Transforming growth factor-β1 blocks the enhancement of tumor necrosis factor cytotoxicity by hyaluronidase Hyal-2 in L929 fibroblasts
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Abstract

Background: Functional antagonism between transforming growth factor beta (TGF-β) and hyaluronidase has been demonstrated. For example, testicular hyaluronidase PH-20 counteracts TGF-β1-mediated growth inhibition of epithelial cells. PH-20 sensitizes various cancer cells to tumor necrosis factor (TNF) cytotoxicity by upregulating proapoptotic p53 and WW domain-containing oxidoreductase (WOX1). TGF-β1 blocks PH-20-increased TNF cytotoxicity. In the present study, the functional antagonism between TGF-β1 and lysosomal hyaluronidases Hyal-1 and Hyal-2 was examined.

Results: Murine L929 fibroblasts were engineered to stably express green-fluorescent protein (GFP)-tagged hyaluronidase (GFP-Hyal-1 or GFP-Hyal-2) or GFP alone. Compared to control cells, Hyal-2-expressing cells had a significantly increased sensitivity to TNF cytotoxicity (~60–110% increase), while Hyal-1-expressing cells were less sensitive to TNF (~20–90% increase). TNF activated NF-κB, along with IκBα degradation, occurred at 20 to 60 min in Hyal-2 cells post stimulation, but at the 20 min time point in both control and Hyal-1 cells. Hyal-2 cells, but not Hyal-1 and control cells, constitutively expressed WOX1, and transiently expressed Hyal-2 enhanced WOX1-mediated cell death. Unlike PH-20, Hyal-1 and Hyal-2 did not induce p53 expression. Hyal-2 translocated from the lysosome to the mitochondria during staurosporine-mediated apoptosis, suggesting that Hyal-2 may damage mitochondria. Finally, Hyal-1 and Hyal-2 blocked TGF-β1-enhanced L929 cell growth. In contrast, TGF-β1 inhibited Hyal-1- and Hyal-2-increased TNF cytotoxicity in L929 cells by 30–50%.

Conclusions: TGF-β1 limits the ability of Hyal-2 to induce TNF cytotoxicity in L929 cells. Hyal-2-increased TNF cytotoxicity in L929 cells appears to be correlated with upregulation of WOX1, a prolonged NF-κB activation, and Hyal-2 translocation to the mitochondria during apoptosis.

Background
Transforming growth factor beta (TGF-β) family proteins are multifunctional cytokines capable of regulating cell growth, extracellular matrix protein synthesis, and immune cell functions [1,2]. Hyaluronidase is an extracellular matrix-degrading enzyme. Interestingly, malignant and metastatic breast and prostate cancer cells frequently overexpress both hyaluronidase and TGF-β proteins [3–
TGF-β family proteins, including β1,β2 and β3, induce TNF cytotoxicity and suppress the PH-20 effect of increasing TNF cytotoxicity in murine L929 fibroblasts [8,11]. TGF-β1 induces a novel extracellular matrix protein that prevents TNF-mediated cell death and blocks the activation of extracellular signal-regulated kinase (ERK; also known as p42/44 mitogen-activated protein kinase, p42/44 MAPK) in L929 cells [8,12]. Additionally, TGF-β1 induces the expression of TIAF1 (TGF-β-induced antiapoptotic factor) [13] and TIF2 (TGF-β-induced factor 2) [14] that inhibit TNF cytotoxicity. Whether these TGF-β1-induced proteins restrict the ability of PH-20 to increase TNF cytotoxicity is unknown.

PH-20 blocks TGF-β1-mediated growth suppression of epithelial cells [8,15]. Additionally, PH-20 rapidly activates ERK in L929 cells and TGF-β1 reduces the PH-20-induced ERK activation [16]. In contrast, TGF-β1 synergistically increases PH-20-mediated inhibition of staurosporine apoptosis [16]. Thus, PH-20 and TGF-β1 are physiological antagonists for only certain cellular responses.

