating onto debrided Bruch's membrane. This cell shows preferential attachment to RPE basement membrane (arrows) vs. the denuded inner collagenous layer (*). Note the presence of a lamellar process (arrowhead) that appears to be wrapping around the basement membrane fragment. Bar = 2·0 μm. (E) TEM 1 hr after plating. Note absence of RPE basement membrane (arrow) and spaces between plasma membrane of adjacent RPE (□). Cells have a more rounded appearance than those attached to basement membrane. The migrating RPE exhibits lamellipodia (arrowhead). Areas of subplasmalemmal fusiform densities are present in the lamellipodia. Bar = 2·0 μm.

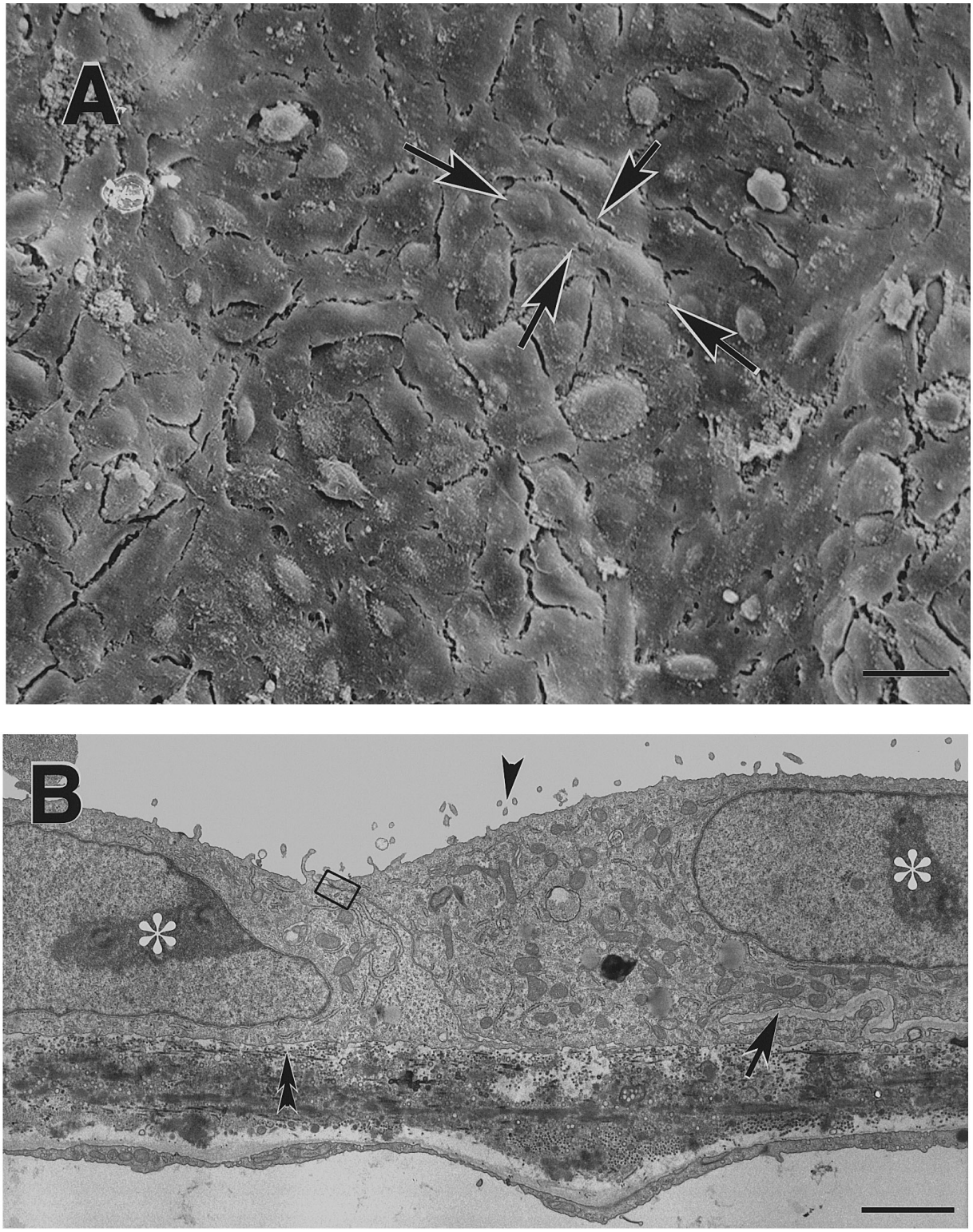

FIG. 4(A) SEM 4 hr after plating. The RPE cells are confluent but have irregular, elongated borders (area marked by arrows). Bar = 20 μm. (B) TEM 4 hr after plating. Note the close apposition of the lateral plasma membranes of adjacent cells. Tight junctions are present (square). These cells appear well attached to the inner collagenous layer (double arrowhead). A piece of RPE basement membrane is present (arrow) which probably represents residual basement membrane following debridement. The nuclei are still flattened, oriented parallel to Bruch's membrane, and exhibit prominent nucleoli (*). Note the slanted cell borders, unlike the cell borders shown in the control (Fig. 2), which are perpendicular to Bruch's membrane. Some apical villous processes are present (arrowhead). Bar = 2·0 μm.

hexagonal shape. By TEM the cells appeared well attached to the denuded BM [Fig. 6(A)–(C)]. However, RPE that were not attached to basement membrane exhibited a greater variety of shapes ranging from elongate, flattened cells with tapered ends [Fig. 6(B)] to rectangular-shaped cells with cell borders oriented

