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Fenretinide-induced neuronal differentiation of ARPE-19 human retinal pigment epithelial cells is associated with the differential expression of Hsp70, 14-3-3, Pax-6, Tubulin β-III, NSE, and Bag-1 proteins

Shanyi Chen,[1] Robert N. Fariss,[2] R. Krishnan Kutty,[1], Ralph Nelson,[3] Barbara Wiggert[1]

[1]Biochemistry Section, Laboratory of Retinal Cell and Molecular Biology; [2]Biological Imaging Core National Eye Institute; [3]Neural Circuits Unit, Basic Neurosciences Program, National Institute of Neurological Disorders and Stroke; National Institutes of Health, Bethesda, Maryland

Purpose: We reported earlier that fenretinide can induce neuronal differentiation of ARPE-19 human retinal pigment epithelial cells in culture. The purpose of this study was to investigate the potential involvement of key proteins involved in gene transcription, signal transduction, cell cycle check point, differentiation, neuronal cell survival, and stress response in the neuronal differentiation of ARPE-19 cells by fenretinide.
Methods: Cells in culture were treated with 1.0 μM fenretinide. Cells were analyzed using antibodies against pax-6, neuronal specific enolase (NSE), tubulin β-III, 14-3-3, bag-1, and Hsp-70 proteins using immunocytochemistry, western blot and ELISA methodologies.
Results: We found that pax-6 and NSE were both expressed in the control ARPE-19 cells. Fenretinide induced neuronal differentiation of ARPE-19 cells led to a decrease in pax-6 protein and an increase in tubulin β-III protein expression after 5 days fenretinide treatment. There was a translocation of 14-3-3 from the cytoplasm to the nucleus, and an increase in nuclear expression of bag-1 after treatment. We also found a time-dependent increase in Hsp70 protein expression in ARPE-19 cells treated with fenretinide. D-407, another human retinal pigment epithelial cell line, but not either Y-79 or PC-12 cells, was also able to be induced into neuronal morphologies by fenretinide.
Conclusions: The fenretinide-induced neuronal differentiation of ARPE-19 cells is associated with an increase in expression of the neuronal specific protein tubulin β-III, and a decrease in expression of the progenitor cell marker pax-6. Neuronal differentiation of ARPE-19 cells is also associated with nuclear translocation of 14-3-3, a protein involved in signal transduction, cell cycle check point and cell growth, and an increase in expression of bag-1, a protein involved in neuronal cell survival and axon elongation. These results suggest that ARPE-19 cells could be a progenitor cell line that can be differentiated into neuronal cells when treated with factors such as fenretinide.

Cell fate determination is dictated by a combination of intrinsic expression or repression of a series of genes [1,2], and environmental growth and differentiation factors [2,3]. The interactions between a target gene and the environment around a neuro-progenitor cell influence cell fate, both in terms of cell survival and morphology [3,4]. Neuroepithelial cells, carcinoma cells and stem cells have been reported to be able to express specific genes and to be able to differentiate into neuronal cells [4,5].

The retinal pigment epithelium (RPE) and neural retina are derived from the same sheet of neuroepithelium during development [6]. Under certain conditions, RPE cells can transdifferentiate into neuronal cells [5,7]. ARPE-19 and D-407 are rapidly growing cell lines that retain the majority of RPE cell components and structural properties [8-10]. These cell lines have been used to study various aspects of cell growth and differentiation [9,11,12].

We have reported earlier that fenretinide (N-(4-hydroxyphenyl)retinamide, 4HPR), a synthetic retinoid, can induce ARPE-19 cells to differentiate into neuronal phenotypes [13]. Fenretinide has been used as a chemopreventive agent against cancer, and has the potential to be used for lipofuscin-based retinal diseases [14-17]. The cells treated with fenretinide showed cell process elongation and expressed neuronal markers [13]. The neuronal differentiation of ARPE-19 cells was associated with differential protein expression as demonstrated by 2D gel electrophoresis followed by mass spectrometry analysis of selected proteins [13].

The mechanism of neuronal differentiation is still unknown. It is a complex cellular process including signal transduction, cell growth, cell adhesion, differentiation, and regulation of ion channels. 14-3-3 is an adaptable protein family involved in the regulation of cell cycle check point, cell growth, apoptosis, development, and cell differentiation [18-22]. Under normal conditions, 14-3-3 proteins sequester or associate with at least 200 proteins and regulate the above cellular func-

Correspondence to: Barbara Wiggert, Biochemistry Section, Laboratory of Retinal Cell and Molecular Biology, Bldg. 7, Room 300, National Eye Institute, National Institutes of Health, 7 Memorial Dr., MSC 0706, Bethesda, Maryland 20892-0706; Phone: (301) 496-5809; FAX: (301) 402-1883; email: bnwigg@helix.nih.gov.

tions by protein-protein interaction in a phosphorylation dependent or independent manner [18,21,22]. Upregulation or downregulation of this protein will change cell growth and cell survival abilities [22]. Factors that have the ability to regulate 14-3-3 expression also have the ability to regulate cell growth, cell survival, and differentiation [22,23]. Therefore, we examined the potential involvement of 14-3-3 protein in fenretinide-induced ARPE-19 cell neuronal differentiation.

