Melanocytes and Iris Color

Electron Microscopic Findings

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Objective: To quantitatively associate iris color with melanocyte pigment content.

Methods: Autopsy eyes were classified as uniform-blue, uniform-hazel, or uniform-brown or showing a darker peripupillary ring. Using electron microscopic images and computerized image analysis, area, number, and size of mature melanosomes within the perinuclear cytoplasmic area only or within perinuclear and peripheral cytoplasmic areas of the superficial stromal melanocytes combined were measured.

Results: Average melanosomal area per perinuclear cytoplasmic area (AMAC) and average number of melanosomes per perinuclear area (AMNC) significantly differed across iris color groups (overall $P<.001$). This result reflects the large difference between blue-uniform and all other color groups. A marginally significant (nominal) trend from blue-ring through brown-ring was also detected ($P=.06$ for AMAC and $P=.07$ for AMNC). The average perinuclear cytoplasmic area was larger in the central iris zone (within 1 mm around the pupillary margin) than in the intermediate iris zone (between 1 and 2 mm around the pupillary margin) ($P=.002$), but AMAC and AMNC did not significantly differ between zones. The average melanosome size did not differ significantly across color groups ($P=.11$).

Conclusion: Differences in iris colors are at least partially attributed to variable AMNC and AMAC within superficial melanocytes.

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A READILY apparent feature in human appearance is the color of the eye known to derive from the pigment content of the iris.[1-3] This pigment consists mainly of melanin, which is contained in melanosomes produced by stromal melanocytes and by the two layers of iris pigment epithelium.[4]

Stromal melanocytes are mainly aggregated in the anterior border layer of the iris. They are oriented parallel to the surface[1-3,5] and, therefore, are the first to influence iris color when observed from the outside through clear cornea. Although densely pigmented, the pigment epithelia presumably provide only a background tint, since they are located on the posterior surface of the iris. They receive and reflect light only through the filter of comparatively thick stroma arranged in front of them. As the pigment-epithelial pigmentation does not vary between different iris colors,[2] the brown appearance of the iris must be attributed to changes in pigmentation of the stroma. "If . . . much pigment is developed in the anterior layer, the iris becomes brown . . ."[1]

A third category of melanin-containing cells consists of clump cells and pigment-laden macrophages. Like the melanocytes, these cells form part of the stroma, but they only store and/or digest melanin, rather than produce it. Their number is small, and their location is usually more posterior to the iris surface. Consequently, in the past, the various shades of iris color have been mainly attributed to the variability in number and distribution of stromal melanocytes.[5,6] However, other studies,[1-3] as well as our companion study,[7] found no color-dependent change in the number of stromal melanocytes.[1-3] This has raised the question whether individual melanocytes vary in their melanin content, showing, eg, varia-

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MATERIALS AND METHODS

SAMPLE SELECTION

Donor eyes from the Wisconsin Eyebank (Madison) were selected using the following exclusion criteria: glaucoma, medication for systemic hypertension, ocular tumor, past eye surgery, and autolysis longer than 6 hours. Twenty-eight representative eyes were selected, and the corneas were removed at the limbus. The anterior segments were then photographed and classified, by observation under a microscope, into three main colors, each being either uniform in color (uniform-blue [n=5], uniform-hazel [n=6], or uniform-brown [n=5]) or showing a darker peripupillary zone (blue-ring [n=3], hazel-ring [n=5], brown-ring [n=4]). Sample characteristics were as follows: mean age, 71 years; females, 8; males, 18; and unknown gender, 2. The study was approved by the University of Wisconsin institutional review board human subject committee (protocol No. 90-702-442).

