Effects of Docosahexaenoic Acid on Retinal Development: Cellular and Molecular Aspects

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ABSTRACT: We have recently shown that docosahexaenoic acid (DHA) is necessary for survival and differentiation of rat retinal photoreceptors during development *in vitro*. In cultures lacking DHA, retinal neurons developed normally for 4 d; then photoreceptors selectively started an apoptotic pathway leading to extensive degeneration of these cells by day 11. DHA protected photoreceptors by delaying the onset of apoptosis; in addition, it advanced photoreceptor differentiation, promoting opsin expression and inducing apical differentiation in these neurons. DHA was the only fatty acid having these effects. Mitochondrial damage accompanied photoreceptor apoptosis and was markedly reduced upon DHA supplementation. This suggests that a possible mechanism of DHA-mediated photoreceptor protection might be the preservation of mitochondrial activity; a critical amount of DHA in mitochondrial phospholipids might be required for proper functioning of these organelles, which in turn might be essential to avoid cell death. Müller cells in culture appeared to be involved in DHA processing: they took up DHA, incorporated it into glial phospholipids, and channeled it to photoreceptors in coculture. Both Müller cells, when cocultured with neuronal cells, and the glial-derived neurotrophic factor (GDNF) protected photoreceptors from cell death. These results suggest that glial cells may play a central role in regulating photoreceptor survival during development through the provision of trophic factors. The multiple effects of DHA on photoreceptors suggest that, in addition to its structural role, DHA might be one of the trophic factors required by these cells.

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The enrichment in docosahexaenoic acid (DHA) of membrane lipids in the nervous system has stimulated the search for the possible functions of this fatty acid in brain and retinal neurons. In the retina, DHA is the major fatty acid of photoreceptor phospholipids (1), and several pieces of evidence suggest that a close correlation exists between this high content of DHA and correct photoreceptor function. A decrease in DHA content in retinal lipids is associated with alterations in the electroretinogram (2,3) and impairment of visual acuity (4,5). DHA plays an important role during the development of the vertebrate retina. A deficiency of this fatty acid or its precursors in infant formulas affects human retinal development (6–8), and supplementation of infant diets with long-chain polyunsaturated fatty acids improves visual development (9). The puzzling question regarding the roles of DHA in the development, structure, and functionality of the retina, and particularly photoreceptor cells, has been the subject of considerable research and has mainly been studied from a structural point of view. Several studies suggest that DHA-enriched membranes can favor rhodopsin conformational changes and function (10–12). However, the precise functions of DHA in the retina are far from being established.

Recent reports from our laboratory have shown a novel role for DHA as a survival factor for photoreceptor cells. This fatty acid is able to rescue photoreceptors from apoptosis, the most frequent pathway of programmed cell death, during the early stages of their development *in vitro* (13–15). Elucidation of the molecular and cellular mechanisms involved in the regulation of photoreceptor apoptosis is one of the major aims in this area of investigation, particularly with the recognition that apoptosis is the main mode of photoreceptor death in mouse models of retinitis pigmentosa (16,17). However, until now the identification of specific trophic molecules able to effectively prevent photoreceptor degeneration has been elusive. Three trophic factors, namely, epidermal growth factor, basic fibroblast growth factor, and ciliary neurotrophic factor, along with other molecules such as laminin, taurine, and retinoic acid have been shown to have either protecting or differentiating effects on photoreceptor cells (18). More recently, the glial-derived neurotrophic factor (GDNF), produced and released by glial cells, has been added to the list of survival-promoting molecules (19).

The mechanism of action involved in DHA protection remains to be established. It is also unclear how retinal cells, and particularly photoreceptors, get the DHA required for building their membrane phospholipids. It has been proposed that DHA is transported from the liver, bound to plasma lipoproteins to reach photoreceptor cells in the retina (20). Non-neuronal cells in the retina may also synthesize and/or process DHA and deliver it to photoreceptors. Glial cells appear to have multiple functions in the retina and have been shown to be a source of trophic factors, including GDNF, required for the survival and maintenance of neuronal cells (21,22).

In the present paper, we review DHA survival- and differentiation-promoting effects; show that GDNF and glial cells,

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Abbreviations: BSA, bovine serum albumin; DAPI, 4,6-diamidino-2-phenylindole; DHA, docosahexaenoic acid; DME, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GDNF, glial-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; PI, propidium iodide.

when cocultured with retinal neurons, can also rescue photoreceptors from cell death; and present evidence suggesting that glia may modulate photoreceptor survival through the provision of DHA and GDNF.

