Subretinal Neovascularization After Naphthalene Damage to the Rabbit Retina

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Purpose. To examine the retinotoxic effect of naphthalene, a powerful oxidative agent and a well-known cataractogenic agent.

Methods. A 10% solution of naphthalene dissolved in paraffin oil was given every other day by gavage to 31 pigmented rabbits for 5 weeks, at a dose of 1 g/kg body weight. Four rabbits who received only paraffin oil served as controls. The eyes were clinically followed up by means of ophthalmoscopy and retinal fluorangiography. At selected intervals, the eyes were examined with light microscopy and transmission electron microscopy.

Results. The first lesions were focal and appeared in the periphery of the fundus about 3 weeks after the beginning of treatment and tended to spread over the entire retina. Histologically, there was a degeneration of photoreceptors, accompanied by a reaction and proliferation of retinal pigment epithelium (RPE) that phagocytized the damaged visual cells. After about 3 months, the proliferation of RPE was followed by subretinal neovascularization (SRN). Both mature fenestrated and thick-walled non-fenestrated capillaries penetrated Bruch’s membrane, enveloped by abundant fibrous extracellular matrix and accompanied by pericytes. As a consequence of this process, the retina was focally transformed into a “neovascular complex” in which a vascular plexus was intermingled with pseudo-acinar cavities lined by RPE. There were no signs of SRN at retinal fluorangiography, possibly because of the dense microenvironment of extracellular matrix and RPE cells of the neovascular complexes.

Conclusions. Naphthalene degeneration of the rabbit retina appears to be a simple model of photoreceptor vulnerability in the first stages and of SRN thereafter. The close chronologic and topographic relationship between the appearance of the anomalous vessels and RPE alteration and the close resemblance with previous models of experimental SRN may support the hypothesis of an experimental model of SRN triggered by the RPE. Invest Ophthalmol Vis Sci. 1994;35:696–705.

During recent years subretinal neovascularization (SRN), a common cause of severe macular pathology, has been the object of many studies that have supplied a series of experimental models. A break in Bruch’s membrane has been considered a prerequisite for the development of SRN.1–3 To this purpose, argon laser photocoagulation has been used to induce the formation of new vessels in primates and rabbit.4–9 The same mechanism may also apply to SRN obtained in rabbit using krypton laser photocoagulation10 and subretinal endophotocoagulation,11 which induce only minor changes in the Bruch’s-RPE complex without any retinal involvement that could be angiogenic per se. On the other hand, experimental new subretinal vessels can also develop in the absence of a break in Bruch’s membrane, as in the case of low intensity cyclic light exposure,12 and the subretinal injection of autologous vitreous.13,14

The ultimate mechanism of new vessel formation in all these models is largely unknown, and a multifactorial pathogenesis, also involving inflammatory factors, immunologic factors, or both, has been suggested.15

In the present study, we report the effects on rabbit retina of naphthalene, a well-known cataractogenic
agent that has a powerful oxidizing effect. After photoreceptor damage, and without any previous lesion of Bruch's membrane, the main feature of its retinal effect is SRN, which thus introduces another SRN model to be used in experimental studies.

MATERIALS AND METHODS

Young adult, male pigmented rabbits weighing approximately 1.2 kg were used. All animals were treated according to the ARVO Resolution on the Use of Animals in Research. The animals were housed in laboratory conditions under a 12-hour light (100–200 lux)/12-hour dark cycle, with food and water ad libitum. Thirty-one animals were assigned to treatment, and four were used as controls. A 10% (wt/vol) solution of naphthalene (99%, Sigma, St. Louis, MO) dissolved in paraffin oil (Sigma, St. Louis, MO) was administered to all treatment animals; controls received only paraffin oil by gavage on alternating days for 5 weeks at a dose of 1 g/kg body weight. The treatment was well tolerated by 90% of the animals that continued to look healthy and normally sighted, did not show any change in their feeding and behavior, and continued to put on weight regularly. Ten percent of the animals developed early complete cataract (15 days) accompanied by severe panuveitis and were sacrificed at that time.

