Prevention of Ornithine Cytotoxicity by Proline in Human Retinal Pigment Epithelial Cells

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PURPOSE. To investigate the relationship between ornithine-δ-aminotransferase (OAT) deficiency and ornithine accumulation and the specific degeneration of retinal pigment epithelial (RPE) cells in gyrate atrophy.

METHODS. Human RPE cells, human hepatoma cells, and human fibroblast cells were treated with 5-fluoromethylornithine (5-FMOOrn), a specific irreversible inhibitor of OAT. Ornithine cytotoxicity was determined by using a [3H]thymidine incorporation assay and immunohistochemical staining for cytokeratin. The effects of various metabolites of ornithine and arginine, such as creatine, creatine phosphate, L-Δ1-pyrroline-5-carboxylic acid (L-P5C), and proline, which may be deficient in gyrate atrophy on RPE cell damage by ornithine, were determined by the same procedures.

RESULTS. When the human RPE cells, HepG2 hepatoma cells, and WI-38 fibroblast cells were treated with 0.5 mM 5-FMOOrn for 30 minutes, which inactivated OAT, ornithine exhibited severe time- and dose-dependent inhibition of DNA synthesis in the human RPE cells but not in the HepG2 hepatoma cells or WI-38 fibroblast cells. The inhibition of DNA synthesis was accompanied by drastic changes in morphologic appearance, disorganization of the cytoskeleton, and cell death. Ornithine or 5-FMOOrn alone did not exhibit such cytotoxicity to the RPE cells. Proline prevented the cytotoxicity of ornithine.

CONCLUSIONS. These findings suggest that an elevated level of ornithine combined with an increased sensitivity to ornithine as a result of OAT deficiency may be crucial to the specific RPE degeneration in gyrate atrophy. They suggest also that abnormalities of proline metabolism may be involved in the progress of gyrate atrophy. (Invest Ophthalmol Vis Sci. 1998;39:820-827)

Gyrate atrophy of the choroid and retina, a rare inborn error of ornithine metabolism transmitted as an autosomal-recessive trait, is characterized by hyperornithinemia and a deficiency of ornithine-δ-aminotransferase (OAT).1 The major clinical problem in patients with gyrate atrophy is a slow loss of vision leading to blindness, usually by the fifth decade of life. Myopia and night blindness are early symptoms, developing during the first decade of life, and posterior subcapsular cataracts and constriction of the visual fields appear a decade or two later.2-4 Simell and Takki5 discovered that the biochemical abnormalities of this disorder are hyperornithinemia and overflow ornithinuria. Plasma ornithine levels in gyrate atrophy range from 400 to 1400 μM, which are 10- to 15-fold above normal, and 0.5 to 10 mmol ornithine is excreted daily into the urine.6-8 The finding of an association between hyperornithinemia and gyrate atrophy led to the discovery of a basic enzyme defect, a deficiency of the mitochondrial matrix enzyme OAT.9-11 OAT is widely expressed in many tissues including the liver, kidney, brain, neural retina, retinal pigment epithelium, and ciliary body.12,13 The human OAT cDNA and its structural gene have been cloned and characterized.14-16 More than 60 mutant OAT alleles, most of which are point mutations leading to unstable or inactive enzymes, have been described in patients with gyrate atrophy, reflecting the genetic heterogeneity of this disease.1.17-19

Recently, a mouse lacking OAT was generated by gene targeting.20 The OAT-deficient adult mouse exhibits chronic hyperornithinemia, massive ornithinuria, and progressive retinal degeneration, which are similar to the symptoms and signs found in patients with gyrate atrophy.20 Further analysis of the OAT-deficient mouse revealed that the retinal pigment epithelial (RPE) cells, a single layer of cuboidal cells lying between Bruch’s membrane and the photoreceptors, are the initial site of insult in gyrate atrophy.21 However, the mechanism by which OAT deficiency and subsequent ornithine accumulation causes the characteristic degeneration in the RPE cells is unknown. To elucidate the mechanism of the specific RPE degeneration in gyrate atrophy, we attempted to establish the in vitro model of gyrate atrophy using in vitro human RPE cells. Here, we showed that increased ornithine in combination with the inactivation of OAT led to the severe inhibition of RPE proliferation and later to cell death. Furthermore, we found that proline, an ornithine metabolite produced by OAT, blocked the cytotoxicity of ornithine.
Involvement of Ornithine-Proline Metabolism in RPE Cell Death

