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Nuclear ferritin mediated regulation of JNK signaling in corneal epithelial cells

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Abstract

Corneal epithelial (CE) cells are exposed to environmental insults (e.g., UV-irradiation), yet they suffer little damage. Our previous studies suggest that chicken CE cells have a novel form of protection involving having ferritin in a nuclear location where it can bind to DNA and sequester free iron. Here we describe another potential nuclear ferritin-mediated protective mechanism: the down-regulation of the JNK signaling pathway. The JNK pathway has been shown by others to promote apoptosis in response to cell damage and also to be activated in CE cell lines following exposure to UV radiation. Here we show in COS7 reporter cell lines that the expression of ferritin in a nuclear localization significantly down-regulates the JNK pathway ($p=5.7 \times 10^{-6}$), but has no effect on the NFkB or the Erk pathways. In organ cultures of embryonic chicken corneas, we observed that inhibiting the synthesis of nuclear ferritin in CE cells, using the iron-chelating molecule deferroxamine, led to an increase in JNK signaling, as measured by phospho-JNK levels compared to CE cells with nuclear ferritin. Furthermore, the chemical inhibition of the JNK pathway using the molecule AS601245 decreased the production of nuclear ferritin. Taken together, these observations suggest that in CE cells a feedback-loop exists in which JNK signaling increases the production of nuclear ferritin and, in turn, nuclear ferritin decreases the activity of the JNK signaling pathway.

Keywords

ferritin; JNK; corneal epithelium; cell survival; UV protection

Ultraviolet (UV) light constitutes a major environmental insult to all exposed tissues of the body, including those comprising the cornea and other underlying ocular structures. The cellular changes resulting from UV and reactive oxygen species (ROS) induced damage can be severe. In skin, for example, UV-induced damage to DNA is thought to be a major factor in the increasing incidence of epidermal cancer (Chen et al., 2013). However, corneal epithelial (CE) cells seem to be refractory to such damage, as primary cancers of these cells are extraordinarily rare, as compared to the incidence of skin cancer – even though this

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tissue is transparent and constantly exposed to ROS-generating UV light (Smolin and Thoft, 1987). This suggests that CE cells have evolved potent defense mechanisms that prevent damage to their DNA. Also, even during embryonic development the cornea is exposed to oxidative stress – as human amniotic fluid contains ROS (Longini et al., 2007) and the eyelids are open during much of the last trimester (Sevel and Isaacs, 1988). But, surprisingly little is known concerning how such protection is afforded, except for the possible involvement of general factors found in many tissues, such as enzymatic scavengers of ROS [i.e., superoxide dismutase, catalase and glutathione peroxidase (Janssen et al., 1993)].

However, we have accumulated evidence from studies on the avian cornea suggesting that chicken CE cells have a novel type of protection from oxidative damage in which the iron-sequestering molecule ferritin (FTN) is localized within the nucleus – rather than the cytoplasmic localization it has in other cell types (Cai et al., 2008).

Additional observations indicate that the nuclear localization of ferritin is effected by a CE-specific nuclear transporter, that we have termed ferritoid (FTD) for its similarity to ferritin (Millholland et al., 2003). Ferritoid not only transports ferritin into the nucleus, but there it also remains associated with ferritin in the form of unique, heteropolymeric complexes that are half the size of a conventional ferritin molecule (Nurminskaya et al., 2009). Nuclear ferritin affords protection from damage to CE DNA by at least two different mechanisms: (1) by preventing the formation of iron-catalyzed OH^- radicals generated through the Fenton reaction (Cai et al., 1998) and (2) through direct physical interaction with DNA (Nurminskaya et al., 2009).

Here we investigated a novel third mechanism involving inhibition of the c-Jun N-terminal kinase (JNK) signaling pathway, through which the nuclear localization of ferritin may protect CE cells. Previous studies by others have shown that cellular damage and oxidative stress can lead to the induction of NF κ -B, Erk or JNK signaling pathways in many cell types (Black et al., 2011; Roos and Kaina, 2006). *in vivo*, the JNK pathway is activated in human skin by both UV-B and UV-C Adachi (Adachi et al., 2003). In the cornea *in vivo*, epithelial function is maintained by the migration of cells from the limbal stem cell niche into the basal epithelial layer. These transit-amplifying cells undergo mitosis in the basal layer and then differentiate corneal epithelial cells in the wing cell layers. These cells then move apically, and are eventually sloughed off the surface of the cornea through the process of desquamation (Thoft and Friend, 1983). Despite the potential for UV-B to cause cellular damage, induce signaling pathways involved in apoptosis and alter the mechanisms of epithelial maintenance, the corneal epithelium appears to be protected against these effects. It is known that the JNK pathway is activated in response to UV irradiation of CE cells *in vitro* (Wang and Lu, 2007) and other studies have also shown that inhibition of JNK signaling can delay the onset of apoptosis (Krilleke et al., 2003), potentially allowing for the repair of damage. However, it is still unclear how signaling in response to UV damage alters the mechanics of corneal epithelial maintenance.