The eyes were examined by means of slit lamp and indirect ophthalmoscopy every week for 5 weeks and at monthly intervals after that. If there was significant change in the fundus, fundus photography and fluorescein angiography were performed. The animals received general anesthesia through intramuscular injections of ketamine hydrochloride and xylazine. Their pupils were maximally dilated with topical 1% tropicamide and 10% phenylephrine hydrochloride. The rabbits were killed by intravenous embolization at different stages of intoxication, that is, 15, 21, 30, 40, 60, 90, and 180 days after the beginning of treatment. The controls were killed at day 30. The eyes were immediately enucleated, intravitreally injected with fixative (0.2 ml) after paracentesis of the anterior chamber, and immersed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4). They were then bisected at the ora serrata, and the posterior cup was kept in the fixing fluid at room temperature. Three hours later, they were divided into small pieces that were further fixed for 1 hour. Twelve hours after fixation in 1% osmium tetroxide and washing and dehydration in graded ethanol, the pieces were embedded in SPURR resin or were directly processed after fixation for embedding in hydrosoluble JB4 resin.

Semithin sections (1 μm) were obtained at the posterior pole, mid-periphery, and periphery (whenever possible at the site of the ophthalmoscopic lesions). They were stained with toluidine blue for light microscopy examination. If there was significant abnormality, the pieces embedded in SPURR resin were ultrathin sectioned (50 to 60 nm), stained with uranyl acetate and either lead citrate or bismuth subnitrate, and then examined by means of a Jeol (Tokyo, Japan) transmission electron microscope.

RESULTS

Clinical Features

In the majority of animals (70%), fundus lesions developed approximately 3 weeks after the beginning of treatment. They were represented by white spots with a "snowflake" aspect scattered in the retinal periphery. In more advanced stages, similar lesions were observed at the posterior pole, but at the periphery they formed white confluent plaques occasionally accompa-
nied by small hemorrhages (Figs. 1a, 1b). After 2 months, there was diffuse retinal atrophy with granular pigmentary disturbance but no clear sign of SRN or dye pooling even during fluorescein angiography. In these animals, the lens remained clear or showed a faint, white, ringed perinuclear opacity. Of the remaining animals (in addition to the 10% that developed early complete cataract and panuveitis), 20% that were sacrificed 15, 21, and 30 days after the beginning of treatment showed no change at the level of the fundus or lens (nor did the controls).

**Morphology**

Treatment with naphthalene led to retinal damage corresponding to the described fundus lesions that occurred in 70% of the animals. The first changes occurred about 3 weeks after the beginning of treatment and led to a shortening of the photoreceptors, the disarrangement and breakdown of their outer segments, and the subsequent degeneration of inner segments and nuclear pyknosis (Fig. 2). Pyknotic and viable nuclei of photoreceptors often prolapsed into the subretinal space, coming into contact with the apical RPE border (Figs. 2a, 2c). Some hypertrophy of Müller's cells, with bumping of cytoplasmic expansions beyond the external limiting membrane, was also apparent. Damage to the photoreceptors was focal, with large areas of normal or less affected retina between the lesions. Within each focus, the maximum damage was in the center and decreased toward the

![FIGURE 2](attachment:image.png)

**FIGURE 2.** (a) Light microscopic aspect of a focus of retinal degeneration 3 weeks after the beginning of treatment, showing damage to photoreceptors with nuclear pyknosis and prolapse and RPE reaction. Magnification bar represents 27 μ. (b, c) Transmission electron microscopy aspect of photoreceptor degeneration. In b, the outer segments have almost completely disappeared, but inner segments are well preserved, whereas in c, there is also swelling and degeneration of inner segments and prolapse of nuclei. (Note: RPE changes are represented by enlargement of cells in b and marked attenuation in c. Notice the absence of any change in Bruch's membrane and the choriocapillaris.) Magnification bars represent 7 μ in b and 4.3 μ in c.
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FIGURE 3. TEM of reactive cells 6 weeks after the beginning of treatment. RPE cells laying on basement membrane retain polarity, basal folds, apical microvilli, and intercellular junctions (arrow), the cell in the subretinal space contains melanin granules. Debris (D) of photoreceptors appears free in the subretinal space or is phagocytated by RPE cells. Notice the presence of a subepithelial fibroblast (arrowhead). Magnification bar represents 2.4 μ.