MATERIALS AND METHODS

Materials. Albino Wistar rats bred in our own colony were used in all the experiments. Plastic 35-mm culture dishes and multichambered slides (Nunc) were purchased from Inter Med. Fetal bovine serum (FBS) was from Centro de Virología Animal (Cevan). Dulbecco's modified Eagle's medium (DME) (Gibco) was purchased from Life Technologies. Bovine serum albumin (fraction V; fatty acid-free; low endotoxin, tissue culture tested) (BSA), poly-DL-ornithine, trypsin, trypsin inhibitor, transferrin, hydrocortisone, putrescine, insulin, polyornithine, selenium, gentamicin, 4,6-diamidino-2-phenylindole (DAPI), fluorescein-conjugated secondary antibodies, propidium iodide (PI), palmitic, oleic, and arachidonic acids, anti-glial fibrillary acidic protein (GFAP) IgG fraction antiserum antibody, and paraformaldehyde were from Sigma Chemical Co. (St. Louis, MO). Secondary antibody, *Alexa 488*-conjugated goat anti-mouse, *Alexa 546*-conjugated goat anti-rabbit IgG, and Mitotracker were from Molecular Probes, Inc. Monoclonal antibody against rhodopsin, Rho-4D2, was generously supplied by Dr. R. Molday (University of British Columbia). Monoclonal antibody HPC-1 was a generous gift from Dr. C. Barnstable (Yale University). DHA was isolated from bovine retinas, by a combination of chromatographic procedures (13). [^{14}C]22:6 (specific radioactivity 160 mCi/mmol) was from NEN (Boston, MA). Solvents were high-performance liquid chromatography grade, and all other reagents were analytical grade.

Retinal neuron cultures. Purified cultures of rat retinal neurons were prepared by methods previously described (13,23), with slight modifications. In brief, 1–2-d-old rat neuroretinas were dissociated for 12 min with trypsin (0.1%) in Ca^{2+}–Mg^{2+}-free Hanks' balanced salt solution, and the resulting cell pellet was incubated with trypsin inhibitor. The cells were sequentially rinsed, resuspended in a serum-free, chemically defined neuronal medium, and then subjected to a gentle dissociation with a glass pipette. About 0.8×10^5 cells/cm^2 were seeded on 35- or 100-mm diameter dishes or on coverslips placed in these dishes. Culture dishes and coverslips had previously been sequentially treated with polyornithine and schwannoma-conditioned medium (24).

Pure glial cell cultures. Pure glial cell cultures were prepared from 1–2-d-old rat retinas dissociated as described above; retinal cells were then resuspended in DME with 10% FBS and seeded at a density of 2.5×10^5–3×10^5 cells/cm^2 on a 35-mm diameter plastic dish, with no pretreatment. The culture medium was routinely replaced every 2–3 d to eliminate neuronal cells.

Coculture of retinal neurons with glial cells. To obtain neuron–glia cocultures, the dissociated cells were resuspended in neuronal medium and seeded at a density of 2.5×10^6–3×10^6 cells per 35-mm diameter plastic dish, with no pretreatment, as described above. After 2 d, the culture medium was replaced by DME with 10% FBS, to allow the growth of glial cells.

Fatty acids and GDNF supplementation. DHA, palmitic, oleic, and arachidonic acids, complexed with BSA, were added to 35- or 100-mm diameter dishes at day 1 in culture, 4–6.7 mM final concentration (13). The same volume of a BSA solution of the same concentration was added to control cultures. After different incubation times, the cells were thoroughly washed and scraped from the culture dishes, and lipids were extracted as described below.

GDNF was added to the cultures immediately after seeding the cells at a final concentration of 10 ng/mL in DME. A similar volume of DME was added to control samples.

Lipid analysis. Rat retinas were excised using a dissecting stereomicroscope and immediately homogenized for lipid extraction (25). To prepare lipid extracts from neurons in culture, the incubation media were removed, and, after the cultures were rinsed, the cells were transferred to glass tubes and centrifuged for 10 min at 1000 rpm (13). Neuronal lipids were then extracted (25). All samples were kept under an N_2 atmosphere.

To analyze neuronal fatty acid composition, free fatty acids were separated by thin-layer chromatography (13); the methyl ester derivatives of phospholipid and triacylglycerol fatty acids were then prepared (26) and analyzed by gas–liquid chromatography (13). Distribution of radioactivity among lipids was determined (15). Unlabeled lipids prepared from bovine retina were added to the samples as carriers.

Labeling of glial and neuronal cells with [^{14}C]DHA. To investigate the possible metabolic coupling between glial cells and retinal neurons, pure glial cell cultures were incubated for 6–10 d until they reached confluence. The serum-containing media were then replaced by chemically defined medium supplemented with [^{14}C]22:6 (0.1 μCi, 6.7 μM). Unlabeled 22:6n-3 was also added, in the amount required to reach the desired concentration. After 1 d, this medium was removed, and glial cells were thoroughly washed to eliminate nonincorporated label. Fresh serum-free, chemically defined culture medium was added (1 mL), and pure neuronal cultures, grown on coverslips, were then placed on top of and facing glial cells and cocultured for 3 d. The coverslips were then removed, and both glial cells and neurons were fixed for 1 h with 2% glutaraldehyde in phosphate-buffered saline (0.9% NaCl in 0.01 M NaH_2PO_4, pH 7.4) and processed for autoradiographic analysis (23).

Cytochemical methods. The cultures were fixed as described above and permeated with Triton X-100 (0.2%). Neuronal cell types were identified by immunocytochemistry with the monoclonal antibodies HPC-1, for amacrine cells (27,28), and Rho-4D2, for photoreceptor cells (29), as previously described (13), using *Alexa 488*-conjugated goat anti-mouse as the secondary antibody. Glial cells were identified by their flat morphology and by their immunoreactivity to GFAP, which selectively recognizes Müller cells in the retina.

Controls for immunocytochemistry were done by omitting either the primary or the secondary antibody. Dead cells were identified by fluorescence microscopy, incubating the cultures with PI (0.5 μg/mL final concentration in culture) for 30 min just before fixation (30). Nuclei integrity was determined with DAPI (14).