TISSUE PROCESSING

Wedges of iris extending from the pupil to the periphery were fixed in 2.5% phosphate-buffered glutaraldehyde for 24 hours, postfixed 2 hours in 1% osmium tetroxide, and embedded in epoxy resin. Ultrathin sections received standardized contrast enhancement by understaining with uranyl acetate and overstaining with lead citrate. The EM was periodically calibrated with a carbon-grated replica to guarantee consistent magnification. Melanocytes from the anterior border layer of the iris were examined up to a depth of 50 μm, and 10 to 11 cells from both a central zone (within 1 mm around the pupillary margin) and an intermediate iris zone (between 1 to 2 mm around the pupillary margin) were photographed at ×4000 magnification. The photograph of each melanocyte included a cross section of a major part of the nucleus.

IMAGE PROCESSING

From each eye, five representative cells from each of the two zones were chosen and their EM negative was scanned as a gray-scale image (1-255) at 472 pixels/cm, with a flatbed scanner (Umax). The image contrast was increased through filters at the level of the scanner. The received image was further enhanced using Adobe Photoshop (Adobe Systems Inc, Mountain View, Calif) to reduce brightness by 15 U and increase contrast by 40 points. The cytoplasmic area (excluding the nucleus) was outlined, and number and area of mature melanosomes, as well as their maximal and minimal diameter, were measured using Image Pro Plus (Media Cybernetics, Silver Spring, Md) as software. Mature (stage IV) melanosomes were defined as uniformly bright objects in a gray scale of a minimum size of 200 pixels. Melanosomes with a discernible filamentous structure corresponding to premature melanosomal stages[4] were excluded from the main part of our evaluation.

To arbitrate uncertainties regarding melanosome stage, to assign size and shape to individual melanosomes located in an apparent cluster of granules, and to clearly distinguish melanosomes from other dense cytoplasmic organelles, the computer image was compared with a magnified view (×5) of the EM negative, which provides better resolution and contrast. Computer images were edited accordingly. A sample of a computer-processed EM positive is shown in **Figure 1**. For statistical evaluation, we compared the average melanosomal area (AMA) per perinuclear cytoplasmic area (AMAC) and the average melanosome number (AMN) per perinuclear cytoplasmic area (AMNC) between the different iris colors and between the two iris zones (central and intermediate).

To include number and area of melanosomes in peripheral parts of the melanocyte cytoplasm (remote from nuclear cross sections), we also measured total AMA and AMN in iris areas instead of just in a single cell. Two areas within both the superficial 10 and 20 μm of two irides from each of the three uniform colors (uniform-blue, uniform-hazel, and uniform-brown) were analyzed. Every cross-sectioned cytoplasmic part of melanocytes within the defined area was included. The image analysis methods used were the same as described above, and AMA per iris area (AMAI) as well as AMN per iris area (AMNI) were evaluated in the superficial 10 and 20 μm of the anterior border layer, respectively, and compared with differences in iris color.

STATISTICAL ANALYSIS

Linear mixed effects models were used to analyze these data. Logarithmic transformations were performed to stabilize variances. Subjects (eyes) were considered random effects, while color group and zone were considered fixed effects. These methods produce accurate estimates of variability that account for the repeated measurements within each eye. Restricted maximum likelihood estimates were obtained using the MIXED procedure (Statistical Analysis Systems, SAS Institute Inc, Cary, NC). Overall comparisons across color groups and zones were based on F statistics. If an overall test result was significant, pairwise comparisons of color groups, using t statistics, were also examined.

tions in number, size, shape, and maturity of their melanosomes.

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The fine structural basis of iris pigmentation in normal eyes has received little detailed attention. Eagle[2] studied normal human eyes and reported a variation of the number of melanin granules within melanocytes from different iris colors. However, his study was limited to a small number of eyes, preventing a formal statistical evaluation. Other researchers[4,8,9] have provided interesting case reports of pathologic eye conditions accompanied by changes in iris pigmentation, possibly related to altered melanosome size and number within stromal melanocytes, but they were also unable to obtain data sufficient for statistical analysis. Moreover, had they been able to collect groups of cases and adequate quantitative data, they

