4-Tertiary Butyl Phenol Exposure Sensitizes Human Melanocytes to Dendritic Cell-Mediated Killing: Relevance to Vitiligo

Tara M. Kroll,* Hemamalini Bommiasamy,* Raymond E. Boissy,† Claudia Hernandez,‡ Brian J. Nickoloff,* Ruben Mestril,§ and I. Caroline Le Poole*

*Department of Pathology/Oncology Institute, Loyola University, Chicago, Illinois, USA; †Department of Dermatology, University of Cincinnati, Ohio, USA; ‡Department of Medicine and §Department of Physiology/Cardiovascular Institute, Loyola University, Chicago, Illinois, USA

The trigger initiating an autoimmune response against melanocytes in vitiligo remains unclear. Patients frequently experience stress to the skin prior to depigmentation. 4-tertiary butyl phenol (4-TBP) was used as a model compound to study the effects of stress on melanocytes. Heat shock protein (HSP)70 generated and secreted in response to 4-TBP was quantified. The protective potential of stress proteins generated following 4-TBP exposure was examined. It was studied whether HSP70 favors dendritic cell (DC) effector functions as well. Melanocytes were more sensitive to 4-TBP than fibroblasts, and HSP70 generated in response to 4-TBP exposure was partially released into the medium by immortalized vitiligo melanocyte cell line PIG3V. Stress protein HSP70 in turn induced membrane tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression and activation of DC effector functions towards stressed melanocytes. Melanocytes exposed to 4-TBP demonstrated elevated TRAIL death receptor expression. DC effector functions were partially inhibited by blocking antibodies to TRAIL. TRAIL expression and infiltration by CD11c+ cells was abundant in perilesional vitiligo skin. Stressed melanocytes may mediate DC activation through release of HSP70, and DC effector functions appear to play a previously unappreciated role in progressive vitiligo.

Key words: autoimmune diseases/skin pigmentation/TNF-related apoptosis-inducing ligand
J Invest Dermatol 124:798–806, 2005

Vitiligo is an acquired skin disorder, involving an autoimmune response against melanocytes (Boissy, 2001; Le Poole *et al*, 2004). It remains to be explained as to what triggers the autoimmune response to melanocytes. Patients frequently refer to skin trauma as an initiating factor for their disease. Melanocyte overexposure to ultraviolet rays may cause deregulation of melanization and/or of mitosis, inducing a stress response in the pigment cell (Jean *et al*, 2001). Sites of mechanical stress will express elevated levels of stress proteins (Kippenberger *et al*, 1999). Burns and cuts have been documented as initiation sites for progressive depigmentation, and the Koebner phenomenon is often observed in vitiligo patients (Le Poole and Boissy, 1997). Finally, in individuals sensitive to bleaching phenols, exposure to phenolic compounds in the workplace can cause what has been coined "occupational vitiligo" (Boissy and Manga, 2004). Skin trauma leads to oxidative stress, and accumulation of H_2O_2 has been observed in vitiligo lesional skin (Schallreuter *et al*, 1999). These conditions will induce expression of stress proteins including heat shock protein (HSP)70 and will enhance the activity of anti-oxidative enzymes to protect skin cell viability (Currie and Tanguay, 1991; Calabrese *et al*, 2001; Renis *et al*, 2003). In this study, 4-tertiary butyl phenol (4-TBP) was chosen as a model compound to address stress protein expression and its involvement in initiating an autoimmune response to melanocytes by dendritic cells (DC).

It has been hypothesized that bleaching compound 4-TBP can serve as an alternative substrate for tyrosinase, which would explain its inhibitory effect on melanin synthesis (Yang and Boissy, 1999). Competitive inhibition of tyrosinase, the rate-limiting enzyme involved in melanogenesis, occurs at low 4-TBP concentrations. Conversion of 4-TBP into semiquinone free radicals can contribute to cellular stress (Boissy and Manga, 2004). Cytotoxic responses occur at a higher concentration of 4-TBP and are independent of the degree of pigmentation of melanocytes (Yang *et al*, 2000). Expression of the A_{2b} receptor for adenosine was enhanced in response to 4-TBP, and expression of this receptor may sensitize melanocytes to apoptosis (Le Poole *et al*, 1999).

Stressed cells are characterized by elevated expression of stress proteins. Stress proteins include the HSP family upregulated in response to elevated environmental temperatures and other forms of stress. Stress proteins are evolutionarily very well conserved, and they function as

Abbreviations: DC, dendritic cell; FACS, fluorescence activated cell sorting; FaSL, Fas ligand; HSP, heat shock protein; IFN, interferon; IL, interleukin; JAM, just another method; 4-TBP, 4-tertiary butyl phenol; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand

Presented in part at the 63rd Annual Meeting of the Society for Investigative Dermatology and at the 11th Annual Meeting of the Pan American Society for Pigment Cell Research.

The study was carried out at Department of Pathology/Oncology Institute, Loyola University, Chicago, Illinois, USA.

chaperone molecules protecting cellular proteins from premature degradation by supporting proper protein folding (Houry, 2001). Cells with elevated levels of stress proteins are protected from the consequences of subsequent stress episodes (Mestril and Dillmann, 1995).

Contrary to the cytoprotective effect of intracellular stress proteins, once released into the extracellular milieu stress proteins can induce an immune response to the very cells from which they were derived. Stress proteins are immunogenic and were shown to serve as antigens in certain autoimmune diseases, which is best explained by the extensive homology observed between human and bacterial stress proteins or "antigen mimicry" (Bell, 1996; Xu, 2003).