Hyaluronidases Hyal-1 and Hyal-2 play a critical role in cancer invasion and metastasis [3–5], although inactivation of HYAL1 gene has been shown in head and neck squamous cell carcinomas [17]. Hyal-1, also known as Laca-1, is a lysosomal enzyme that is secreted from cells. Hyal-1 is a candidate tumor suppressor [5,18], although it enhances extravasation and metastasis of prostate cancer cells [3]. Also, mutations in Hyal-1 may contribute to the pathogenesis of a lysosomal disorder, mucopolysaccharidosis IX [19]. Hyal-2 is a lysosomal protein, which is active at low pH [20]. This protein is also known to be a glycosylphosphatidylinositol (GPI)-anchored cell-surface receptor for jaagsieke sheep retrovirus [21].

Testicular hyaluronidase PH-20 counteracts TGF-β1-induced TNF-resistance in L929 cells [8]. The goal of the present study was to examine whether lysosomal Hyal-1 and Hyal-2 enhance TNF cytotoxic function and counteract TGF-β1-mediated TNF-resistance in L929 cells. It was determined that stable expression of murine Hyal-2 in L929 cells increased their sensitivity to TNF-mediated death, whereas Hyal-1 was somewhat less effective. TGF-β1 blocked this increased sensitivity. In contrast, Hyal-1 and Hyal-2 did not enhance L929 cell death by anticancer drugs such as daunorubicin, actinomycin D, staurosporine and camptothecin. Overexpressed Hyal-1 and Hyal-2 induced the expression of proapoptotic WOX1, but not p53, suggesting a role of WOX1 in the increased cellular susceptibility to TNF cytotoxicity. Additional mechanisms that are associated with the increased TNF cytotoxicity were also investigated.

**Results**

As summarized in Figure 1, PH-20 induces the expression of proapoptotic p53 and WOX1 [9,10] and downregulates antiapoptotic matrix inter-α-inhibitor [9], thereby enhancing TNF susceptibility in L929 cells. Also, PH-20 rapidly activates ERK and c-Jun N-terminal kinase (JNK1 and JNK2) [16]. Whether these signaling events lead to induction of p53 and WOX1 is unknown.

In contrast, TGF-β1 protects L929 cells from TNF cytotoxicity by upregulating antiapoptotic TIAF1, TIF2 and a novel matrix protein [11–14]. TGF-β1 counteracts PH-20-induced TNF-susceptibility in L929 cells [8]. TGF-β1 suppresses PH-20-mediated ERK activation [16], whereas ERK activation is not related with PH-20-induced TNF susceptibility in L929 cells [15]. Whether the proapoptotic p53 and WOX1 block the antiapoptotic function of TIAF1 and TIF2 is not known.

![Figure 1](http://www.biomedcentral.com/1471-2121/3/8)
PH-20 inhibits TGF-β1-induced TNF-resistance [15]. TGF-β1 suppresses PH-20-mediated ERK activation [16]; however, the ERK activation is not related with PH-20-increased TNF susceptibility in L929 cells [15]. Whether the proapoptotic p53 and WOX1 block the function of antia-poptotic TIAF1 and TIF2 is not known. In this study, experiments were designed to determine whether lysosomal Hyal-1 and Hyal-2 enhance TNF cytotoxic function and counteract TGF-β1-mediated TNF-resistance in L929 cells.

The full-length murine hyaluronidase *Hyal1* and *Hyal2* cDNAs were found in the EST database (GenBank accession AA688635 and BF139787, respectively) and the determined DNA sequences have been deposited in the GenBank (*Hyal1*, AF422176; *Hyal2*, AF422177). The *Hyal1* cDNA encodes a 463-amino-acid protein, which has 99.1% identity to a reported murine sequence (AF011567) [18]. Differences in the protein sequences are Gly-247, Gly-284 and Cys-450 in our Hyal-1 protein, compared to Arg-247, Glu-284 and Arg-450 in AF011567. Transitions in these amino acid residues are non-conservative.