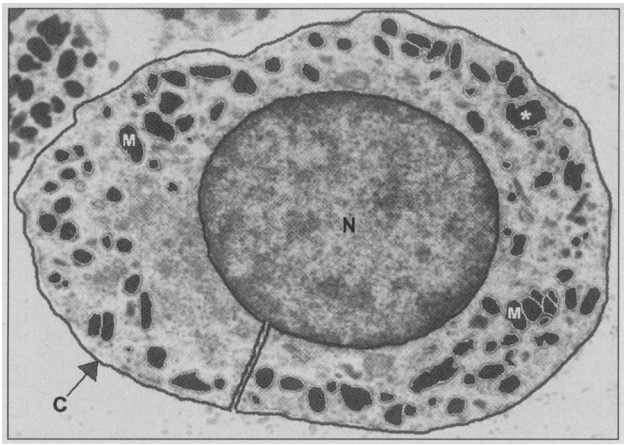

Figure 1. *An image of a stromal iris melanocyte as processed by the software. N indicates nucleus; M, mature melanosome; C, outline of the cytoplasmic area excluding the nucleus; and asterisk, a cluster of unseparated melanosomes.*

would still have lacked a definitive ultrastructural investigation of normal eyes with which to compare their findings. Thus, this study was undertaken with two purposes in mind: (1) to expand the findings of our companion study[7] to a level of detailed examination possible only with electron microscopy (EM) and (2) to provide formal baseline data for any future evaluation of pathologic conditions that alter the melanin content of iris stromal melanocytes and the externally observable color of the eye.

RESULTS

These results are based on 27 897 counted melanosomes. There was no significant difference (P=.11) in the average size of melanosomes across color groups (1.43 nm^2 for uniform-blue, 1.36 nm^2 for blue-ring, 1.42 nm^2 for uniform-hazel, 1.38 nm^2 for hazel-ring, 1.46 nm^2 for uniform-brown, and 1.43 nm^2 for brown-ring eyes). The minimum ratio required to detect differences in melanosome size between color groups at the 5% level ranged from 1.04 to 1.05, depending on the number of eyes in each color group.

The values of AMAC and AMNC by color group are given in **Table 1** and displayed in **Figure 2**. The overall comparisons of all six color groups for AMAC and AMNC each were significant (P<.001). The uniform-blue group differed significantly from all other color groups (see Table 1). A marginally significant (nominal) trend from blue-ring through brown-ring was also detected (P=.06 for AMAC and P=.07 for AMNC).

Similar results for the uniform-color groups were obtained in a subset of six eyes (two eyes from each uniform color), in which the sample zone was restricted to the superficial 10 and 20 μm of the iris, respectively, but included every part of the melanocytic cytoplasm (results not shown). For this subset for the superficial 10 μm, the AMNI and the AMAI were also significantly lower in uniform-blue vs uniform-hazel eyes (P<.04 vs P.02) or uniform-blue vs uniform-brown eyes (P=.02 vs P=.009). Uniform-brown and uniform-hazel eyes did not differ significantly (P=.56 and P=.22, respectively). The data from the superficial 20 μm gave comparable results to those from the superficial 10 μm. Furthermore, AMAC and AMNC analyses for this subset of six eyes yielded similar results to the AMAI and AMNI analyses.

Table 1. Summary of AMAC and AMNC*

Color Group (No. of Eyes)	Estimates (95% CI) AMAC,†‡ $nm^2/\mu m^2$	AMNC,†§ No./μm^2
Uniform-blue (n=5)	1.16 (0.76-1.77)	0.81 (0.54-1.22)
Blue-ring (n=3)	3.32 (1.93-5.74)	2.44 (1.44-4.14)
Uniform-hazel (n=6)	3.64 (2.47-5.35)	2.57 (1.76-3.74)
Hazel-ring (n=5)	4.66 (3.05-7.11)	3.37 (2.24-5.08)
Uniform-brown (n=5)	6.01 (3.93-9.17)	4.13 (2.74-6.22)
Brown-ring (n=4)	5.54 (3.45-8.90)	3.87 (2.44-6.14)

**AMAC, indicates average melanosomal area per perinuclear cytoplasmic area; AMNC, average number of melanosomes per perinuclear cytoplasmic area; and CI, confidence interval for the estimate.*

†Logarithmic transformations of data were used in analyses; estimates are provided in original units. The AMAC and AMNC significantly differed across color groups (overall P=.001). Pairwise comparisons of the estimates at the 5% level showed that uniform-blue differed from all the other color groups. None of the remaining pairwise comparisons was significant at the 5% level. The overall difference may simply reflect the differences between uniform-blue eyes and all other color groups. A marginally significant (nominal) trend from blue-ring through brown-ring was also detected (P=.05 for AMAC and P=.07 for AMNC).