During the investigation of fenretinide-induced ARPE-19 cell neuronal differentiation, we found that more than ten proteins, including Hsp70, are upregulated, and more than eight proteins were downregulated over five-fold in ARPE-19 cells treated with fenretinide [13]. Hsp70 is a chaperone protein that is inducible by heat, heavy metals, and other chemicals [24]. It is also involved in folding or unfolding newly synthesized proteins during translation and transportation [25,26]. Studies have reported that Hsp70 is related to cell differentiation and development and involved in cell fate control [27-29]. The potential involvement of Hsp70 and one of its binding proteins, bag-1, in the fenretinide-induced neuronal differentiation of ARPE-19 cells is investigated in the current study.

In this study, we also compared the effect of fenretinide treatment on ARPE-19 cells, D-407 cells, another human RPE cell line [10], and two cancer cell lines, human Y-79 retinoblastoma cells and rat pheochromocytoma cells (PC-12) that have been shown to undergo neuronal differentiation upon treatment with growth factors [30,31].

METHODS

Cell culture: ARPE-19, Y-79, and PC-12 cells were obtained from ATCC (Manassas, VA). D-407 cells were a gift from Dr. R. C. Hunt (University of South Carolina School of Medicine, Columbia, SC). These cells were treated with 0-5 μM fenretinide as previously described [13], and were used within 4 passages after being obtained from ATCC or other sources. Fenretinide was obtained from Biomol (Plymouth Meeting, PA) and was dissolved in DMSO to a concentration of 10 mM prior to adding to the cell culture medium. The controls received the same amount of DMSO. ARPE-19, D-407, and PC-12 cells were plated at a density of $2.2x10^3/cm^2$ on culture dishes or cover slips in Medium A containing DMEM, fetal bovine serum, L-glutamine (GIBCO, Carlsbad, CA), sodium pyruvate (Mediatech, Inc., Herndon, VA), MEM non-essential amino acids (GIBCO), penicillin and streptomycin (GIBCO) at 37 °C incubator with 5% CO_2. Y-79 cells were grown suspended in flasks in a medium without serum, then were transfered to a culture dish to observe cell elongation as described by Tombran-Tink et al. [30]. After one day, the culture medium was replaced with medium B containing all the components in Medium A except that charcoal/dextran treated fetal bovine serum 3% (Hyclone, Logan, UT) was used instead of fetal bovine serum. Cells were judged to be differentiated into neuronal phenotype when the cell processes were longer than or equal to the longest axis of the cell body as described previously [13].

Western blot analysis: Control and treated cells were homogenized in 50 mM Tris-buffer, pH 7.4, containing complete protease inhibitors (Roche, Basel, Switzerland). The protein concentration was estimated using a Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA). Samples containing equal amounts of 10 μg of total protein were subjected to SDS-PAGE analysis using 10-20% Tricine gradient gels (Invitrogen, Carlsbad, CA). The protein bands were transferred to a nitro-