Mitochondrial activity was determined by incubating the cultures for 30 min before fixation with the fluorescent probe Mitotracker (0.1 μg/mL), which selectively stains active mitochondria in culture. Active mitochondria, displaying a bright red fluorescence, and damaged mitochondria, showing a pale red fluorescence, were identified by microscopy.

Statistical analysis. For cytochemical studies, 10 fields per sample were analyzed in each case. Each value reported from cytochemical, compositional, and lipid labeling studies represents the average of at least three experiments ± SD. Statistical significance was determined by Student's two-tailed *t*-test.

RESULTS

Neuronal and glial cultures. When retinal cells were incubated in chemically defined medium and in the absence of specific trophic factors, they developed and differentiated mainly as photoreceptors and amacrine neurons (13,14). Photoreceptors have a small, round cell body with a single neurite at one end and sometimes display a connecting cilium at the opposite end, but they failed to develop their characteristic outer segments (Fig. 1A). Opsin was diffusely distributed over the cell body, as observed with the Rho4-D2 monoclonal antibody. Amacrine neurons are bigger than photoreceptors and have multiple neurites (Fig. 1B). Both cell types developed normally for 3–4 d. Photoreceptors then started an apoptotic pathway leading to the death of most of these cells by day 14 (14). In contrast, amacrine neurons continued their survival and differentiation for up to 17 d *in vitro*.

Dissociated retinal cells resuspended in 10% FBS in DME and seeded at high density on low adhesive substrata developed mainly as flat cells, with big oval nuclei, that survived in culture for several days. Using the GFAP antibody, these cells were identified as Müller cells (Fig. 1C).

DHA had multiple effects on photoreceptor survival and differentiation. Analysis of the fatty acid composition of neuronal lipids showed that DHA amounted to only 4–6% of the total esterified fatty acids; this proportion was the same as found in the 1–2-d-old retinas used for the cultures. While the percentage of DHA in retinal lipids increased steadily during development *in vivo*, it remained constant in neurons *in vitro* (Fig. 2A). However, addition of DHA to the culture medium showed that neurons had active mechanisms for taking up and esterifying this fatty acid, because the proportion of DHA in neuronal lipids increased to over 20%, reaching similar values to those found *in vivo* (Fig. 2A,B).

Unexpectedly, this increase in the proportions of DHA in neuronal lipids was paralleled by an increase in photoreceptor survival (13). Addition of DHA had a protective effect on photoreceptors, rescuing them from the degeneration pathway observed in control conditions. At day 10, about 80% of photoreceptors were apoptotic in control cultures, whereas in DHA-treated cultures this percentage was reduced to nearly 60% (Fig. 3A). This effect was specific for photoreceptors; amacrine cells showed no signs of apoptosis and were unaffected by DHA addition.