The murine *Hyal2* cDNA (AF422177) encodes a 473-amino-acid protein. The deduced protein sequence is identical to a reported murine sequence (AF302844), but has an Ile-355 to Val-355 transition compared to another clone (AF302843). Variation in the protein sequence is also found in a murine Hyal-2 homologue (NP_034619) [22], which possesses three unique 2-, 5- and 7-amino-acid segments, not identical to the above Hyal-2 proteins.

L929 cells were engineered to stably express GFP-Hyal-1 or GFP-Hyal-2 fusion proteins. The presence of these proteins in these cells was demonstrated in Western blotting using anti-GFP antibody (data not shown). Exogenous bovine testicular hyaluronidase PH-20 stimulates rapid activation of c-Jun N-terminal kinases (JNK1 and JNK2) in L929 cells [16]. In contrast, the stably expressed Hyal-1 and Hyal-2 could not mediate constitutive activation of JNK1 and JNK2 in L929 cells (data not shown).

Exposure of the Hyal-2-expressing L929 cells to TNF for 16–24 hr resulted in increased cell death by ~60–110%, compared to the control GFP-expressing cells (Fig. 2). These results are similar to the bovine testicular PH-20-increased TNF cytotoxicity of L929 cells [15]. In contrast, there were no significant differences in the extent of cell death when GFP-Hyal-2 and control GFP-expressing cells were exposed to staurosporine, actinomycin D, daunorubicin, or camptothecin for 16–24 hr (Fig. 2).

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There was also an increased susceptibility to TNF cytotoxicity in the Hyal-1-expressing L929 cells (approximately 20–90% increase; also see Fig. 7), compared to the GFP control cells. However, there were no significant differences among these cells in the extent of death caused by staurosporine, actinomycin D, daunorubicin and camptothecin (data not shown).

In similar experiments, the effect of transient expression of Hyal-1 and Hyal-2 on cellular sensitivity to anticancer drugs was examined. L929 cells were electroporated with the GFP-Hyal-1 or GFP-Hyal-2 construct, cultured for 48 hr and then exposed to actinomycin D, daunorubicin or...
Bovine testicular PH-20 enhances TNF sensitivity in L929 cells by increasing the expression of proapoptotic p53 and WOX1 [9,10]. The ability of Hyal-1 and Hyal-2 to induce the expression of p53 and WOX1 in L929 cells was examined. L929 cells were electroporated with nothing (null), GFP, GFP-Hyal-1 or GFP-Hyal-2 constructs and cultured for 48 hr. Transient expression of GFP-Hyal-1 or GFP-Hyal-2 in L929 cells induced WOX1 expression, whereas no WOX1 expression was observed in non-transfected cells or control GFP-expressing cells (Fig. 3A). The 46-kDa band is full-length WOX1, while the 31-kDa band is a degraded WOX1 [10]. No detectable p53 expression was observed in these cells (Fig. 3A).

In comparison, the cytosolic WOX1 (46 kDa) was constitutively expressed in the L929 cells stably transfected with the GFP-Hyal-2 construct, while TNF reduced WOX1 expression in a time-dependent manner (Fig. 3B). This reduction is probably due to TNF-mediated WOX1 nuclear translocation from the mitochondria [10]. In contrast, WOX1 was not expressed in L929 cells stably transfected with the GFP-Hyal-1 construct, and TNF induced WOX1 expression in 40 min (Fig. 3B). TNF did not induce WOX1 expression in the control GFP-expressing cells (Fig. 3B).

