DISTRIBUTION OF SULFHYDRYL AND DISULFIDE GROUPS IN OCULAR TISSUES

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The localization and concentration of protein-bound sulfhydryl groups and disulfide linkages in mouse ocular tissues were examined with a new fluorescent thiol reagent, N-(7-dimethylamino-4-methylcoumarinyl)-maleimide (DACM). Corneal epithelium and endothelium, conjunctival epithelium, lens cortex, retinal plexiform layers and inner segment and pigment epithelium were rich in SH groups. Descemet's membrane and lens capsule contained many S-S linkages. Corneal stroma, sclera and retinal outer segment contained a moderate number of S-S linkages. There was a unique distribution of SH groups and S-S linkages in the eye; that is, tissues abundant in SH groups contained few S-S linkages, and tissues abundant in S-S linkages contained few SH groups. The role of SH groups and S-S linkages in the eye was discussed.

The number of disulfide (S-S) linkages and sulfhydryl (SH) groups is changed in several ocular diseases, such as radiation cataract, senile cataract, photokeratoconjunctivitis, etc. (1, 7, 9, 23). It is important to study the localization and concentration of protein-bound SH groups and S-S linkages in the normal ocular tissue to clarify the etiology of ocular diseases. There are few histochemical studies of SH and S-S groups in ocular tissues (2, 20, 21, 22). Previous studies have problems in the specificity of reagents and stability of reaction.

Recently, a new fluorescent thiol reagent, N-(7-dimethylamino-4-methylcoumarinyl) maleimide (DACM), has proved to be a useful tool in histochemical studies of sulfhydryl groups (17, 24). DACM is much more highly specific for SH groups than are several other reagents (4, 25). DACM quickly reacts with sulfhydryl groups in cryostat sections. Using this method, we clarified the distribution of protein-bound SH groups and S-S linkages in the ocular tissues.

MATERIALS AND METHODS

The eyes of 10 healthy 3-month-old ddY-strain mice were immediately frozen in acetone-dry ice mixture, and 2 μm slices were prepared in cryostat. SH groups and S-S linkages were stained as described by Ogawa et al. (17). Briefly, for detecting SH groups, a section was stained with 0.01 mM DACM (Teika Seiyaku Co., Ltd., Toyama, Japan) solution for 1 min. DACM is nonfluorescent by itself but will react readily with SH groups to form highly fluorescent addition products.
(exciting wave length (ex) 400 nm; emission wave length (em) 485 nm). For detecting S-S linkages, SH groups were first blocked by 0.15 mM NEM at 37°C for 10 min, then S-S bonds were reduced to SH groups with 40 mM dithiothreitol at 37°C for 3 min, and the preparation was stained with DACM and examined under a Nikon Fluoophot fluorescence microscope, equipped with epillumination with a 200 W super high pressure mercury lamp, an excitor filter (IF 385–425) which excites wavelengths between 385 and 425 nm, and barrier filters which suppress waves below 450 nm.

RESULTS

The lens cortex, especially epithelial cells and subcapsular cortex, contained many SH groups (Fig. 1a). In the lens nucleus, there were slightly fewer SH groups, and S-S linkages were abundant, while the lens capsule and zonular fibers (not shown) contained few SH groups and many S-S linkages (Fig. 1b). Inner and outer plexiform layers, inner segment, pigment epithelium of the retina and choroid contained many SH groups (Fig. 2a), and the outer segment of

Figs. 1a, b. The distribution of sulfhydryl and disulfide groups in the mouse lens. DACM staining.
- **a.** -SH fluorescence: Brilliant granular fluorescence is observed in the lens cortex, especially epithelial cells and subcapsular cortex. Fluorescence diminishes slightly in the lens nucleus. No fluorescence on lens capsule (cap).
- **b.** S-S fluorescence: Strong fluorescence in the lens capsule and lens nucleus. a, b ×250

Figs. 2a, b. The distribution of sulfhydryl and disulfide groups in the mouse retina. DACM staining.
- **a.** -SH fluorescence: The fluorescence is seen in the nerve fiber layer, inner and outer plexiform layers, rod inner segment, pigment epithelium and choroid. Very poor fluorescence is present in the cell nucleus and rod outer segment.
- **b.** S-S fluorescence: The fluorescence is observed in the rod outer segment and Bruch’s membrane (arrow). a, b ×250

Figs. 3a, b. The distribution of sulfhydryl and disulfide groups in the mouse cornea. DACM staining.
- **a.** -SH fluorescence: Fluorescence is seen in the corneal epithelium, keratocyte and endothelium. Very poor fluorescence is present in the epithelial cell nucleus.
- **b.** S-S fluorescence: Homogeneous strong fluorescence in the Descemet’s membrane. Fluorescence is also seen in the corneal stroma. Very poor fluorescence in the epithelial cell. a, b ×250