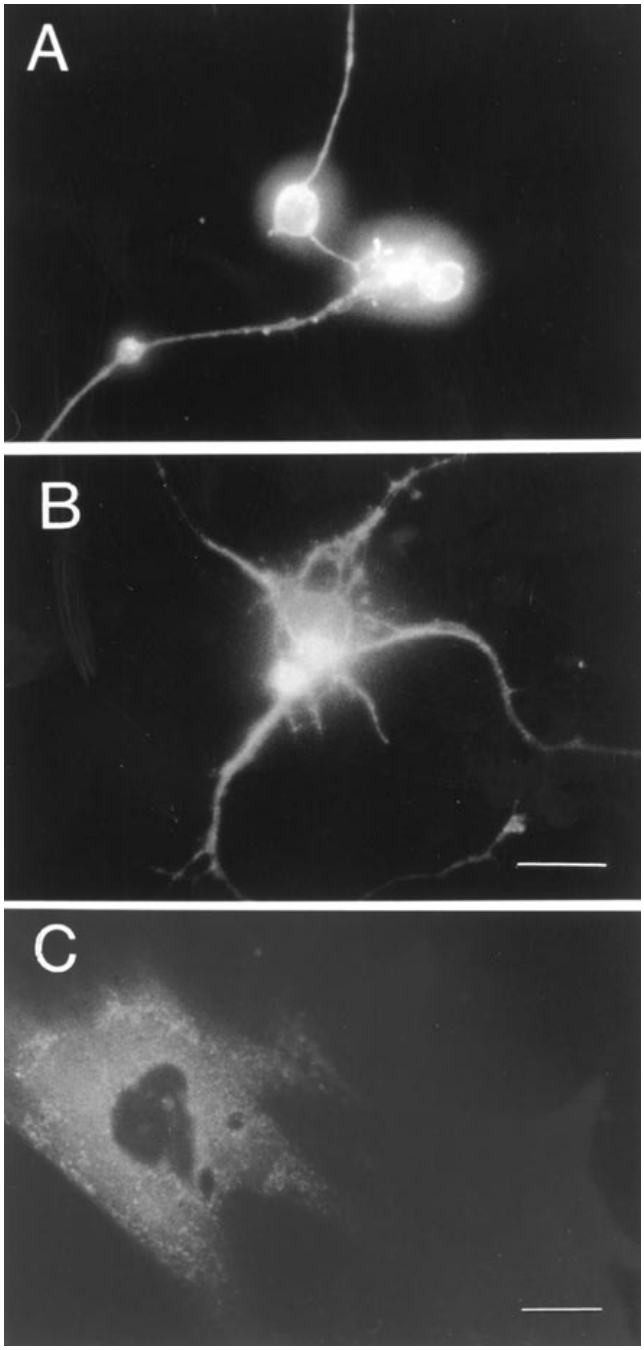

FIG. 1. Neuronal and glial cell identification. Fluorescence photomicrographs of (A) photoreceptors labeled with the Rho4-D2 monoclonal antibody, (B) amacrine cells visualized with the HPC-1 monoclonal antibody, and (C) glial cells, identified with antibodies to glial fibrillary acidic protein (GFAP). Rat retinal neurons were cultured for 7 d in a chemically defined medium; glial cells were cultured until they were confluent in 10% fetal bovine serum in Dulbecco's modified Eagle's medium. The bars represent 10 μm (A and B) and 20 μm (C).

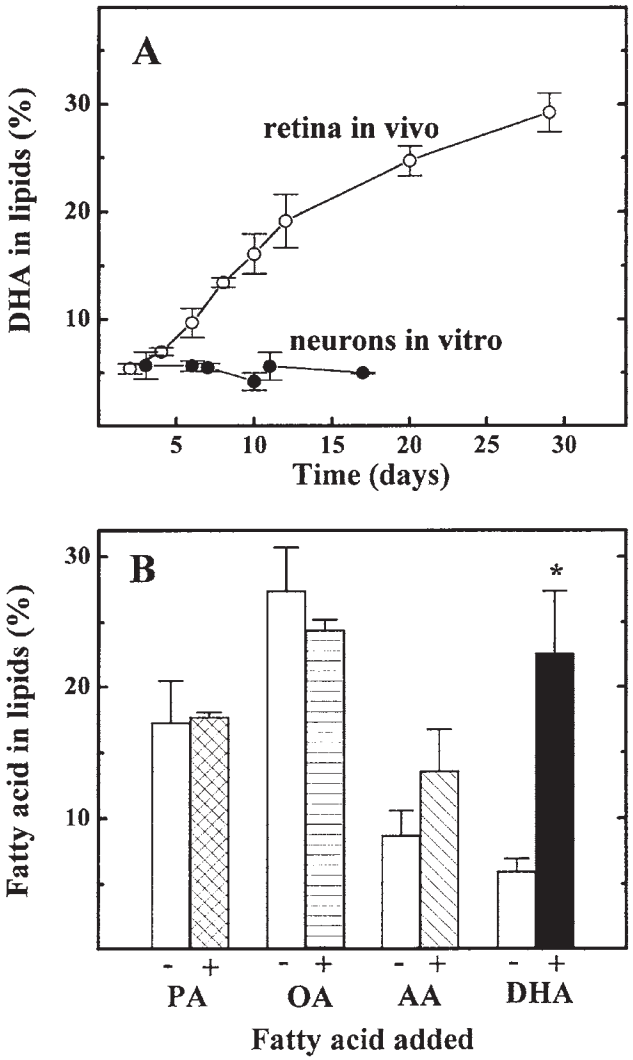

FIG. 2. Fatty acid composition of the retina *in vivo* and of retinal neurons in culture. Whole rat retinas were excised at different postnatal days *in vivo*, and retinal neurons were collected at different times of development *in vitro*. In fatty acid-supplemented cultures, the fatty acid (4 μM) complexed with bovine serum albumin (BSA) was added at day 7 *in vitro*; the same volume of a BSA solution was added to control cultures. At day 11, lipids were extracted, and the combined fatty acid composition of phospholipids and triacylglycerols was analyzed. (A) Variations in the percentage of docosahexaenoic acid (DHA) in lipids in the retina *in vivo* and retinal neurons *in vitro* during development. Values represent the means ± SD of three to seven samples. (B) Comparison of fatty acid composition of neuronal membranes in control cultures and cultures supplemented with palmitic acid (PA), oleic acid (OA), arachidonic acid (AA), or DHA. Bars represent the percentage of each fatty acid in lipids of retinal neurons in control cultures (–) and in cultures supplemented (+) with same fatty acid. Mean values ± SD of at least three samples are given. *Statistically significant difference with respect to control cultures ($P < 0.05$).

