Cross-Linked Actin Networks (CLANs) in the Trabecular Meshwork of the Normal and Glaucomatous Human Eye In Situ

Mary-Jo Hoare,1 Ian Grierson,1 Daniel Brotchie,1 Natalie Pollock,1 Kathy Cracknell,1 and Abbot F. Clark2

PURPOSE. A percentage of trabecular meshwork (TM) cells in tissue and organ culture have been shown to contain cross-linked actin networks (CLANs) when exposed to dexamethasone, as have TM cultures derived from glaucomatous individuals. The purpose of this study was to determine whether CLANs exist within TM cells in situ in tissue un manipulated by culturing, thereby eliminating the possibility that CLANs are artifacts of culture conditions, and to determine their numbers and dimensions in normal and glaucoma TM cells.

METHODS. Twelve human donor eyes (five normal and seven with glaucoma) provided the TM tissue. Each eye was disected, and the TM tissue was exposed either by microdissection (qualitative studies) or cryo-sectioning (quantitative analysis). The actin cytoskeleton was visualized using a high-affinity probe and viewed using confocal microscopy.

RESULTS. Qualitative examination of the microdissected tissue revealed that CLANs and CLAN-like structures were a common finding in the TM cells in every specimen, irrespective of whether they were from normal or glaucomatous eyes. CLAN size and phenotype were variable, with the same variations occurring in both normal and glaucomatous eyes. Quantitative analysis showed that there were more CLANs in glaucoma TM specimens than normal TM specimens, but this difference was not statistically significant. The mean number of CLANs/TM cell in our glaucoma tissue was estimated to be 1.03, while in the elderly normal controls it was 0.67.

CONCLUSIONS. This study showed for the first time that CLANs exist in cells of TM tissues from both normal and glaucomatous eyes that have not been manipulated by either tissue or organ culture procedures. It also provides quantitative data on CLAN prevalence in organized TM tissue, which indicates that CLANs are far more common than predicted (even from tissue culture) and there may be one in every cell in the glaucomatous TM in situ. (Invest Ophthalmol Vis Sci. 2009;50:1255–1263) DOI: 10.1167/iovs.08-2706

The common patterns for F-actin microfilament organization in the cell cytoplasm are peripheral actin bands, actin bundles called stress fibers, and diffuse actin networks. A fourth, far less common pattern, is a polygonal arrangement of actin filaments that can form a geodesic dome-like structure. Polygonal actin arrangements were first described in tissue culture cells by Lazarides1,2 and later found in a range of cell types as transient structures, mostly occurring when trypsinized cells were plated onto substrates. This polygonal arrangement was subsequently lost when the cultured cells spread and became established.1–6 The functional consequences of polygonal actin arrangements are unknown, but Ingber7 suggested that polygonal geodesic structures have intrinsic rigidity and contribute to cellular tensegrity. To date, such actin polygons are thought to be exclusive to the in vitro environment, with no clear evidence that they exist in vivo.

Trabecular meshwork (TM) cells have certain features in common with smooth muscle cells8–10; this may be of functional significance, because the TM contractile action may play a part in the regulation of aqueous humor outflow.11–15 A crucial part of the contractile cytoskeleton of the TM cell is their F-actin distribution in the cytoplasm, and the TM actin arrangements have been examined in tissue culture,16–21 organ culture,22 and in situ.23–28 Diffuse actin networks are evident in TM cells, but the dominant organizational pattern both in vitro21,22 and in situ23–28 are prominent stress fiber bundles. On exposure to glucocorticoids such as dexamethasone, however, some cells in human TM cultures (from both normal and glaucomatous eyes) develop complex polygons of actin that have been called cross-linked actin networks (CLANs).29–31 The glucocorticoid-induced CLANs that form in well-established confluent TM cell cultures29,31 appear to have architectural similarities to those polygonal F-actin arrangements that are associated with freshly established cultures from many cell types.1–6,52 Both are made up of units that consist of a central hub, which others have called a vertisome52, from which radiate at least five thin actin filament bundles or spokes that connect to adjacent hubs. Since dexamethasone-CLAN induction seems to be exclusive to TM cells, it is not entirely clear whether the dexamethasone CLANs and the transiently formed settlement-associated polygonal actin arrangements are the same thing. CLAN distinctiveness is supported by numerous other features; for example, the structures persist in the presence of glucocorticoids, CLANs are associated with confluence rather than settlement, and they form in cultured glaucomatous TM cells without dexamethasone exposure.31

To date, CLAN structures have only been found in some TM cells under cell culture conditions29–31 or after organ culture perfusion procedures.22 The question remains whether or not CLANs are present in non-cultured, non-perfused normal and glaucomatous TM tissues or indeed, whether it requires dexamethasone exposure for them to be found in any great numbers in situ. Studies of CLANs in organized TM tissue to date lack essential quantitative data.22,28 Although the confocal microscopic investigation by Read et al.28 qualitatively examined

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glaucoma tissue, it was restricted to the endothelium of Schlemm’s canal and the juxtacanalicular connective tissue (JCT), where the qualitative identification of polygonal actin arrangements was not definitive, so that the term “CLAN-like” structure was thought more appropriate.

