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Restoration of immune response gene induction in trophoblast tumor cells associated with cellular senescence

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Abstract

Trophoblast cells and many cancer cells that harbor foreign antigens may evade immunity by epigenetic silencing of key immune response genes, including MHC class I and II and CD40. Chromatin active agents, such as histone deacetylase inhibitors (HDACi), induce immune response gene expression but often the expression levels are low and the cells lack a robust antigen presentation response. We show here that pre-treatment of trophoblast cells and certain cancer cells with agents that activate stress pathways (Ras oncogene, PMA or H₂O₂) and induce senescence can substantially enhance the induction of immune response genes (MHC class II, CD40, MICA, MICB) by HDACi and restore a vigorous IFN- γ response in trophoblast cells and tumor cells. These results could potentially impact the development of novel anti-cancer therapeutic strategies.

Keywords

Epigenetic regulation; ROS; IFN- γ ; HDACi; choriocarcinoma; MHC

1. Introduction

The ability of the semi-allogeneic fetus to persist during a normal pregnancy has been attributed to multiple factors (Hunt et al., 2005; Moffett and Loke, 2006; Trowsdale and Betz, 2006; Guleria and Sayegh, 2007). Prominent among the mechanisms suggested is that normal trophoblasts ‘escape’ maternal immunity by epigenetically repressing MHC and costimulatory gene expression. A generally similar concept for the escape of tumors from host immunity has also been recognized and found to be associated with the epigenetic silencing of immune response genes involved in both the initiation and effector phases of immunity (reviewed in Tomasi et al., 2006). In support of this, histone deacetylase inhibitors (HDACi), such as trichostatin A (TSA), which have broad HDAC specificity can directly alter the chromatin at immune response genes inducing MHC class II and CD40 genes and enhancing their cell

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surface expression (Magner et al., 2000; Khan et al., 2004; Chou et al., 2005). This induction of MHC and costimulatory molecules on tumors has been shown to elicit effective antigen presentation in vitro and durable immunity, mediated by both T and NK cells, in vivo (Chou et al., 2005; Khan et al., 2008; Khan et al., 2004; Khan et al., 2007).

In mammalian cells, histone H3 is phosphorylated following exposure to stressors that activate the MAPK and stress-activated protein kinases (SAPK). Both pathways lead to the downstream activation of MSK1 and MSK2 kinases which phosphorylate serine 10 and 28 of histone H3 and are associated with changes in chromatin accessibility (Clayton and Mahadevan, 2003). Histones that are phosphorylated at H3S10 are much more sensitive to lysine acetylation in response to the histone deacetylase inhibitor TSA (Clayton and Mahadevan, 2003). This suggests that histone modifications may be synergistically coupled. Terminal kinases may also directly phosphorylate transcription factors and co-factors and, as shown in recent studies, p38 MAPK can be recruited by transcription factors and co-factors to promoter complexes (Clayton and Mahadevan, 2003). Such recruitment could therefore lead to direct phosphorylation of histone H3, transcription factors and co-factors, as well as enhanced histone acetylation and methylation and chromatin remodeling.

Cellular senescence is induced in non-transformed as well as cancer cells by various cell stressors, including DNA damage, ROS (reactive oxygen species), irradiation, hypoxia, and by the activation of oncogenes, such as Ras (Bihani et al., 2007). Ras proteins elevate intracellular H₂O₂ which is thought to be a significant factor in mediating senescence (Lee et al., 1999). Ras-induced senescence is associated with activation of Raf/MEK/MAPK (Lin et al., 1998) and PI3K pathways (Malumbres and Barbacid, 2003). Noteworthy is that the senescence pathway must be bypassed for carcinogenesis to occur (Bihani et al., 2007; Collado et al., 2005) and therefore senescence, like apoptosis, is thought to represent a barrier to tumorigenesis (Braig et al., 2005). A role of chromatin in the initiation and maintenance of the senescent state has been suggested (Ogryzko et al., 1996; Bandyopadhyay et al., 2002; Munro et al., 2004; Braig et al., 2005) and HDACi have been shown to induce senescence (Ogryzko et al., 1996). HDACi activation of ATM (ataxia telangiectasia, mutated) (Gasser et al., 2005) likely contributes to a form of senescence similar to that which occurs following DNA damage and oncogene-induced senescence (Bartkova et al., 2006). HDACis can directly activate stress pathways, including MAPK, PI3K/TOR and NF- κ B (Ruefli et al., 2001; Mayo et al., 2003), and these pathways are often associated with senescence.