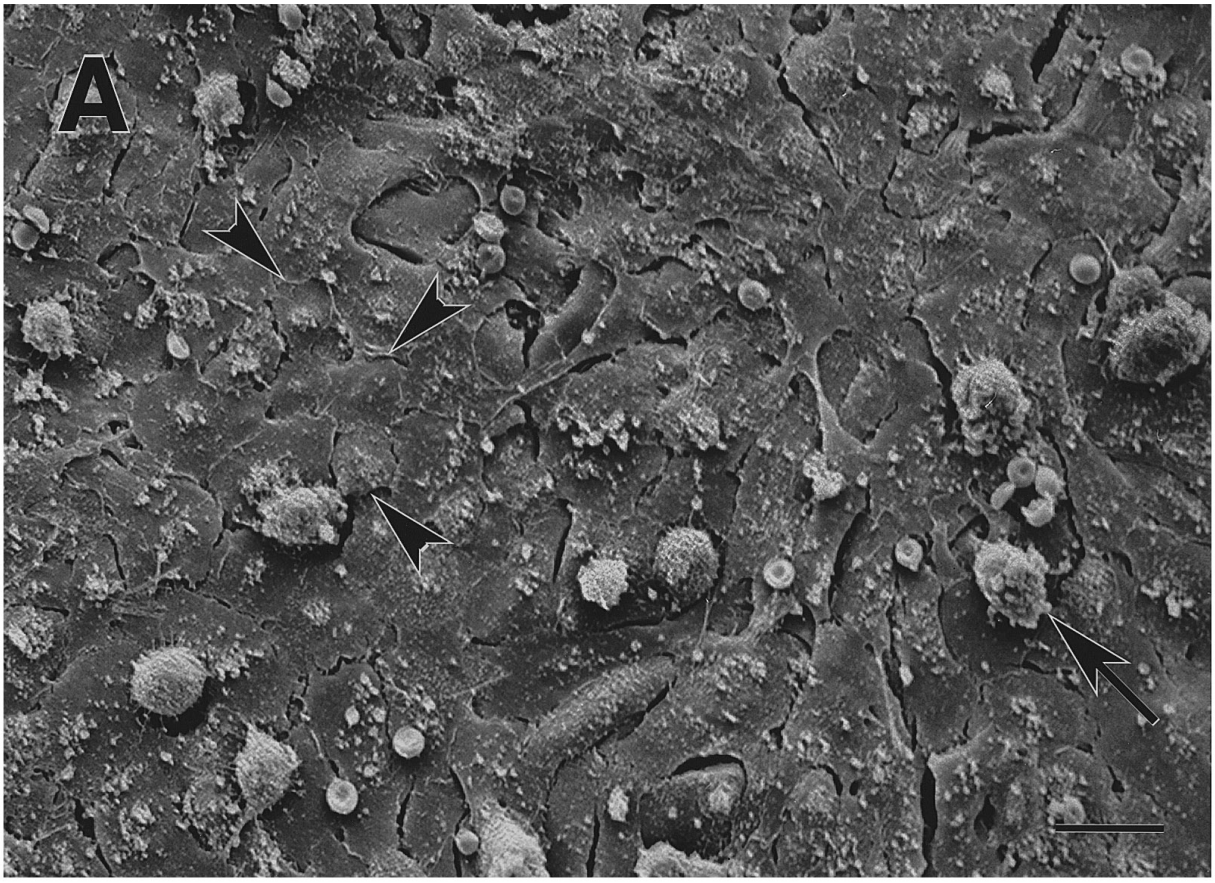

FIG. 5. For legend see facing page.

closer to the vertical meridian. The RPE showed closer apposition to the inner collagenous layer than at earlier time points. Some cells (with and without subjacent basement membrane) appeared flatter than at earlier time points and in some areas were very flat, as if they had flattened to cover areas that were not initially covered with cells [Fig. 6(C)]. Foci of cell bilayers were present in some areas, but foci of more extensive multilayering, seen at earlier attachment times, were not observed. The cells at the top of the bilayer were elongate. At this time we saw variable expression of microvilli, and the cells were closely apposed and connected by junctional complexes. No newly formed basement membrane was observed. A few cells showed the beginning of intracellular vacuole formation, including cells attached to basement membrane as well as those attached to the inner collagenous layer.

After 12 hr incubation, a RPE monolayer with coverage ranging from 97 to ~ 100% (mean 99%, median 99·8%) was observed (Tables I, II and Fig. 1). The cells still showed variable shapes ranging from flattened to hexagonal, similar to the 6 and 8 hr time points. TEM confirmed that the cells still had a variety of shapes, from spindle to rectangular in profile. At this time point, this difference in cell shape appeared to be density dependent. Light and electron microscopic examination showed that areas covered by a low density of RPE cells had cells that were extremely flattened [as in Fig. 6(C)] whereas in areas with a higher density of RPE cells, the cells tended to have a more rectangular shape. All cells appeared closely apposed against BM. Foci of cell bilayers were present. Apical microvilli and tight junctions were present. The lateral cell borders were not perpendicular to BM, and no new basement membrane was seen. Vacuoles were more frequent and often were larger than at earlier incubation times (Fig. 7).

At 24 hr incubation, a confluent monolayer totally covered denuded BM [Fig. 8(A)]. Hexagonal cell shape was more common. The mean and the median percentage of coverage was 99·76% and 100%, respectively. By TEM the cells were well attached to BM. The cells were slightly taller and less flattened than before, but compared to their in situ morphology (Fig. 2), the RPE nuclei were still elongated and oriented parallel to BM [Fig. 8(B)]. At this time the cells resembled fetal RPE cells in culture (Fig. 9), although cells cultured on BCE-ECM-coated dishes exhibit more apical villous processes and prominent basal infoldings. Microvilli were variably expressed. The lateral cell borders were not quite perpendicular to BM. They were closely apposed to each other and were joined with apical lateral tight junctions [Fig. 8(B)]. No newly formed basement membrane was noted.

We questioned whether the increase in percent coverage over the first 8 hr was linear. Lowess and cubic spline smoothers that we applied to the re-