If CLANs are to be found in the normal and glaucomatous TM cells, what is the CLAN prevalence, distribution and location? The imaging of CLAN structures, especially in organized TM tissue, is particularly difficult, which has confounded effective morphometrics and estimation of their numbers and dimensions to date. In this study, we have addressed these issues using confocal microscopy to systematically reconstruct many series of sequential images of phalloidin-stained TM tissue taken from normal and glaucomatous donor eyes. A masked, quantitative analysis was conducted to determine the prevalence of CLANs in TM tissues, and a qualitative assessment was made on the appearance of the CLANs.

We report herein for the first time that CLANs are present (and are common) in TM tissues obtained from normal and glaucomatous donor eyes that had not been subjected to the manipulations of organ or tissue culture. In addition, we provide quantitative data on CLAN prevalence in organized TM tissues in situ even in the absence of glucocorticoids.

**Materials and Methods**

**Subjects**

Twelve human donor eyes (five normal and seven with primary open-angle glaucoma) with an age range of 81 to 94 years (mean, 85 ± 4.53), were acquired by Alcon Research, Ltd. (Fort Worth, TX) from Central Florida Lions Eye Bank (see Table 1). Acquisition of these eyes was in strict adherence with the Declaration of Helsinki. The eyes were enucleated, placed on ice in fixative (10% formaldehyde) within 8 hours of death, fixed for 24 hours, and then stored in 0.1% PBS. Patient details were masked at Fort Worth before shipment to Liverpool to avoid observer bias. All the glaucoma donors had a documented history of primary open-angle glaucoma (POAG), and six of the seven patients with glaucoma were being treated with anti-glaucoma medications (timolol, betaxolol, alphagan, betagan, xalatan, or dorzolamide). One patient had a history of uncontrolled POAG, but did appear to be receiving any glaucoma therapy.

In Liverpool, the anterior segment was dissected from each eye and divided into quadrants, after which the iris was removed (Fig. 1a). The nasal quadrant was selected (where appropriate) for cryo-sectioning and confocal microscopic quantitative analysis of TM CLANs, while the temporal quadrant was subjected to TM tissue microdissection and

**TABLE 1. Details of the 12 Samples Used in the Quantitative and Qualitative Studies**

<table>
<thead>
<tr>
<th>Eye Specimen Code</th>
<th>Gender</th>
<th>Age</th>
<th>Cause of Death</th>
<th>Time from Death to Preservation in Fixative in Hours</th>
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</thead>
<tbody>
<tr>
<td>CS2</td>
<td>Male</td>
<td>84</td>
<td>Acute cardiac crisis, renal failure</td>
<td>5</td>
</tr>
<tr>
<td>CS3</td>
<td>Female</td>
<td>94</td>
<td>Pneumonia, hypoxia</td>
<td>6</td>
</tr>
<tr>
<td>CS4</td>
<td>Female</td>
<td>94</td>
<td>Pneumonia, hypoxia</td>
<td>6</td>
</tr>
<tr>
<td>CS5</td>
<td>Male</td>
<td>89</td>
<td>Acute cardiac crisis, anemia</td>
<td>5</td>
</tr>
<tr>
<td>CS6</td>
<td>Male</td>
<td>89</td>
<td>Acute cardiac crisis, anemia</td>
<td>5</td>
</tr>
<tr>
<td>CS7</td>
<td>Male</td>
<td>87</td>
<td>Gastrointestinal bleed, hypertension</td>
<td>7</td>
</tr>
<tr>
<td>CS8</td>
<td>Male</td>
<td>81</td>
<td>Chronic obstructive pulmonary disease, pneumonia</td>
<td>8</td>
</tr>
<tr>
<td>CS9</td>
<td>Male</td>
<td>81</td>
<td>Acute cardiac crisis</td>
<td>5</td>
</tr>
<tr>
<td>CS10</td>
<td>Male</td>
<td>87</td>
<td>Congestive heart failure, respiratory failure</td>
<td>5</td>
</tr>
<tr>
<td>CS11</td>
<td>Female</td>
<td>81</td>
<td>Pneumonia, pulmonary edema</td>
<td>7</td>
</tr>
<tr>
<td>CS12</td>
<td>Male</td>
<td>86</td>
<td>Cancer of the esophagus, Alzheimer disease</td>
<td>2</td>
</tr>
<tr>
<td>CS14</td>
<td>Male</td>
<td>80</td>
<td>Cerebrovascular accident, coronary artery disease</td>
<td>5</td>
</tr>
</tbody>
</table>

**FIGURE 1.** (a) Macrophotography showing the TM (arrows) partly exposed for dissection. The cornea (C) and the ciliary body (CB) are evident, and the iris has been removed. The ciliary tissue will be taken off before peeling the TM. (b) A large microdissected TM strip viewed by inverted phase microscopy. The TM is evident along with the scleral spur (arrows). (c) Low power confocal microscopy showing cytoplasmic actin in green and nuclei in red. This section is from the most anterior portion of the TM. At this point, it thins to make contact with the endothelium of the peripheral cornea, which is a region rich in cells known as Schwalbe’s line (SL). A single optical section is shown. (d) A plan view of part of the uveal trabecular meshwork (TM) with the actin stained green and nuclei red. The reticular nature of the tissue is evident and this orientation is how we routinely approach the tissue for our studies. A single optical section is shown. All images are from normal eyes. Scale bars: (a) 2 mm; (b) 1 mm; (c) 100 μm; (d) 50 μm.
qualitative confocal analysis. The inferior quadrant was set aside for morphologic quality control evaluation of the outflow system and adjacent structures by light (LM) and transmission electron microscopy (TEM), whereas the superior quadrant was reserved as a backup for additional cryo-sectioning and TM cell CLAN quantitation.