Figs. 4a, b. The distribution of sulfhydryl and disulfide groups in the mouse sclera, ciliary body and iris. DACM staining.
- **a.** -SH fluorescence: Fluorescence is seen in the ciliary body, scleral fibroblast and corneal endothelium (e).
- **b.** S-S fluorescence: Strong fluorescence in the Descemet’s membrane (D). Moderately strong fluorescence in the sclera and corneal stroma. Very poor fluorescence in the ciliary body and iris (i). a, b ×250

Figs. 5a, b. The distribution of sulfhydryl and disulfide groups in the mouse conjunctiva. DACM staining.
- **a.** -SH fluorescence: Fluorescence is seen in the conjunctival epithelial cell and stromal cell. CS: conjunctival stroma, S: sclera.
- **b.** S-S fluorescence: Brilliant fluorescence is seen in the stromal collagen fiber. Poor fluorescence in the epithelial cell. a, b ×250
FIGS. 1a, b. 2a, b.
the photoreceptors and Bruch's membrane many S-S linkages and few SH groups (Fig. 2a and 2b). There were few SH and S-S groups in the ganglion cells, inner and outer cell nuclear layers.

In the corneal epithelial cells, there were many SH groups in the cell cytoplasm, but only a few in the cellular nuclei. A moderate number of SH groups was present in corneal stromal keratocytes. In endothelial cells there were many SH groups
Corneal stroma contained a moderate number of S-S groups, while Descemet's membrane had many (Fig. 3b).

In the ciliary body and iris there were many SH groups, but only a few in the sclera and cell nucleus (Fig. 4a). In the sclera, there were moderate number of S-S linkages (Fig. 4b).

Conjunctival epithelial cells had many SH groups and a few S-S linkages (Fig. 5a, 5b). Collagen fibers in the conjunctival stroma had many S-S linkages (Fig. 5b). The histochemical findings were summarized in Table 1.

DISCUSSION

Our findings differ from previous studies (2, 20–22) in the following respects: 1) The cytoplasm of the epithelial cells was the most rich in SH groups. 2) There are a few SH and S-S groups in the cell nucleus. 3) Rod outer segment contained many S-S linkages and a few SH groups. The difference between previous results and ours may be due mainly to the fact that they used paraffin-embedded specimens. It was pointed out that the results differed according to the kind of fixative used.
and that thiol stainability was lost during storage of paraffin-embedded tissues (21). We also stained paraffin-embedded tissues with DACM (data not shown). In the paraffin-embedded specimens, it took more than 60 min for DACM to react with SH groups, and non-specific fluorescence was frequently observed. These results indicate that paraffin specimens are not suitable for precise localization and semiquantitative studies.

Descemet's membrane, lens capsule and Bruch's membrane showed brilliant homogeneous S-S fluorescence. This finding agrees well with high S-S content in the basement membrane (11). Corneal stroma and sclera have fewer S-S linkages than do Descemet's membrane and lens capsule. This difference in distribution of S-S linkages may be related to compactness of the tissue or difference in type of collagen in the tissue (3, 10, 14, 16, 18). These S-S linkages were thought to contribute greatly to the strength of tissues.

There are many reports that senile cataract lenses contain many S-S linkages (1, 23). Raman spectroscopic studies have shown that in the aging rat lens nucleus there is a decrease in SH and a corresponding increase in S-S (6), these can be supported by our findings that SH was high in the cortex and was low in the nucleus, vice versa was the S-S content. The details of aging change of the SH and S-S in lens are now under study (Entani et al., in preparation).

In the retina, rod outer segment contained many S-S linkages and few SH groups. It is suggested that S-S linkages play an important role to stabilize the protein molecules (8). The S-S in the disc membrane may play the same role. It has been shown that disulfide bonds are quite labil to disruption following UV-induced excitation (12). Indeed, rods are surprisingly sensitive to damage by a prolonged exposure to light (13). In disc membranes, there exist numerous number of rhodopsins which contain six SH groups and two S-S linkages per mole (5). Raman spectroscopic studies about opsin structure have shown that both SH and S-S vibration were weak (19). From these results, we suggest that proteins in the disc other than rhodopsin contain many S-S linkages.

Eyes, as well as skin, are directly affected by sunlight. The cornea filters out UV radiation below 295 nm (15). High content of SH groups in the corneal epithelium and lens may contribute to eliminate UV damage to the retina.

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REFERENCES

4. Curtis, S. K. and Cowden, R. R.: Demonstration of sulfhydryl and disulfide groups by a fluo-