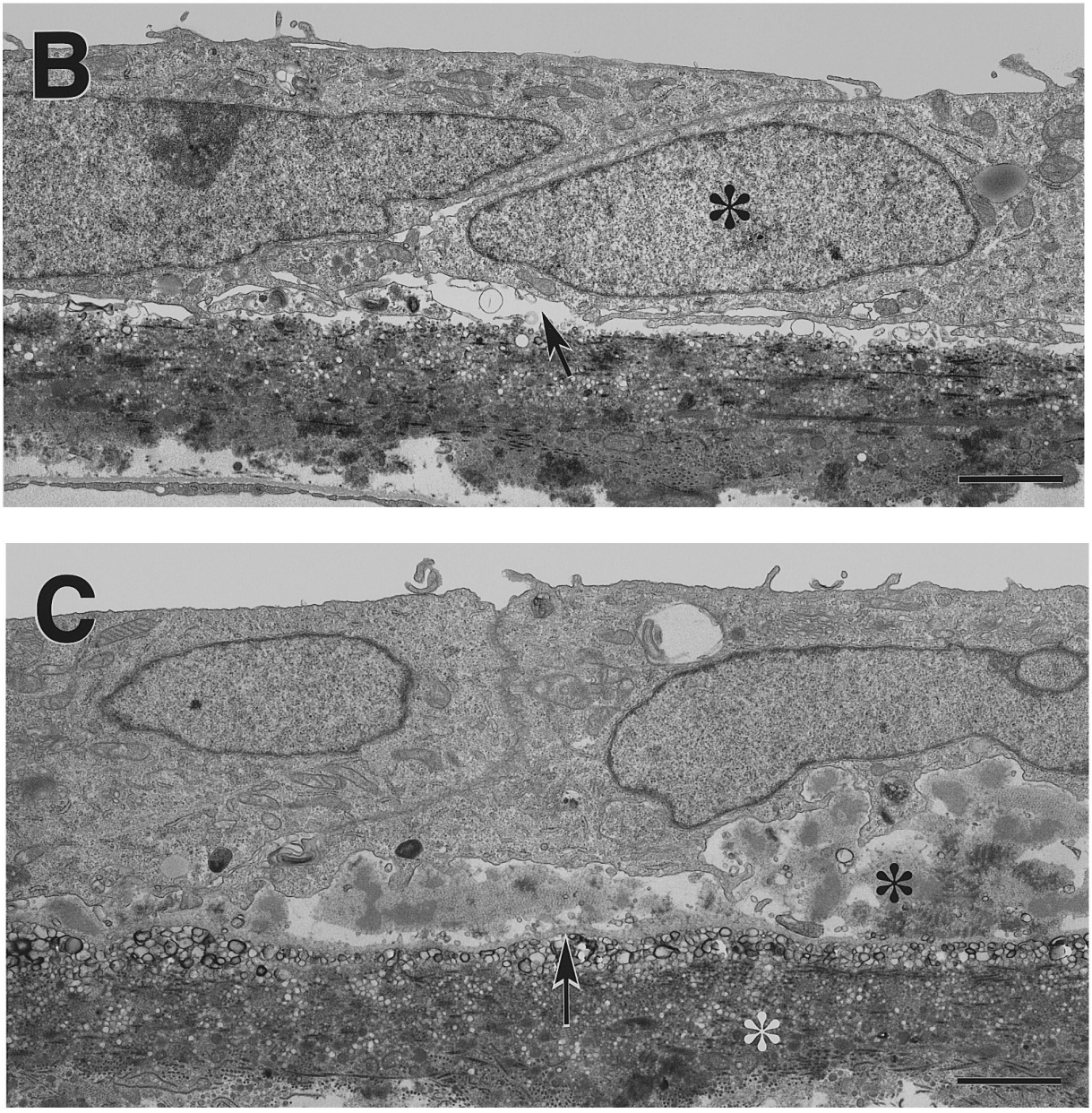

FIG. 5(A) SEM 6 hr after plating. Cells are confluent, and some exhibit hexagonal shape (arrowheads). Supernumerary cells are still present (arrow). Bar = 20 μm. (B) TEM 6 hr after plating. Note the absence of basement membrane and the gap present between the cells and the inner collagenous layer (arrow). Cells are flattened with elongated nuclei (*). Cells are closely apposed with slanted cell borders. Bar = 2·0 μm. (C) TEM 6 hr after plating. These cells are attached to residual basal laminar deposit (*). RPE basement membrane is present (arrow). This specimen also exhibits basal linear deposit (white *). The presence of basal laminar deposit does not seem to affect the attachment of the cells nor their morphology. Bar = 2·0 μm.

expressed data indicated a curvilinear relationship between percentage of coverage and time whereby percent coverage is nearly 80% by the first hour and increases smoothly in a quasi hyperbolic manner to above 99·9% by 8 to 24 hr. Welch's analysis of variance (ANOVA) test of the re-expressed data indicated that the variances were unequal ($P >$ 0·0001). Subsequently, a Tukey-Kramer multiple showed that differences observed between the early hours and some of the later hours were significant; adjacent pairwise differences became less significant as the percent coverage neared 100%.

To test the difference in attachment of cells with and without the presence of basement membrane, we performed further studies. Macular buttons were debrided gently, wiping 3–5 times with a wet surgical sponge (Alcon), or firmly (10–15 wipes). We seeded the cells as above and analysed the specimens after 1 hr incubation. If BM was debrided gently, leaving large areas of RPE basement membrane intact, the RPE cells attached to the remaining basement membrane, and by 1 hr we saw almost complete coverage ($92{\cdot}3 \pm 9\%$, $n = 7$) of the debrided macular button [Fig. 10(A)]. The RPE cells were not well attached (i.e., were not present) with coverage of $36{\cdot}7 \pm 32{\cdot}8\%$ ($n =$ 7) in areas from which the RPE basement membrane

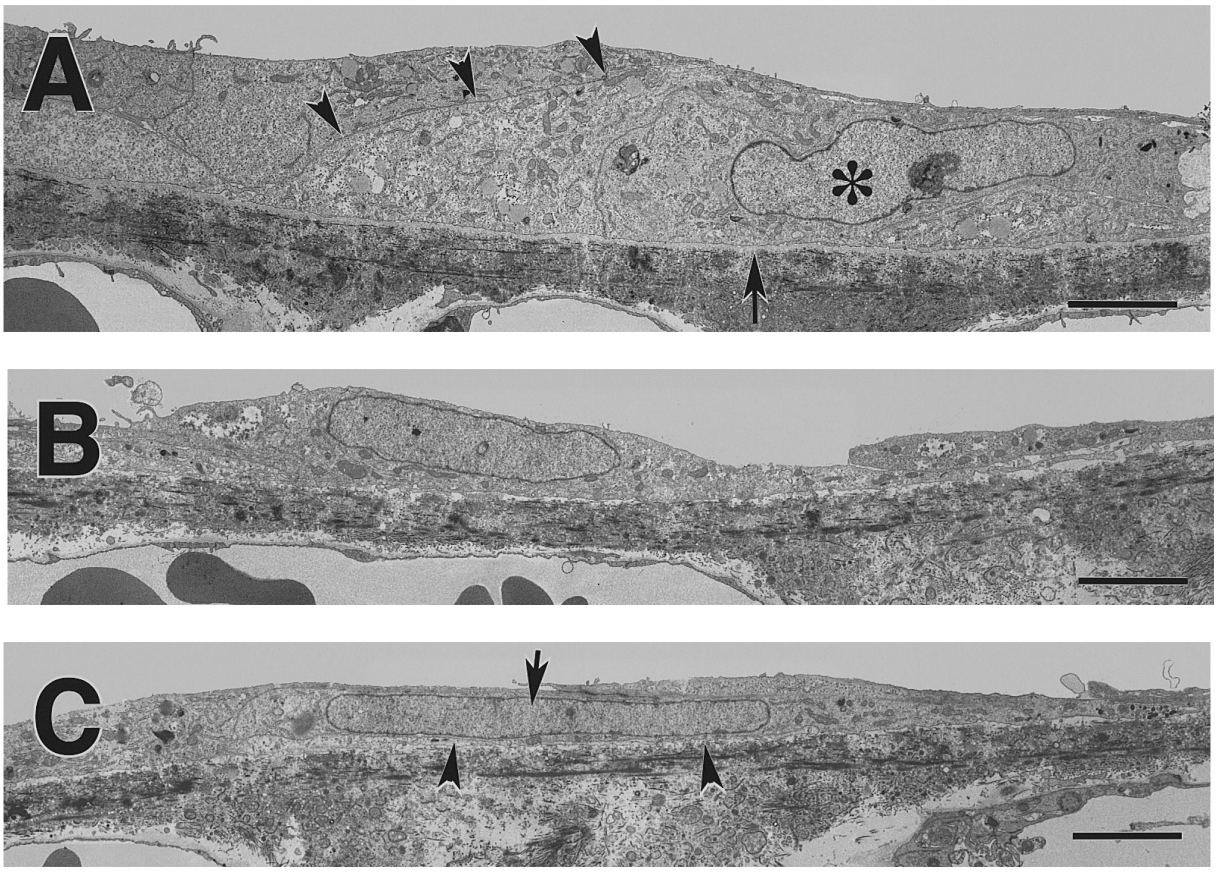

FIG. 6(A) TEM 8 hr after plating showing cells still exhibit elongated nuclei (∗) parallel to Bruch's membrane with prominent nucleoli. Basement membrane is present under these cells (arrow). The horizontal cell diameter is still greater than normal. Apical slanting of cell borders is evident (arrowheads). Bar = 5·0 μm. (B) TEM 8 hr after plating onto the inner collagenous layer. The cells are flatter than cells with subjacent RPE basement membrane [Fig. 6(A)]. Bar = 5·0 μm. (C) TEM 8 hr after plating onto the inner collagenous layer (arrowheads) in an area of low cell density. Cells show extreme flattening with very elongated nuclei (arrow). Bar = 5·0 μm.