FIGURE 1. Time course of inactivation of ornithine-δ-aminotransferase (OAT) in human cell lines by 5-fluoromethylornithine. Bars for SD, which are less than 10%, are not shown to avoid crowding.

MATERIALS AND METHODS

Cell Cultures of Human Cell Lines

The human RPE cell lines 240, 286, and 297 were established as described by Del Monte and Maumenee.22 The human HepG2 cell line and fibroblast cell line WI-38 were obtained from the Riken cell bank (Tsukuba, Japan). The composition of the culture medium was the following: Ham's F-12 nutrient medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum and 2 mM L-glutamine for the RPE cells; Dulbecco's modified Eagle's medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum for the HepG2 cells; and minimum essential medium (Gibco) supplemented with 10% fetal bovine serum for the WI-38 cells. All culture media contained 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were incubated in an atmosphere of 5% CO2-95% air at 37°C in a 75-cm2 flask and were supplied with fresh medium every 3 days. When the cells became confluent, they were subcultured at a split ratio of 1:3.

Ornithine-δ-Aminotransferase Enzyme Assay

OAT activities from the human cell lines were assayed by the method described previously.23 The standard assay mixture contains 175 mM L-ornithine, 35 mM α-ketoglutarate, 50 μM pyridoxal phosphate, 50 mM potassium phosphate buffer (pH 8.0), and 300 μl cell extract in a 1-ml volume. The reaction mixture was incubated at 37°C for 20 minutes. The reaction was stopped by the addition of 100 μl 40% trichloroacetic acid and 400 μl of an aqueous solution of o-aminobenzaldehyde (0.125%). The color reaction was developed by further incubation for 10 minutes. After centrifugation (3000g, 10 minutes) the supernatants were measured at 440 nm by a spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol Δ¹-pyrroline-5-carboxylic acid (P5C)/hour using its molar extinction coefficient value of 2.71 × 10³.

Inactivation of Ornithine-δ-Aminotransferase by 5-Fluoromethylornithine

5-Fluoromethylornithine (5-FMOrn), an irreversible specific OAT inhibitor,23 was generously provided by the Marion Merrell Dow Research Institute (Strasbourg, France). It was demonstrated that this inhibitor inactivated none of the other enzymes involved in ornithine metabolism such as L-ornithine carbamoyltransferase, L-ornithine decarboxylase, and γ-amino butyric acid-α-ketoglutaric acid transaminase.23 To confirm the inactivation of OAT in human cell lines, 0.5 mM 5-FMOrn was added to the cells that had been plated onto culture dishes (100 × 20 mm; Falcon, Franklin Lakes, NJ) at 2 × 10⁶ cells/dish. After a defined time, the cells were lysed by repeated freezing and thawing in a lysis buffer (10 mM potassium phosphate buffer, pH 8.0, 50 μM pyridoxal phosphate, and 0.1% Triton-X [Wako Pure Chemicals, Osaka, Japan]). The residual OAT activity was determined as described above.