A total of 12 eyes were available for our use (five normal and seven with primary open-angle glaucoma). Four of these eyes, chosen without bias (one from the normal group and three from the glaucomatous group) were used for qualitative investigation only and also for optimizing our analytical methods. Initial analysis involved taking multiple images at different magnifications and determining the appropriate balance between area sampled and numbers of 'images' in each z-series stack. Thereafter, by the time we had optimized and standardized our sampling approach for future quantitative studies, sufficient sample tissue did not remain from these specimens to include them in the quantitative studies. Quantitative studies were conducted on each of the remaining eight eyes (four glaucomatous and four normal eyes), whereby the number of CLANs were counted and evaluated in at least one quadrant. For the purpose of this study, a CLAN was considered to be an actin-containing cytoskeletal structure with at least one triangulated actin arrangement, consisting of a minimum of actin spokes and three identifiable hub points. Three adjacent connected hub points make up what we call a triangulated structure. Figure 5b shows three of these triangulated structures, shown by the red outline. Spokes were defined as long filamentous aggregates of actin, and hubs as roughly spherical structures at the intersection point of two or more actin spokes.

Tissue Preparation

Resin Sectioning and Staining. Tissue from the inferior quadrant was processed in epoxy-resins for LM and TEM to evaluate the level of postmortem degeneration in the tissue samples for quality control purposes. The protocol involved secondary fixation in 1% osmium tetroxide, dehydration through alcohol, and embedding in an Araldite/Epon resin (Agar Scientific, Stansted, UK). Thin LM sections of 1 μm to 2 μm and ultrathin TEM sections of 90 nm were cut on a microtome (Reichert Ultracut E; Leica Microsystems, Wetzlar, Germany). Thin sections were stained with 1% toluidine blue; ultrathin sections were stained with uranyl acetate and lead citrate. All sections were viewed in sequence to assess the quality of the tissue for further study.

Microdissection. Microdissection was conducted as previously reported22 and entailed the removal of a length of the TM (Fig. 1b) by peeling the tissue from the scleral sulcus. A razor blade knife was used to cut posterior and anterior slices toward Schlemm's canal. Then strips of tissue, containing sclera spur, uveal TM, corneoscleral TM, and some JCT, were peeled and removed using fine pointed forceps (Agar Scientific) using fine-pointed forceps (Fig. 2a). The tissue was then mounted in fluorescence mounting medium (Dako Cytomation, Ely, UK). Each microdissection and cryo-section was transferred onto a glass microscope slide and positioned on top of a 50-mesh gold TEM grid (Agar Scientific) using fine-pointed forceps (Fig. 2a). The tissue was then mounted in fluorescence mounting medium (Dako Cytomation, Ely, UK). The grid served in the qualitative part of the investigation as the means by which we maintained a consistent and systematic approach to the TM tissue sampling. It also allowed us to return to areas of particular interest (by noting the relevant vectors). Our approach was initially to obtain low magnification single images of representative tissues areas from all the TM regions. Then we produced higher power z-series of varying depths (numbers of optical sections) in specific regions of interest. With respect to CLANs, we adopted a "seek and find" strategy.

For quantitation, using cryo-sections only, the relevant grid squares were labeled A to D and, within each square, five areas (1 to 5) were designated, as shown in Figure 2b. For each of the specimens, the 20 designated locations were viewed using confocal microscopy. The actin architecture in the uveal and corneoscleral TM (along with the nuclei) were visualized by optically sectioning the tissue with the confocal microscope and creating a z-series. Each z-series consisted of 11 images wherein each image was taken consecutively as a 1-μm optical slice. Two z-series image collections were taken from the same sample tissue did not remain from these specimens to include them in the quantitative studies. Quantitative studies were conducted on each of the remaining eight eyes (four glaucomatous and four normal eyes), whereby the number of CLANs were counted and evaluated in at least one quadrant. For the purpose of this study, a CLAN was considered to be an actin-containing cytoskeletal structure with at least one triangulated actin arrangement, consisting of a minimum of actin spokes and three identifiable hub points. Three adjacent connected hub points make up what we call a triangulated structure. Figure 5b shows three of these triangulated structures, shown by the red outline. Spokes were defined as long filamentous aggregates of actin, and hubs as roughly spherical structures at the intersection point of two or more actin spokes.

Tissue Staining for Confocal Microscopy

The cryo-sections and microdissected strips were viewed with a microscope (Nikon Optiphot-2; Nikon, Tokyo, Japan) under a ×40 objective. The best microdissected strips of TM and cryo-sections with obvious TM present were selected for confocal microscopy. To visualize Factin, the tissue and sections were stained with phalloidin-Alexa 488 (Molecular Probes Europe, Leiden, The Netherlands) and to identify nuclei they were treated with propidium iodide (PI; Sigma-Aldrich Company Ltd, Dorset, UK). Our protocol involved three washes of one minute each wash in PBS containing Tween to remove fixatives and to make the tissue permeable. Thereafter, our samples were incubated for 20 hours in the dark at 4°C in phallloidin (diluted to 1/20 with 0.1% PBS). Next, three washes with 0.1% PBS were done and the samples were incubated for 4 hours at 4°C with PI (diluted 1/10 with 0.1% PBS). Finally, an additional three washes in 0.1% PBS were performed.