Whether Hyal-2 enhances the apoptosis-inducing function of WOX1 was determined. In transient transfection experiments, L929 cells were electroporated with GFP-Hyal-2 and/or WOX1 constructs. GFP-Hyal-2 enhanced WOX1-mediated L929 cell death, as determined 48 hr post-transfection using crystal violet staining (Fig. 3C). In contrast, GFP-Hyal-1 did not enhance WOX1-mediated cell death (Fig. 3C). GFP-Hyal-1 and GFP-Hyal-2 alone did not mediate cell death (Fig. 3C).

Next, the effect of TNF-mediated IκBα degradation and NF-κB activation was examined in both Hyal-1 and Hyal-2 cells. The GFP-Hyal-1 and GFP-Hyal-2 stable transfectants and control cells were exposed to TNF for various times. IκBα degradation and NF-κB activation (or NF-κB nuclear translocation) were observed at 20 to 60 min in Hyal-2 cells post TNF stimulation, but at the 20 min time point in both control and Hyal-1 cells (Fig. 4).

To further understand how Hyal-2 participates in the apoptosis pathway, the translocation of Hyal-2 to the mitochondria was examined during cell death. Transient expression of GFP-Hyal-2 in COS7 fibroblasts showed the localization of the expressed protein in the lysosome but not in the mitochondria, as determined by staining the cells using LysoTracker and Mitotracker Red (data not shown). These observations are consistent with other reports [5,20]. COS7 cells are TNF-resistant. The reasons for using this cell type is its large size (which provides better resolution under fluorescence microscopy) and the ease of transfection using crystal violet staining (two representative rows of cell stains shown at left and the analyzed data shown at right; mean ± standard deviation, n = 4). Cell death. In controls, GFP-Hyal-1 or GFP-Hyal-2 alone failed to induce cell death.

camptothecin for 24 hr. There were no differences in cell death in these Hyal-1 and Hyal-2-expressing cells, compared to control cells (data not shown). These findings are consistent with the results for stable transfecants presented above (Fig. 2).
of transfection and gene expression using liposome-based reagents such as FuGene 6 and GeneFector. Exposure of the GFP-Hyal-2-expressing COS7 cells, as well as control L929 cells, were exposed to TNF (50 ng/ml) for 0, 20, 40, 60 and 120 min, followed by analysis of protein expression by Western blotting. TNF mediated a prolonged cytosolic IκBα degradation, as well as a nuclear presence of NF-κB p65 (or NF-κB activation), over a 20 to 60 min time period in Hyal-2 cells. A similar effect was seen only at 20 min in Hyal-1 and control cells.

Next, whether TGF-β1 antagonizes the function of Hyal-1 and Hyal-2 in growth regulation was examined. TGF-β1 inhibits epithelial cell growth but promotes fibroblast growth [15]. TGF-β1 inhibition of epithelial cell growth is blocked by bovine testicular PH-20 [15]. In agreement with our previous observations [11], TGF-β1 promoted L929 cell growth by approximately 20% in 24–48 hr (Fig. 6). However, TGF-β1 did not promote the growth of L929 cells stably expressing Hyal-1 or Hyal-2 (Fig. 6).

Finally, the functional antagonism between TGF-β1 and Hyal-1 or Hyal-2 in regulating TNF susceptibility in L929 cells was examined. The Hyal-1 and Hyal-2 stable transfectants were pretreated with TGF-β1 for 1 hr and then treated with TNF overnight. Both Hyal-1- and Hyal-2-increased TNF cytotoxicity were suppressed by TGF-β1 (30–50%) (Fig. 7). However, TGF-β1 blocked TNF cytotoxicity of the GFP-expressing control cells to a greater extent (50–70%) (Fig. 7).

