

Prostate cancer cells undergoing ER stress *in vitro* and *in vivo* activate transcription of pro-inflammatory cytokines

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Background: Several micro-environmental and cell-intrinsic stimuli cause tumor cells to undergo endoplasmic reticulum (ER) stress *in vivo*. The occurrence of an ER stress response has been associated with tumor progression and angiogenesis. Recently, we found that pharmacological induction of ER stress in B lymphoma cells upregulates the transcription of several pro-inflammatory cytokines.

Results: Here, we show that transgenic adenocarcinoma of the mouse prostate (TRAMP) C1 murine prostate cancer cells induced to undergo ER stress *in vitro* activate the transcription of interleukin 6 (IL-6), interleukin 23p19 (IL-23p19), and tumor necrosis factor α (TNF- α). Furthermore we show that TRAMP C1 tumors growing *in vivo* spontaneously experience ER stress and that transcription of IL-6, IL-23p19, and TNF- α correlates with the *in vivo* ER stress response.

Conclusions: These results suggest that an ER stress response in prostate cancer cells activates a program of pro-inflammatory cytokine transcription. A possible implication of this finding is that cancer cells may use the ER stress response to modify their microenvironment.

Keywords: unfolded protein response, tumorigenesis, inflammation

Background

The endoplasmic reticulum (ER) is the initial checkpoint for the biosynthesis, folding, assembly, and modification of membrane-bound and secreted proteins in all eukaryotic cells. Stimuli that cause the accumulation of un/misfolded proteins in the ER lumen result in a condition known as ER stress.¹ Eukaryotic cells have evolved a set of intracellular signaling pathways known collectively as the unfolded protein response (UPR) that facilitates cellular adaptation to ER stress. In mammalian cells, the UPR is initiated by three ER membrane-bound sensors (inositol-requiring protein 1 α (Ire1 α), Activating transcription factor 6 (Atf6), protein kinase-like endoplasmic reticulum kinase (PERK)), which in unstressed cells, are maintained in an inactive state through association with the ER chaperone molecule Grp78.² When a cell experiences ER stress, Grp78 disassociates from each of the three sensor molecules to preferentially bind un/misfolded proteins, causing each sensor to activate downstream signaling cascades, which ameliorate ER stress via several mechanisms, including selective translation inhibition and upregulation of genes that encode enzymes that aid in protein folding, maturation, and degradation.³ Involved in this homeostatic/regulatory cascade are two target genes *Myd116* (growth arrest and DNA damage-inducible protein (Gadd34)) and C/EBP homologous protein (CHOP) that are associated with translational recovery and apoptosis, respectively.¹

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Tumor cells *in vivo* are continuously exposed to ER stress in their microenvironment through hypoxia, low nutrient supply, and low pH. Tumor-intrinsic causes of ER stress include oxidative stress, defective glycosylation, and defects in calcium homeostasis.⁴ Evidence suggests that the ability to mount the UPR confers upon tumors a growth advantage. Primary human tumor cells of many different origins, including breast,⁵ lung,⁶ liver,⁷ colon,⁸ and prostate,⁹ have been shown to upregulate various elements of the ER stress response, including GRP78. In primary human melanoma specimens, the level of GRP78 positively correlates with tumor progression.¹⁰ Conversely, Grp78 hemizygous mice crossed with MMTVPyVT heterozygous transgenic mice display significantly decreased tumor proliferation, survival, and angiogenesis compared to Grp78^{+/+}, PyT mice.¹¹ Additionally, the inactivation of ER stress signaling by mutations of PERK, or by the introduction of a dominant-negative PERK, in human colon cancer cells, results in tumors that are smaller, grow less rapidly, and display abnormal angiogenic ability, as compared to their normal counterparts when implanted into mice.^{12,13}

Since Virchow's original suggestion of a link between inflammation and tumorigenesis, the idea that inflammation in the tumor microenvironment serves as a potent driver of tumor progression has been validated by epidemiological and molecular evidence. For instance, gastrointestinal carcinogenesis is associated with *Helicobacter pylori* infection, and lung cancer correlates with exposure to smoking and asbestos.^{14,15} Tumor necrosis factor α (TNF- α) produced by stromal cells causes adjacent hepatocytes to undergo transformation into malignant cells via nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) activation¹⁶ and, conversely, deletion of NF- κ B in hepatocytes reduces the incidence of liver tumors.¹⁷ An additional source of inflammation in the tumor microenvironment is infiltrating leukocytes, most notably, tumor-associated macrophages.^{18–20}