Confocal Microscopy

The microscope used for our investigations was a confocal laser scanning microscope (CLSM) imaging system (MRC-600; BioRad, Hemel Hempstead, UK). Each microdissection and cryo-section was transferred onto a glass microscope slide and positioned on top of a 50-mesh gold TEM grid (Agar Scientific) using fine-pointed forceps (Fig. 2a). The tissue was then mounted in fluorescence mounting medium (Dako Cytomation, Ely, UK). The grid served in the qualitative part of the investigation as the means by which we maintained a consistent and systematic approach to the TM tissue sampling. It also allowed us to return to areas of particular interest (by noting the relevant vectors). Our approach was initially to obtain low magnification single images of representative tissues areas from all the TM regions. Then we produced higher power z-series of varying depths (numbers of optical sections) in specific regions of interest. With respect to CLANs, we adopted a "seek and find" strategy.

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Cryo-sectioning. The nasal quadrant was halved meridionally, and in the absence of the iris, the exposed TM could be identified (Fig. 1a). The tissue segments were placed in 10% phosphate buffered sucrose solution overnight, then frozen using cooled isopentane (VWR International Ltd, Lutterworth, UK). A cryostat (2800 Frigocut-E Cryostat; Leica Microsystems, Milton Keynes, UK) was used to cut 60 μm thick cryo-sections at −20°C and stored in individual wells of 24-well culture dishes containing 0.1% PBS/PSF.

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The cryo-sections and microdissected strips were viewed with a microscope (Nikon Optiphot-2; Nikon, Tokyo, Japan) under a ×40 objective. The best microdissected strips of TM and cryo-sections with obvious TM present were selected for confocal microscopy. To visualize Factin, the tissue and sections were stained with phalloidin-Alexa 488 (Molecular Probes Europe, Leiden, The Netherlands) and to identify nuclei they were treated with propidium iodide (PI; Sigma-Aldrich Company Ltd, Dorset, UK). Our protocol involved three washes of one minute each wash in PBS containing Tween to remove fixatives and to make the tissue permeable. Thereafter, our samples were incubated for 20 hours in the dark at 4°C in phallloidin (diluted to 1/20 with 0.1% PBS). Next, three washes with 0.1% PBS were done and the samples were incubated for 4 hours at 4°C with PI (diluted 1/10 with 0.1% PBS). Finally, an additional three washes in 0.1% PBS were performed.
area, one to image the actin architecture and the other to image the nuclei. All the images were obtained using a ×60 oil immersion objective on the confocal laser scanning microscope. z-Series images were processed using an image analysis program (Confocal Assistant version 4.02; Indiana Center for Biological Microscopy, Indianapolis, IN), and each individual image was subjected to morphometric analysis to identify CLANs and to determine their size and complexity. For all the CLANs that occupied more than one z-series (1 μm) image, the images were aligned in register; composites of the CLANs were re-constructed using the appropriate software and appropriate projections were analyzed and recorded. This allowed us to have a more robust inclusion/exclusion strategy for CLAN identification and a more precise measurement of the dimensions of these structures. Intra observer and inter observer errors for our counts of CLANs were less than 10% and less than 15%, respectively.

Meshwork Area Estimation

The LM sections of the outflow system in meridional orientation were imaged (Nikon Optiphot-2 microscope using a ×10 objective lens; Nikon). Digital images were recorded with a color video camera (JVC TK-1280; JVC, Yokohama, Japan) and stored using a software archiving system (Aequitas IDA 1.3, DDL Ltd, Cambridge, UK). The TM sectional area was measured using calibrated image analysis software (Aequitas). Viewed in the meridional plane, the TM is a triangular structure with the anterior apex ending at Schwalbe’s line (Fig. 1c), the posterior limit demarcated by the scleral spur. The inner limit was the aqueous chamber angle and the outer limit was Schlemm’s canal. We estimated the total volume of the TM using the averaged measurement of the area of the TM for our specimens multiplied by a value for the circumference of the TM, which we took to be 36 mm. These two values allowed us to determine a total volume of the TM, which we worked out for the purpose of the present study to be 1.97 mm³.

Estimates of the Number of CLANs and Nuclei in the Whole TM

For each specimen, a calculation was made to determine the total number of CLANs present in the whole of the TM. We used our total counts of CLANs and of TM nuclei in a known volume of TM tissue. The tissue volume was calculated from the area of the z-series image field of view multiplied by the number and depth of optical thickness of each confocal slice imaged in the z-series multiplied by the number of individual sections analyzed. For purposes of calculation, we assumed that the average TM nucleus and CLAN territory was roughly the same size. Our measured estimate of the total TM volume was determined and other important reference points were published calculations of the total nuclear numbers in the TM. Specifically, we used our own published data from earlier studies, which showed that the number of nuclei (cells) in the TM was age and disease dependent. If we use the number of nuclei counted as an estimate of the number of cells in the tissue, there are around 400,000 cells in the TM of normal people in their 80s, and glaucoma patients of a similar age have approximately 50% of that number.