‡The minimum ratios (larger to smaller) required to detect a difference in AMAC between color groups with specified number (n) at the 5% level were 1.77 (n=6 vs n=5), 1.82 (n=5 vs n=5), 1.85 (n=6 vs n=4), 1.88 (n=5 vs n=4), 1.95 (n=6 vs n=3), 1.99 (n=5 vs n=3), and 2.06 (n=4 vs n=3).

§The minimum ratios (larger to smaller) required to detect a difference in AMNC between color groups with specified number (n) at the 5% level were 1.75 (n=6 vs n=5), 1.79 (n=5 vs n=5), 1.81 (n=6 vs n=4), 1.85 (n=5 vs n=4), 1.92 (n=6 vs n=3), 1.95 (n=5 vs n=3), and 2.02 (n=4 vs n=3).

Comparison of the two iris zones (central and intermediate) in the complete sample of 28 eyes is provided in **Table 2**. The average perinuclear cytoplasmic area for the overall sample (N=28) was significantly larger in the central zone (P=.002). The ratios of the average perinuclear cytoplasmic area did not significantly differ across color groups (P=.50). For the overall sample (n=28), AMAC and AMNC did not differ significantly between zones (P=.79 and P=.75, respectively). The ratios of AMAC and of AMNC did not significantly differ across color groups (P=.82 and P=.81, respectively).

Only mature (stage IV) melanosomes were evaluated, although a few premature melanosomes of stages I through III were encountered. Estimating their numbers, we saw no obvious difference in percentage of premature melanosomes between different iris color groups. Aberrant forms of melanosomes like giant, granular, or balloon melanosomes[4] were not found in any of the examined irides.

COMMENT

Because of the exploratory nature of this study, there were no established morphological criteria on which to base power calculations. Instead, we have presented the minimum ratios required for detecting differences with P<.05 in Tables 1 and 2. The magnitude of this ratio is a function of the variability and the sample