[³H]Thymidine Incorporation Assay

The effect of ornithine on DNA synthesis was evaluated by the incorporation of [³H]thymidine into the acid-insoluble fraction by using [methyl-³H]thymidine (Amersham, Tokyo, Japan). The RPE cells (240) were plated onto culture plates (24 wells, Falcon) at 1 × 10⁵ cells/well. [³H]Thymidine (1.6 μCi/0.4 ml/well) was added to the culture medium at 0, 4, 8, 12, and 16 hours after the addition of various concentrations of ornithine. After 4 hours of incubation with [methyl-³H]thymidine, the cells were washed three times with phosphate-buffered saline (PBS) and 5% trichloroacetic acid and were lysed with 1 ml 0.5 M NaOH. Aliquots of the lysate were neutralized with 0.5 M HCl and were added to a vial containing scintillation fluid to measure the radioactivity. Data were normalized by a percentage of tritium compared with an untreated control. Control values taken as 100% at each incubation period of one experiment, for example, were 3.48 ± 0.21, 7.12 ± 0.60, 16.4 ± 0.57, 30.2 ± 1.8, and 40.0 ± 2.1 × 10³ dpm/4 hours/well for 0 to 4 hours, 4 to 8 hours, 8 to 12 hours, 12 to 16 hours, and 16 to 20 hours, respectively.

FIGURE 2. Time-dependent inhibition of DNA synthesis by ornithine. (□) control, (◆) 10 mM ornithine, (□) 0.5 mM 5-fluoromethylornithine (5-FMOrn) without ornithine, and (◆) 0.5 mM 5-FMOrn and 10 mM ornithine. P < 0.01 and P < 0.001 versus control.
FIGURE 3. Effect of ornithine on DNA synthesis in (A) retinal pigment epithelium cells, (B) HepG2 cells, and (C) WI-38 cells with (D) or without (•) 0.5 mM 5-fluoromethylornithine (5-FMOrn) at 12 to 16 hours after the addition of ornithine. P < 0.001 versus each value in the absence of 0.5 mM 5-FMOrn.

Enzymatic Synthesis of \( \alpha \Delta^3 \)-Pyrroline-5-Carboxylic Acid

\( \alpha \)-P5C was synthesized enzymatically by a modification of a procedure described previously.\(^{24}\) The reaction mixture (200 ml) consisted of 12.5 mM potassium phosphate buffer (pH 8.0), 20 mM ornithine, 20 mM \( \alpha \)-ketoglutarate, 50 \( \mu \)M pyridoxal phosphate, and 20 \( \mu \)g purified thermostable bacterial OAT.\(^{25}\) After incubation at 37°C for 8 hours, concentrated HCl (12.5 ml) was added, and the sample was lyophilized. The sample was dissolved in distilled water and was applied onto a gel (Dow-X; Bio-Rad, Hercules, CA) (60 ml, H-form). The gel was washed with 300 ml distilled water and 320 ml 0.3 M HCl, and \( \alpha \)-P5C was eluted with 0.35 M HCl and was collected in 5-ml fractions. The \( \alpha \)-P5C fractions were determined by thin-layer silica chromatography (butanol/water/acetic acid ratio, 4:1:1) and staining with ninhydrin, comigrated with \( \alpha \)-\( \alpha \)-P5C (Sigma).

Effects of Metabolites of Ornithine and Arginine on Retinal Pigment Epithelial Cells Damaged by Ornithine

The human RPE cells (240) were preincubated with 0.5 mM 5-FMOrn for 30 minutes, after which 10 mM ornithine together with various metabolites such as creatine, creatine phosphate, \( \alpha \)-P5C, and proline were added to the medium. The effects were estimated by the \( ^{3} \)Hthyminde incorporation assay, and morphologic changes were estimated by using immunofluorescence staining of the cytokeratin as described above.

Statistics

Data are expressed as the mean ± SD of four separate experiments. The unpaired Student's t-test was used for statistical analysis. P < 0.05 was considered statistically significant.

RESULTS

Inactivation of Ornithine-\( \alpha \)-Aminotransferase in Various Human Cell Lines by 5-Fluoromethylornithine

We used the human RPE cell line 240 in this study, which was previously established from a primary culture of human eyes. We also examined the human hepatoma cell line HepG2 and human fibroblast cell line WI-38, which are well documented in their ability to retain various properties of normal tissues as a comparison.