RESULTS

The microdissection procedure was found to be particularly suited for qualitative investigation of the distribution of F-actin arrangement patterns within TM cells. Microdissections of TM provided information on the distribution of CLANs, variation in the pattern and organization of individual CLANs, and their relationship to other F-actin arrangements. However, as microdissection involved peeling the TM tissue from its normal location, the specimens were irregularly shaped and of variable thickness (Fig. 1b). Cryo-sectioning produced sections of consistent thickness, which allowed accurate volume estimations. The sectioned tissue was associated with more uniform and consistent staining appropriate for reliable counting, with clear and easily visualized actin-rich crossover points (hubs) that could be used as CLAN “identifiers.”

Qualitative Observations on Dissected Segments of Human Trabecular Meshwork

Examination of the TM by confocal microscopy showed considerable detail of actin and nuclear distribution even at low magnification (Figs. 1c and 1d). Higher magnification revealed that F-actin staining was so pronounced and extensive within the TM cells that the architectural distinguishing features of the TM sub-regions (uveal meshwork, corneoscleral meshwork and the JCT) could still be appreciated. The TM cell F-actin arrangement was dominated by stress fibers (Fig. 3).

**Figure 3.** Confocal microscopic views, at a medium power magnification, of the three main regions of the TM. (a) Uveal meshwork (actin staining only). The cytoplasmic actin stress fibers are clearly seen, these are wrapped around the trabeculum such that the chord-like nature of these trabeculae is evident (one optical section). (b) Corneoscleral meshwork (actin staining only). The trabeculae in this region are in the form of sheets. The density of actin staining and the prominence of stress fibers are such that the anatomy of the tissue is evident without the need for any other counter staining, and even trans-trabecular pores for the drainage of aqueous humor can be identified. (Projection of 8 optical sections through a thickness of 8 μm.) (c) In the juxtacanalicular connective tissue, orientation is more difficult and nuclear PI staining helps. There are myriads of stress fibers that form a complex 3D arrangement in this loose connective tissue. (Projection of nine optical sections through a thickness of 9 μm.) Images taken from a glaucoma specimen. Scale bars, 10 μm.
In the uveal meshwork, the cord-like nature of the trabeculae was easily recognized. The stress fibers in the TM cells were oriented in different directions but, generally, wrapped around the trabecular like string around a pole (Fig. 3a). In the corneoscleral meshwork the F-actin staining of the TM cells was an arrangement of thick and thin stress fiber bundles. The fibers formed more complex arrangements (Fig. 3b) than were evident in the uveal meshwork. Conventional stress fibers were straight structures but there were also F-actin bundles that were wavy and rope-like. These wavy ropes often came together in knot-like arrangements that formed a tangle of fibers. These knots and tangles were evident in all specimens and were particularly common in some (Fig. 4a), but rare in others.

The meshwork portion of Schlemm’s canal inner wall, the JCT, was only present in small segments or was not present at all in the microdissected tissue. When present, the JCT was identified as a loose connective tissue zone rich in stellate interconnecting cells that contained a myriad of small stress fibers running in a multiplicity of differing directions (Fig. 3c). Stress fibers were usually straight but occasionally folded arrangements of wavy fibers that formed tangles were observed. Tangles of F-actin fibers often formed complex arrangements in the JCT and, when identified, they seemed to be at least as abundant as those found in the corneoscleral meshwork.

CLANs (Fig. 5) and CLAN-like structures (Fig. 4) were found in many TM cells. The prevalence of CLANs was variable, but they were identified in the TM of every eye in our series irrespective of if they were from glaucomatous or normal individuals (Fig. 5). In addition, CLANs were found in every TM tissue sample examined. Our impression was that CLANs were identified as a loose connective tissue zone rich in stellate interconnecting cells that contained a myriad of small stress fibers running in a multiplicity of differing directions (Fig. 3c). Stress fibers were usually straight but occasionally folded arrangements of wavy fibers that formed tangles were observed. Tangles of F-actin fibers often formed complex arrangements in the JCT and, when identified, they seemed to be at least as abundant as those found in the corneoscleral meshwork.

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The CLANs in the TM occurred in a number of different architectural arrangements, and they were of variable size and complexity. The number of hub points within each CLAN territory ranged from as few as 2 up to 40 or more. Exceptionally, CLANs were found with over 100 hub sites (Fig. 5a). It is possible (although it is difficult to be certain) that these massive structures may well be several CLANs in adjacent cells of such close proximity to each other that they appeared to be one structure. The CLAN volume varied from a few cubic microns to territories occupying the majority of the TM cytoplasm. The CLAN phenotype was also quite variable in our tissue samples. Some CLANs were made up of highly regular polygonal subunits usually consisting of 5 or 6 F-actin spokes emanating from a central hub site. Adjacent actin spokes were interconnected to form higher order structures such as triangles (Fig. 5b). The individual units linked together sometimes to form geodesic domes but far more often they were flatter geodesic structures that we describe as being “geodesic sowers”. In the uveal and corneoscleral regions of the TM, it was these classic geodesic CLANs that were most frequently identified.