Here we addressed the hypothesis that pre- and co-treatments with stress-inducing agents may alter the subsequent response of cells which are refractory to immunostimulatory factors. We also show that stressors that activate kinase pathways and promote senescence enhance the sensitivity of trophoblast cell lines to HDACi. Additionally, pre-treatment with several agents that induce stress were shown to restore IFN- γ -mediated immune response gene induction in the trophoblast cells JAR and JEG-3. We also demonstrate that stressors that have an enhancing effect also induce cellular senescence and propose that senescent cells could have altered immune reactivity.

2. Materials and Methods

Cells and Reagents

The human trophoblast cell lines BeWo, JAR and JEG-3, mouse trophoblast cell line SM9, human cervical carcinoma cell line HeLa, and human fibroblast cell lines MRC-5 and IMR90 were from American Type Culture Collection (ATCC) (Manassas, VA) and cultured according to ATCC's instructions. Ras-transduced IMR90 cells were provided by A.W. Lin (Lin et al., 1998). Early passage (15 PDL) IMR90 lung fibroblasts were transduced with H-RasV12 or an empty vector (pBabe-puro) via retroviral gene transfer as previously described (Mason et al.,

2004). Cells were selected with puromycin (2.5 µg/ml, 3 days), cultured for an additional 6 days, and subsequently treated with 250-500 nM TSA from Wako Biochemical (Richmond, VA) or a vehicle control (ethanol) for 24 hours. Valproic acid, hydrogen peroxide (H₂O₂), phorbol-12-myristate-13-acetate (PMA), and ionomycin were obtained from Sigma (St Louis, MO). JAR cells were treated individually or in combination with 500 ng/ml PMA/10 µM ionomycin or 50 nM TSA. PMA/ionomycin treatments were 6 hours and TSA 24 hours. The Mek1/2 inhibitor U0126 (25 µM) and the p38 inhibitor SB203580 (20 µM) were from Calbiochem (Gibbstown, NJ). Human IFN-γ was obtained from R&D Systems (Minneapolis, MN). For pre-treatment experiments, JAR cells were treated with TSA (25 nM) for 4 hours prior to IFN-γ (100 U/mL) (20 hours), valproic acid (500 µM) for 4 hours prior to IFN-γ (100 U/mL) (20 hours), or H₂O₂ (250 µM) for 15 minutes, washed, and then treated with IFN-γ (100 U/mL) (24 hours). Senescence associated β-galactosidase staining of JAR cultures was carried out with the “Senescence β-Galactosidase Staining Kit” (Cell Signaling Technology, Danvers, MA) according to the manufacturer’s instructions. As a positive control, cells were γ-irradiated (5 Gy) and cultured for 72 hours prior to SA-β-gal staining.

Western blotting

Cells harvested for whole cell lysates were pelleted and lysed on ice for 30 minutes in RIPA lysis buffer (Sigma) supplemented with protease inhibitor cocktail (Sigma), 1 mM dithiothreitol (DTT, Sigma), and 2 mM sodium orthovanadate. The extracts were centrifuged at 10,000 x g for 10 minutes and the supernatants collected. For all proteins, with the exception of β-actin, 40 µg of cell lysates were resuspended in SDS sample buffer plus 0.13 M DTT, separated on 10% SDS-PAGE gels and transferred to Immobion-P membrane (BioRad, Hercules CA). For β-actin, 10 µg of protein was used. The antibodies employed were anti-Stat1, anti-IRF1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-β-actin (Abcam, Cambridge MA) and goat anti-rabbit IgG-horseradish peroxidase (Promega, Madison WI). Blots were developed with West Pico Chemiluminescent Kit (Pierce, Rockford IL). All immunoblot data presented in this manuscript are representative of two or more independent experiments.