Recently, ER stress has been linked both to several inflammatory diseases and cancer.^{3,4} Support for the idea that ER stress signaling activates an inflammatory program comes from evidence demonstrating that signaling through each of the three ER stress sensors can activate NF- κ B, a master regulator of inflammation.^{21–23} Previous work from this laboratory suggested a link between ER stress and the transcription of pro-inflammatory cytokines *in vitro*. Notably, genome-wide array of RNA of murine B lymphoma cells subjected to ER stress induced by thapsigargin, a canonical ER stressor, showed increased transcription of pro-inflammatory cytokines, including interleukin (IL)-6, IL-23p19, and TNF- α .²⁴ Here

we present evidence that prostate cancer cells undergoing ER stress *in vitro* or *in vivo* also activate a program of pro-inflammatory cytokine transcription.

Results and discussion

We used quantitative PCR (qPCR) to analyze the effect of thapsigargin on murine transgenic adenocarcinoma of the mouse prostate (TRAMP) C1 prostate cancer cells *in vitro*. As expected, cells exposed to thapsigargin (0.3 μ M) upregulated the ER stress-responsive genes Grp78, Gadd34, and CHOP relative to vehicle-treated cells (Figure 1A). IL-6 and IL-23p19 transcript levels also increased 26- and 53-fold, respectively, over vehicle-treated cells at the latest time point measured (Figure 1B). TNF- α transcript increased at 8 hours and declined thereafter. Increased transcription of TNF- α was observed but its kinetics *in vitro* seems to follow a pattern inverse to that of Grp78, Gadd34, and CHOP (Figure 1, data not shown), suggesting that it may be regulated differently than IL-6 and IL-23p19 by UPR signaling.

Corroborating these data, we observed an increase in intracellular IL-6 protein in TRAMP-C1 cells treated for 18 hours with thapsigargin (data not shown). Taken together, these data suggest that induction of ER stress in prostate cancer cells activates a program of pro-inflammatory cytokine transcription. It should be pointed out that we observed the same phenomenon in diverse tumor cell lines representative of melanoma, ovarian cancer, and T- and B-cell lymphoma (Gonzalo Almanza, unpublished data).

To corroborate this notion and assess whether a similar phenomenon would occur spontaneously *in vivo*, we utilized a heterotopic tumor transplant model. Briefly, 5×10^6 TRAMP C1 cells were injected subcutaneously into syngeneic C57BL/6 mice, and the tumors excised 7 days later to assess the activation of ER stress markers and cytokine transcription by qPCR. All but one tumor showed increased expression of the ER stress marker Grp78 (data not shown) and tumor IL-6, IL-23p19, and TNF- α expression highly correlated with Grp78 expression (Figure 2). These data suggest that TRAMP C1 tumors undergo spontaneous ER stress *in vivo* and upregulate the transcription of pro-inflammatory cytokine genes.

Admittedly, the exact source of these cytokines was not determined and is presently unknown. However, since *in vitro* cultured TRAMP C1 cells activate the transcription of IL-6, IL-23p19, and TNF- α under ER stress, we argue that ER-stressed tumor cells are a likely source of these cytokines *in vivo*. A contribution by tumor-associated myeloid cells²⁵ to