There were several other CLAN-like arrangements in our TM tissue dissections. One such CLAN phenotype was a “network CLAN,” which often had many actin filament spokes and hub points but with far less geometric regularity than seen in classic geodesic CLANs. The network CLANs were made up of highly irregular polygonal and triangular subunits linked together to form “web-like” (Fig. 4c) or “string basket-like” (Fig. 4b) structures. The cytoplasmic territories occupied by the more irregular network structures (in common with classic CLANs) were often poorly demarcated, so their areas were difficult to measure accurately, but the network CLANs and classic CLANs seemed to have a similar size range. The final frequently identified CLAN-like arrangement was a “star-burst” (Fig. 4d) consisting of a single, but very prominent, central hub with up to 10 or 12 particularly thick F-actin spokes radiating out from it.

Our qualitative investigation of phalloidin staining patterns in the cells of the TM did not reveal any F-actin arrangement that might be a fingerprint that distinguished the glaucomatous TM from normal specimens. CLANs of various sizes and CLAN-like structures were evident in both. However, although actin tangles (Fig. 4a) were present in the cytoplasm of TM cells in both normal and glaucoma tissues, these structures appeared to be more evident and extensive in the glaucoma TM.
measure with any degree of accuracy and reproducibility, the number of hubs in each CLAN territory was determined as an alternative size parameter.

Basically, the CLANs seen by confocal microscopy in the frozen sections (Figs. 5c and 5d) had a similar appearance to those identified in the microdissected TM preparations (Fig. 5b). However, the actin spokes tended to be slightly less obvious but, on the other hand, the hubs were usually a little more prominent than had been usual in the microdissected TM preparations. CLANs were present in every section of every specimen examined, although the prevalence of CLANs varied threefold between specimens, ranging from 59 to 164 in the sample areas subjected to quantitative analysis (Table 2). The overall mean and SD from the eight specimens that made up the CLAN count was 95.5 ± 39.2. On unmasking it was found that three of the top four specimens with the highest CLAN counts were from glaucomatous eyes. However, one of the glaucomatous eyes was ranked seventh in the overall CLAN counts. The mean number of CLANs in the glaucoma specimens (110.5 ± 42.9) was higher than in the normal specimens (80.5 ± 33.8).

Within the limits of the present analysis, we found CLANs ranged from small structures with 2 hubs to large ones containing more than 26 hubs with the distribution skewed toward the small CLANs. We attempted to count spokes and they ranged from 5 to >100 but it should be said that, because of lack of staining intensity, spokes were much more difficult to count accurately than hubs. The area occupied by CLANs was also extremely variable but we tended to classify them as being large (100–500 μm²), medium (35–99 μm²), and small (<34 μm²); however, the very smallest CLANs with territories less than 5 μm² were difficult to discriminate. The largest CLANs identified in this quantitative part of the study were not as vast as the huge CLANs identified in the qualitative part of the investigation (see Fig. 5a). To an extent this difference can be explained by the rarity of truly large CLANs, which we could only find when specifically searching for them, but they escaped detection when a bias-free, objective sampling procedure was adopted.

The trabecular meshwork of every eye within this series contained a mixture of small, medium and large CLANs, so that the averaged hub count showed far less variation than had been the case for CLAN prevalence (Fig. 6). In addition, there was no obvious difference in the distribution or the mean size of the CLANs obtained from glaucoma specimens when compared to the normal specimens. Assessment of the z-series showed that most CLANs occupied sections of between 1 μm and 3 μm in depth with a few occupying 4 × 1 μm optical sections. All images are from glaucomatous specimens. Scale bars, 5 μm.

**Quantitative Analysis of CLANs (Frozen Sections)**

Frozen sections (Fig. 2a) were appropriate for this analysis because the thickness—and therefore, the section volume—was known and there was no preferential bias in the cutting orientation. From each image in the z-series, the number of CLANs was recorded and, as the size of a CLAN was difficult to classify them as being large (100–500 μm²), medium (35–99 μm²), and small (<34 μm²); however, the very smallest CLANs with territories less than 5 μm² were difficult to discriminate. The largest CLANs identified in this quantitative part of the study were not as vast as the huge CLANs identified in the qualitative part of the investigation (see Fig. 5a). To an extent this difference can be explained by the rarity of truly large CLANs, which we could only find when specifically searching for them, but they escaped detection when a bias-free, objective sampling procedure was adopted.

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**TABLE 2. CLAN and Nuclei Counts in Normal and Glaucomatous TM Tissues**

