Dietary Restriction Slows Age Pigment Accumulation in the Retinal Pigment Epithelium

Martin L. Katz,* Heather A. White,* Chun-Lan Gao,* George S. Roth,† Joseph J. Knapka,‡ and Donald K. Ingram†

Purpose. The accumulation of age pigment, or lipofuscin, in postmitotic cells appears to be a universal feature of the aging process in animals. In mammals, the lipofuscin content of the retinal pigment epithelium (RPE) increases progressively during senescence. Dietary restriction has been shown to slow the rate at which many biologic parameters change during aging. Experiments were conducted to determine if dietary restriction alters the rate of age pigment accumulation in the RPE.

Methods. Male Wistar rats were placed on one of three dietary regimens starting at weaning. One group was fed a nutritionally complete diet ad libitum. Another group was fed the same diet but was only allowed to consume 60% as much food daily as the ad libitum group ate. The final group was fed ad libitum a nutritionally complete diet that had a lower caloric density per gram than the diets fed to the other animals primarily because of the replacement of carbohydrate with oat fiber. Ultrastructural morphometric analysis was used to determine the RPE age pigment content in the first group at 6 months of age, and in all of the groups at 18 months of age.

Results. Dietary restriction, achieved either by reducing total food intake or by reducing the caloric content of the diet, resulted in significant decreases in RPE lipofuscin accumulation.

Conclusions. Dietary restriction provides a relatively simple means by which RPE age pigment content can be modulated. This should prove useful in assessing the role of RPE lipofuscin accumulation in age-related retinal disorders. That the oat fiber diet fed ad libitum was almost as effective as restriction of total food intake in slowing RPE age pigment accumulation indicates that the effect of restricted caloric intake is not mediated by almost constant hunger.
If dietary restriction does indeed slow aging processes this manipulation can be used to identify the individual cellular and molecular changes that are components of senescence and that may underlie age-related disorders. The effects of dietary restriction on numerous biologic parameters have been investigated, and the rates of change during senescence in many but not all of these parameters do decrease in rodents fed calorie-restricted diets.4

Dietary restriction refers to a wide range of nutritional manipulations that can be employed to reduce caloric intake compared to that of control groups. Such manipulations can include pair-feeding the same diet at a percentage level (20% to 50%) below that of ad libitum-fed controls, intermittent feeding (e.g., every other day), reducing a major component of the diet (e.g., fats), or increasing the nondigestible or inert content of the diet (e.g., cellulose).4 The latter types of regimens have been employed to a very minor extent, but they are of great significance because such manipulations can indicate whether the effects of dietary restriction on aging processes are due directly to a reduction in caloric intake or to other physiological consequences resulting from a constant state of hunger. Recent reports have indicated that diets enriched in cellulose and provided ad libitum can reduce caloric intake and growth rates, increase maximum life span, and retard age-related behavioral and neurobiologic parameters of aging in rats relative to a conventional diet fed ad libitum.10,11 The current study was designed to extend those findings by evaluating the effects dietary restriction achieved by feeding a diet rich in insoluble fiber ad libitum compared to the effects of restricting intake of a conventional diet.

One of the most universal age-related changes at the cellular level is the progressive accumulation in postmitotic cells of membrane-bound inclusion bodies that appear to be derived from secondary lysosomes.12,13 Based on their accumulation during senescence, these cellular inclusions are customarily designated age pigments. Alternatively, this class of intracellular inclusions are referred to as lipofuscin, a name based on their histochemical staining properties.12 The lipofuscin content of the retinal pigment epithelium (RPE) increases during aging in both humans and rodents.14,15 The accumulation of this pigment may impair RPE cell function and thereby lead to secondary senescent changes in the retina. If lipofuscin accumulation is a fundamental component of senescence, and if dietary restriction slows the overall aging process, rather than prolonging life span by preventing specific disorders, it was hypothesized that dietary restriction would slow the rate of accumulation of age pigment in the RPE. Experiments were conducted to evaluate this hypothesis.

MATERIALS AND METHODS

Animals and Diets

All procedures involving animals were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male Wistar rats were selected at approximately 2 months of age from a colony maintained at the Gerontology Research Center (Baltimore, MD). At the outset of the experiment, the groups were balanced in terms of body weight. The rats were assigned randomly to three dietary groups: controls fed the NIH-07 diet16 ad libitum (AL); a dietary restriction group fed the same diet but that was allowed to consume only 60% as much as that eaten by controls (DR); and a group fed ad libitum a special diet (Table 1) of which 34.5% by weight was oat bran fiber (OF). The NIH-07 diet is approximately 3.7% crude fiber. Caloric intake of rats fed the OF diet was estimated to be 40% that of animals in the AL group. The experiment was initiated with 32 rats in each dietary group.