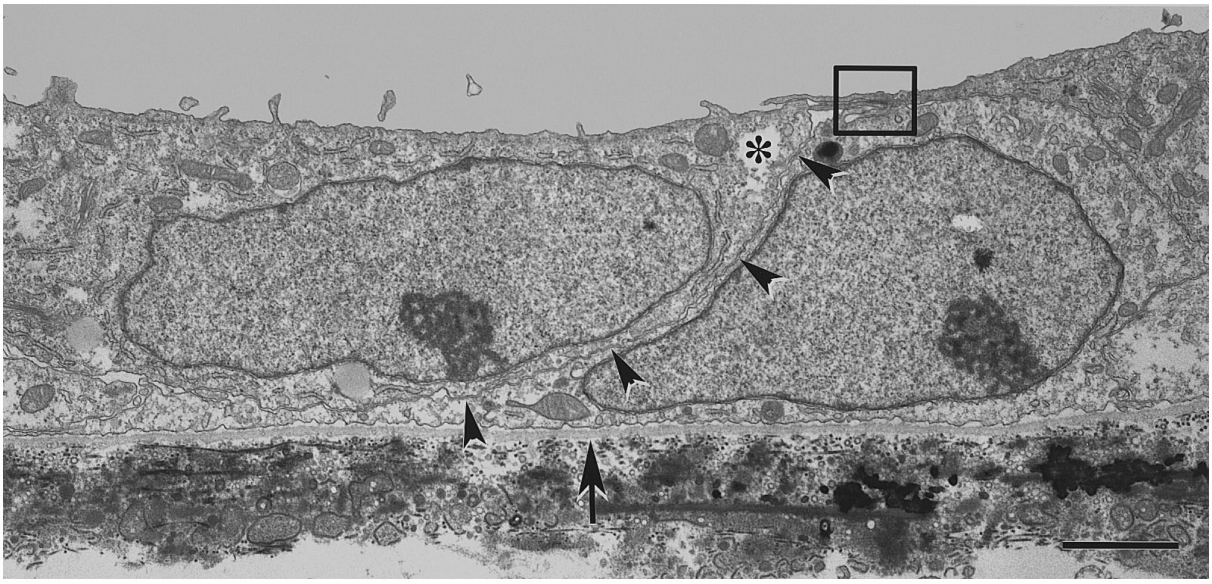

FIG. 7. TEM 12 hr after plating. Basement membrane is present in this specimen (arrow). Beginning of vacuole formation is evident (∗). Cells are assuming a more vertical orientation although cell borders are still slanted (arrowheads). Tight junctions are present (□). Bar = 2·0 μm.

had been removed via firm debridement with exposure of the underlying inner collagenous zone of BM [Fig. 10(B)]. Pilot studies suggested that the attachment of cultured fetal RPE was very poor after debridement deep enough to expose the elastic or outer collagenous layers of Bruch's membrane, even 48 hr after seeding [Figs. 11 (A)–(D)].