Figure 1 shows the inactivation of OAT in the studied human cell lines by 5-FOMOrn, an irreversible specific inhibitor of OAT. The specific activities for the RPE, HepG2, and WI-38 cells were 0.78 ± 0.04, 0.63 ± 0.04, and 0.45 ± 0.03 (mean ± SD, n = 4) U/mg protein, respectively, before the addition of 5-FOMOrn. 5-FOMOrn quickly inhibited OAT activity, inasmuch as it was almost absent in all cell lines at 30 minutes after the addition of 0.5 mM 5-FOMOrn, and the inactivation lasted at least 48 hours regardless of the continued presence of 5-FOMOrn. Therefore, we first incubated the experimental cells with 0.5 mM 5-FOMOrn for 30 minutes before adding ornithine to examine the effect of ornithine in the cells lacking OAT.

Immunofluorescence

Cells grown on glass chamber slides (Nunc, Naperville, IL) were fixated in 100% methanol for 10 minutes at −20°C, washed three times with PBS, incubated in 1% normal goat serum in PBS for 30 minutes, and incubated for 60 minutes at room temperature with anti-cytokeratin 8.13 antibody (Sigma, St. Louis, MO) diluted 1:50 with 1% normal goat serum in PBS. The cells were washed three times with PBS and were incubated for 60 minutes at room temperature with rhodamine-conjugated goat antimouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted 1:50 with 1% normal goat serum in PBS. A negative control for immunostaining consisted of the omission of the primary antibody. After immunostained slides were washed three times with PBS, they were processed for fluorescence microscopy (Olympus GB 200 microscope, Tokyo, Japan).
Involvement of Ornithine–Proline Metabolism in RPE Cell Death

Figure 4. Cell death induced by 5-fluoromethylornithine (5±FMOrn) and ornithine. Control (A). Cells incubated with 0.5 mM 5±FMOrn and 10 mM ornithine for 12 hours (B), 18 hours (C), and 24 hours (D), and with 0.5 mM 5±FMOrn (E) and 10 mM ornithine (F) for 24 hours. Arrows indicate dying and detaching cells. Phase-contrast photomicroscopy; original magnification, ×100.

Figure 5. Dose-dependent prevention of ornithine-induced inhibition of DNA synthesis by proline. (□) Control. Cells incubated with 20 mM proline (□) and with 20 mM proline and 5-fluoromethylornithine (5-FMOrn) (□). (■) Cells incubated with 0.5 mM 5-FMOrn and 10 mM ornithine without (None) or with proline, L-P5C, creatine, or creatine phosphate. *P < 0.05; †P < 0.01; ‡P < 0.001 versus None.

Inhibition of DNA Synthesis by Ornithine in 5-Fluoromethylornithine–treated Retinal Pigment Epithelial Cells

To evaluate the effect of ornithine on DNA synthesis in 5-FMOrn–treated cells, we performed a [3H]thymidine incorporation assay. As shown in Figure 2, 10 mM ornithine inhibited [3H]thymidine incorporation in a time-dependent manner. Ornithine inhibited DNA synthesis after 16 to 20 hours of exposure. Neither 5-FMOrn–treated cells without ornithine nor cells incubated with 10 mM ornithine alone demonstrated inhibition of [3H]thymidine incorporation, although 5-FMOrn alone had a tendency to enhance DNA synthesis.

The inhibitory effect on DNA synthesis in the 5-FMOrn–treated RPE cells, estimated after 12 to 16 hours of ornithine exposure, is dose dependent (Fig. 3A), and has the maximal effect with 10 mM ornithine. In contrast, ornithine had no effect on DNA synthesis in HepG2 and WI-38 cells pretreated with 5-FMOrn (Figs. 3B, 3C).