<table>
<thead>
<tr>
<th>Eye Specimen Code</th>
<th>Number of CLANs Counted</th>
<th>Total Estimated TM CLANs/Eye</th>
<th>Number of Nuclei Counted</th>
<th>Total Estimated TM Nuclei/Eye</th>
<th>Ratio of CLANs/TM Nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS3</td>
<td>151</td>
<td>217,527</td>
<td>113</td>
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<tr>
<td>CS12</td>
<td>67</td>
<td>111,254</td>
<td>85</td>
<td>141,000</td>
<td>0.79</td>
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<tr>
<td>CS7</td>
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<td>107,934</td>
<td>144</td>
<td>239,000</td>
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<td>CS9</td>
<td>59</td>
<td>97,970</td>
<td>203</td>
<td>336,000</td>
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<tr>
<td>Mean Std Dev</td>
<td>80.5</td>
<td>133,750 ± 56,188</td>
<td>156.25</td>
<td>226,000 ± 83,542</td>
<td>0.67</td>
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<tr>
<td></td>
<td>33.8</td>
<td></td>
<td>50.6</td>
<td></td>
<td></td>
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<tr>
<td>Glaucoma</td>
<td></td>
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<tr>
<td>CS8</td>
<td>164</td>
<td>272,324</td>
<td>120</td>
<td>199,000</td>
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<tr>
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<td>169,573</td>
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<td>CS11</td>
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<td>99,631</td>
<td>58</td>
<td>96,300</td>
<td>1.02</td>
</tr>
<tr>
<td>Mean Std Dev</td>
<td>110.5</td>
<td>183,500 ± 71,196</td>
<td>110.25</td>
<td>183,000 ± 63,626</td>
<td>1.03</td>
</tr>
<tr>
<td></td>
<td>42.9</td>
<td></td>
<td>38.4</td>
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</table>
sections. The very largest CLANs (found only in the qualitative study such as shown in Fig. 5a) did not occupy more than seven optical sections (depth 7 μm). The vast majority of CLANs, therefore, were flattened dome-like or, more accurately, saucer-like structures. A large CLAN occupying a territory with a diameter of 18 μm (based on our data) would have, at most, a height that was sixfold lower (approximately 3 μm).

CLAN prevalences for the whole meshwork were calculated using an estimate of the TM volume and the recorded mean values of CLANs. For the series of eight specimens, the mean CLAN prevalence in the TM was estimated to be around 160,000. When the glaucoma cases were considered separately, it was found that their approximate mean CLAN prevalence was 184,000 per eye. The controls had a slightly lower mean total of 134,000 CLANs per eye. It is known however, that glaucomatous eyes have fewer TM cells compared to aged matched non-glaucomatous eyes.35–39 We found that there were fewer TM nuclei in our glaucoma specimens compared to controls (we projected from our sample numbers that there were, on average, 183,000 nuclei in the whole glaucoma TM against an average of 226,000 in the normal TM; Table 2). Therefore, more CLANs and fewer nuclei (i.e., cells) in the glaucoma specimens gave us a higher number of CLANs per cell in the glaucoma TM than those in the normal specimens (the average ratio of CLANs to nuclei came to 1.05 in glaucomatous specimens and was 0.67 in the normal specimens in our series). If we did our calculations based on the literature total TM nuclear prevalences,34–37 then the CLAN/nuclear ratio for the glaucoma TM was much the same at just under 1 (0.92), while for the normal TM the ratio was much less (0.35). The present study lacks the power to make definitive statements about glaucoma and the significant increase in CLANs. We have calculated that to make the study statistically significant we would need to analyze a total of 40 specimens (20 normal controls and 20 with glaucoma).

**DISCUSSION**

Polygonal F-actin arrangements were found to be induced in confluent, monolayer cultures of human TM cells when exposed to glucocorticoids such as dexamethasone for several days. Clark et al.29 called these structures cross-linked actin networks or CLANs. CLANs were not particularly evident in established TM cultures from normal eyes lacking dexamethasone, but were prominent in TM cultures established from donors with glaucoma.31 Organ culture preparations of the anterior segment showed that CLANs develop in the TM cells of organized outflow tissue when perfused with dexamethasone.22 In the present study, we have demonstrated for the first time that CLANs are abundant in the organized TM from both normal and glaucomatous eyes.

The appearance of the CLANs varied and we identified architectural F-actin arrangements that we classified in our specimens as being either “true” CLANs (identical with the polygonal arrangements of hubs and spokes first noted by Clark et al.29), or somewhat similar structures that we called CLAN-like (network CLANs, basket CLANs, and starbursts). CLANs and CLAN-like arrangements were found in all regions of the TM, but they appeared to be more prevalent in the uveal and corneoscleral regions than in the JCT region. Read et al.28 used confocal microscopy to examine the actin structure of Schlemm’s canal endothelium and the JCT in normal specimens and glaucoma patients. The group identified CLAN-like arrangements but were not entirely convinced they were seeing “true” CLANs. We could be more emphatic partly because of our systematic quantitative approach but mostly because the identification of CLANs is much easier in the trabecular regions of the TM. We consider that our current findings resolve the issue of whether CLANs exist in situ and demonstrate conclusively that they are not an artifact associated with the manipulations of cell or organ culture.

Tissue culture studies of TM cell lines derived from glaucomatous donors have higher basal levels of CLANs (between 18 and 40%) when compared to normal TM cells (with an average close to 4%).31 As a result, from the outset of the present investigation, we suspected that CLAN prevalence from the glaucoma TM in situ would be high and that turned out to be the case. Our calculations of average CLAN prevalence in the whole glaucomatous TM gave a value of over 180,000 of these structures. The abundance of CLANs in the glaucoma specimens was highlighted when we calculated CLAN to nuclear ratios that were close to parity, irrespective of whether we used the nuclear counts generated in the present study or the nuclear counts for the glaucomatous TM previously published by us.35–37 Our CLAN calculations predicted, assuming one nucleus per cell, that every TM cell in a glaucomatous outflow system was likely to have a CLAN present in its cytoplasm. In addition, the vast majority would be “true” CLAN structures because our counting inclusion criteria emphasized hub points (distinctive in CLANs) rather than spokes (common to all CLAN-like arrangements). CLANs are more common in the uveal and corneoscleral TM, and TM cell number in this region is decreased in glaucoma. Therefore, we are likely underestimating the CLANs in POAG TM tissues.