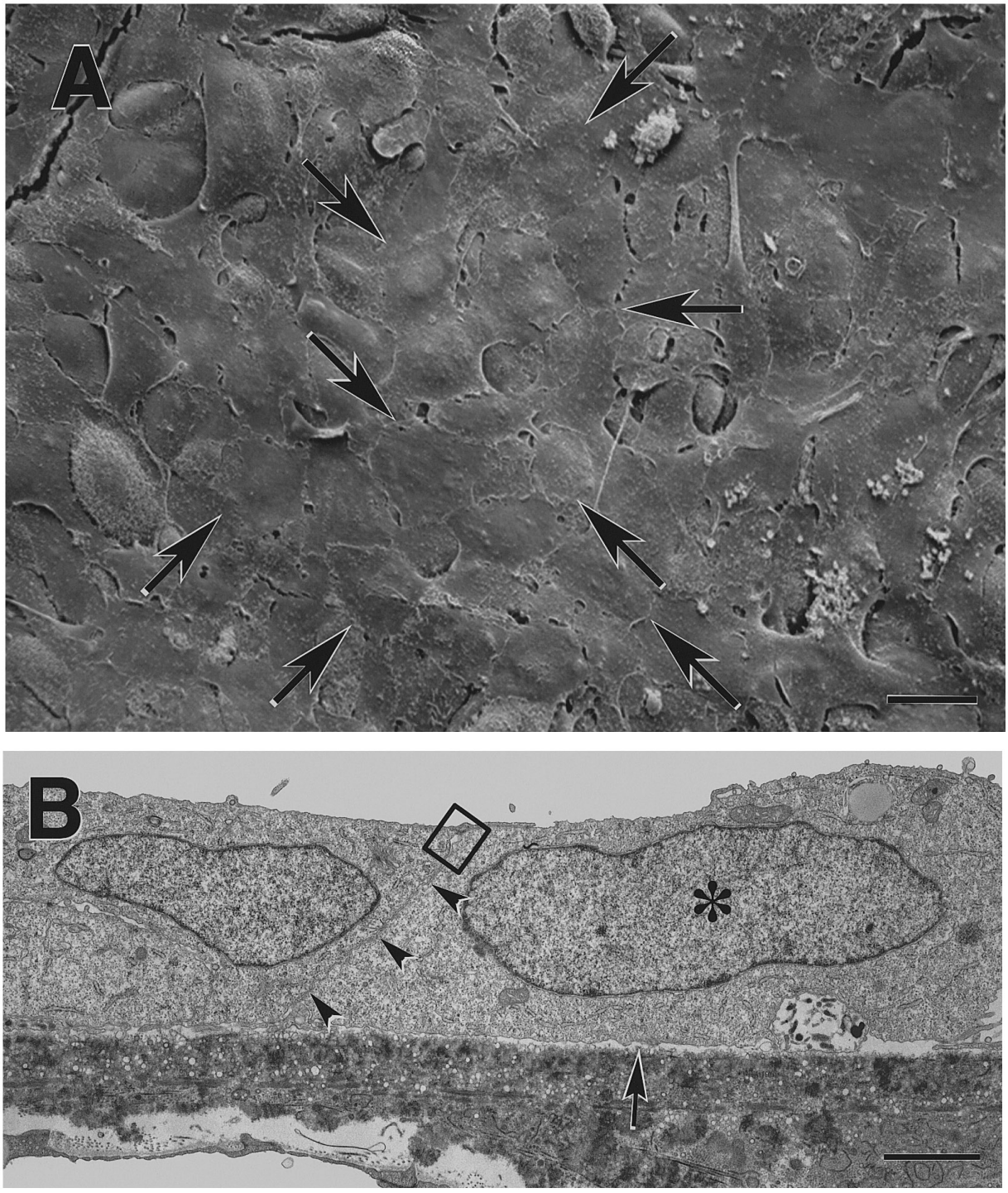

FIG. 8(A) SEM 24 hr after plating. A confluent layer of cells is present on Bruch's membrane. More cells show hexagonal shape than at earlier time points (arrows). Bar = 20 μm. (B) TEM 24 hr after plating. Lateral cell borders are more perpendicular to Bruch's membrane than at earlier time points (arrowheads), and cells have assumed a more vertical orientation. Cells are closely apposed against Bruch's membrane regardless of the presence or absence of RPE basement membrane. This specimen shows attachment in the absence of basement membrane (arrow). Tight junctions are present between cells (□). Nuclei (asterisk) are still elongate. Bar = 2·0 μm.

4. Discussion

The in vitro RPE-BM culture system we used is attractive because it allows one to quantitatively evaluate the behavior of human RPE cells on human BM specimens that may resemble the substrate encountered in vivo (i.e., BM from aged eyes with a thickened inner collagenous layer) during RPE transplantation in AMD patients. Miceli and Newsome (1994) used a similar in vitro assay to study rod outer segment uptake in fetal human RPE transplanted onto BM-choroid explants and other ECM preparations. They found that the substrate to which the RPE cells are attached may affect their morphology and differen-

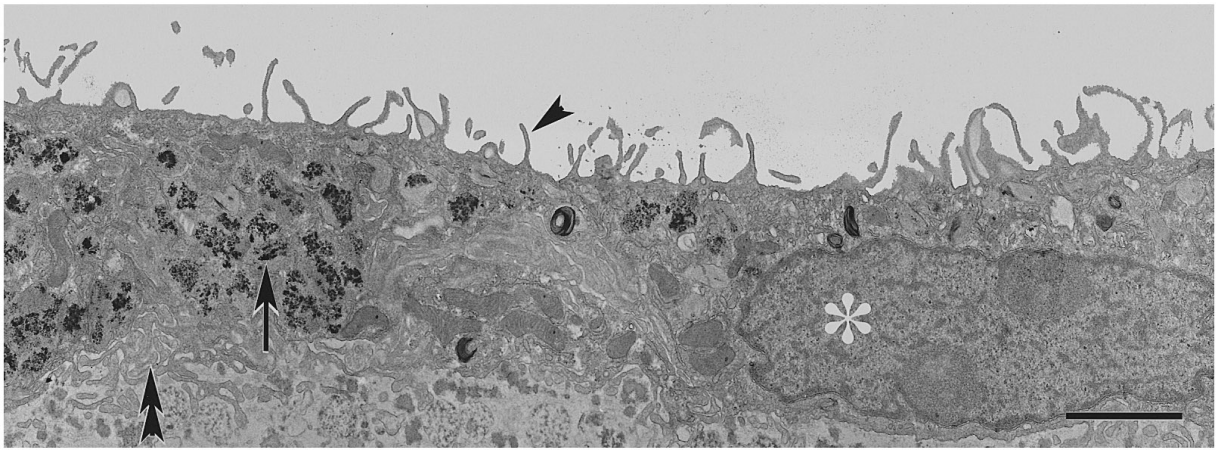

FIG. 9. Fetal human RPE cells in culture. Third passage. Apical villous processes (arrowhead), melanin granules (arrow), and basal infoldings (double arrowhead) are present. Note the flattened horizontal nucleus (*). Bar = 2·0 μm.

tiation. Our experiments focused on the time course of RPE adhesion to macular BM and used BM specimens that appeared normal clinically (although some specimens exhibited microscopic evidence of AMD changes). Disadvantages of this in vitro paradigm include the inability to assess an inflammatory or immune response to the planned interventions/substances and the inability to study the role of and effect on photoreceptors in these experiments.

As the incubation time increased, the degree of coverage of the debrided surface increased and coverage became more uniform (Fig. 1). Areas with RPE basement membrane present showed cells closely apposed to the underlying basement membrane. Cells attached to the inner collagenous layer showed variable apposition with some closely attached to the collagen and others separated by a distinct space. The loose attachment of these cells may have resulted in high variability in the percent coverage due to displacement of some of these cells during processing. At times between 1 and 4 hr after seeding, there was a clear tendency of the cells to attach to basement membrane instead of bare collagen.

At the earliest incubation times, cellular morphology varied greatly, ranging from rounded and globular to flattened and spread out. Cells were used during their third to fifth passage for these experiments, and all the cells were from the same cell line (Song and Lui, 1990). Thus there are no systematic generational or cell line differences between experiments assessing RPE adhesion to debrided BM at different incubation times. The variability in morphology may be due to the presence or absence of subjacent RPE basement membrane and/or a consequence of variable cellular recovery after trypsinization. If the cells have not had time to attach with close apposition of the plasma membrane against BM, manipulation of the tissue may provoke the displacement of the cells from BM which in turn may influence their survival. In addition to the vulnerability to external manipulation at times shortly after seeding and before adherence to the underlying substrate, different recovery times of enzymatically dispersed cells might explain the variability in cellular attachment to BM at the early times (e.g., < 4 hours) after seeding. These findings may be relevant to in vivo transplant studies using dispersed cells (Algvere et al., 1996).

Cellular morphology varied greatly at the earliest incubation times but was more uniform at later times. Later time points showed that the shape of cells may be density dependent (see also Tezel and Del Priore, 1997). In areas where there was a high cell density, RPE assumed a horizontal rectangular shape. In areas where there were few cells, the RPE were flattened. The observed differences in cell shape and density may be due to the preferential attachment of cells to basement membrane. Areas with RPE basement membrane had higher RPE cell density and therefore more normal cell shape. Areas lacking RPE basement membrane had lower cell density, and therefore the cells may have spread out more to effect 100% coverage of the BM explant. As pointed out above, variable cell shape may also be due to variable recovery from trypsinization. Loss of anomalous cells might also lead to greater morphological uniformity after longer incubation periods. At later times (e.g., $>$ 6 hr incubation) and in areas where the basement membrane was present, in addition to having hexagonal shape, the cells tended to have a smaller horizontal diameter, exhibit apical microvilli, and have lateral cell borders oriented more perpendicular to BM. These cells resembled RPE in situ although they were not as hexagonal as native RPE and were slightly larger and more flattened. Also, their microvilli were shorter, and their apical-basal polarity was less pronounced than that of native RPE.