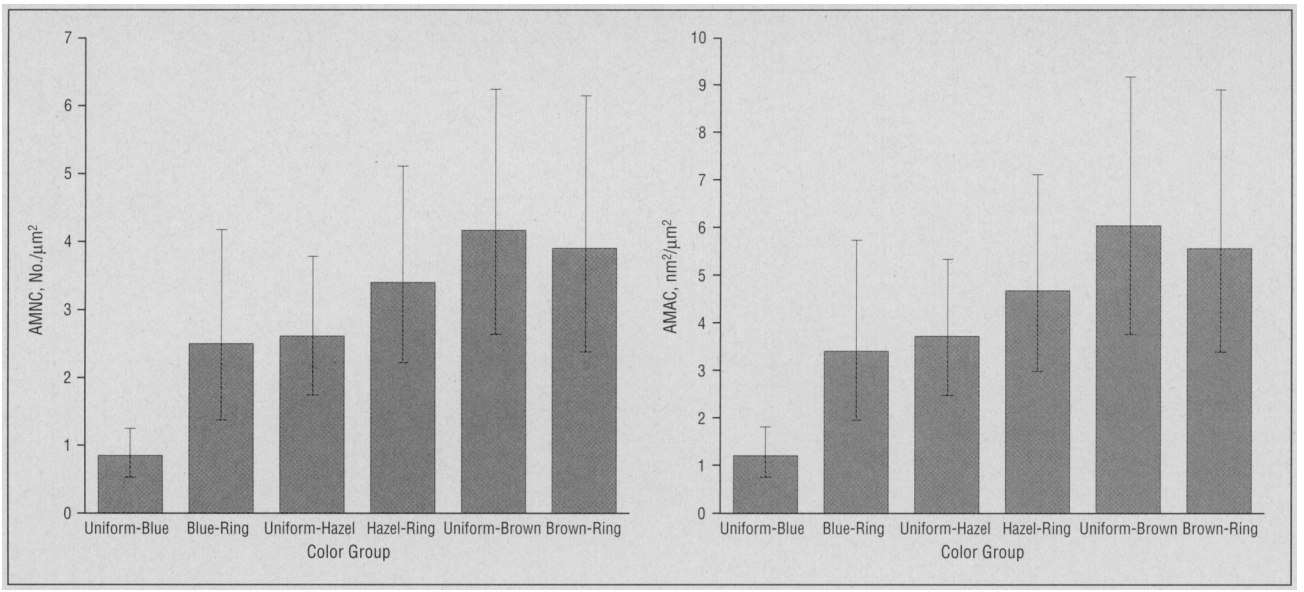

Figure 2. *Average number of melanosomes per perinuclear cytoplasmic area (AMNC) (left) and average melanosomal area per perinuclear cytoplasmic area (AMAC) (right) by color group. The height of the shaded area is the estimate, and the whiskers represent the 95% confidence interval.*

Table 2. Summary of Zonal Relationship*

Color Group (No. of Eyes)	Ratio of Central to Intermediate Zone: Estimate† (95% CI)		
	Average Perinuclear Cytoplasmic Area	AMAC	AMNC
Uniform-blue (n=5)	1.24 (0.97-1.58)	0.98 (0.63-1.52)	0.98 (0.64-1.49)
Blue-ring (n=3)	0.96 (0.70-1.31)	1.26 (0.71-2.23)	1.22 (0.71-2.11)
Uniform-hazel (n=6)	1.18 (0.94-1.48)	0.92 (0.61-1.39)	0.92 (0.62-1.36)
Hazel-ring (n=5)	1.25 (0.98-1.59)	1.05 (0.68-1.63)	1.05 (0.69-1.60)
Uniform-brown (n=5)	1.17 (0.92-1.50)	0.95 (0.61-1.47)	0.98 (0.64-1.49)
Brown-ring (n=4)	1.45 (1.09-1.91)	0.76 (0.46-1.25)	0.75 (0.46-1.21)
Overall (N=28)	1.20 (1.08-1.33)	0.98 (0.81-1.18)	0.97 (0.81-1.17)

**CI indicates confidence interval for the estimate; AMAC, average melanosomal area per perinuclear cytoplasmic area; and AMNC, average number of melanosomes per perinuclear cytoplasmic area.*

†Logarithmic transformations of data were used in analyses; estimates are provided in original units. If the 95% CI does not include 1.0, the ratio is significantly different from 1.0 (P<.05), ie, zonal differences are present. The average perinuclear cytoplasmic area for the overall sample (N=28) was significantly larger in the central zone (P=.002). The ratios of the average perinuclear cytoplasmic area did not significantly differ across color groups (P=.50). AMAC and AMNC for the overall sample (N=28) did not differ significantly between zones (P=.79 and P=.75, respectively). The ratios of AMAC and of AMNC did not significantly differ across color groups (P=.82 and P=.81, respectively).

size. Ratios smaller than this minimum ratio may be clinically observable but could not be detected in this study. This detailed ultrastructural analysis of 28 human eyes shows an association between iris color and both the AMNC and the AMAC in a perinuclear section of superficial stromal melanocytes. Since the average melanosome size did not change significantly between different iris colors, the increase in AMAC in pigmented eyes is presumably owing to a higher number of pigmented granules. Other authors[2] have described an increase in melanosome diameter between light and dark irides, however without statistical analysis. Our study had sufficient power to detect differences in melanosome size of 4% to 5% or larger, depending on sample size. To investigate smaller differences, further studies using our technique on a larger population would be needed.