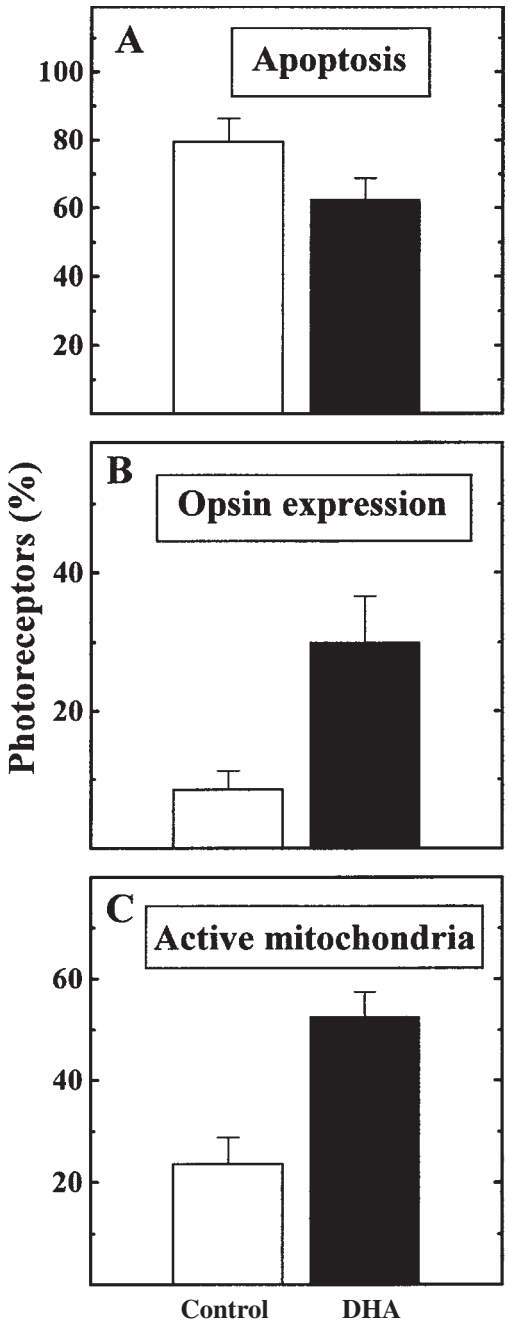

FIG. 3. Effects of DHA on photoreceptor survival, differentiation, and mitochondrial activity. Pure neuronal cultures were supplemented at day 1 with either 6.7 μM DHA or with a BSA solution (control), as described in Figure 2. Cells were fixed at day 10, and (A) photoreceptor apoptosis was determined by counting the number of these cells with fragmented nuclei, labeled with 4,6-diamido-2-phenylindole (DAPI); (B) opsin expression was evaluated by counting the number of Rho-4D2-positive photoreceptors; and (C) mitochondrial activity was analyzed with the fluorescent probe Mitotracker. Mean values ± SD of at least three samples are shown. See Figure 2 for abbreviations.

Even when photoreceptors displayed such characteristic apoptotic features as fragmented nuclei, they maintained some basic functions for several days. At early stages of apoptosis, most cells had a normal appearance and even expressed opsin. Depending on the severity of the damage, nonviable cells could be visualized by the pale to bright red fluorescence they exhibited after incubation with PI. At day 1, about 40,000 photoreceptors/dish were nonviable; by day 10, this number had sharply increased, and about 700,000 cells/dish were PI-positive (Fig. 4). Addition of DHA markedly reduced the number of nonviable cells, to about 470,000 photoreceptors/dish (Fig. 4).

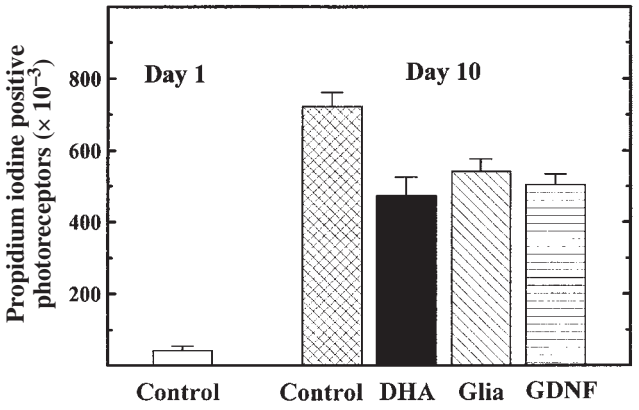

FIG. 4. Effect of DHA, glial cells, and glial-derived neurotrophic factor (GDNF) on photoreceptor survival. Pure neuronal cultures supplemented with either DHA, as described in Figure 2, or GDNF (10 ng/mL), added immediately after seeding the cells or neuron–glia cocultures, were incubated for 10 d. Dead photoreceptors were identified with propidium iodide (PI). The bars represent the number of dead, PI-positive photoreceptors in each culture condition, as means ± SD of at least three samples. See Figure 2 for abbreviations.

To find out if these effects were specific to DHA, neuronal cultures were supplemented with other fatty acids, such as palmitic, oleic, or arachidonic acid. The fatty acid composition of neuronal membranes remained the same upon addition of these fatty acids (Fig. 2), because none of them was able to accumulate in membrane lipids. Concomitantly, these fatty acids were unable to stop photoreceptor degeneration and had no effect on apoptosis (14). Hence, DHA was the only fatty acid having an anti-apoptotic effect and this effect, was consistent with its increased proportions in neuronal lipids.

Searching for clues to understand the anti-apoptotic activity of DHA, we analyzed characteristic parameters of photoreceptor differentiation and found that both the development and the differentiation of these cells were enhanced upon DHA addition. As described above, under control conditions, a small percentage of photoreceptors expressed opsin, and the photoceptors did not develop their characteristic outer segments (Fig. 1A). However, upon DHA supplementation, the proportion of photoreceptors displaying apical processes at the end of their cilium (13,15) and showing opsin expression was markedly increased (Fig. 3B). Thus, the percentage of photoreceptor cells expressing opsin at day 10, either in the cell bodies or in their apical processes, increased from 8.6% in control cultures to 30% in DHA-treated cultures (Fig. 3B). In addition, in DHA-treated cultures, opsin tended to lose its axon localization and to concentrate either in photoreceptor cell bodies or in their apical processes. All these changes were indicative of photoreceptor differentiation.