RPE cell flattening is observed as a normal process during ocular development (Ts'o and Friedman, 1968; Streeten, 1969), in eyes with glaucoma (Ueno and Naumann, 1989), in AMD (Green, McDonnel and Yeo, 1985; Sarks 1976), beneath sensory retinal

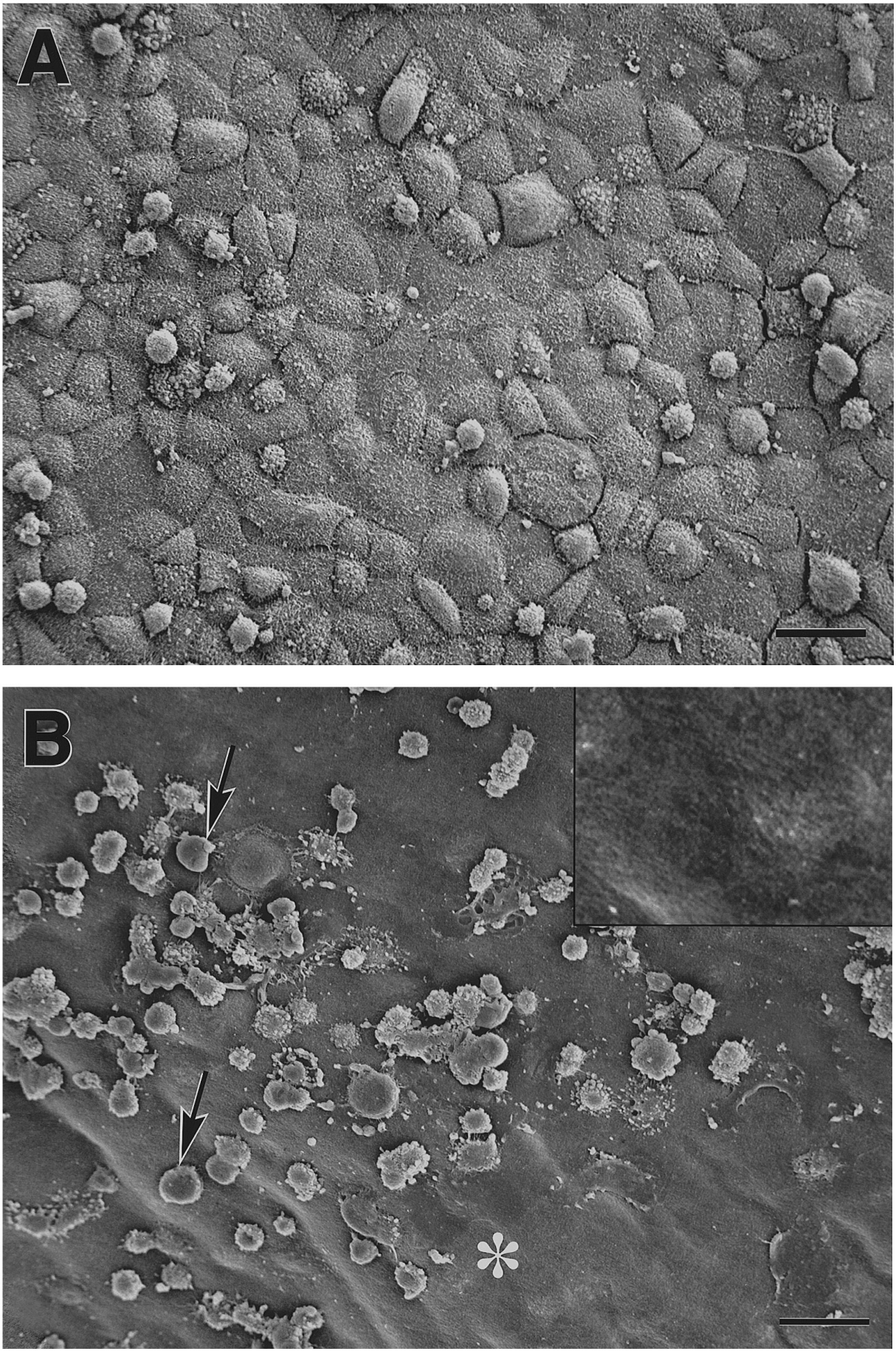

FIG. 10. For legend see facing page.

detachment (Machemer and Laqua, 1975), and in wound healing following thermal injury (Apple, 1977; Lincoff and Kreissig, 1979) or localized RPE debridement (Heriot and Machemer 1992; Lopez et al., 1995; Leonard et al., 1997; Valentino et al., 1995; Del Priore et al., 1995). This morphology also characterizes human RPE growing on plastic (Israel et al., 1980; Del Monte and Maumenee, 1981; Campochiaro and Hackett, 1993). Flattened appearance and reduction in the number of microvilli was reported by Nicolaissen et al. in another model of in vitro ingrowth of RPE after as long as 4 weeks in culture on cadaver BM (Nicolaissen et al., 1988; Overskott and Nicolaissen, 1988; Fiskaadal et al., 1992; Nicolaissen et al., 1989). Also, we transplanted the cells from a petri dish coated with BCE-ECM to cadaver BM obtained approximately 24–48 hr after enucleation and later incubated at 37°C. The change in growth environment may play an important role in the transformation of the RPE into a flattened fibroblast-like morphology. Contamination of the inner surface of BM by choroidal fibroblasts is unlikely in these experiments due to the early times after harvest and plating at which the preparations were examined. In otherwise mitotically quiet cells, 12–72 hr are required before proliferation occurs in response to a stimulus (Baserga and Wiebel, 1969). Choroidal fibroblasts might have influenced the behavior of the seeded RPE. The RPE nature of the cells was determined by their morphology in culture and the reliability of the RPE isolation technique. We judged RPE cell differentiation by morphological criteria alone. Additional information regarding differentiation might be obtained by testing for cellular retinaldehyde binding protein expression in the cells at various times after plating (Campochiaro and Hackett, 1993). The lack of overlying photoreceptors might explain why the cultured RPE do not tend to express many apical microvilli and might also contribute to their flattened appearance. Some of the tight junctions seen in the one hour incubation experiment might reflect incomplete cell separation after trypsinization.

Our pilot studies showed that if the debridement exposed the elastic or the outer collagenous layers of BM, the cultured fetal RPE cells seemed to adhere poorly. If debridement left intact part of the native RPE basement membrane or exposed the inner collagenous layer, the fetal RPE cells tended to attach faster with closer apposition of the plasma membrane and subjacent BM. The best RPE attachment was observed when the basement membrane was left intact [Figs 10(A), (B)]. The coverage of the debrided BM explants with little or no basement membrane may have been underestimated due to cell detachment from BM during specimen preparation. In some SEM specimens we observed what appeared to be lifted or torn edges of the sheets of transplanted cells. We also observed an edge of cells lifted above the debrided surface in some plastic embedded specimens.