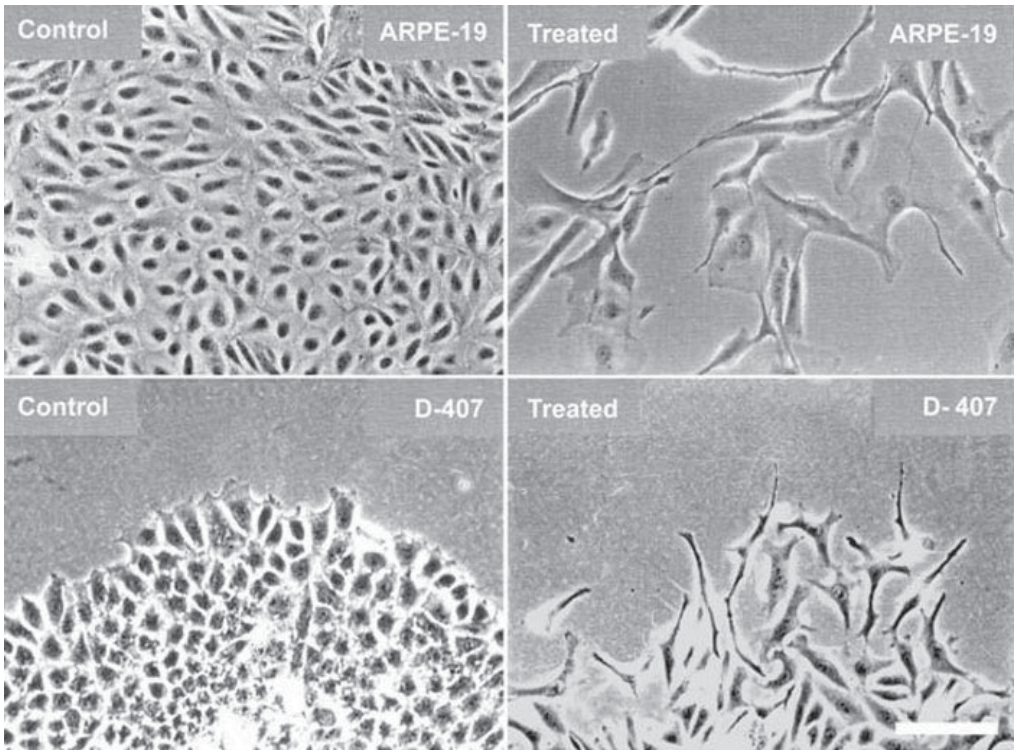

Figure 1. Effect of fenretinide treatment of retinal pigment epithelial cells. ARPE-19 and D-407 cells were treated with 1.0 μM fenretinide for 5 days. Scale bar represents 100 μm.

cellulose membrane (Invitrogen, Carlsbad, CA). The membrane was incubated in 5% non-fat milk in Tris-buffered saline for 1 h. After washing in Tris-buffered saline, the membrane was incubated in the buffer containing blocking reagent with anti-pax-6 (Chemicon International, Inc. Temecula, CA), anti-NSE (Calbiochem-Novabiochem Corporation, San Diego, CA), anti-tubulin β-III (Chemicon International), anti-14-3-3 (BD Biosciences, San Jose, CA), anti-bag-1 (Stressgen), anti-Hsp70 (Stressgen, San Diego, CA), or anti-actin (Sigma, St. Louis, MO) at room temperature. After washing in Tris-buffered saline, the membrane was incubated with alkaline phosphatase-conjugated anti-mouse IgG or anti-rabbit IgG (Pierce Biotechnology Inc., Rockford, IL). Immunoreactivity was subsequently visualized by incubating the membrane in a solution containing BCIP and NBT (Pierce).

Enzyme Linked Immunoassays: A Hsp70 ELISA kit from Stressgen Biotechnology was employed, and a protocol provided by the company was followed. In brief, cells were washed with PBS then lysed in lysis buffer and centrifuged. Supernatant from control and treated cells containing 30 mg of total protein and the Hsp70 standard were loaded onto a 96-well plate provided by the company. After incubation, the wells were washed and then incubated with biotin conjugated anti-Hsp70 antibody. Wells were then washed before being incubated with avidin-HRP and followed by color development. Analysis was done using a Wallac 1420 Multilabel Counter (PerkinElmer Life Sciences, Wellesley, MA).

Immunocytochemistry: Expression and subcellular localization of 14-3-3, bag-1, and Hsp70 proteins were investigated using immunocytochemistry on ARPE-19 cells before and after fenretinide treatment. The cells were grown on 35 mm diameter culture dishes and treated with 1.0 μM fenretinide for one week as described above. Control and treated cells were washed with PBS, fixed in 4% paraformaldehyde for 5 min, and blocked with 1% normal goat serum containing 0.05% Triton X-100 for 30 min after wash. The cells were then incubated overnight at 4 °C with diluted primary antibody: mouse anti-14-3-3 (BD Biosciences), mouse anti-bag-1 (Stressgen)

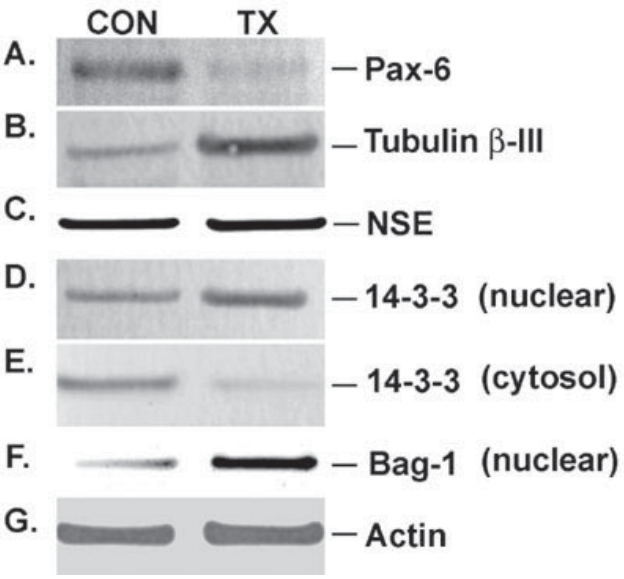

Figure 3. Effect of fenretinide treatment on pax-6, tubulin β-III, NSE, 14-3-3, and bag-1 protein expression. ARPE-19 cells were treated with 1.0 μM fenretinide for 5 days. Western blotting was used to analyze protein expression in control and treated cell extracts from: total protein (**A**-**C**, **G**); nucleus (**D**, **F**); and cytoplasm **E**. 10 μg of protein were loaded per lane.

