Cytotoxic Effects of Transthyretin Aggregates in an Epidermoid Cell Line

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Abstract

Objective: Skin amyloid deposits can occur as part of systemic amyloidoses including those involving misfolded-aggregated transthyretins (agTTR). Pathological effects of agTTR on the skin are not well understood. The main objective of the current study was to examine the toxicity of agTTR upon a human keratinocyte cell line. **Methods:** Cells were analyzed for indicators of oxidative stress after treatment with normal soluble TTR or the same pre-aggregation concentration of agTTR. Hydrogen peroxide production was analyzed as an indicator for the involvement of reactive oxygen species. **Results:** Treatment of cells with agTTR significantly increased hydrogen peroxide production ($p < 0.05$ vs. controls). Glutathione (GSH) and catalase were analyzed as indicators of endogenous cellular antioxidant activity. Treatment of cells with agTTR resulted in significant decreases in both catalase activity and GSH levels ($p < 0.05$ vs. controls). **Conclusion:** agTTR disrupts redox balance and induces oxidative stress in these epidermoid cells.

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Introduction

The protein transthyretin (TTR) is present in extracellular fluid, such as plasma and cerebrospinal fluid, and participates in the transport of thyroid hormones and vitamin A, the latter through association with retinol-binding protein. In addition to nutrient/hormone transport, a growing number of novel TTR physiological functions, such as modulation of signal transduction and proteolysis, are being investigated [1–4]. TTR is produced by the liver, choroid plexus, and some other tissues, including the skin; the skin is also a major site of TTR degradation [5, 6]. Misfolded-aggregated TTR (agTTR) contribute to amyloidotic diseases, such as familial amyloidotic polyneuropathy and senile systemic amyloidosis, which, in some cases, include TTR-positive deposits in skin tissue [7–12]. The molecular and cellular toxicity mechanisms of agTTR are not well understood, and an objective of our current work is to examine the potential of agTTR to disrupt the normal cell redox balance and promote oxidative stress.

More generally, amyloidogenic polypeptides contribute to various age-related diseases, including neurodegenerative disorders, and for some of these, amyloid (A)- β (Alzheimer) and α -synuclein (Parkinson), there

is evidence for the involvement of prooxidative reactions [13–15]. A β peptides A β _{1–40/42}, for example, can lead to increased production of both reactive oxygen and reactive nitrogen species (ROS) in vivo [16, 17]. Moreover, cells with increased activities of antioxidant enzymes such as catalase and glutathione (GSH) peroxidase are more resistant to the toxic, prooxidative effects of A β peptides [18]. We have previously reported on the prooxidative actions of agTTR in terms of ROS production [19]. In some patients with familial amyloidotic polyneuropathy, protein nitration in nerve tissue and other oxidatively modified proteins and lipids have been reported [20, 21]. We now present a novel study examining the involvement of ROS and cellular antioxidant factors in agTTR-mediated toxicity in an epithelial cell line. Comparisons are also made between prooxidant effects of agTTR and other amyloidogenic polypeptides in epidermoid cells with those in other cell types [19, 22, 23].

Materials and Methods

Reagents and Cells

agTTR was prepared (purified human TTR subjected to acidic buffer conditions⁹ and characterized (turbidity and thioflavin binding) by standard methods [24, 25]; we have previously reported on the method as well as agTTR characterization parameters [26]. The concentration of agTTR (pre-aggregation) used in our study is close to physiological TTR concentrations and within ranges reported in other aggregate toxicity studies [24, 27–29]. All the reagents used were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA), unless noted otherwise. A431, a human epithelial carcinoma cell line, was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Waltham, MA, USA). The medium was supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin (complete medium). Cells were maintained in a humidified 5% CO₂ incubator at 37°C.

Hydrogen Peroxide Production

Hydrogen peroxide assays were performed based on the Amplex Red method according to the manufacturer's instructions (Molecular Probes). The conversion of Amplex Red (N-acetyl-3,7-dihydroxyphenoxazine) to resorufin was quantified. Equal numbers of cells were treated with TTR (2.4 μ M) or agTTR (same pre-aggregate concentration), or no TTR (another control) in Krebs-Ringer phosphate buffer (KRPB) for 30 min at 37°C. A reaction mixture (100 μ L) containing 50 μ M Amplex Red reagent and 0.1 U/mL horse radish peroxidase (HRP) in KRPB buffer was added to the above samples, and to hydrogen peroxide standards and blank controls (buffer alone). The reaction was allowed to proceed for 30 min at 37°C. Light absorbance (A) was measured using a microplate reader, and results were expressed as $100 \times (A_{\text{sample}} - A_{\text{blank}})/(A_{\text{control}} - A_{\text{blank}})$.