In more advanced stages, about 6 weeks after the beginning of treatment, the photoreceptors almost completely disappeared in the foci of degeneration or left only a few rows of mainly pyknotic nuclei. In the most damaged areas, the degenerated retina was completely substituted by fibroglial tissue with obliteration of the subretinal space.

It was only at this stage that limited changes at the level of Bruch's membrane and the choriocapillaris began, mainly represented by the extrusion of RPE-cell cytoplasmic processes often containing microfilaments and surrounded by abundant lamina-like material that crossed the basement membrane and extended into the inner collagen layer of Bruch's membrane (Fig. 4a). In this layer, occasional spindle-shaped, fibroblast-like cells and winding expansions of the choriocapillaries, often interrupting the elastic lamina, were also apparent (Fig. 3). At this stage, occasional thrombi were also found in the choriocapillaries and some retinal folds were formed in which the neural retinal layers were raised inwardly, the opposed photoreceptors degenerated, and mounds of proliferating pigmented cells clumped at the base of the fold. There was a great variability among different foci in the severity of lesions as they increased in number and extent proceeding toward the periphery of the fundus.

All the animals sacrificed after 3 months showed vascular profiles in the degenerated areas that were abnormal in appearance and location. Both mature fenestrated and immature nonfenestrated capillaries were found (Figs. 4b, 5a, 5b). The latter were characterized by thick walls, a narrow lumen, and many free ribosomes and Golgi complexes in the cytoplasm of the endothelial cells. In most cases, they also presented a basement membrane that was generally multilayered, corresponding to the mature vessels.

Pale, elongated pericytes almost invariably accompanied the anomalous vessels breaking through interruptions in Bruch's membrane (particularly the elastic lamina). Toward the inner side, the anomalous vessels merged with the proliferating RPE, and variable amounts of extracellular matrix were interposed between the basement membranes of RPE and the abnormal vessel. There was also a marked irregularity of Bruch's membrane, with an increase in the number of RPE cytoplasmic expansions creeping through the basement membrane and in the number of cells resembling fibroblasts, macrophages, and pericytes.

The final appearance observed in all the animals sacrificed 6 months after the beginning of treatment (Fig. 6), was characterized by areas in which the retina had completely disappeared and was substituted by a neovascular complex consisting of a plexus of newly formed vessels, as well as of pigmented cells, a few fibroglial cells, and pericytes embedded in variable amounts of extracellular matrix (Fig. 6). The pigmented cells often forming pseudo-acinar cavities...
FIGURE 4. (a) TEM illustrating damages at the level of Bruch's membrane 6 weeks after the beginning of treatment. A cytoplasmic process of RPE enveloped by abundant lamina-like material (arrow) projects toward Bruch's membrane. Notice intracytoplasmic filaments, flattening (F) and attachments (A) of basal surface of RPE cells. Magnification bar represents 1.25 μ. (b) Electron micrograph of an immature vessel interrupting the elastic lamina (E) of Bruch's membrane. The endothelial cells are thick and without fenestrations, and they contain free ribosomes and mitochondria. Large interruptions of the basement membrane and small sprouts are also evident (arrowhead). P, pericytes. Magnification bar represents 4 μ.

containing amorphous material maintained the typical RPE cell cytologic features, including residual polarity, reciprocal junctions, and melanin granules (Fig. 7). These cells had basal attachments and a regular basement membrane that projected linear septa toward the cells. Often, small amounts of extracellular matrix and whorled deposits of lamina-like material were interposed between the basement membrane and the basal cell surface (Fig. 8).

Besides these neovascular complexes, there was an epithelial monolayer of heavily pigmented RPE cells, often facing a detached degenerated retina that in some instances still showed rows of photoreceptor nuclei (Figs. 6c, 6d). The basement membrane of this RPE layer had an irregular course that was focally tugged by bundles of extracellular filaments with the fine structural features of elastic fibrils (Figs. 8a, 8b). On their inner side, these bundles were anchored to the epithelial basement membrane; on their outer side, they seemed to fuse with the elastic lamina of Bruch's membrane or with the cell membrane of the fibroblast-like cells. Less often, bundles of fibrils with the same characteristics were also seen stretching the basement membrane toward RPE (Fig. 8c). Bruch's membrane was markedly disorganized, and the number of choriocapillaries had decreased. Even at this stage RPE macrophages were never seen on the outer side of the retinal basement membrane, and there was a virtual absence of inflammatory cells.