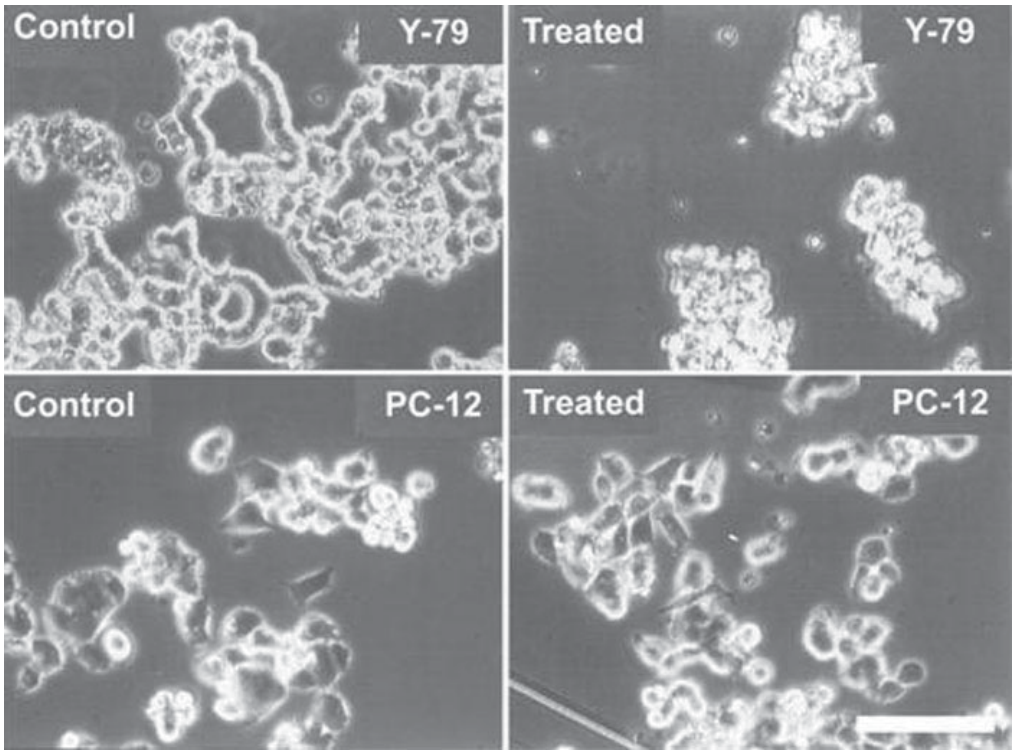

Figure 2. Effect of fenretinide treatment on two cancer cell lines. Y-79 and PC-12 cells were treated with 1.0 μM fenretinide for 2 days. Scale bar represents 100 μm.