Catalase Assays

Catalase activity was estimated using the Amplex Red method according to the manufacturer's instructions (Molecular Probes) through the measurement of unreacted, exogenous hydrogen peroxide. Equal numbers of cells were treated with TTR (2.4 μ M), or agTTR (same, pre-aggregate concentration), or no TTR (another control) in 2% FBS-DMEM for 4 h. Cells were then collected by centrifugation (1,000 g, 4°C, 5 min) after being detached from the plate with trypsin-EDTA. Cell pellets were resuspended in an ice-cold extraction buffer containing 0.1% Triton-X (in 0.1 M potassium phosphate buffer, KPE) and subjected to 3 freeze-thaw cycles. After centrifugation of the cell suspension (3,000 g, 4°C, 4 min), 25 μ L of supernatant, or catalase standard, were added to the wells of a 96-well microtiter plate, followed by the addition of 25 μ L of 40 μ M hydrogen peroxide. After incubation for 30 min at room temperature, 50 μ L of a mixture containing 100 μ M Amplex Red reagent and 0.4 U/mL HRP were added to each well. Light absorbance was measured using a microplate reader. Values of catalase activity were interpolated from standard catalase curves and expressed by subtracting the sample value from that of the no-TTR control.

GSH Assays

Quantitative assays for GSH were based on the enzymatic recycling method in which GSH is oxidized by 5,5'-dithio-bis(2-nitrobenzoic acid) and leads to the formation of 5'-thio-2-nitrobenzoic acid [30]. Equal numbers of cells were treated with TTR (2.4 μ M), or agTTR (same, pre-aggregate concentration), or no TTR (another control), for 4 h at 37°C. Cells were then collected by centrifugation (1,000 g, 4°C, 5 min) after being detached from the plate with trypsin-EDTA. Pellets were resuspended in ice-cold extraction buffer containing 0.1% Triton-X and 0.6% sulfosalicylic acid (in 0.1 M KPE) and lysed and centrifuged as above for the catalase assays. A solution of 120 μ L containing 0.34 mg/mL DTNB and 1.65 units/mL GSH reductase was added to the samples and GSH standards for 30 s in a 96-well microtiter plate. β -NADPH (60 μ L of 1.5 mg/mL) was then added, and light absorbance was measured at 412 nm. Values of total GSH were obtained using GSH standard curves.

Statistical Analyses

Data are presented as means $\pm$ SEM, unless noted otherwise. Statistical analyses were performed using either the Student *t* test, or one-way analysis of variance (ANOVA) with SPSS 17.0 software. The α level for statistically significant differences was 0.05.

Results

Cellular Production of Hydrogen Peroxide and Cellular Catalase Activity

As shown in Figure 1a, treatment of A431 cells with agTTR (2.4 μ M pre-aggregate concentration) resulted in a statistically significant increase of about 75% in the cellular levels of hydrogen peroxide versus untreated controls. Cells treated with normal, soluble TTR (sTTR, 2.4 μ M) did not exhibit significantly different levels of hydrogen perox-

Fig. 1. a Cellular hydrogen peroxide production was significantly increased in A431 cells treated with aggregated transthyretins (agTTR; 2.4 μ M pre-aggregate concentration) than in those treated with the same concentration of normal soluble TTR (sTTR) or those with no TTR treatment (C). For each treatment, $n = 17$. **b** Residual catalase (CAT) activity was significantly lower in A431 cells treated with agTTR (2.4 μ M pre-aggregate concentration)

than in those treated with the same concentration of normal sTTR, or those with no TTR treatment (C). For each treatment, $n = 9-10$. **c** Glutathione (GSH) levels were significantly lower in A431 cells treated with agTTR (2.4 μ M pre-aggregate concentration) than in those treated with the same concentration of normal sTTR, or those with no TTR treatment (C). For each treatment, $n = 15$. **a-c** * $p < 0.01$ versus each of both controls (C and sTTR).

Fig. 2. Effects of aggregated transthyretins (2.4 μ M pre-aggregate concentration) on hydrogen peroxide production, residual catalase activity, and glutathione (GSH) levels were compared among the A431 epidermoid cells and nonepidermoid (Schwann-like) sNF94.3 cells [19]. Results are shown versus normal, wild-type soluble transthyretin controls. For each treatment, $n = 6-17$.

ide production versus untreated controls. Assays of catalase activity in extracts prepared from A431 cells that had been treated with agTTR, sTTR, or no TTR, reveal a statistically significant agTTR-dependent enzyme activity depletion of about 22% (Fig. 1b). Cells treated with normal sTTR did not exhibit statistically significant changes in residual catalase activity versus untreated controls.