Discussion
In this study, the novel function of the lysosomal hyaluronidases Hyal-1 and Hyal-2 in increasing cellular sensitivity to TNF cytotoxicity is reported. TGF-β1 counteracted Hyal-1- and Hyal-2-increased TNF killing of L929 fibroblasts. TGF-β1 increased L929 growth, while Hyal-1 and Hyal-2 blocked this enhancement of cell growth. These observations are consistent with our previous finding that exogenous bovine testicular hyaluronidase PH-20 enhances TNF cytotoxicity in various cancer cells, and that TGF-β1 blocks the increased TNF cytotoxicity [8,15]. As summarized in the Figure 8, the underlying mechanisms whereby Hyal-2 induces TNF cytotoxicity appear to be associated with 1) Hyal-2 induction of the proapoptotic WOX1, 2) Hyal-2 migration to the mitochondria during apoptosis, and 3) the prolonged period of IκBα degradation and NF-κB activation in TNF-treated Hyal-2-expressing cells.

Upregulation of p53 and WOX1 contributes, in part, to the PH-20-enhanced cytotoxic function of TNF [9,10,23]. WOX1 is a p53-binding protein. Ectopic expression of both p53 and WOX1 cDNA constructs results in colocalization of these proteins in the mitochondria [23]. Increased synthesis and complex formation between p53 and WOX1 in the cytoplasm are observed during apo-
sis [23]. Both p53 and WOX1 mediate apoptosis synergistically. Overexpressed WOX1 alone mediates apoptosis independently of p53 [10]. However, p53 apoptosis requires the participation of WOX1. Blocking WOX1 expression with antisense mRNA abolishes p53-mediated cell death, suggesting that WOX1 is a potential partner of p53 in apoptosis [10,23].

Both Hyal-1 and Hyal-2 induced WOX1 expression when transiently expressed in L929 cells. However, only Hyal-2 induced WOX1 expression following stable transfection of L929 cells. This correlates with the greater extent of Hyal-2 enhancement of TNF killing compared to Hyal-1. WOX1 expression was induced by TNF in the Hyal-1 stable transfectant. In transient transfection experiments, Hyal-2 enhanced WOX1-mediated L929 cell death, while Hyal-1 had no effect. Both Hyal-1 and Hyal-2 alone failed to induce cell death. Thus, induction of WOX1 expression contributes, in part, to Hyal-1- and Hyal-2-increased TNF sensitivity in L929 cells.

TNF rapidly induces phosphorylation of IκBα, which normally peaks at 5–10 min, followed by proteasome-dependent degradation (10–20 min) [10,24]. IκBα degradation and NF-κB activation occurred, as deter-

**Figure 6**

TGF-β1 promotes L929 cell growth but not the growth of Hyal-1 and Hyal-2-expressing L929 cells. Non-transfected L929 cells or the stable GFP-Hyal-1 and GFP-Hyal-2-expressing cells were treated with TGF-β1 (2 ng/ml) for 24 hr. The extent of cell growth was measured by staining the cells with crystal violet (mean ± standard deviation; n = 8).

**Figure 7**

TGF-β1 suppresses Hyal-1 and Hyal-2-increased TNF cytotoxicity in L929 cells. L929 cells stably expressing GFP, GFP-Hyal-1 or GFP-Hyal-2 were cultured in 96-well plates overnight, treated with TGF-β1 (2 ng/ml) for 2 hr, and then cotreated with TNF-α (5–40 ng/ml) for 24 hr. TGF-β1 inhibited TNF-α killing of L929 cells expressing GFP, and GFP-Hyal-1 or GFP-Hyal-2 (mean ± β1 inhibited TNF-α killing of the control GFP L929 cells to a greater extent (50–70%) than the GFP-Hyal-1 or GFP-Hyal-2 cells (30–50%).