The rats were housed in groups of four in stainless steel suspended cages and were maintained in the same groupings for the duration of the experiment, except that several rats in the DR group were regrouped to eliminate fighting. At approximately 4:00 PM daily, the allotted food was placed in three hoppers located at the front of each cage. Every 5 weeks during the first 3 months of the study and then every fourth week thereafter, food consumption in the AL group was assessed by weighing the food when placed into the hoppers and when removed the next day, taking care to include the food droppings under the cage. These estimates for the AL group were used to adjust the food allotments provided to the DR rats. All rats

<table>
<thead>
<tr>
<th>Table 1. Oat Fiber Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ingredient</strong></td>
</tr>
<tr>
<td>Fishmeal (60% protein)</td>
</tr>
<tr>
<td>Soybean meal (48% protein)</td>
</tr>
<tr>
<td>Corn gluten meal (60% protein)</td>
</tr>
<tr>
<td>Ground whole hard wheat</td>
</tr>
<tr>
<td>Ground #2 yellow shelled corn</td>
</tr>
<tr>
<td>Ground whole oats</td>
</tr>
<tr>
<td>Wheat middlings</td>
</tr>
<tr>
<td>Brewers dried yeast</td>
</tr>
<tr>
<td>Soy oil</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
</tr>
<tr>
<td>Sodium chloride</td>
</tr>
<tr>
<td>Oat bran</td>
</tr>
<tr>
<td>NIH-31 mineral mix*</td>
</tr>
<tr>
<td>NIH-31 vitamin mix*</td>
</tr>
</tbody>
</table>

were weighed weekly. The mean weekly food intake per rat for the first year of the study was approximately 125 g for controls (AL), 60 g for the rats in the DR group, and 140 g for the OF animals. Water was available ad libitum from an automated hyperchlorinated (10 ppm) water system. The rats remained in one vivarium of the Gerontology Research Center colony for the duration of the study. Air was circulated at a rate of 12 changes per hour. Room temperature was controlled at 22 ± 2°C and relative humidity was maintained at 45%. The photoperiod was regulated as a 12 hour:12 hour light/dark cycle with lights on at 6:00 AM. By using a sentinel animal system during the experiment, the vivarium was determined to be generally free of infectious pathogens, except for pneumonia virus of mice. The cages were inspected daily, and moribund or dead rats were removed.

Quantitation of RPE Lipofuscin
At 18 months of age, five animals were randomly selected from each of the treatment groups for determination of RPE lipofuscin content. Analyses were also performed on five animals in the AL group at 6 months of age. RPE lipofuscin content was quantitated using ultrastructural morphometry. The rats were killed by decapitation and the eyes were immediately enucleated and placed in a cacodylate-buffered aldehyde fixative.17 The corneas were slit and the eyes were fixed at room temperature with gentle agitation for at least 12 hours. The eyes were then stored in fixative at 4°C until they were further dissected.

The eyes were dissected to obtain a region containing an area of the retina extending between 0.5 and 2 mm superior to the optic nerve head along the superior-inferior meridian. This portion of each eye was washed in 0.17 mol/l sodium cacodylate (pH 7.4), postfixed with OsO4, and embedded in epon resin.14 Ultrathin sections of the embedded tissue were obtained with a Sorvall MT5000 (DuPont, Wilmington, DE) ultratome and were collected on 200 mesh copper grids. Sections were cut in planes parallel to the long axes of the photoreceptor cells. The sections were stained with uranyl acetate and lead citrate and were examined with a JEOL 12000EX electron microscope (JEOL Ltd., Tokyo, Japan). A series of electron micrographs of adjacent nonoverlapping regions of the RPE from each sample was made at a magnification of 4000 X. These micrographs were obtained from a minimum of 200 mm of RPE length (measured along the RPE basal lamina) from each sample. The number of lipofuscin granules in each measured area was determined, and the area of each granule was measured with the assistance of an Image-1 image analysis system (Universal Imaging, West Chester, PA).

Statistical Analyses
Determination of whether diet or age significantly influenced RPE lipofuscin content was accomplished using analysis of variance. Comparisons among individual treatment groups were performed using the Newman-Keuls procedure.18

RESULTS
Reduced caloric intake in both the DR and OF groups resulted in a decrease in maximum body weight. In the AL group, mean maximum body weight was 625 g. Conversely, the DR rats achieved a mean maximum body weight of only 400 g, whereas in the OF animals, mean maximum body weight was 500 g. Median survival times (50% survival) were 18, 27, and 22 months for the AL, DR, and OF groups, respectively. Maximum survival times have yet to be determined for the three dietary groups.