These vascular and RPE changes distributed patchily throughout the retina and, even at the end stage, they were more frequent and wider at the periphery. In the center, large areas of normal retina remained.

DISCUSSION

The results of the present investigation clearly show that the administration of naphthalene to pigmented rabbits causes the degeneration of photoreceptors and an RPE reaction subsequently followed by vascular changes, suggesting SRN. Even if there are obvious limitations in describing a dynamic event such as the growth of new vessels from sequential morphologic observations, the occurrence of SRN appears to be substantiated by the fact that the features of the anomalous vessels closely resembled those reported in many other examples of SRN and that they were located in the inner layer of Bruch's membrane and within the proliferated RPE, where they are normally absent.11,18-21 The photoreceptor damage that closely resembles the class 1 photochemical damage of Kremers and Van Norren22 preferentially affects the outer and inner segments, whereas the nuclear portion appears to be more resistant. The central fundus areas are less affected than the periphery, and the border between the affected and nonaffected retinal areas is often sharp, suggesting that the toxic effect of naphthalene may have a critical threshold. Confirming previous observations,18 the response to naphthalene toxicity was
variable among the treated animals: A small group (10%) had very severe reaction, and among those sacrificed at days 21 and 30, there were affected and non-affected animals. Oxidative stress might have been the cause, as it is when there is light damage, because naphthalene is a powerful oxidative agent known to induce experimental cataract.16 In this case, impairment of the redox system of the lens is caused not only by 1,2 naphtoquinone but also by its precursors, among them 1,2 diol (1,2 dihydroxynaphthalene), 1,2 dihydro-1,2 diol, and 1,2 naphthalene oxide.23,24 Naphtoquinone has been shown to reach the rabbit retina through the choroidal circulation even after a single administration of naphthalene.25 Naphthalene is also a murine Clara-cell cytotoxicant, metabolized to unstable chiral epoxide metabolites by cytochrome P-450 mono-oxigenases. These metabolites can conjugate with glutathione in the presence of glutathione transferases.26 Preincubation with piperonyl butoxide, a cytochrome P-450 mono-oxigenase inhibitor, has been reported as preventing naphthalene-induced cytotoxicity in an explant of bronchiolar epithelium.27 Given that the ciliary body and the RPE show the highest activity of cytochrome P-450 dependent mono-oxigenases in the eye, this may be able to catalyze the xenobiotic biotransformation of naphthalene at a high rate.28 Because of the high concentration of polyunsaturated fatty acids in their outer segments, photoreceptors are particularly sensitive to oxidative stress, and lipid peroxidation has already been considered a frequent mechanism of outer segment damage.29,30

In the present study, photoreceptor degeneration was constantly associated with RPE reactive changes, which appeared to be a necessary prerequisite for the vascular changes seen at the level of the choriocapillaris and the proliferating RPE. Although the changes in RPE cell phenotype during the course of such events led in previous studies to prudent classification, such as RPE macrophage, their origin from RPE was proven in experimental studies31,32 and is also indicated by cytoplasmic morphology, residual polarity, surface specialization, junctional complexes, and attachments to basement membrane not observed in regular macrophages. During the first stage, phenotypic RPE changes seem to be dedicated to scavenging the degenerated cells but, after the disappearance of outer and inner segment debris, they may play a role in the somewhat excessive reparative process leading to fibrovascular proliferation.

An imbalance in the production of the diffusible extracellular modulating factors that regulate adjacent structures on the part of altered RPE33 can be one
of the reasons for SRN. Moreover, macrophage-like RPE cells, such as those found in the foci of naphthalene degeneration, have been shown to participate in reparative processes and, perhaps to express angiogenic effects. The relationship between neovascular in-growth and RPE cells in the different stages of SRN is probably complex. It is particularly noteworthy that the in-growing new vessels that perforated Bruch's membrane did not perforate the RPE epithelial layer nor its basement membrane. On the contrary, the morphologic sequence suggests that the RPE layer retreats in the face of the growing vessel accompanying it in its intraretinal course, with the constant interposition of extracellular matrix between the respective basement membranes.