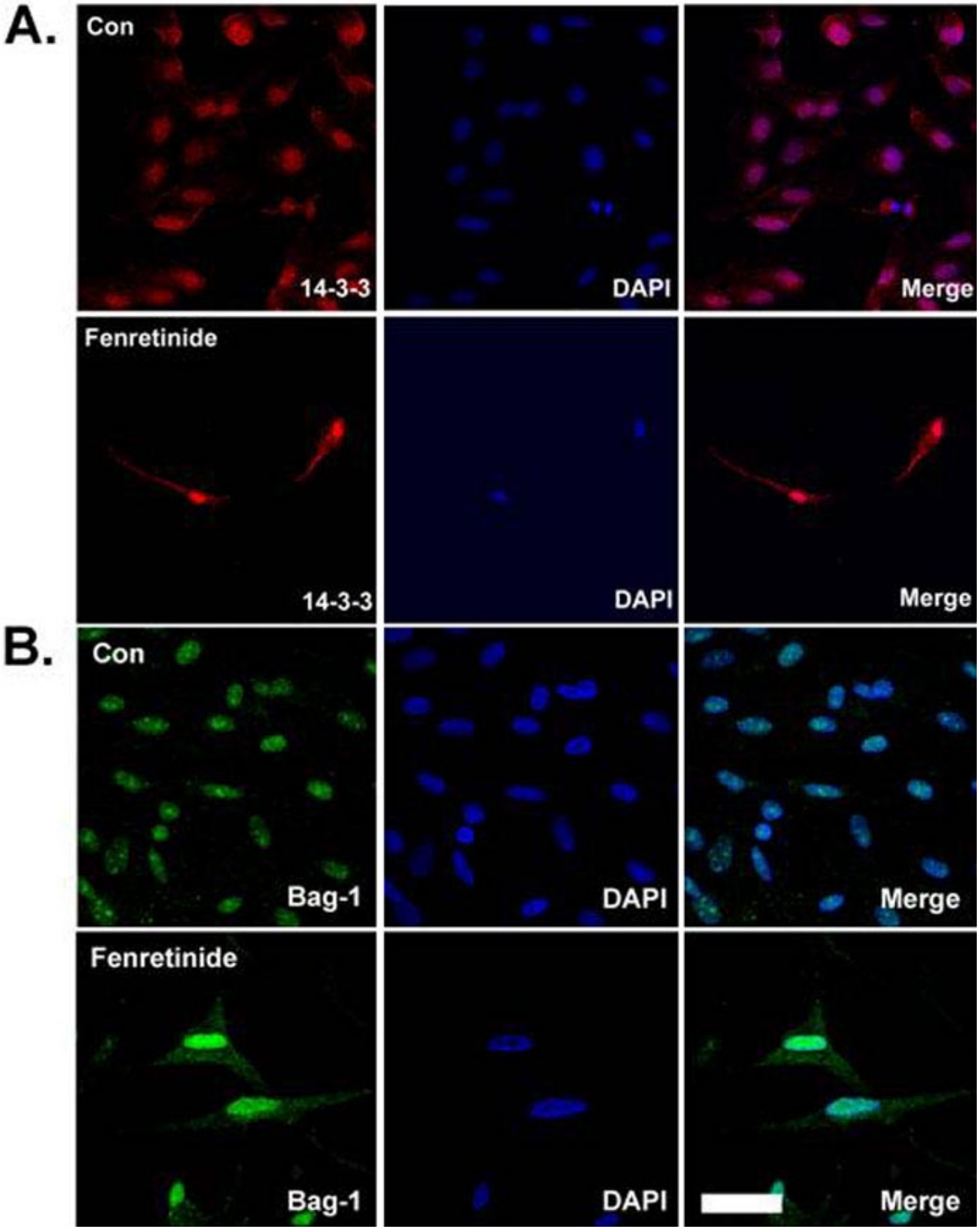

Figure 4. Distribution of 14-3-3 and bag-1 proteins in ARPE-19 cells treated with fenretinide. ARPE-19 cells were treated with 1.0 μM fenretinide for 5 days and analyzed by confocal microscopy. Cells were stained with **A**: anti 14-3-3 antibody and Cy3 conjugated secondary antibody; **B**: anti bag-1 antibody and Alexa Fluor 488 conjugated secondary antibody. Cell nuclei were stained with DAPI (blue). Scale bar represents 50 μm.

or mouse anti-Hsp70 (Stressgen). After washing in PBS, cells were incubated with Cy3 conjugated secondary antibody (Molecular Probes, Eugene, OR) or Alexa Fluor 488 antibody (Molecular Probes, Eugene, OR). Cells were then analyzed under a confocal microscope (Leica SP2, Exton, PA) or a fluorescent microscope (Olympus BH2, Tokyo, Japan). In some experiments, cell nuclei were stained with DAPI (Sigma).

RESULTS

Fenretinide induced neuronal differentiation of RPE cells, but not Y-79 or PC-12 cells: ARPE-19 cells are known to be induced into neuronal morphology by fenretinide and exhibit neuronal characteristics [13]. This phenomenon has not been studied on other cell types. Therefore, we studied the effects of fenretinide on another RPE cell line (D-407), and on Y-79 and PC-12 cells. Both Y-79 and PC-12 cells have been reported to be induced into neuronal differentiation by growth factors [30,31].

In Figure 1, ARPE-19 and D-407 cells were treated with 1.0 μM fenretinide for 2 days. D-407 cells differentiate into neuronal morphology as previously reported for ARPE-19 cells [13], with their cell processes longer than or equal to their cell body. No effect was seen in control cells without fenretinide treatment.

In order to investigate the effect of fenretinide on Y-79 and PC-12 cells, we treated these cells with 1.0 μM fenretinide for up to 10 days as described above. Figure 2 demonstrates that fenretinide does not induce neuronal differentiation of Y-79 or PC-12 cells after 2 days treatment. In fact, Y-79 and PC-12 cells died after 5 days of fenretinide treatment.

Decrease in pax-6 and increase in tubulin β-III expression following fenretinide treatment: We used immunoblotting to investigate the expression in ARPE-19 cells of pax-6, a homeobox gene and a transcriptional activator expressed in neuroretina progenitor cells, and tubulin β-III, a protein specifically expressed in neuronal cells, with and without fenretinide treatment. ARPE-19 cells were treated with 1.0 μM fenretinide for 5 days, and the same amount of total protein from control and treated cells were used for western blotting. In Figure 3A and B, we found that pax-6 protein was decreased by fenretinide treatment (Figure 3A), and tubulin β-III was increased after treatment (Figure 3B). NSE protein expression was similar in both control and treated cells (Figure 3C). There was no change in the amount of actin protein, used as a loading control, between control and treated cells. Experiments were repeated at least three times.