**Figure 8**

A summarized scheme for Hyal-2-induced TNF susceptibility in L929 cells. Upregulation of proapoptotic WOX1 but not p53 was observed in the Hyal-2-expressing cells. Unlike PH-20, Hyal-2 could not induce constitutive JNK activation. TNF cytotoxicity was enhanced in these Hyal-2-expressing cells. The enhancement of TNF function is likely due to 1) the induced proapoptotic WOX1, 2) the likely translocation of Hyal-2 to the mitochondria in response to TNF, and 3) the prolonged period of IκBα degradation and NF-κB activation in as mediated by TNF.
minded 20 min after exposure of control and Hyal-1 cells to TNF. However, there was an extended period of TNF-mediated IκBα degradation and NF-κB activation in Hyal-2 cells, ranging from 20–60 min. It is unclear whether this extended period of IκBα degradation and NF-κB activation directly contributes to the ability of Hyal-2 to enhance TNF-mediated apoptosis. IκBα is a physiological inhibitor of NF-κB activation; IκBα complexes with NF-κB and prevents its translocation to the nucleus [10,24]. NF-κB activation is believed to induce antiapoptotic proteins to block cell death [8]. However, in several studies activated NF-κB cannot protect cells from apoptosis [8]. Also, NF-κB appears to be essential in the p53-mediated apoptosis in tested cell lines [25].

The mitochondrion is a reservoir of proapoptotic proteins and plays a key role in apoptosis [[26,27]; reviews]. One of the pathways that leads to the release of proapoptotic proteins in the mitochondria has been determined. For example, the TNF/TNF receptor-signaling complex activates caspase 8, which leads to the activation and cleavage of Bid, a proapoptotic protein of the Bcl-2 family. Activated or truncated Bid (tBid) in turn activates Bak and Bax. Bak migrates to the mitochondrial outer membrane where it generates transmembrane pores; Bax induces the opening of the mitochondrial permeability transition pores. Cytochrome c is then released from the mitochondria into the cytosol, where it interacts with downstream proapoptotic proteins such as Apaf-1 and caspase 9. Activation of these proteins further activates nucleases, such as caspase-activated DNase (CAD), which cause DNA fragmentation.

During staurosporine-mediated apoptosis, a portion of the lysosomal Hyal-2 migrates to the mitochondria. Whether Hyal-2 causes mitochondrial membrane damage is unknown. Presumably, Hyal-2 interacts with a specific protein target on the mitochondrial surface that changes the membrane permeability. This notion is supported by our recent screening of Hyal-1 and Hyal-2 interacting proteins in yeast two-hybrid interactions (Chang et al, unpublished).

The functional property of Hyal-2 in degrading hyaluronan is controversial. Hyal-2 is found in the lysosome and is active under conditions of low pH [20]. Hyal-2 is also found on the cell surface as a glycosylphosphatidylinositol (GPI)-anchored receptor for jaagsiekte sheep retrovirus [21]. Nonetheless, the enzymatic activity of Hyal-2 has not been detected [21]. Whether the increased TNF cytotoxicity in L929 cells caused by Hyal-1 and Hyal-2 is associated with their enzymatic activities is being studied in this laboratory.

The TGF-β1 inhibition of Hyal-1 and Hyal-2 effects could be abolished by cycloheximide or actinomycin D (data not shown). That is, inhibition of protein synthesis or gene transcription reduced the inhibitory effect of TGF-β1 in the prevention of Hyal-1- or Hyal-2-increased TNF cytotoxicity. Whether TGF-β1-induced antiapoptotic proteins such as TIAF1 and TIF2 inhibit the function of Hyal-1 and Hyal-2 remains to be determined.

Conclusions

Like testicular PH-20, lysosomal Hyal-1 and Hyal-2 enhanced TNF susceptibility in L929 cells. TGF-β1 suppressed the induced TNF sensitivity. Furthermore, TGF-β1-mediated L929 cell proliferation was blocked by Hyal-1 and Hyal-2. These data indicate that there is a functional antagonism between TGF-β1 and Hyal-1 or Hyal-2. Hyal-2-increased TNF susceptibility in L929 cells appears to be associated with 1) Hyal-2 upregulation of the proapoptotic WOX1, 2) Hyal-2 migration to the mitochondria during apoptotic cell death, and 3) the prolonged IκBα degradation and NF-κB activation in Hyal-2-expressing cells stimulated with TNF.