Mitochondria have been proposed to have a central role in triggering apoptotic death (31). Impairment in mitochondrial activity seems to be closely related to the onset of cell death. Looking for possible molecular pathways leading to the protective effect of DHA, we investigated mitochondrial functionality in control and DHA-treated cultures. Most amacrine cells had several active mitochondria at every time studied (not shown). In contrast, mitochondrial activity was severely reduced in photoreceptor cells under control conditions: by day 10, only about 25% of these cells were left with active mitochondria. DHA supplementation seemed to ameliorate mitochondrial damage: after the same time in culture, 53% of photoreceptors still displayed active mitochondria (Fig. 3C). Hence, the anti-apoptotic effect of DHA might be related to the maintenance of mitochondrial activity.

Glial cells ameliorated photoreceptor cell death in vitro. We investigated the effects of glial cells on neuronal survival by coculturing retinal glia with neuronal cells. The presence of glial cells led to a significant increase in photoreceptor survival: the amount of PI-positive photoreceptors at day 10 was reduced from 700,000 cells/dish in pure neuronal control cultures to about 550,000 cells/dish in neuron–glia cocultures (Fig. 4). Glial cells seemed to exert this effect by delaying the onset and retarding the progression of apoptosis, in a similar manner to DHA (Insua, M.F., Rotstein, N.P., and Politi, L.E., unpublished data). Moreover, as observed in DHA-supplemented cultures, glial cells increased the formation of apical processes in photoreceptors (Insua, M.F., Rotstein, N.P., and Politi, L.E., unpublished results).

We then analyzed glial cell fatty acid composition and the ability of glia to take up DHA from the culture medium. The major fatty acids present in glia total lipids were oleic and palmitic, with arachidonic as the major polyunsaturated fatty acid; almost 15% of the fatty acids were DHA (Table 1). The proportion of this acid was considerably higher than that found in neuronal lipids. When glial cells were supplemented with 6.7 μM DHA, the percentage of this fatty acid in lipids increased to about 27% with a concomitant decrease of oleic acid. Therefore, glial cells were able to take up DHA and accumulate it in their lipids.

TABLE 1
Effect of Docosahexaenoic Acid (DHA) Supplementation on the Fatty Acid Composition of Glial Cells *in vitro*[a]

Fatty acid	–DHA (%)	+DHA (%)
16:0	19.25 ± 1.33	17.26 ± 1.54
16:1	3.42 ± 1.48	1.80 ± 0.03
17:0	1.21 ± 0.27	0.97 ± 0.05
18:0	15.21 ± 2.66	20.10 ± 2.67
18:1	23.78 ± 7.66	12.33 ± 0.31
18:2n-6	1.37 ± 0.18	0.80 ± 0.06
20:4n-6	18.59 ± 4.80	15.50 ± 1.78
20:5n-3	0.59 ± 0.45	2.07 ± 0.22
22:4n-6	0.93 ± 0.44	0.69 ± 0.12
22:5n-6	0.81 ± 0.06	0.52 ± 0.10
22:6n-3	14.73 ± 3.18	27.13 ± 0.65
24:5n-3	0.06 ± 0.09	0.31 ± 0.08
24:6n-3	0.04 ± 0.04	0.51 ± 0.24

[a]Glial cells were cultured for 6 d in 10% fetal bovine serum in Dulbecco's modified Eagle's medium until they reached confluence and were then incubated in neuronal medium either with 6.7 μM DHA complexed with bovine serum albumin (BSA) (+DHA) or with the same volume of a BSA solution (–DHA). After 2 d, the cells were collected and centrifuged, their lipids were extracted, and the fatty acid composition was analyzed as described in Figure 2. The values are percentages of total fatty acids and are shown as means ± SD from three or four samples.

Glial cells can transfer DHA to neurons in coculture. The question remaining was whether glial cells could channel DHA to neurons. Glial cells previously labeled with [^{14}C]DHA were cocultured with neurons grown on coverslips, and then glial cells and neurons were again separated. Autoradiographic analysis showed a diffuse distribution of the silver grains in glial cells, indicating [^{14}C]DHA uptake and dispersion over the entire cells (Fig. 5). In contrast, silver grains were more densely concentrated in neuronal cells, preferentially in photoreceptors; moreover, in these cells, the most intense staining was observed in the regions corresponding to apical processes. Overall, these results suggest that glial cells incorporated the fatty acid and managed to convey [^{14}C]DHA to neurons in coculture and that photoreceptors avidly took it up and concentrated it in the newly formed apical processes.

GDNF protects photoreceptors from cell death. Glial cells might rescue photoreceptors from cell death by releasing trophic factors other than DHA, such as GDNF. The possibility that this molecule might protect photoreceptors from degeneration *in vitro* was investigated. When cell death was evaluated (Fig. 4), the number of PI-positive photoreceptors at day 10 was reduced from about 720,000 cells/dish in control cultures to 500,000 cells/dish in GDNF-supplemented cultures. This reduction was similar to that induced by glial cells and DHA (Fig. 4). Therefore, GDNF, DHA, and glial cells were able to rescue photoreceptors from cell death triggered by the absence of trophic factors.