Distribution of 14-3-3 and bag-1 proteins following fenretinide treatment: ARPE-19 cells were treated with 1.0 μM fenretinide for 5 days. In order to investigate the 14-3-3 and bag-1 protein expression in ARPE-19 cells as well as the regulation of these protein by fenretinide, an equal amount of protein extracted from cell nuclei and cell cytoplasm before and after treatment was used for western blot analysis. In Figure 3D,F, we show that 14-3-3 (Figure 3D) and bag-1 (Figure 3F) immunoreactivity in the cell nucleus was increased by fenretinide treatment. 14-3-3 protein was decreased in the cell cytoplasm by the treatment (Figure 3E), and bag-1 protein was undetectable in the cell cytoplasm extract in both control and treated cells (data not shown).

To investigate the distribution of 14-3-3 and bag-1 protein in ARPE-19 cells with and without fenretinide treatment, the cells were treated with fenretinide for 5 days, then incubated with 14-3-3 antibody against the full length protein, or with bag-1 antibody and analyzed by confocal microscopy. Figure 4A shows that 14-3-3 was generally present throughout the ARPE-19 cells without fenretinide treatment. After fenretinide treatment, the distribution of 14-3-3 was localized primarily to the cell nucleus and the intensity of 14-3-3 antibody immunostaining in the cell nucleus was increased. In Figure 4B, we found that bag-1 protein expression was located in the cell nucleus in ARPE-19 cells with and without fenretinide treatment, and was increased after fenretinide treatment.

Distribution and increased expression of Hsp70 following fenretinide treatment: Immunofluorescence analysis was used to investigate the distribution of Hsp70 in ARPE-19 cells and the regulation of this protein by fenretinide. The cells were treated with fenretinide for 5 days, then incubated with antibodies against Hsp70, and analyzed by immunofluorescence microscopy. Figure 5 shows Hsp70 was present at low levels

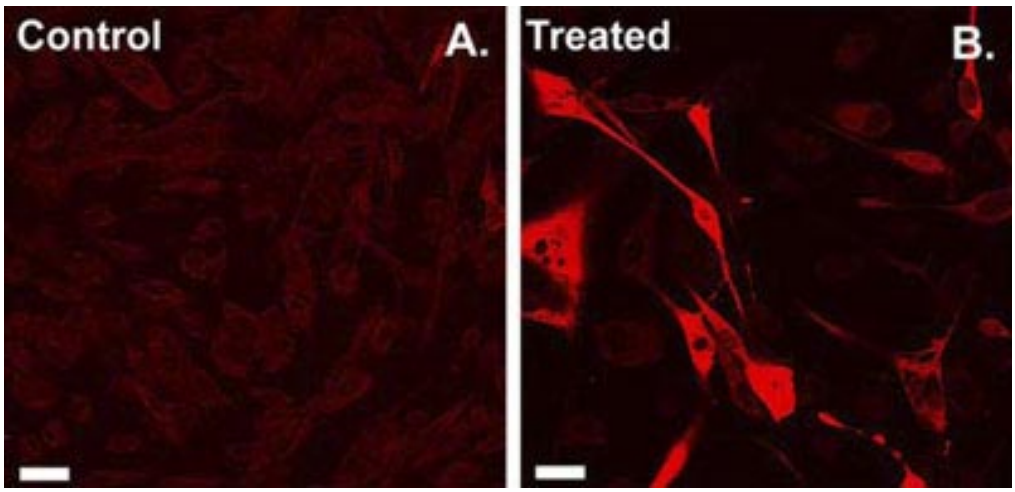

Figure 5. Expression of Hsp70 in ARPE-19 cells treated with fenretinide. ARPE-19 cells were treated with 1.0 μM fenretinide for 5 days and analyzed by immunofluorescence microscopy. Cells were stained with anti-Hsp70 antibody. Scale bar represents 50 μm.

throughout the cytoplasm of untreated cells. Increased immunoreactivity of Hsp70 was seen in treated cells, with the strongest immunostaining associated with cells that were differentiated into a neuronal morphology after fenretinide treatment.

Time-dependent increase in Hsp70 protein expression: The expression of Hsp70 protein was investigated in ARPE-19 cells at different time points following fenretinide treatment. In Figure 6, ARPE-19 cells were treated with 1.0 μM fenretinide for 2, 4, 7, 10, and 14 days. Proteins extracted from treated and control cells were analyzed by western blot, using an antibody against Hsp70 and actin. Expression of Hsp70 protein was increased at all time points.

In Figure 7, ARPE-19 cells were treated with fenretinide for 4, 8, and 16 h, and 1, 2, 3, and 5 days. Protein extracts were analyzed by ELISA using a pre-coated Hsp70 monoclonal antibody. There was a sharp increase (4-6 fold) in Hsp70 protein expression in treated cells, reaching a maximum level within 24 h.