Differences in the degree of coverage of firmly vs. gently debrided BM specimens may indicate that at early time points, the attachment and colonization of transplanted RPE cells can be affected by the presence or absence of RPE basement membrane. In vitro experiments have shown that normal, untransformed epithelial cells can attach rapidly and spread on attachment factors such as fibronectin, laminin, β-1 integrin, and epibolin (Stenn, 1981; Song and Lui, 1990; Ohji et al., 1993, Chu and Grunwald, 1991 a, b). In general epithelial cells do not appear to have an absolute requirement for only one attachment protein such as laminin (Stenn et al., 1983; Paulsson, 1992; Ohji et al., 1993). Studies of RPE cells have shown that attachment and proliferation of these cells can be facilitated by combinations of laminin, fibronectin, collagen type IV, and other components of the extracellular matrix as well as by heparin and growth factors (Heth et al., 1987; Song and Lui, 1990; Campochiaro and Hackett, 1993; Ho and Del Priore, 1997). This lack of absolute specificity for attachment factors suggests that RPE cells are capable of interacting with multiple factors, and therefore a combination of the various attachment factors in appropriate proportions may facilitate the attachment of transplanted RPE cells onto denuded BM. This combination is likely present in the areas of residual RPE basement membrane. In fact, studies by Campochiaro and Hackett (1993) show that RPE cells not only

FIG. 10(A) SEM of specimen debrided, leaving RPE basement membrane intact, 1 hr after plating. Cells have formed a continuous layer with fairly uniform hexagonal shape. Bar = 30 μm. (B) SEM of specimen debrided, removing RPE basement membrane, 1 hr after plating. Few cells (arrows) are present on the debrided surface exposing the inner collagenous layer (*); insert (3 × magnification) corresponds to area near the asterisk. The cells are rounded in appearance. Bar = 30 μm.

FIG. 11(A) SEM of specimen debrided, leaving RPE basement membrane intact, 48 hr after plating. For this experiment, $2{\cdot}42 \times 10^5$ cells/0·5 ml of this suspension was pipetted into the trephine onto the explants. Thus, some supernumerary cells are present (arrow). RPE cells have formed a continuous layer with hexagonal shape (arrowheads). Bar = 10 μm. (B) TEM of specimen debrided, leaving RPE basement membrane intact, 48 hr after plating. RPE cells are closely apposed against the basement membrane (arrowheads). Vacuoles (*) and microvilli (arrow) are present. Bar = 2 μm. (C) SEM of specimen debrided, removing RPE basement membrane and portions of the inner collagenous layer 48 hr after plating. Cells do not form a confluent monolayer, and, due to the deep debridement, disorganized collagen fibrils are exposed (*). A gap is present between the cells and the subjacent collagen fibrils (arrowheads). Some supernumerary cells are present (arrow). Bar = 10 μm. (D) TEM of specimen debrided, removing RPE basement membrane and most of the inner collagenous layer, 48 hr after plating. Large gaps are present between the cells and subjacent Bruch's membrane (*). Lamellopodia (arrowhead) and microvilli (arrow) are visible. The lamellopodia may reflect an ongoing attempt of the RPE to establish contact with the underlying surface. Bar = 2 μm.

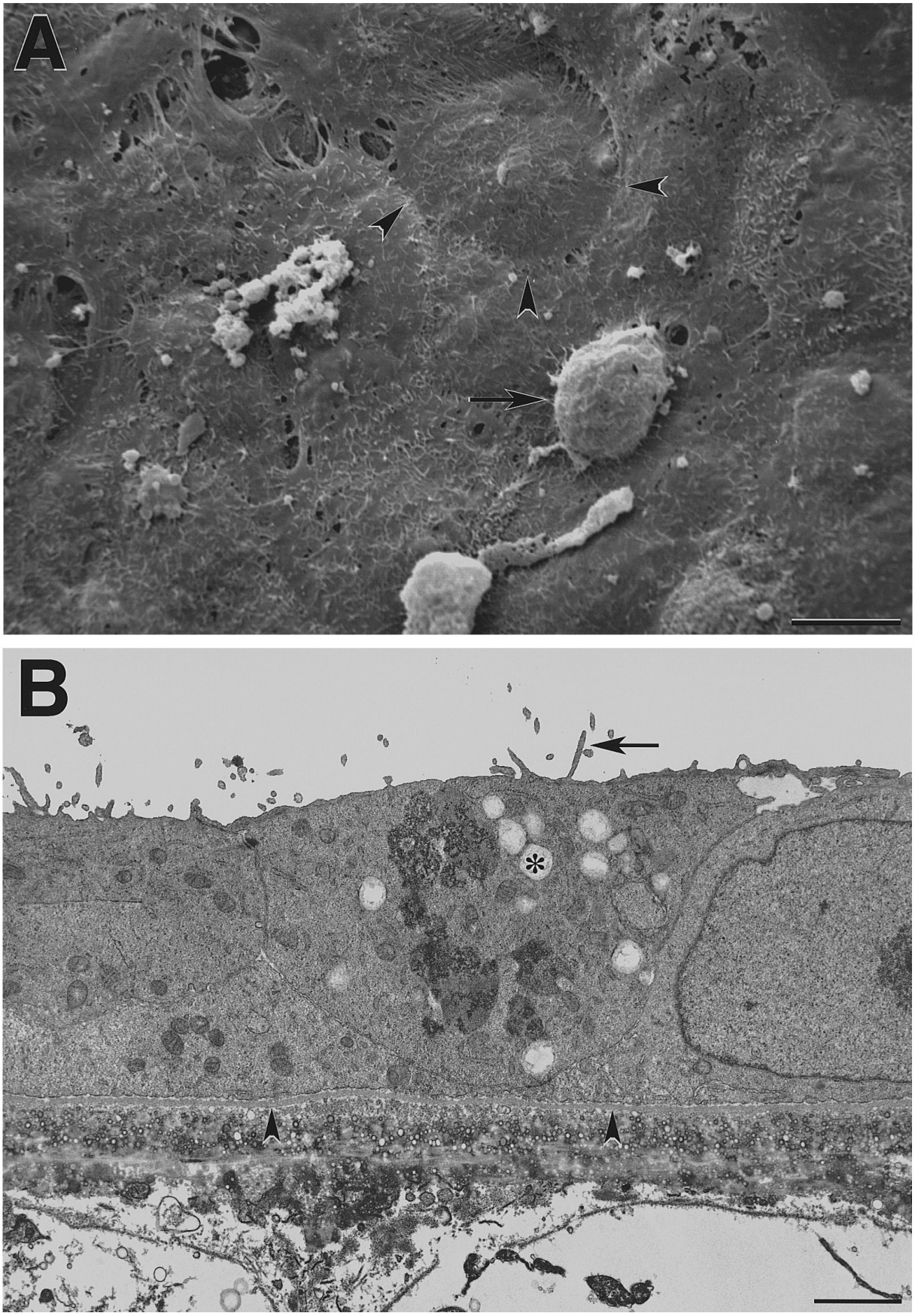

FIG. 11. For legend see p. 63.