Whether more deeply pigmented irides produced or stored more melanosomes than less pigmented irides cannot be concluded from this study with certainty. However, an obviously increased number of immature melanosomes typical for accelerated melanogenesis was not seen in the more deeply pigmented eyes. Darker irides sometimes showed clusters of melanosomes that were difficult to separate by the image-processing software. Therefore, despite editing, AMNC might, at times, provide a falsely too low count. This is not the case when measuring AMAC, however, as this parameter measures the area of mature granules regardless of their separation into different organelles. The AMAC, the potentially more accurate parameter, showed comparable statistical trends to AMNC, suggesting low impact of undercounting in this study.

Evaluation was based on the assumption that melanosome distribution is uniform throughout the melanocyte cytoplasm. However, iris melanocytes may

have the ability to transport and accumulate melanosomes in some areas of their cytoplasm as skin melanocytes do.[10] If this were true, differences in AMAC between iris colors should become greater if one measured AMAC in cross sections of entire cells, rather than in cross sections of only the perinuclear area. Therefore, AMA was also assessed within a defined area of the iris surface (ie, AMAI), including all parts of the cell. Low-power electron micrographs of a layer 10 or 20 μm thick were measured in a limited sample size of six eyes whose irides were uniform in color. Interestingly, results were similar to those obtained by comparing iris color and AMAC of only the perinuclear area. The association of AMAC or AMAI and iris color was significant between blue and hazel or brown, but not between hazel and brown. The similarity of results for AMAC and AMAI suggests that the possible melanosome transport from perikaryon to periphery of iris melanocytes does not result in obviously different melanosome distribution within cells, at least not in older humans.

In the main sample of 28 eyes, both parameters, AMNC and AMAC, did not show significant differences between a darker central and a lighter intermediate iris zone, even though this color difference was distinguishable by clinical observation, particularly after removal of the cornea. Failure to detect a difference between the two zones could be caused by the limits of our methods. Alternately, absence of a detectable difference in AMNC or AMAC could be true. In that case, the clinically observed color differences might be explained by the following: (1) The translucency of the surface layer of superficial melanocytes seems to be crucial, particularly in blue irides. The dense sphincter muscle located in the subsurface iris stroma can then more readily contribute to the perception of a darker central zone.[11] (2) The central zone might contain more overall melanin than the intermediate zone because, in our study, the cytoplasmic area of melanocytes in the central zone has been found to be significantly larger than the cytoplasmic area of melanocytes in the intermediate zone. On the other hand, if a larger cell size of central melanocytes were the reason for a darker clinical appearance of the central zone in some eyes, the differences in cell size between the central and intermediate zone should be greater in ringed than in nonringed (uniform-color) eyes. No evidence for such a difference was found.

The absence of a significant difference between the different shades of pigmented eyes (eg, hazel vs brown) was puzzling. We had used a larger sample size than had previous studies and had measured well-defined features expecting that color differences readily observable through the dissecting microscope would be clearly reflected by AMAC and AMAI and, thus, become structurally quantifiable. Instead, we found no statistically significant difference in AMNC, AMAC, or AMAI within all different groups of pigmented eyes. However, the presence of a marginally significant trend in AMAC within all groups of pigmented eyes excluding blue suggests a relationship between higher AMAC values and darker iris colors. The lack of significant differences between the individual pigmented colors may, therefore, be owing to the low statistical power to detect smaller, but clinically important, differences. Given the variability of the data, a larger sample size and/or a more refined color classification may be needed to detect statistically significant relationships between morphologic parameters and clinically discernible shades of iris color.

In summary, this study shows iris color in older humans to be associated with the number and area of melanin granules within superficial iris stromal melanocytes. The granules were of comparable size regardless of color, and there was no evidence for accelerated melanogenesis. Among morphologic parameters, AMAI and AMAC were the most informative and reliable. The ease of obtaining AMAI values suggests that this parameter would be particularly efficient for further studies attempting to quantify changes of iris color.

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