Cell Death by Ornithine in 5-Fluoromethylornithine–treated Retinal Pigment Epithelial Cells

The inhibition of cell proliferation in the RPE cells by ornithine was accompanied by drastic morphologic changes (Fig. 4). After 12 hours, cells became rounder and more swollen (Fig. 4B). At 16 to 20 hours, when DNA synthesis was inhibited (Fig. 2), the cells began to die and to detach from the culture dish (Fig. 4C); half the cells died within 24 hours (Fig. 4D). Neither 10 mM ornithine nor 5-FMOrn alone had any effect on the RPE cell morphology (Figs. 4E, 4F). The HepG2 and WI-38 cells were not affected by 10 mM ornithine even when their OAT was inactivated by the inhibitor (data not shown). The RPE cell death by ornithine...
Prevention of ornithine-induced time-dependent inhibition of DNA synthesis by proline.

![Graph showing prevention of ornithine-induced time-dependent inhibition of DNA synthesis by proline.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933429/)
Involvement of Ornithine-Proline Metabolism in RPE Cell Death

Retinal Pigment Epithelial Cell Lines with Features Similar to Cell Line 240

To examine whether the effects of ornithine and proline on RPE cell line 240 observed here are generalizable in human RPE cells, we tested two other human RPE cell lines, 286 and 297, in the same way. They started to degenerate in 24 hours by the combination of 5-FMOrn and 10 mM ornithine, and almost all cells died in 4 days (Figs. 9D, 9J); ornithine or 5-FMOrn alone had no effect (Figs. 9B, 9C, 9G, 9I) on those cells. Similar to cell line 240, 20 mM proline blocked the ornithine damage in these cells (Figs. 9E, 9I).

FIGURE 8. Prevention of 5-fluoromethylornithine- and ornithine-induced disorganization of cytokeratin by proline. (A) Control cells. Cells were incubated with 0.5 mM 5-FMOrn and 10 mM ornithine for 24 hours without (B) or with (C) 20 mM proline. Phase-contrast photomicroscopy; original magnification, ×600.

Immunofluorescence staining with antibody against cyto-keratin 18 was performed to examine whether the protective effect of proline is reflected in the structural organization of the cytoskeleton (Fig. 8). The typical fibrillar structure was apparent because of the characteristic polarity in the RPE cells (Fig. 8A). In the cells damaged by ornithine, the staining was diffuse and the fibrillar structure disappeared (Fig. 8B). However, the clear fibrillar structure of the cytokeratin was retained in the 5-FMOrn-treated cells incubated with proline and ornithine (Fig. 8C).

DISCUSSION

In this study, we demonstrated that ornithine exhibits severe cytotoxicity when OAT is inactivated by a specific inhibitor, 5-FMOrn, in proliferating cultured human RPE cells. The ornithine cytotoxicity was characterized by the inhibition of cell proliferation and by marked morphologic changes, which were followed finally by cell death. The inhibition of cell proliferation by ornithine was dose and time dependent (Figs. 2, 3). Ornithine, 10 mM, which is an approximately 10-fold higher concentration than that in the plasma of patients with gyrate atrophy and markedly inhibited [3H]thymidine incorporation 12 hours after its addition, caused morphologic changes in the cells and resulted in 50% cell death after 24 hours of exposure (Figs. 4C, 4D). Although HepG2 hepatoma cells and WI-38 fibroblast cells had OAT activity comparable to that of RPE cells (Fig. 1), they were not affected by ornithine even when OAT was inactivated (Figs. 3B, 3C). We obtained similar results when the three cell lines were cultured in the same medium (Dulbecco's modified Eagle's medium, 10% fetal bovine serum; data not shown), indicating that the sensitivity of RPE cells to ornithine is not caused by the difference of culture condition. Because the urea cycle is present in the hepatocytes,1,27 it may consume accumulated ornithine to protect HepG2 cells from ornithine damage. However, in the fibroblasts, as in the RPE cell enzymes associated with the urea cycle such as ornithine transcarbamylase, carbamoyl-phosphate synthase and arginase are not expressed,1 suggesting that the mechanism of ornithine-related cell death in the RPE cells is based on some innate RPE metabolism that is associated with the absence of OAT.