Real-time Quantitative RT-PCR

Total RNA was isolated using the mirVana™ kit from Ambion (Austin, TX) and 2 µg was used for reverse transcription with Superscript™ II (Invitrogen). Real-time PCR was performed as previously described on an ABI7900HT (Applied Biosystems, Foster City, CA) (Magner et al, 2000). Amplification of cDNA samples was carried out with either Taqman PCR Master Mix or SYBR Green Master Mix (Eurogentec, San Diego, CA) according to the manufacturer’s protocol. Primer and probe sequences are available upon request. All assays were carried out in triplicate with standard deviation (SD), variance and Z-scores calculated within each experiment. Relative fold change in gene expression was determined by the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001) and plotted with error bars representing variance. Statistical significance, determined by Z-test, is indicated in the figures for values with $p < 0.005$ (Sadek et. al., 2008). Student t tests were used to assess the significance of differential expression in paired treatments (pairs of independent means). Figures incorporate data from individual experiments representative of at least two independent experiments.

3. Results

Enhancement of the HDACi responses by pre- and co-treatments that activate MAPK pathways

Cellular stressors may alter the cell’s ability to respond to the chromatin remodeling activity of HDACi and MAPK pathway activation which have been shown to directly phosphorylate histones and enhance the subsequent sensitivity of genes to HDACi. To explore the role of

kinase signaling pathways in HDACi activation of immune response genes we first employed a well-established system of activating the MAPK pathway by transduction of activated Ras into the human MRC-5 and IMR-90 non-transformed human fibroblast cells (Mason et al., 2004). TSA treatment induced significant expression of each of these genes relative to their respective controls ($p < 0.005$). Figure 1 demonstrates that transduction with activated Ras prior to TSA treatment resulted in significantly enhanced HLA-DR α and CD40 gene expression compared to vector transduced cells ($p < 0.05$). The phorbol ester PMA is a Ras agonist and pre-treatments with PMA were also found to enhance expression of these immune response genes in MRC5 (data not shown).

Since activation of the MAPK pathway with Ras and PMA enhanced MHC class II and CD40 induction by TSA in fibroblast cells, we investigated whether JAR trophoblast cells showed a similar enhancement. As shown in Figure 2, stress induced by PMA and ionomycin (PMA/ionomycin) synergizes with TSA in the activation of HLA-DR α in JAR cells. While there is a low level of CIITA induction, our previous work has demonstrated that TSA induction of MHC class II is CIITA-independent and that the CIITA induced in JAR cells is an inactive splice variant similar to the CIITA clone 10 previously described (Magner et al, 2000; Chou et al, 2005; Riley et al, 1995; Tomasi et al unpublished data). The CIITA clone 10 has an internal deletion of 147 bp as well as a C-terminal truncation which renders it inactive (Riley et al, 1995). Additionally, there is a robust enhancement of the induction of costimulatory molecules CD40, MICA and MICB, when PMA/ionomycin was employed prior to and in combination with TSA (Fig. 2). PMA/ionomycin treatment alone induced these same genes but at very low levels (Fig. 2). The MEK/ERK inhibitor U0126 blocked PMA/ionomycin enhancement of immune response gene induction, while the p38 inhibitor SB203580 did not (Fig. 2 and data not shown). The HDACi valproic acid also induced immune response genes in JAR, but to a lesser extent than TSA, and its activity was also enhanced by PMA/ionomycin (data not shown). These data suggest a contribution of the MAPK pathways in the epigenetic regulation by HDACi and a role for the MEK/ERK pathway in HDACi sensitization.