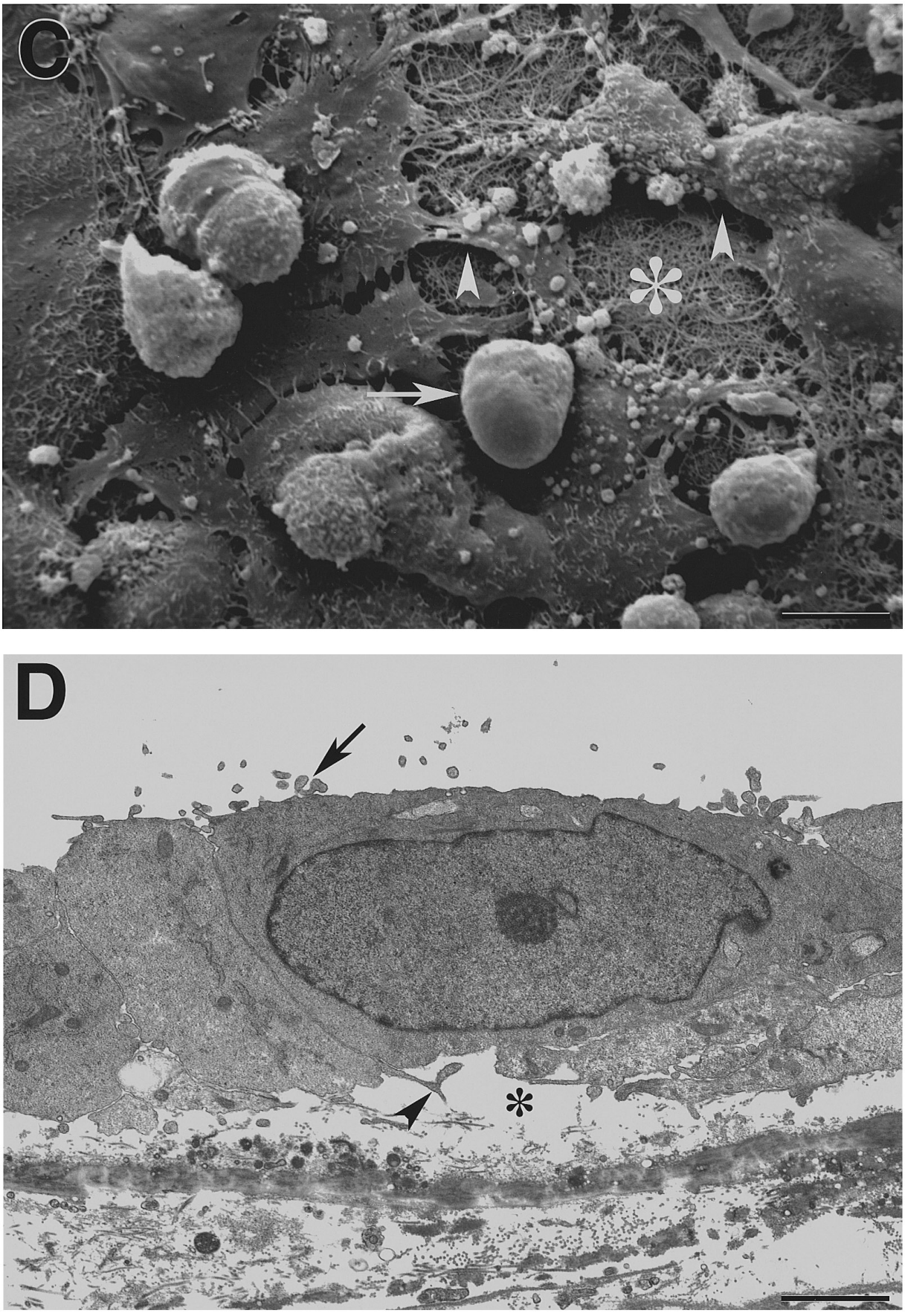

FIG. 11. For legend see p. 63.

attach and proliferate in culture but also differentiate and show density dependent inhibition when grown on laminin supplemented with bFGF and heparin.

We did not observe signs of cell proliferation (by SEM or TEM) in the transplanted cells perhaps because the cultures were followed only for 24 hr. We did not, however, study markers of proliferation to specifically address this issue. We also did not observe basement membrane synthesis. Ultrastructural analysis allowed us to exclude the presence of newly formed basement membrane after 24 hr incubation based on our observation that newly formed basement membrane is thinner than aged basement membrane. (We have found that basement membrane under metaplastic RPE in recurrent CNVs in AMD patients is approximately 0·1 μm in thickness (Castellarin, Zarbin and Sugino, unpublished observation) compared to thickness range of 0·3–0·6 μm in the specimens of the present study.) Further investigations are planned to establish whether and when the transplanted cells are able to produce new basement membrane. Campochiaro, Jerdan and Glaser (1986) showed that human RPE synthesize basement membrane components in culture (e.g., laminin, type IV collagen), but the cells had to be maintained for several days before these proteins were detected immunohistochemically. Also, Campochiaro and Hackett (1993) showed that cultured human RPE did not exhibit features of differentiation optimally unless grown on ECM taken from human RPE cultures at least 3 weeks old. These studies imply that more than 24 hr is required for cultured human RPE to elaborate basement membrane.

The eyes used for these experiments did not have signs of AMD on post mortem gross pathological examination, but the average age of the donors was 78·8 years. Nonetheless, in several cases we noted the presence, in variable quantities, of basal linear and basal laminar deposit which are histopathological signs of AMD (van der Schaft et al., 1991, 1992; Green and Enger, 1993; Green, McDonnel and Yeo, 1985; Sarks, 1976). We cannot determine how many BM specimens had basal laminar deposit because in most cases the material was probably wiped off by the RPE debridement. We did not examine enough specimens to determine whether basal laminar or basal linear deposit per se affect RPE attachment to BM although the cells did appear to attach in areas with basal laminar deposit. Nicolaissen et al. (1989) showed that the presence of drusen or cell debris did not affect RPE cell attachment or spreading on BM explants.

In summary, enzymatically dispersed, cultured fetal human RPE can grow in vitro on human BM (from elderly donors) debrided of native RPE. By 4–6 hr incubation, a near monolayer of hexagonal cells with tight junctions and apical microvilli, and nearly 100% coverage is established. The attachment of cells and their subsequent morphology may depend on the presence or absence of subjacent basement membrane, especially at early times after seeding. At 24 hr, the cells still do not exhibit fully differentiated morphology. This in vitro system provides a reproducible way to study the adherence of RPE to normal and diseased human BM.

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