DISCUSSION

We have previously reported that the treatment of human ARPE-19 cells in culture with low concentrations of fenretinide led to a neuronal-type differentiation of these cells that occurred in a time- and dose-dependent manner [13]. In the present study, we have investigated the effect of fenretinide on another human RPE cell line, D-407 [10], and on two cancer cell lines, human Y-79 retinoblastoma cells and rat pheochromocytoma cells (PC-12) that have been shown to undergo neuronal differentiation upon treatment with growth factors [30,31]. We found that D-407 cells, but not Y-79 or PC-12 cells, differentiated into a neuronal phenotype by treatment with fenretinide. Both Y-79 and PC-12 cells were, in fact, killed by fenretinide treatment beyond 5 days, indicating a toxic effect of this drug on these cell lines. Thus, ARPE-19 cells are not unique among RPE cell lines in their response to fenretinide, and it is possible that cultured RPE cells in general possess the ability to differentiate into a neuronal phenotype with fenretinide treatment.

We also investigated pax-6, NSE, and tubulin β-III protein expression following fenretinide treatment. Pax-6 is a homeobox gene and transcriptional activator [32,33]. It is expressed in neuroretinal progenitor cells [33] and regulates embryonic cell development [33]. Pax-6 increases during development and then decreases when embryonic cells start

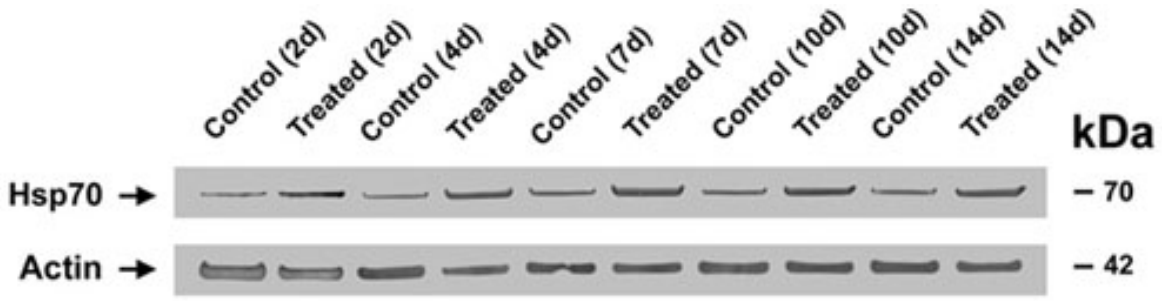

Figure 6. Timecourse of fenretnide treatment on Hsp70 expression in ARPE-19 cells. ARPE-19 cells were treated with 1.0 μM fenretinide for the indicated duration after SDS-PAGE and the total proteins from cell extracts were analyzed by immunoblotting from 2 to 14 days. 10 μg of protein were loaded per lane.

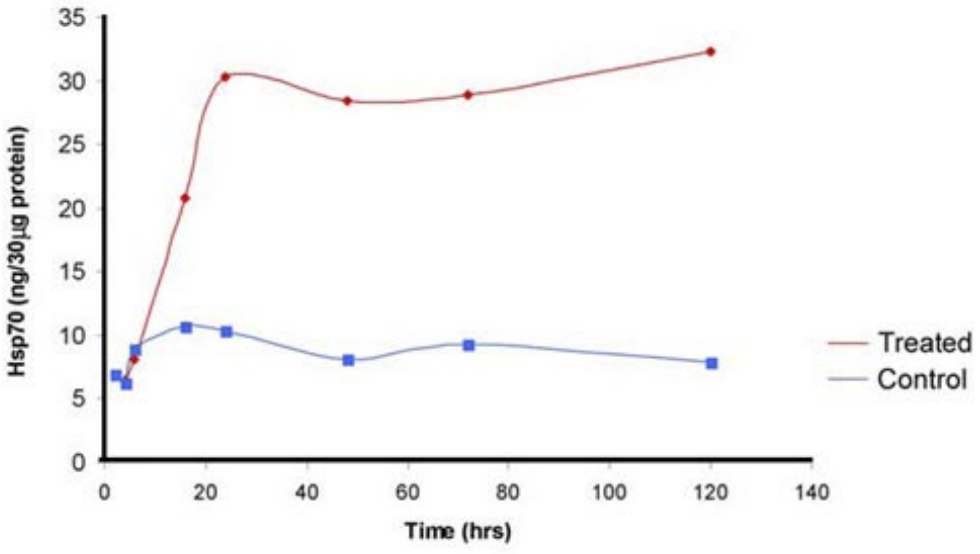

Figure 7. Increase in Hsp70 protein expression in fenretinide-treated ARPE-19 cells. ARPE-19 cells were treated with 1.0 μM fenretinide for the indicated time intervals and the Hsp70 protein content was estimated by ELISA. The treatment resulted in a sharp increase in the Hsp70 protein expression.