Enhancement of the IFN- γ response by pre- and co-treatments with HDACi

A potentially related issue is whether the cellular silencing pathways described above could be involved in the well-established defects in the IFN- γ induction of immune response genes in trophoblast tumor cells (Murphy and Tomasi, 1998; Morris et al., 2002; Tomasi et al., 2006). Our initial studies indicate that BeWo and JEG-3 trophoblast cells are capable of activating the classical IFN- γ responsive gene IRF-1 and yet CIITA and MHC class II are poorly inducible by IFN- γ compared to human kidney and HeLa cells (Table 1) (Chou et al., 2005). This suggests that a major target of silencing in trophoblast cells is CIITA. As shown in Figure 3A, pre-treatment with TSA followed by co-treatment with IFN- γ for 24 h leads to robust expression of CIITA, MHC class II and CD40 in JAR cells. This pre-treatment significantly enhanced the induction of each of these genes relative to the individual treatments ($p < 0.05$). The level of CIITA and MHC class II mRNAs obtained following this pre-treatment are similar to those found after IFN- γ treatment of HeLa or the macrophage line Raw 264.7 (data not shown). It is noteworthy that protein levels of IRF-1 and Stat1 are also enhanced by co-treatments (Fig. 3B). The ability to restore the IFN- γ pathway to a level equivalent to that of classical IFN- γ responsive cells (HeLa, Raw 264.7) suggests that the epigenetic silencing of MHC class II expression is essentially completely reversible in these trophoblast tumor cells.

Valproic acid, a common anti-epileptic therapeutic, has HDACi activity and was shown to have effects similar to TSA, although of lesser magnitude, in enhancing MHC, CIITA, Stat and IRF expression when combined with IFN- γ (Fig. 4A, B). While the induction of CIITA by sequential valproic acid and IFN- γ treatments was reproduced in multiple experiments, the small changes in CIITA transcript levels shown here from IFN- γ and valproic acid individual

treatments were not significant in multiple experiments. Thus, like TSA, the induction of MHC class II by valproic acid is also CIITA-independent. Moreover, the induction of CIITA and class II transcripts by valproic acid was enhanced ($p<0.05$) by the combination treatment relative to IFN- γ alone. Oxidative stresses that activate the MAPK pathway following H₂O₂ treatments also enhanced IFN- γ induced IRF-1, CIITA and MHC class II (Fig. 5). Our data indicates that low levels of CIITA are activated by pre-treatments and, while these may be responsible for some of the enhanced MHC class II expression following IFN- γ , it is likely that there is a direct synergistic effect of these pre-treatments at the level of the MHC class II gene. Importantly, the expression of CIITA and MHC class II transcripts was significantly enhanced ($p<0.05$) by the H₂O₂ and IFN- γ combination treatment relative to either individual treatment.

Induction of senescence

The pathways activated by the various stresses employed here are complex likely with unique features to each type of stress but with certain common characteristics including the activation of NF- κ B, MAPK and PI3K/TOR and potentially the production of intracellular inflammatory cytokines. It is of interest that the above stresses also induce cellular senescence. Ras elevates intracellular H₂O₂ which is a major factor in mediating senescence (Lee et al., 1999; Chen et al., 2000). Figure 6 and Table 2 illustrate that the TSA treatments employed in this study induces the senescence-associated β -galactosidase (SA- β -gal) marker in JAR cells. Of note is that the PMA/ionomycin treatment did not consistently induce significant levels of SA- β -gal staining in JAR cells but did show molecular markers characteristic of a senescent profile (Mason et al., 2004) with enhanced p21 and HDAC9 and decreased TopoII α (data not shown).