The findings in our model could suggest a possible mechanism for the specific RPE degeneration in gyrate atrophy. The deficiency or reduced activity of OAT caused by the mutation of the OAT gene would provide two essential pathologic disease factors at the intracellular and extracellular levels of the RPE cells. Increased RPE sensitivity to elevated pathologic disease factors at the intracellular and extracellular levels of the RPE cells. Increased RPE sensitivity to elevated extracellular ornithine, which accumulates because of a lack of OAT, is caused by unique features of ornithine metabolism, which are different from those of other tissues such as hepatocytes and fibroblasts. This sensitivity is exacerbated by an accumulation of ornithine in the plasma caused by the disorder of ornithine metabolism. The combination of both pathologic factors could result in the characteristic RPE degeneration seen in gyrate atrophy. This view is consistent with clinical studies from patients with gyrate atrophy. The reduction of plasma ornithine levels by an arginine-restricted diet may delay or cease the retinal degeneration and improve vision in some cases.28,29
indicating that the RPE cells of patients with gyrate atrophy possess a special sensitivity to ornithine.

Although the mechanism of ornithine-induced inhibition of cell proliferation, morphologic change, and cell death in the RPE cells lacking OAT activity is unknown, we demonstrated that proline, a secondary metabolite of ornithine catalyzed by OAT, prevents this cytotoxicity, including cell death (Figs. 5, 6, 7, 8). The protective effect of proline is specific for ornithine cytotoxicity because this amino acid could not prevent RPE cell death induced by chloroquine, sodium iodate, or serum starvation.

Concerning the pathologic mechanism for specific degeneration in gyrate atrophy, the deficiency of some metabolites has been suggested. Sipilä and coworkers proposed that a deficiency of creatine and creatine phosphate may be critical for gyrate atrophy, which is supported by the evidence that ornithine inhibits arginine-glycine aminotransferase, the first enzyme in creatine biosynthesis. Mammalian cells possess two proline biosynthetic pathways and share a common intermediate, L-P5C, which is formed from ornithine by OAT or from glutamic acid by P5C synthase. Lodato et al. showed that a near-physiological concentration of ornithine inhibited P5C synthase in a Chinese hamster cell line and suggested that a deficiency of P5C or proline may be pathophysiological as a result of the loss of proline biosynthetic pathways. Our findings that proline, but not creatine or creatine phosphate, prevents the degeneration of the RPE cells induced by ornithine may support the latter suggestion. However, the concentration of proline required to exhibit the protective effect in the damaged RPE cells is far greater than a physiological occurrence (plasma proline is approximately 150 μM), implying that the effect is not simply the result of the provision of proline to a cell with impaired proline synthesis.

We demonstrated that although two other human RPE cell lines (286 and 297) require a little longer time to degenerate, they were also severely damaged by ornithine in the presence of 5-FMOm and were rescued by proline (Fig. 9), suggesting that the effects of ornithine and proline may be generalizable in RPE cells. Further investigation of the mechanism(s) of RPE cell death using this cell culture system may provide insights into the pathophysiological mechanism of gyrate atrophy.

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References

Involvement of Ornithine–Proline Metabolism in RPE Cell Death 827


ANNOUNCEMENT

Age-Related Macular Degeneration Education Program Jointly Sponsored by Wilmer Ophthalmological Institute at Johns Hopkins and the Foundation Fighting Blindness

The international symposium, Pathogenesis and Treatment of Age-Related Macular Degeneration, will be held June 4–6, 1998, in the Turner Building located at Johns Hopkins Medical Institutions, Baltimore, Maryland. Designed for ophthalmologists and basic scientists, the meeting will review current knowledge concerning the pathogenesis and treatment of AMD and explore the most promising leads for future research. AMA Category 1 credit available. Attendance fee for physicians is $400. Basic scientists, residents, and fellows pay $250 if letter verifying status is provided. For more information, contact the Program Coordinator at Johns Hopkins Medical Institutions, Office of Continuing Education, Turner 20, 720 Rutland Avenue, Baltimore, MD 21205. Tel: 410-955-2959; fax: 410-955-0807; e-mail: cmenet@som.adm.jhu.edu.