morphologic differentiation [4,32]. In the mouse, it is expressed in the optic cup and declines in the neuroretinal region when cell differentiation starts around embryonic day 18 and progenitor cells begin morphological changes. Pax-6 becomes undetectable when progenitor cells differentiate into prospective neuronal cells or RPE cells [4,32]. We found in both undifferentiated and differentiated RPE cells expression of NSE, a protein that is found in many cell types, c.f., rapidly growing neoplastic cells [34-36] and neuronal cells [37]. The positive pax-6 and NSE expression in control ARPE-19 cells suggests that this cell line could be a progenitor cell line and that can differentiate into RPE cells [8,9] or neuronal cells [13,38]. In contrast to pax-6, we report here that the fenretinide-induced neuronal differentiation of ARPE-19 cells is associated with an increase in the expression of tubulin β-III, a protein that is specifically expressed in neuronal cells when they are fully differentiated [39,40]. These results demonstrate that neuronal morphological differentiation of ARPE-19 cells by fenretinide coincides with the expression of neuronal specific proteins.

The mechanisms underlying the neuronal differentiation of ARPE-19 cells by fenretinide are unknown. In this study we found that the expression of 14-3-3 protein is increased and that 14-3-3 is translocated from the cytoplasm to the cell nucleus after fenretinide treatment. The 14-3-3 family was identified as contributing to neuronal development [19] and localized in neurons in the human cerebral cortex, but found at low levels in most human tissues [41]. 14-3-3 has no enolase, ATPase, or cyclic nucleotide-dependent protein kinase activity, does not bind calcium, and appears unrelated to tubulin, neurofilament, or tropomyosin proteins [41]. 14-3-3 proteins have binding sites for proteins including protein kinase C, calmodulin-dependent kinase, Cdc25, Cdk5, Raf, Bad, and Ron, and function as indirect activators of many important cell biological functions [20]. They contribute to the regulation of cellular processes such as signal transduction, cell cycle control, apoptosis, protein trafficking, transcription, and cell differentiation [20,42]. 14-3-3 is involved in the development of *Drosophila* photoreceptors via the Ras/Raf pathway [42]. 14-3-3 is also a regulator of intracellular protein localization, and the binding of 14-3-3 appears to sequester many proteins and stop their further downstream biological activities [20,21]. The translocation of 14-3-3 from the cell cytoplasm to the nucleus following fenretinide treatment suggests that 14-3-3 may be involved in the neuronal differentiation of ARPE-19 cells induced by fenretinide treatment. Translocation of 14-3-3 after fenretinide treatment may also result in the regulation of other proteins involved in cell cycle, cell growth and cell differentiation, which inhibit cell division as is seen in the ARPE-19 cells after neuronal differentiation [13]. We are currently working on the downstream molecules that bind to or are released from 14-3-3 protein by fenretinide in order to further understand the mechanism of fenretinide induced neuronal phenotypic differentiation of ARPE-19 cells. In other cell types, 14-3-3 forms a complex with heat shock transcription factor 1 that plays a role in heat shock protein gene transcription [43,44], so it is possible that 14-3-3 may be involved in regulating Hsp70 expression in ARPE-19 cells.

Hsp70 has been reported to be involved in folding or unfolding newly synthesized proteins during translation and transportation through the organelle membranes [25,26]. Hsp70 also interacts with misfolded or partially folded polypeptides in order to prevent protein aggregation under stress conditions [45]. We previously observed a significant increase in Hsp70 protein expression in the differentiated ARPE-19 cells by treatment with fenretinide [13]. In this study we found that Hsp70 protein is upregulated by fenretinide in a time-dependent manner similar to that of fenretinide induced neuronal differentiation of the ARPE-19 cells [13]. The increased expression of this protein may be involved in protecting the cells from possible toxic effects of fenretinide treatment.

A protective mechanism against cell death involving Hsp70 is associated with bag-1, an anti-apoptotic molecule that binds to Hsp70 [46,47]. Bag-1 is also associated with raf-1 and 14-3-3 proteins in regulation of cell growth and cell differentiation [48]. It has been reported as a neuroprotectant and a regulator and marker of neuronal differentiation [49,50]. Overexpression of bag-1 in the neuronal cell line CSM14.1 led to elongation of axon-like processes and formation of dense nests between cells [50]. In this study, we found increased Hsp70 and bag-1 expression in ARPE-19 cells with fenretinide treatment suggesting that these molecules play roles in ARPE-19 cell growth and differentiation into neuronal cells.

In summary, this study demonstrates that ARPE-19 cells express specific proteins that have been observed in rapidly growing progenitor cells, and that the neuronal differentiation of ARPE-19 cells by fenretinide is associated with proteins that play roles in cell cycle check point, cell growth and cell differentiation and with increased expression of tubulin β-III that is characteristic of fully developed neurons. These results suggest that ARPE-19 cells may be progenitor cells that can differentiate into neuronal cells when treated with fenretinide. Further studies are warranted to elucidate the mechanisms underlying the neuronal differentiation of ARPE-19 cells and the functional characteristics of the differentiated cells.

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