In addition to its effects as an iron chelating molecule cytoplasmic ferritin can alter cellular signaling in cortical neurons (Abt and Meucci, 2011). However, little is known about the effects of nuclear localized ferritin on signaling pathways. Therefore, we sought to

determine the effects of nuclear ferritin on the NFκ-B, Erk and JNK signaling pathways, i.e. those pathways involved in the cellular response to damage. To do this we first generated stable luciferase reporter cell lines, each responsive to either the Erk, NFκB or JNK signaling pathway using the Cignal lentireporter system (Qiagen, Valencia, CA). For these initial screenings, the reporter cell lines were constructed in COS-7 cells as they have high transfection efficiency (Chen and Okayama, 1987) and their endogenous ferritin is cytosolic and expressed at low levels (Corsi et al., 1998). Each of the COS-7 reporter lines was transfected using FuGene HD (Promega, Madison, WI) with an expression construct (nFTN) encoding ferritin heavy chain linked to which a conventional nuclear localization signal (NLS) from the SV40 large T antigen that we have previously shown translocates to the nucleus (Cai and Linsenmayer, 2001). For these screens, we considered nFTN to be the simplest, most direct way to achieve ferritin in a nuclear location, as other approaches would require multiple components such as co-transfecting ferritin and the nuclear transporter ferritoid that could complicate interpretation of the results. Following transfection relative luminescence was measured following the manufacturers instructions. Relative luciferase activities in the COS-7 cells are shown (figure 1) as the ratio between their luciferase activity and standard, both of which were normalized to renilla as a control for infection efficiency.

In comparison to mock-transfected controls (figure 1), transfection of nFTN did not significantly affect the activity of either the Erk or NFκB pathways. However, the reporter cell line for the JNK signaling pathway showed a highly significant decrease in JNK activity as compared using an unpaired students t-test to control cells transfected with the empty vector ($p=5.7\times 10^{-6}$). These data with COS-7 cells demonstrate that having ferritin in a nuclear localization can down-regulate the basal activity levels of the JNK signaling pathway. However, other studies have shown differences in the signaling pathways that respond to oxidative stress and DNA damage in different cell types and tissues (Pham et al., 2004). Therefore, we next sought to determine whether a similar effect on JNK signaling would occur with the endogenous nuclear ferritin in the CE cells of intact corneas.

During normal embryonic development of the chicken the expression of ferritin is not detected in corneal epithelial cells until embryonic day 10.75 (E10.75). Shortly after (at E11), ferritin is then translocated to the nucleus by ferritoid. Previously, we observed that embryonic corneas cultured free floating in medium undergo these same developmental changes in the transport of ferritin to the nucleus (Beazley et al., 2008). So, here we used this cornea culture system to test whether the localization of ferritin in the nucleus had a similar effect on JNK signaling as was observed in the COS-7 cells.

For this, fertilized chicken eggs (Hy-Line NA, Elizabethtown, PA) were incubated at 37 degrees C for 9 days and then the embryos were staged according to the Hamburger-Hamilton stages of chicken development (Hamburger and Hamilton, 1951). All animal experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Corneas were cultured endothelium side down in 12 well culture dishes in organ culture medium and then prepared for immunohistochemistry as described previously (Beazley et al., 2008). Briefly, following culture the corneas were fixed for 2 hours at 4

degrees C in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). Corneas were then embedded in OCT (Sakura Finetek, Torrance, CA) and 8µm serial sections were cut on a Microm HM560 cryostat (Fisher Scientific, Pittsburg, PA). As shown in Figure 2A, when E9 pre-ferritin stage corneas were cultured for 48 hours in organ culture medium the production of ferritin (red) was detected in cryosections of cultured corneas using an antibody specific to chicken ferritin (Cai et al., 1997). As has been shown previously, the expression of ferritin was localized within the nuclei of the corneal epithelial cells as demonstrated here in the merged image of the ferritin signal with Hoechst staining of the nuclei (Fig. 2B). It has been shown previously that the expression of ferritin is tightly regulated by the presence of iron (Lash and Saleem, 1995). Here, we prevented the synthesis of ferritin in the cultured corneas with the addition of the iron chelating agent deferoxamine (DFO; Sigma-Aldrich, St. Louis, MO) to the culture medium at a concentration of 100µM. As shown in figure 2C and 2D very little ferritin (red) is detected in the CE of the DFO treated corneas.