RPE lipofuscin content increased approximately twofold in the AL animals between the ages of 6 and 18 months (P = 0.005; Figs. 1, 2). The number of lipofuscin granules per unit RPE length, however, increased only 49% between these two ages (P < 0.01; Fig. 3). Thus, the age-related increase in RPE lipofuscin content resulted from increases in both lipofuscin granule number and size. Limiting the animals’ access to food (DR group) resulted in a substantial reduction in the age-related increase in RPE lipofuscin content. At 18 months of age, the mean RPE lipofuscin content in the DR group was only 64% of that in the AL animals (P = 0.02; Fig. 2). This decrease was due to decreases in both RPE lipofuscin granule number (Fig. 3) and size. When animals were fed ad libitum the diet with reduced caloric content (OF group), the age-related increase in RPE lipofuscin content was also reduced. At 18 months, the amount of RPE lipofuscin in the OF group was a mean of only 71% of that in the AL animals (P = 0.04; Fig. 2). This was primarily due to a decrease in the age-related increase in the number of lipofuscin granules per unit RPE length (Fig. 3). There was no significant difference in the mean RPE lipofuscin content between the DR and OF groups at 18 months of age (Fig. 2), indicating that both methods of dietary restriction were equally effective in retarding the age-related accumulation of lipofuscin in the RPE.

DISCUSSION
The apparent slowing of senescence by dietary restriction has provided a tool for assessing whether time-dependent changes in specific biologic parameters are integral components or markers of aging. Manipulations that slow the overall aging process would be ex-
pected to slow changes that are basic components of senescence. The data from this study and others indicate that lipofuscin accumulation is intimately associated with senescence. Thus, it is possible that underlying factors that determine the maximum life-span potential of a species also determine the rates of age pigment accumulation in the RPE and other tissues. Understanding the mechanisms involved in lipofuscin accumulation may therefore contribute to our understanding of general aging processes.

A variety of data suggest that lipofuscin formation involves oxidative damage to cellular constituents. For example, dietary deficiency in antioxidant nutrients results in the accumulation of lipofuscin-like autofluorescent pigments in numerous tissues. It is possible that dietary restriction decreases the rates of both senescence and age pigment accumulation by decreasing oxidative damage to tissues. Indeed, it has been reported that antioxidant defense systems are enhanced in animals fed restricted diets.

Proteins are one class of molecules that may be modified as a result of nonenzymatic oxidation in cells. One means by which cells compensate for oxidative damage to proteins is to degrade those molecules that have been oxidatively modified. If protein turnover is impaired by treating tissues with protease inhibitors there is a rapid accumulation of lipofuscinlike pigments in the treated cells. This suggests that modified proteins constitute a significant component of lipofuscin granules. It therefore is possible that dietary restriction increases life span and reduces lipofuscin accumulation by enhancing protein turnover. This possibility is supported by a number of studies that indicate that protein turnover is accelerated in dietary-restricted animals.

More is probably known about the mechanisms underlying lipofuscin accumulation in the RPE than in any other tissue. It appears that the major precursors of RPE lipofuscin are molecular constituents of the photoreceptor outer segments. If RPE degradation of phagocytosed outer segment proteins is inhibited, partially degraded phagosomes with lipo-
Dietary Restriction Slows RPE Age Pigment Accumulation

Fuscin-like fluorescence properties rapidly accumulate in the RPE. This suggests that outer segment proteins are directly involved in RPE lipofuscin fluorescence formation. The fluorescence probably develops as a result of covalent modification of the proteins, most likely by visual cycle retinoids. These covalent modifications may prevent the proteins from being degraded by lysosomal enzymes, and thus account for their accumulation as lipofuscin granule contents. Apparently outer segment proteins must be degraded very quickly to prevent these covalent modifications from occurring, or at least from progressing to the stage at which protein degradation is impeded. Dietary restriction may slow RPE lipofuscin accumulation by enhancing outer segment protein degradation by these cells. If this is so, it may be possible to develop a means other than dietary restriction by which to accelerate degradation of phagocytosed outer segment proteins by the RPE. This could slow RPE lipofuscin accumulation, and thereby help determine whether buildup of this pigment is involved in the development of age-related retinal disorders in humans.

The retardation of RPE age pigment accumulation was similar in the DR and OF groups. This observation supports the possibility that diets that are consumed ad libitum, but provide lower caloric intake, may be as effective as restriction of total food intake in retarding age-related processes. In terms of application to human health, this finding is quite important, because in developed countries it is easier to restrict caloric intake by lowering the caloric content of diets consumed ad libitum than it is to restrict intake of nutritionally adequate diets.

Key Words
Aging, lipofuscin, retina, caloric restriction, dietary fiber

Acknowledgments
The authors thank J. Scott Christianson for his assistance with image analysis and A. Carter and N. Williams for excellent management of the nutritional protocols.

References