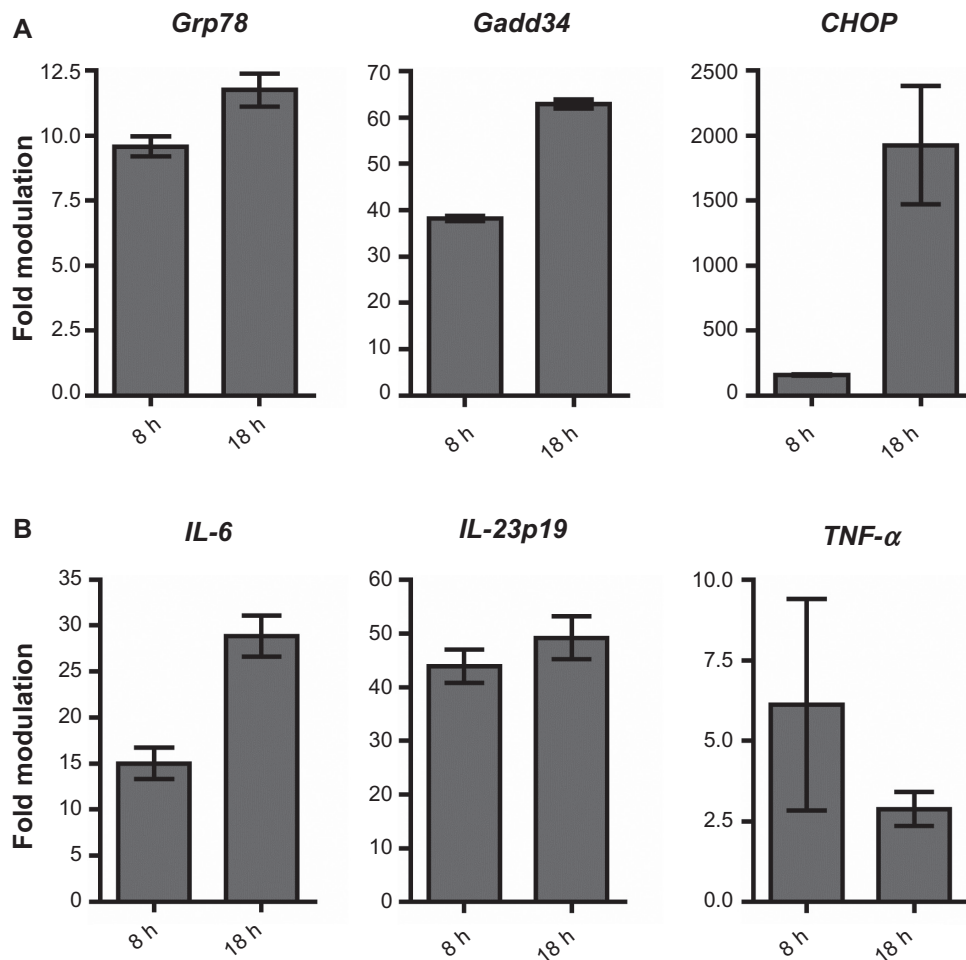


Figure 1 TRAMP C1 cells activate pro-tumor inflammatory cytokines during ER stress *in vitro*. TRAMP C1 cells were treated with thapsigargin (Tg) or vehicle control for the indicated times and assayed for **(A)** ER stress marker expression, and **(B)** pro-inflammatory cytokine transcription, by qPCR. Data columns indicate the mean fold difference in transcript level between Tg- and vehicle-treated TRAMP C1 cells. Error bars represent standard error of the mean (sem.) of 2 independent experiments.

the observed transcriptional activation of IL-6, IL-23p19, and TNF- α cannot be formally excluded; however, since the accumulation of myeloid cells in tumors on day 7 post-implantation (as was the case in this study) is negligible, if any,²⁶ we maintain that the present results can be explained on the basis of tumor cells being the source of pro-inflammatory cytokine transcription. Future experiments based on cell sorting, coupled with

phenotype characterization, will be needed to precisely assess the role of other cells in tumors *in vivo*.

The tumorigenic nature of IL-6, IL-23, and TNF- α ²⁷⁻²⁹ as well as of other molecules released by tumor cells and tumor infiltrating immune cells under ER stress,³⁰ makes it plausible to suggest that one of the mechanisms by which ER stress aids tumor growth is by mediating the release of