Using the addition of DFO to control the expression of ferritin in these cultured corneas we then assayed by Western blot for the presence of phosphorylated JNK (p-JNK), as a measure of JNK signaling pathway activity. For this, 12 corneas were cultured for 48 hours with or without the addition of 100µM DFO to the media. Also as a control for any off-target effects of DFO and DMSO (see JNK inhibitor experiments below), another set of 12 corneas was cultured in 100µM DFO and equimolar ferrous sulfate (FeSO_4) in a final media also containing 0.0001% DMSO. This experiment was repeated 3 times. Following a brief rinse in Hanks Buffered saline solution (HBSS, ThermoFisher, Waltham, MA) the CE was then removed from the underlying stroma using Dispase as previously described (Beazley et al., 2008). The corneal epithelia were homogenized in RIPA buffer containing protease inhibitors (Roche Diagnostics, Indianapolis, IN) and phosphatase inhibitors (HALT, ThermoFisher, Waltham, MA). The lysates were centrifuged at 16000×g for 20 minutes at 4°C and the protein concentrations of the supernatants were quantified by Bradford assay (Coomassie Plus, ThermoFisher, Waltham, MA). CE lysates were then subjected to SDS-PAGE (15µg protein/lane) as described previously (Beazley et al., 2009) and immunoblotted for the presence of ferritin, p-JNK (Cell Signaling Technology, Danvers, MA), total JNK (Cell Signaling Technology, Danvers, MA) and, as a loading control, alpha-tubulin (α-Tubulin, Abcam, Cambridge, MA). Densitometry was performed and analyzed on the immunoblots using NIH imageJ software (Schneider et al., 2012).

As shown in the immunoblot in figure 2G, in the CE of untreated cultures (no trtmt) ferritin (FTN) was clearly detectable, whereas the cultures treated with DFO contained little ferritin, which is consistent with the results observed by immunofluorescence in figure 2C and described above. Also, the addition of equimolar FeSO_4 along with the DFO in control cultures (fig. 2G: DFO/ FeSO_4) negated the inhibitory effect of DFO on ferritin synthesis, demonstrating that the effect of DFO on ferritin synthesis results from iron sequestration. Further, densitometry of the bands for p-JNK in the untreated and DFO/ FeSO_4 treated corneas determined that the levels were not significantly different ($p=0.18$, unpaired students- t-test; Fig. 2G, “p-JNK”, “No trtmt” and “DFO FESO4”), showing that the JNK signaling pathway activity is the same under these two conditions. However, the levels of p-JNK showed a clear, statistically significant increase ($p<0.001$) in corneas where the

expression of ferritin and its translocation to the nucleus were inhibited by DFO (fig. 2G, “p-JNK”, “DFO”), compared to either the untreated or DFO/FeSO₄ treated corneas. As is also shown in figure 2G, the overall levels of total JNK protein (JNK) remained the same for all of the culture conditions examined ($p>0.25$ for all treatments compared to untreated controls using un-paired students t-test), showing that the increase in p-JNK in DFO treated corneas is not due to just an overall increase in the amount of total JNK and consistent with activation, not expression, of the JNK pathway being regulated by ferritin.

These results suggest that for CE cells, the levels of ferritin within the nucleus control the levels of JNK signaling, such that when ferritin is present, JNK signaling activity is suppressed, and when levels of ferritin in the nucleus decreases, JNK activity increases. We have previously shown that DNA damage is increased in corneal epithelial cells when FTN is prevented from entering the nucleus in response to oxidative stress (Cai et al., 2008) or UV (Cai et al., 1998). Importantly though, in the studies presented here we did not specifically examine the effects of inhibiting the JNK pathway on apoptosis within the chicken CE at the molecular level in response to UV or oxidative stress. This is the subject of future studies.