Cellular GSH Levels

As shown in Figure 1c, treatment of A431 cells with agTTR (2.4 μ M pre-aggregate concentration) resulted in a statistically significant decrease of about 57% in the cellular levels of GSH versus untreated controls. Cells treated with normal sTTR (2.4 μ M) did not exhibit significantly different GSH levels versus untreated controls although sTTR treatment appeared to result in minor decreases in GSH levels.

Comparison of agTTR Effects in Human Epidermoid and Schwann-Like Cells

Figure 2 shows that agTTR induces similar effects – increased hydrogen peroxide production, decreased catalase activity, and GSH depletion – in both the epidermoid keratinocyte line and a Schwann-like human cell line. As detailed in the Discussion, such toxicity of agTTR may contribute to Schwann cell degeneration.

Discussion

The current study provides evidence for agTTR toxicity in a human epidermoid cell line through oxidative stress and involvement of ROS. Increased production of hydrogen peroxide and other ROS can lead to cellular depletion of endogenous antioxidant factors, such as reduced GSH and the catalase enzyme. Prooxidative factors lead to depletion of GSH and catalase activity which, in turn, can result in higher susceptibility to high ROS production and increased oxidative damage [31–34]. Our re-

sults in epidermoid cells are consistent with such a scenario: GSH levels and catalase activity were both significantly depleted by agTTR treatment ($p < 0.05$ vs. controls).

A comparison between the A431 epidermoid cells and a nonepidermoid, human, Schwann-like cell line (Fig. 2) indicates similar effects of agTTR in both cell types, especially in terms of GSH levels. Interestingly, agTTR is also a likely factor in Schwann cell degeneration observed in some patients with familial amyloidotic polyneuropathy [34]. The main difference between the epidermoid and Schwann-like cells was a greater resistance of the former to depletion of catalase activity. In this context, it has previously been reported that keratinocytes have higher catalase-mediated resistance to prooxidants than fibroblasts [22]. We conclude from our comparison of both cell types (Fig. 2) that agTTR prooxidative effects are not specific to epidermoid cells. In another comparison, both A431 cells treated with agTTR and M17 neuroblastoma cells treated with A β_{1-42} (low μ M concentrations) exhibit major decreases in metabolic activity as gauged by MTT reduction assays [23, 26]. It will be of interest to examine the prooxidative effects of agTTR in human primary keratinocytes and to compare them with those reported for the cell lines (above).

Previous studies have implicated prooxidative factors in the pathology of age-related amyloidotic diseases caused by misfolded and aggregated proteins and peptides [13–17, 20, 21, 35, 36]. Pheochromocytoma cells selected for resistance to toxic A β peptides, for example, have elevated catalase activity and do not exhibit the amyloid-induced accumulation of hydrogen peroxide that is characteristic of nonresistant cells [37]. Moreover, cells treated with peptides or chemical compounds that prevent amyloid-induced cellular production of hydrogen peroxide are protected against toxic effects of the amyloid [23].

Our results suggest that increased production of ROS and other reactive chemical species, and decreased levels of cellular antioxidant components, may contribute to the disruption of the cellular redox balance and increased oxidative damage in amyloidotic diseases involving agTTR. Such prooxidative factors may damage critical biomolecules and organelles involved in regulating basic cell functions, such as energy metabolism, signal transduction, and transport [38–40]. Disruption of such basic functions is likely to contribute to the toxic effects of agTTR and other amyloidogenic factors. It will also be of interest in future studies to further examine the molecular mechanisms of agTTR-related toxicity in the skin; for example, do keratinocytes or other epidermoid cells participate in the clearance of agTTR? Is a keratinocyte TTR receptor involved in agTTR uptake and in mediating agTTR-dependent oxidative stress? How does such oxidative stress influence endocytic transport pathways of skin cells [40, 41]?

Conclusions

The results provide novel evidence for the involvement of ROS (hydrogen peroxide) in prooxidative effects of agTTR on epidermoid cells. We provide additional evidence that agTTR can disrupt the normal redox balance and induce oxidative stress by decreasing the levels of endogenous cellular antioxidant factors, GSH and catalase.

Disclosure Statement

The authors declare that they have no competing interests.

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