Despite the CLAN abundance in the glaucomatous TM in situ, there was no significant difference in CLAN prevalence between the glaucoma and normal groups; however, the glaucoma group did tend toward having higher CLAN numbers (110.5 ± 42.9 in the glaucomatous eyes; 80.5 ± 33.8 in the normal controls). The laborious nature of the counting (over 750 CLANs were reconstructed during the study) meant that relatively few eyes were evaluated, this and the large variation between specimens helped to confound statistical analysis. The wide variation between specimens may have been predicted based on our previous in vitro31 and ex vivo32 experiences, but the remarkably high prevalence of CLANs in all our normal specimens was a surprising finding. The projected prevalence of CLANs in this group was over 130,000 and,
depending on how the calculation was done, that predicted a frequency somewhere between one third and two thirds of normal TM cells with CLANs. The fact that all our eyes came from people over 80 years of age may be a factor but, as yet, an evaluation of CLAN prevalence and age has not been undertaken.

In the TM tissue in situ, CLANs were variable both in size and territory, but this corresponded fairly well to the variation reported in cultured human TM cell lines. The other CLAN-like phenotypes we described in the TM cells of our tissue preparations have also been noted in TM cells in tissue culture (unpublished data). The size distribution in our tissue samples was heavily skewed as there were many more small than large CLANs; this also was consistent with findings in vitro and after steroid treatment ex vivo. The true size diversity of these structures was only fully appreciated through our initial CLAN-hunting qualitative approach, where we searched specifically for the biggest structures exhibiting the greatest architectural complexity and identified CLANs with over 100 hubs in a single territory (but these huge structures were not a common finding).

Tall, dome-shaped CLANs in the TM cells of organized tissue were also uncommon, and most CLANs were fairly flat structures occupying three or fewer three optical sections (3 μm) at most. These flattened structures were more saucer-like than dome shaped. The physiological significance of these saucer-shaped CLANs is currently unknown, but probably reflect overall cellular tensegrity, the degree of adhesion to the extracellular matrix, the shape of the adhesive substrate (which is different in TM tissue compared to cultured TM cells), and the overall stress/strain on the cells. Although precise quantitative data is lacking, many of the CLANs seen in human TM cell cultures are also fairly flat structures. Little is known about the molecular mechanisms responsible for CLAN formation, but CLAN hub sites (also called vertisomes) are particularly rich in α-actinin, an actin cross-linking protein that belongs to the spectrin family. The hubs have also been reported to contain syndecan-4, and CLAN formation in freshly plated TM cells appears to be regulated by β1 and β3 integrins. At the present time much more work is required to begin to understand the mechanisms involved and to identify all the factors responsible for CLAN formation.

The significance of CLANs in the TM cells of the outflow system in health and disease is currently unknown. However, unusual actin arrangements of this type may have many potential consequences for cell and tissue function that are not entirely predictable. TM cells in culture, that have been stressed by stretching, reorganize their F-actin arrangement and some of these cells seem to form CLAN-like structures. Whether or not the CLANs are protective or detrimental to the cells expressing them during or after physical deformation still needs to be established. In addition to contractility, phagocytic activity, proliferation and migration are key TM cell activities that require a dynamic actin cytoskeleton. Most of these functions are modified by glucocorticoid treatment, but whether or not CLAN induction is an important key step requires substantial clarification.

CLAN changes, if they occur in glaucoma, are not qualitatively different and there is still is a long way to go to find definitive evidence of glaucoma-related increased numbers, despite the findings of this study and previous tissue culture investigations. On the other hand, it has long been suggested that the contractile proteins in TM cells are altered in glaucoma. Much more recently, Read and colleagues reported that the F-actin arrangement in inner wall and JCT cells of the outflow system was more disordered in glaucoma, and they observed an abundance of F-actin tangles among the stress fibers. We can confirm the presence of F-actin tangles not just in the JCT, but throughout the TM of both the glaucomatous and normal samples. If actin-related cytoskeletal pathology is, as it appears to be, an important TM adverse event in glaucoma then it will be important to establish whether CLANs, F-actin tangles or even some other actin-containing entities are crucial upstream events in the functional failure of the outflow system.

In the present study, we have shown that CLANs are a common feature of human TM tissues (both from glaucoma donors and normal donors) and the majority are identical with those described in cultured TM cells. To our knowledge, this is the first demonstration that these cytoskeletal structures are an intrinsic component of a tissue in situ, ocular or otherwise. A common supposition has been that CLANs are exclusive to the in vitro tissue culture environment. However, as we have shown such high numbers of CLANs in both normal and glaucoma TM specimens, we are now faced with the interesting prospect that instead of tissue culture up regulating CLAN formation, it may be that the culture environment is actually suppressing CLANs. Another example of this type of phenomenon is the downregulation of myocilin expression in cultured TM cells, which is reactivated by glucocorticoid treatment. Clearly additional studies are needed to discover what regulates CLAN formation and to determine the functional consequences of TM cell CLANs.

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References