Although a detailed description of new vessel formation was beyond the scope of this paper, it can be suggested that the new vessels originate from the choriocapillaris and that the proportion of immature to mature vascular profile may be related to the stage of SRN (early or late) or to its location (central or peripheral) in the foci of retinal degeneration. With respect to previous models of SRN in which both fenestrated and nonfenestrated capillaries have also been found, it is possible that naphthalene-induced SRN exhibits a different course and features because it is not preceded by endothelial damage and recanalization as in the previously reported models of iodate and laser-induced SRN. In our model, dilution of any morphologically recordable proliferative feature (e.g., mitosis, immature cells) could be the consequence of chronicity and the slow rate of SRN progression involving a small number of endothelial cells (unlike the rapidly progressing laser-induced SRN, there is an interval of about 2 months between the appearance of naphthalene-induced retinal damage and that of putative SRN). To this, we may add that new vessel formation could also follow cytoplasmic expansion of mature parent cells, a case in which mitoses are unlikely to occur.

Other factors could be involved in naphthalene-induced SRN. It might be stimulated by the migration of such organisms as blood-borne macrophages or pericytes into the subpigment epithelial and, less frequently, the subretinal space. These cells produce angiogenic factors and may be chemoattracted by the
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FIGURE 7. TEM aspect of a pseudoacinar cavity in a neovascular complex lined by RPE cells with residual polarity, basal infoldings, apical microvilli, and intercellular junctions (arrowhead). Magnification bar represents 3.3 μ.

cytokines (such as beta-type transforming growth factor) released by RPE. The release or lack of inhibition of lytic enzymes (such as protease urokinase) on the part of altered RPE may facilitate neovascular ingrowth by means of the degradation of the extracellular matrix. Changes in the microenvironment of the foci of degeneration (particularly the extracellular matrix) may influence cell interactions and support cell migration and proliferation. In the present study, an increase in the extracellular matrix and newly formed collagen was regularly found in the foci of neovascularization.

A special feature of the modified RPE-Bruch’s membrane junction observed in the advanced stages of the degeneration was represented by the elastic microfibrils connected to the RPE basement membrane. As far as we know, such an aspect—regularly found at the level of the dermo-epidermal junction—has never been described in Bruch’s membrane, where it could represent a modification of the attachment system of RPE basement membrane to the profoundly altered and irregular Bruch’s membrane.

As observed in previous rabbit models of SRN, the newly formed vessels do not leak fluorescein despite their regular fenestrations. The reason may be that the extracellular matrix and RPE-cell coating (which create a dense microenvironment within the neovascular complexes) leave no available space for the pooling of extravasated fluid. However, contrary to the results of previous investigations showing involution of SRN to be the result of RPE proliferation, a florid vascular net characterized the neovascular complexes.

FIGURE 8. (a, b, c) TEM aspects of the bundles of microfilaments, resembling elastic microfibrils, closely related to the RPE basement membrane. In a, on the outer side, the bundle appears connected with the elastic lamina (black arrow). In b, collagen (C) is present on the inner side of RPE basement membrane. In c, the bundles of extracellular microfilaments project toward the cell. Notice the basal attachments of RPE cells and the presence of lamina-like material close to the microfilaments (arrowhead). Magnification bars represent 1.5 μ in a and b, 1.25 μ in c.
of naphthalene degeneration even at the end of our observation.

Naphthalene-induced degeneration of the rabbit retina offers a new and simple model of the vulnerability of photoreceptors to oxidative stress in the first stages and of SRN in advanced stages. With respect to other SRN experimental models, it occupies a special place because the neovascularization is not a consequence of an initial lesion or break in Bruch’s membrane but probably only results from the reaction of RPE after photoreceptor damage. Although it represents an experimental paradigm different from that of SRN in the clinical setting (where degenerative changes in Bruch’s membrane play an important role), it may offer a good opportunity for investigating the role of RPE that, in any case, represents a common and fundamental factor of SRN.

Key Words
subretinal neovascularization, retinal pigment epithelium, retinal degeneration, oxidative retinal damage

References
28. Schwartzmann ML, Masferrer J, Dunn MW, McGiff JC, Abraham NG. Cytochrome P450, drug metaboliz-
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