DISCUSSION

The mechanisms by which photoreceptor cells avoid natural programmed cell death during normal development in the retina are still unknown. The identification of the possible trophic factors involved in this process is of paramount interest, not only to increase our understanding of retinal functioning but also to prevent or treat human inherited degenerative

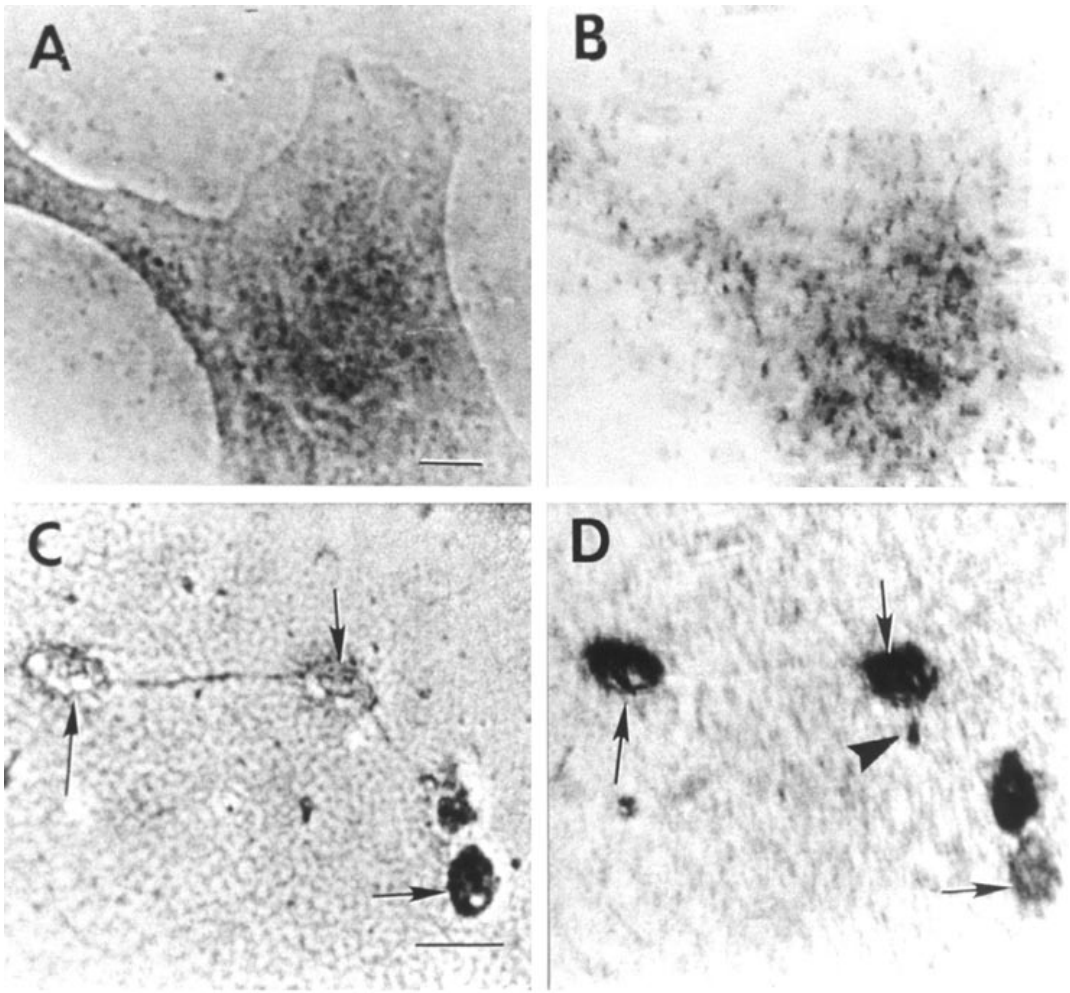

FIG. 5. Channeling of [^{14}C]DHA from glial to neuronal cells in coculture. Phase-contrast (left) and bright-field (right) pictures of autoradiographies of glial (A, B) and neuronal (C, D) cells. Glial cultures were incubated with [^{14}C]DHA for 1 d and, after thorough removal of the fatty acid, cocultured with pure neuronal cultures for 3 d. Both cell types were then separated, and [^{14}C]DHA labeling was analyzed by autoradiography. In glial cells, silver grains were diffusely distributed, whereas these grains were concentrated in photoreceptors in neuronal cultures. The bars indicate 10 μm. For abbreviation see Figure 2.

diseases affecting the retina, such as retinitis pigmentosa. The multiple effects of DHA on photoreceptor survival and differentiation shown in the present and previous reports (13–15) suggest a novel role as a neurotrophic factor for this lipid molecule. DHA rescues photoreceptors by delaying the onset and slowing down the progression of apoptosis. Moreover, our present results confirm previous reports that DHA, like other trophic factors, advances the differentiation of this single neuronal cell type. In the mature retina, photoreceptors must have highly differentiated characters at both the molecular and structural levels to accomplish their visual function: they must develop apical processes to fully differentiate the outer segments where opsin is to be concentrated for phototransduction to occur. Under control conditions *in vitro*, the diffuse distribution of opsin over the entire plasmalemma and the lack of apical differentiation mirrored immature stages of photoreceptor differentiation (14,15). DHA addition promoted the formation of apical processes, enhanced opsin expression, and favored its localization in these newly formed apical processes of photoreceptor cells.