To begin to more fully understand the relationship between JNK signaling and nuclear ferritin though, we inhibited the JNK pathway using the chemical inhibitor, AS601245 (Merck KGaA, Darmstadt, Germany). Upon addition of this inhibitor at a concentration of 10uM and 0.0001% DMSO in media to the CE cultures (Fig. 2G, “JNK inhib”) the level of p-JNK decreased dramatically, as compared to all of the other types of cultures. In dose response experiments increasing (30uM) or decreasing (1uM) the concentration of AS601245 in the culture medium led to a slight increase or decrease in the level of p-JNK, respectively (not shown). Again, in these experiments densitometry of the western blot determined that the level of total JNK protein did not change ($p=0.26$, unpaired students t-test), indicating that only the activation of the JNK pathway was inhibited, not the overall production of JNK protein. Interestingly, the inhibition of the JNK signaling pathway resulted in a significant decrease ($p<0.001$) in the amount of ferritin protein in the cultures compared to controls as shown by western (Fig. 2G, “JNK inhib”) and immunofluorescence (fig. 2E and 2F). These data suggest an interaction between levels of ferritin and JNK activity in chicken corneal epithelial cells. In K562 and HeLA cells in vitro the down-regulation of ferritin has been associated with increased reactive oxygen species (Cozzi et al., 2004; Kakhlon et al., 2001), which then in turn increase the activity JNK and lead to apoptosis (Pham et al., 2004). Similarly, the data described here show that decreased ferritin expression in CE cells leads to an increase in p-JNK. Here, we did not test whether the down-regulation of FTN by DFO also led to an increase in reactive oxygen species within the cornea epithelium. However, the observation that ferritin levels are also controlled by JNK signaling suggests a feedback loop. Furthermore this feedback loop may be specific to the cornea epithelium, as inhibition of JNK signaling does not appear to alter ferritin levels in lens epithelial cells (Lall et al., 2013).

Overall, in the present study the results reveal another potential mechanism through which nuclear ferritin could ameliorate cellular damage from environmental insults to the CE – such as those involving oxidative stress and UV damage. In many tissues, including the

cornea, UV insult causes damage to multiple cellular components like DNA, induces transcriptional changes and potentially leads to apoptosis. We hypothesize that nuclear ferritin helps to protect the cells of the corneal epithelium by down-regulating JNK signaling. The data also suggest that, potentially, the nuclear localization of ferritin may regulate JNK signaling through conserved mechanisms in some other cell types and tissues as well, as it occurs in both COS7 cells and CE cells, but not lens epithelium or retinal pigmented epithelium (Lall et al., 2013). While the mechanisms responsible for the prevention of the phosphorylation of JNK by the nuclear localization of ferritin remain to be elucidated, it has been shown that in the mouse there is enhancer region 4.1 kilobases upstream of the ferritin heavy chain gene that contains an AP-1 binding site (Tsuji et al., 1998). This region may be involved in the regulation of ferritin expression by JNK observed in our studies. However, a model involving the recruitment of c-Jun to the ferritin promoter through p300 and nuclear factor Y has been suggested as another potential alternative (Faniello et al., 2002). The mechanisms involved, as well as their ability to protect corneal epithelial cells from damage will be the subject of future studies. Taken together, the data presented here show a complex but potentially powerful mechanism that may also be involved in the protection and survival of CE cells.

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Highlights

- Localization of ferritin to the nucleus decreases the activity of the JNK pathway in COS-7 cells.
- Inhibition of ferritin synthesis in intact corneal epithelia leads to increased phosphorylation of JNK.
- Inhibition of the JNK pathway in corneal epithelial cultures decreases the expression of nuclear ferritin in avian corneal epithelial cells

Figure 1. Nuclear ferritin down-regulates JNK signaling in COS-7 cells

COS-7 cells were stably transfected with the Cignal luciferase lenti-reporter for the ERK, NFkB or JNK pathways and 1×10^4 cells were plated in 96-well plates. Cells were transiently transfected with ferritin with a nuclear localization signal (nFTN) or treated with transfection reagent alone (mock) as a control. After 24 hours the luciferase activity was quantified and the level of Firefly luciferase was normalized by Renilla luciferase. No significant difference was observed in either the ERK or NFkB pathways. Transfection of nuclear ferritin resulted in a significant decrease in JNK pathway activity. This experiment was repeated for three times and the values were shown as mean $\pm$ SD. (*: students t-test; $p=5.7 \times 10^{-6}$)

Figure 2. Ferritin expression in corneal epithelial cells regulates JNK signaling

Immunohistochemistry was performed on 8µm cryostat sections of E9 corneas that had been cultured for 48 hours either alone (A and B), with DFO (C and D) or with JNK inhibitor (E and F). Panels A, C and E show ferritin (red) for each condition and panels B, D and F show this labeling merged with the Hoechst staining (blue) of the nuclei. BL designates Bowman's Layer. In untreated corneas (A and B) ferritin is localized in the nuclei of corneal epithelial (ce) cells but not in the corneal stroma (cs). Very little ferritin labeling was observed in DFO (C) or JNK inhibitor (E) treated corneas. Western blots on corneal epithelia from cultured corneas (D) show decreased FTN expression in DFO treated and JNK inhibitor treated corneas compared to controls (No Trtmt, DFO FeSO₄). DFO treated corneal epithelia show increased pJNK compared to controls (No Trtmt, DFO FeSO₄) and JNK inhibitor (JNK inhib) treated. No differences were observed in protein expression for total JNK (JNK) or alpha-tubulin (a-Tub).