3. Discussion

The data demonstrate that pre-treatments with several agents that induce cellular stress are accompanied by an enhanced expression of certain immune response genes following HDACi or IFN- γ activation. Moreover, stress pre-treatments restore the ability of IFN- γ to induce MHC class II in trophoblast tumor cells. Co-treatments also are effective but generally less so than the pre-treatment combination. The detailed mechanisms by which diverse stresses activate the immune response genes are complex and uncertain. MAPK cooperate with other pathways and are subject to feedback and crosstalk which ultimately generate specific signals and cellular events which could alter immune phenomena (reviewed in Johnson and Lapadat, 2002). In addition, multiple MAPK phosphatases regulate the immune response (Liu et al., 2007) and various stresses could modulate phosphatase activity. It has been suggested that the IFN- γ response is dampened in trophoblast cells by phosphatase inhibition of the JAK/Stat1 pathway (Choi et al., 2007). For example, in our studies, stresses or direct acetylation by TSA of one or several protein phosphatases could inhibit enzyme activity and enhance Stat and IRF-1 pathway signals resulting in the effects noted here. An explanation for the result of Choi et al. (Choi et al., 2007) is that the reagent (vanadate) that was used to show the specific inhibition of phosphatase activity might itself generate H₂O₂. This may not have been fully inhibited by the catalase treatments employed. As demonstrated here H₂O₂ is a stress which enhances IFN- γ induction of IRF-1 and MHC class II. As noted in Table 1, variations in the level of the IRF-1 response to IFN- γ occur in different cell lines and the IRF-1 induction is present but at lower levels in JAR cells suggesting that, in this trophoblast cell, IRF-1 is relatively refractory to activation. There are likely several mechanisms operating in different cells, and even in the same cell under varying environmental conditions. However, a common theme in the trophoblast cells examined, which is also found in many tumor cells, is a diminished response of CIITA to IFN- γ (Chou et al., 2005).

Senescence is an irreversible proliferative arrest which is induced by a number of stressors including oxidative DNA damage as well as oncogene induced arrest, as employed here. However we emphasize that whether the enhanced expression of immune response genes following pre-treatment is a result of the senescence process per se, or only accompanies senescence, is uncertain. Moreover we do not know the detailed mechanisms involved and whether the enhanced HDACi activity and restoration of the IFN- γ response are mechanistically related. Since the induction of tumor growth arrest by senescence has been suggested as a potential therapeutic goal in cancer treatments (Shay and Roninson, 2004; Bihani et al., 2007), it will be important in future studies to determine whether the immune response genes of senescent tumor cells are more readily activated by HDACi and IFN- γ and if in vivo senescent tumor cells are more sensitive to immune elimination. In addition to inducing senescence, the pre-treatments employed here may also induce autophagy and apoptosis. These are distinct processes that can be distinguished morphologically and by markers but are interrelated and often occur in the same cell. For example, HDACi have been shown to induce senescence (Chen et al., 2000) and both autophagy and apoptosis occur in HeLa cells treated with HDACi (Shao et al., 2004). Whether the enhancement phenomenon leading to increased expression of immune response genes is a specific characteristic of senescence, autophagy, or apoptosis is not yet known.

The data obtained raise several other important questions. Does the inherent capacity of HDACi to activate chromatin, and the level of gene expression obtained with a given HDACi, depend on the ability of the HDACi to activate stress pathways? Is this a factor in determining some of the observed differences in the inherent activity of different HDACis in inducing immune response genes? Do endogenous histone acetyltransferase (HAT)/HDAC pathways behave similarly following changes in the cell's environment? For example, could there be stress-enhancing effects on the endogenous HAT activity, such as by CBP/p300. Importantly, would it be clinically advantageous to use combinations of chemotherapeutic drugs that activate stress pathways and senescence in conjunction with chromatin active agents? Additionally, are senescent trophoblast and tumor cells more readily eliminated by normal cellular mechanisms such as activated macrophages? Our current hypothesis is that the environment of the trophoblast and many tumor cells is repressive for immune response gene expression and that cellular stresses alter the sensitivity of the cells to IFN- γ and epigenetic agents.