Materials and Methods

cDNA constructs, transient expression and stable transfectants

The full-length murine hyalurondase Hyal1 and Hyal2 cDNAs were found in the EST database (Genbank accession AA688635 and BF139787, respectively) and purchased from Incyte Genomics (St. Louis, MO). The determined DNA sequences have been deposited in the GenBank (Hyal1, AF422176; Hyal2, AF422177). The coding regions were tagged with a C-terminal green fluorescent protein (GFP) sequence by subcloning into the EcoR1 site of the pEGFP-N1 vector (Clontech, Palo Alto, CA). Murine L929 cells were transfected with the constructed Hyal1-pEGFP-N1 or Hyal2-pEGFP-N1 vector, or a control pEGFP-N1 vector by electroporation (40 μg DNA / 5 × 10^6 cells; 250 volt and 960 Fd), as previously described [10]. The cells were cultured in the continuous presence of G418 (200 g/ml) for approximately 2 weeks, and the resulting total cell colonies were harvested. The presence of GFP-Hyal-1 and GFP-Hyal-2 proteins in these stable transfectants was examined by Western blotting using anti-GFP antibodies (Clontech). Where indicated, COS7 fibroblasts were transfected with the above constructs using a liposome-based reagent FuGene 6 (Roche, Indianapolis, IN) or GeneFector (Venn Nova, Pompano Beach, FL). Protein expression was visualized by fluorescence microscopy. Also, the cells were counterstained with LysoTracker or Mitotracker Red (Molecular Probes, Eugene, OR) to determine whether the expressed protein was located in the lysosome or mitochondria.

Cell lines and chemicals

Murine L929 fibroblasts and monkey kidney COS7 fibroblasts were cultured as previously described [10]. Stau-
rosporine, daunorubicin, actinomycin D, camptothecin and DAPI were from Calbiochem (San Diego, CA), Sigma (St. Louis, MO) and Biomol (Plymouth Meeting, PA). Purified human platelet TGF-β1 was from R&D (Minneapolis, MN). Antisera against WOX1 were generated in rabbits against a synthetic peptide [10]. Antibodies against p53, IκBα, NF-κB p65 and α-tubulin were from Santa Cruz Biotechnology (Santa Cruz, CA) and Accurate Chemicals (Accurate Chemical, Westbury, NY).

**TNF cytotoxicity assay and Western blotting**

The above established cell lines were treated with TNF-α (2.5–20 ng/ml; Pepro-Tech, Rocky Hill, NJ) for 16–24 hr. The extent of cell death was determined by crystal violet staining [10]. Where indicated, the cells were also exposed to staurosporine, actinomycin D, daunorubicin, and camptothecin for 16–24 hr, before determining the extent of cell death. To determine whether TGF-β1 blocked Hyal-1 and Hyal-2-induced TNF killing, the cells were pretreated with TGF-β1 for 2 hr and then exposed to TNF-α in the continued presence of TGF-β1 for 16–24 hr. The extent of cell death was then determined. Where indicated, GFP-Hyal-1, GFP-Hyal-2 and GFP stable transfectants were treated with TNF (50 ng/ml) for 0, 20, 40, 60 and 120 minutes. The cells were extracted using NE-PER Nuclear and Cyttoplasmic Extraction Reagents (Pierce, Rockford, IL) to separate the cytoplasmic and nuclear fractions. These preparations were subjected to SDS-PAGE and Western blotting using specific antibodies against IκBα, WOX1 and NF-κB.

**Gene transfection and fluorescent microscopy**

COS7 cells were transfected with the GFP-Hyal-2 vector using the liposome-based FuGene 6 or GeneFector, cultured for 24–48 hr, and treated with staurosporine (1 M) for 0, 1, 2 and 4 hr. Mitochondria were stained with the membrane potential sensitive Mitotracker Red. Nuclei were stained by DAPI. The cells were then examined by fluorescence microscopy.

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