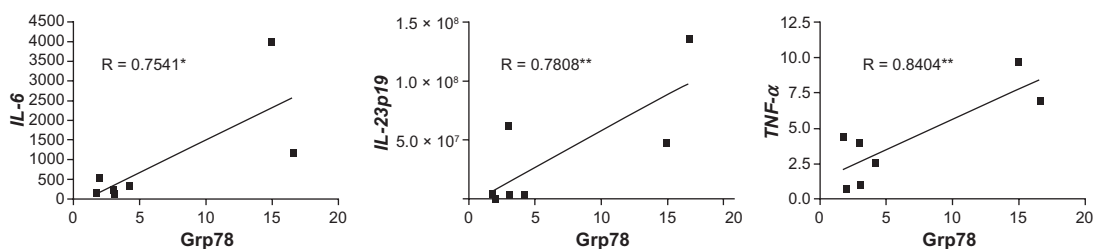


Figure 2 TRAMP C1 cells forming tumors *in vivo* undergo ER stress and transcriptional activation of pro-inflammatory cytokine genes. TRAMP C1 cells (5×10^6) were injected subcutaneously into 12- to 14-week-old male C57BL/6 mice. Seven days after injection, tumors were surgically excised, mechanically dissociated, and assayed for Grp78 and pro-inflammatory cytokine transcription, by qPCR. Data points refer to individual tumors, and indicate the fold modulation in transcript level between tumor samples and spleen cells from tumor-bearing mice. Correlation was sought using a two-tailed Pearson correlation test. * $P < 0.05$, ** $P < 0.05$.

these molecules, which in turn shape the immune landscape of the microenvironment to favor tumor progression. It will be interesting to see whether tumor cells under ER stress can influence surrounding immune cells, such as macrophages. In light of the fact that IL-6 and IL-23 have been shown to be involved in the differentiation and maintenance of the Th17 lineage,³¹ it is also possible that ER stress mediated IL-6 and IL-23 production by tumor cells may enable them to modulate CD4⁺ T-cells in their microenvironment.

Methods

Cells

TRAMP C1 cells were originally obtained through the courtesy of Dr Andrew Weinberg (Providence Portland Medical Center) and grown in complete RPMI-1640 medium supplemented with 10% fetal calf serum. Cells were treated with thapsigargin (Alexis Biochemicals) initially dissolved in 100% ethanol and diluted in culture medium to a final concentration of 0.3 μ M, or an equal volume of vehicle only, for the indicated times.

Mice and *in vivo* experiments

C57BL/6 Mice were purchased from Charles River and housed at the Moores Cancer Center Animal Facility and handled in accordance with University of California, San Diego Animal Subjects Program Guidelines (San Diego, CA, USA). For tumor inoculation, 5×10^6 TRAMP C1 cells were injected subcutaneously into the flank of 12–14 week old, male, wild-type C57BL/6 mice. Mice were sacrificed 7 days after tumor injection when ~4 mm tumors were visible. Tumors were surgically excised and mechanically dispersed into cell suspensions. Spleen cells from tumor bearing mice C57BL/6 mice were similarly prepared and used as controls.

Quantitative RT-PCR

RNA was isolated from cell suspensions using the Nucleospin RNA II Kit (Macherey-Nagel). Genomic DNA was digested by on-column treatment with DNase. Concentration and purity of RNA was determined by analysis on a NanoDrop spectrophotometer (Thermo Scientific). cDNA was obtained using the High Capacity cDNA Synthesis kit (Applied Biosystems) and quantitative PCR was performed in triplicate on an ABI StepOne system using TaqMan reagents. Target gene expression was normalized to β -actin, and analyzed using the $-\Delta\Delta C_t$ relative quantification method. Validated FAM-labeled mouse IL-23p19, IL-6, TNF- α , Ddit3 (CHOP), Myd116 (Gadd34), Hspa5 (Grp78), and VIC-labeled mouse

β -actin TaqMan primer/probe sets (Applied Biosystems) were used.

Statistical analysis

Statistical analysis was performed using two-tailed Pearson correlation test with 95% confidence with the aid of GraphPad Prism software (GraphPad Software, Inc., California, USA).

Conclusion

This report shows that murine prostate cancer cells undergoing ER stress initiate a program of pro-inflammatory cytokine transcription. TRAMP C1 cells treated *in vitro* with a canonical inducer of ER stress upregulate transcription of IL-23p19 and IL-6 concomitantly with upregulation of ER stress markers. It also shows that TRAMP C1 tumors *in vivo* spontaneously upregulate markers of ER stress which is strongly correlated with the transcription of IL-6, IL-23p19, and TNF- α .

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Disclosure

The authors report no conflicts of interest in this work.

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