The DHA-promoted survival and differentiation of photoreceptors contrast with the well-known harmful and proapoptotic effects of arachidonic acid in other nervous tissues (32). The protective effects of DHA paralleled an increase in its content in neuronal lipids upon DHA supplementation, similar to the accretion of this fatty acid in the retina *in vivo* during early stages of development (Fig. 2). Although the enzymes required for the esterification and turnover of several fatty acids are active in retinal neurons (15), DHA was the only fatty acid able to accumulate in neuronal lipids and modify their acyl chain composition when added to the culture media (Fig. 2). This was consistent with DHA being the only fatty acid having a neurotrophic activity (13–15). This suggests that the ability to form new DHA-containing phospholipids might be somehow related to the survival-promoting and, even more likely, the differentiating actions of DHA. The availability of such phospholipids, essential components of rod outer segments, might help redirect opsin to its correct localization (33,15), and the right combination of protein and phospholipids might allow the formation of apical processes. In patients with retinitis pigmentosa, and in animal models of this disease, a decrease in DHA content in plasma (34) and in both retina and photoreceptors (35) has been described. This decrease might contribute to the failure of photoreceptors to develop their outer segments and the ultimate death of photoreceptors in retinitis pigmentosa.

However, roles played by DHA are probably not only structural. Addition of DHA also has a protective action on monocytes, reducing tumor necrosis factor-induced apoptosis in these cells (36). These protective effects suggest that DHA could induce the release of a survival signal or, alternatively, it could prevent the release of a death signal during apoptosis development. Death signals, such as cytochrome c, are known to be released from damaged mitochondria during apoptotic processes (reviewed in Ref. 31). Our results show that an increase in the number of apoptotic photoreceptors was consistent with increased mitochondrial impairment in these cells. Mitochondrial damage has also been shown in other apoptotic processes affecting photoreceptors (37). Addition of DHA to the cultures partially prevented the loss of mitochondrial activity, suggesting a possible role for this acid in sustaining mitochondrial function. Mitochondrial failure is closely related to alterations in membrane properties, such as transmembrane potential and H^+ gradient, mainly due to the opening of a large-conductance channel (31). Polyunsaturated fatty acids have been shown to be involved in the regulation of ion channels, thus regulating neuronal survival in other systems (38,39). Accumulation of DHA in mitochondrial lipids might similarly participate in the modulation of the aperture of mitochondrial channels, partially preventing or postponing the changes in membrane properties and thus slowing down the apoptotic death of photoreceptors. Further research to obtain a better understanding of the mechanisms involved in DHA protection of mitochondrial function by DHA is warranted.

The essentiality of DHA for photoreceptor survival makes it essential for retinal cells to accumulate this fatty acid. DHA is tenaciously retained in the retina, even upon prolonged dietary deprivation (2,40), and efficient mechanisms have been developed to avoid loss of DHA during the daily recycling of photoreceptor discs (41). The liver has been proposed to be in charge of DHA provision to the retina (20). This fatty acid can also be synthesized by both the pigment epithelium (42) and the retina itself (43–45), although experiments conducted with the whole retina have not determined whether this synthesis was performed by neurons or glial cells. In the brain, astrocytes take charge of DHA synthesis and delivery to neurons (46). Our results showed that glial cells had a protective effect on photoreceptors, delaying cell death in a similar fashion to DHA. In addition, the proportion of DHA was higher in glial than in neuronal lipids. This prompted us to investigate whether glia could provide DHA to neurons in coculture, and we found that not only did glial cells transfer [^{14}C]DHA to neurons but these cells avidly incorporated it and esterified it in their lipids as well. Radioactive DHA was particularly concentrated in photoreceptors, suggesting that either glial cells preferentially channeled it to these neurons or that photoreceptors had the most efficient mechanisms for the uptake of DHA. Glial cells have been shown to modulate several neuronal functions, including photoreceptor survival through the supply of trophic factors (21,22). Provision of DHA might be at least one of the mechanisms involved in the protective role played by glial cells in the regulation of photoreceptor survival in the retina.

There is growing consensus that a combination of trophic factors is probably required for sustaining photoreceptor survival (47,48). DHA is undoubtedly required at a precise developmental period to postpone the triggering of photoreceptor apoptosis; however, this process eventually starts, with only a small population of photoreceptors being spared from apoptotic death (14). This suggests that other survival factors are required, acting in a sequential or synergistic manner to allow these cells to survive for longer time periods and

acquire fully differentiated characters. Our results show that GDNF was able to protect photoreceptors from cell death in the absence of other trophic factors *in vitro*, having a survival-promoting effect similar to those of DHA and glial cells. We have also shown that GDNF diminished apoptosis, acting coordinately with DHA (49). Since glial cells are known to release GDNF, this trophic factor may provide glia with another way of controlling photoreceptor apoptosis. Hence, glial cells might play a central role in the regulation of photoreceptor survival, by modulating the release of GDNF and DHA during development.

In conclusion, mounting evidence supports the hypothesis that a lipid molecule like DHA, acting in a coordinated, developmentally regulated fashion with other trophic molecules, may behave as a trophic factor essential for sustaining photoreceptor survival and differentiation.

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