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Figure 1. Ras activation enhances HDACi mediated immune response gene induction

Real time RT-PCR analyses demonstrate that TSA-mediated upregulation of CIITA, HLA-DR α , and CD40 is enhanced in Ras transduced IMR-90 fibroblast cells. Normal human IMR-90 lung fibroblasts were transduced with H-RasV12 or an empty vector and treated with TSA or a solvent control for 24 hours prior to real time RT-PCR analysis of gene expression. Fold changes in gene expression calculated from the real time RT-PCR data are shown with error bars representing SD and all expression levels shown here are significantly different from their respective controls ($p < 0.005$) (see Materials & Methods for details). The treatments were also analyzed to determine whether the differences were significant when the same treatment was applied to Ras transduced or control vector transduced cells. These analyses were carried out as pairs of independent means (pairs shown by bars) and those in which the t-test indicated significance at $p < 0.05$ are indicated with a double asterisk (**).

Figure 2. MAPK agonist treatment enhances TSA induction of immune response genes
JAR choriocarcinoma cells were treated individually or in combination (as indicated) with 500 ng/ml PMA/10 μ M ionomycin or 50 nM TSA. PMA/ionomycin treatments were 6 hours and TSA 24 hours. The Mek1/2 inhibitor U0126 was used at 25 μ M. Gene expression was analyzed by real time RT-PCR as described for Figure 1. Statistical significance ($p < 0.005$) relative to untreated control cells is indicated by asterisk (*). Student t-tests were used, as described for Fig. 1, to determine the significance of PMA/ionomycin enhancement of TSA responses. The pairs for which $p < 0.05$ are indicated with a bar and double asterisk.

Figure 3. TSA treatment of JAR cells enhances the IFN- γ induction of MHC class II

A. JAR cells treated with TSA (25 nM) for 4 hours prior to IFN- γ (100 U/mL) (20 hours), labeled TSA-IFN- γ in the figure, induce 5-fold more HLA-DR α than TSA alone (24 hours) as shown by real time RT-PCR. The individual treatments were 24 hours each. CIITA and CD40 induction was also enhanced with pre-treatment. **B.** Western blotting demonstrated that Stat1 and IRF1 protein levels were also enhanced by co-treatment. Statistical significance ($p < 0.005$) relative to untreated control cells is indicated by asterisk (*).

Figure 4. Valproic acid enhances IFN- γ induction of HLA-DR α in JAR cells

A. JAR cells treated with 500 μ M valproic acid for 4 hours prior to IFN- γ (100 U/mL 20 hours) induced considerably more HLA-DR α than valproic acid alone (24 hours). **B.** Western blotting demonstrated that Stat1 expression levels were enhanced by pre-treatment and IRF1 protein levels were slightly. The results shown are representative of duplicate experiments. Statistical significance ($p < 0.005$) relative to untreated control cells is indicated by asterisk (*). VA = valproic acid.

Figure 5. ROS enhance IFN- γ induction of MHC class II

A. JAR cells were pre-treated with 250 μ M H₂O₂ for 15 minutes, washed, and then treated with 100 U/mL IFN- γ for 24 hours. Pre-treatment enhanced CIITA and HLA-DR α RNA expression as shown by real time RT-PCR. **B.** Western blotting demonstrated that pre-treatment with H₂O₂ enhanced Stat1 and IRF1 protein expression. Statistical significance ($p < 0.005$) relative to untreated control cells is indicated by asterisk (*). DR α = HLA-DR α .

Figure 6. TSA treatment induced senescence

To determine whether the stresses employed in these studies induced senescence, JAR cultures were treated and stained for senescence-associated β -galactosidase (SA- β -Gal). **A.** Negative control - untreated cells. **B.** Positive control - cells stained 72 hours after irradiation (5 Gy). **C.** Culture treated with 25 nM TSA for 24 hours. Images shown are representative fields (magnification 50x).

Table 1IFN- γ responses represented as fold change from real time RT-PCR determinations

	CIITA	HLA-DRα	IRF-1
BeWo	0.8	0.4	25.3
JAR	2.2	2.0	8.0
JEG-3	2.7	0.4	27.0
Human kidney	18.9	99.7	26.0
HeLa	1154	1918	38.8

Table 2Quantitation of SA- β -gal staining of JAR cultures

Treatment	Blue cells/total counted	SA- β -gal pos (%)
Untreated	15/164	9
Irradiated (5 Gy)	79/118	67
25 nM TSA	71/173	41