Besides serving as antigens, stress proteins also enhance an immune response by inducing phagocytosis and processing of chaperoned antigens by DC (Noessner *et al*, 2002). Consequently, stress proteins have been included as adjuvants in tumor vaccines (Srivastava and Amato, 2001).

Recently, it was reported that DC can specifically kill tumor cells whereas surrounding, healthy cells are left untouched (Janjic *et al*, 2002; Lu *et al*, 2002). DC-mediated killing was found to be mediated by expression of tumor necrosis factor (TNF) family members on the DC surface, accompanied by the expression of the appropriate receptors by tumor cells (Lu *et al*, 2002). Healthy control cells do not express the same levels of such receptors, and are thus protected from DC-mediated killing (Lu *et al*, 2002). The hypothesis under study is that DC are equally capable of killing stressed melanocytes to initiate an autoimmune response resulting in progressive depigmentation of the skin.

The direct effects of 4-TBP exposure on cell viability of control and vitiligo-derived melanocytes was measured. Induction of HSP70 induction was assessed, and expression of HSP70 was artificially elevated by adenoviral overexpression to evaluate its cytoprotective effect. DC exposed to activating stress proteins or activated by interferon-γ (IFN-γ) were reacted with stressed and unstressed melanocytes, and resulting melanocyte death was measured. The cytotoxicity observed was correlated to membrane expression of TNF family members by DC, and to corresponding death receptors on stressed melanocytes. Finally, the results were correlated to observations in vitiligo skin by immunohistology. These studies were performed to evaluate a possible role of stress proteins and of DC in initiating depigmentation.

Results

Viability of cells in the presence and absence of 4-TBP In Fig 1, the viability of normal melanocyte culture Mc0009 P12, as well as immortalized cell lines PIG1 and PIG3V, and normal fibroblast culture Ff9929 P7 was shown in the presence or absence of 4-TBP. At relatively low concentrations of 4-TBP (250 μM), the viability of both immortalized cell lines, PIG1 and particularly PIG3V, was significantly reduced (to 59.1% and 37.5%, respectively). The difference in viability among PIG1 and PIG3V cells was not considered significant at $p = 0.11$ in a *t* test. The viability of primary fibroblast and melanocyte cell cultures was not affected at 250 μM of 4-TBP. Overall, fibroblasts were less sensitive to 4-TBP than melanocytes and a significant reduction in fibroblast viability was noted only at 1 mM of 4-TBP ($p = 0.001$).

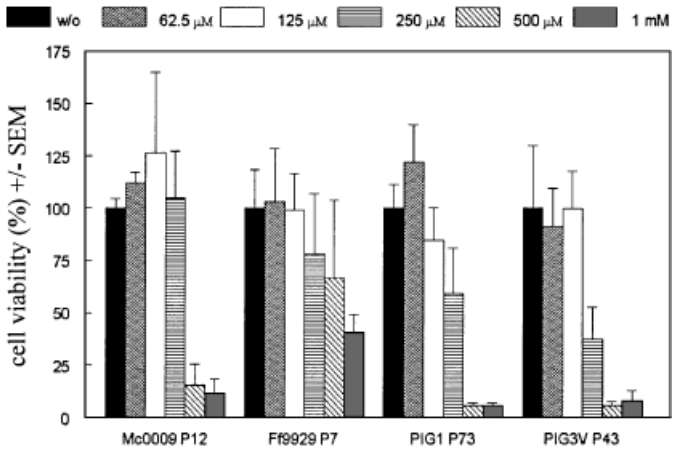

Figure 1
Reduced viability of skin cells in the presence of 4-tertiary butyl phenol (4-TBP). Cultured melanocytes Mc0009 P12, fibroblasts Ff9929 P7, immortalized normal PIG1, and vitiligo PIG3V melanocyte cell lines were subjected to 4-TBP exposure at different concentrations for 72 h. Cell viability ($\pm$ SEM) was measured in a just another method (JAM) assay. At 250 μM of 4-TBP, both immortalized cell lines experienced significantly reduced viability compared with untreated cells ($p = 0.013$ or 0.009 for PIG1 cells and PIG3V cells, respectively). Representative experiment of three performed.

Induction of HSP70 expression by 4-TBP Expression of HSP70 by immortalized melanocytes cultured in the presence or absence of 4-TBP is shown in Fig 2*A*. It can be observed that the level of intracellular HSP70 increased up to 6.1-fold in PIG1 control melanocytes and 5.2-fold in PIG3V vitiligo melanocytes in the presence of 4-TBP when compared with untreated cells. Interestingly, a 3.3-fold increase in the release of HSP70 was also observed for PIG3V vitiligo melanocytes following treatment with 500 μM 4-TBP as shown in Fig 2*B*. Moreover, a 5.3-fold increase in the HSP70 content of the medium was noted for PIG3V *versus* PIG1 melanocytes, further supporting that the vitiligo melanocytes secrete a relatively larger proportion of the stress proteins.

Protection from 4-TBP exposure by adenoviral overexpression of HSP27 or HSP70 Melanocytes overexpressing HSP27 or HSP70 were treated with 4-TBP in the range of 0–1000 μM for 72 h prior to measuring cell viability. Adenoviral overexpression of HSP70 by melanocytes following adenoviral infection was confirmed by western blotting as shown in Fig 3. A 3.7-fold increase in HSP70 content was demonstrated only for cells exposed to AdHSP70, with no increase observed following exposure to other adenoviruses. Western blot analysis of HSP27 expression revealed that the stress of the adenoviral infection procedure *per se* upregulated HSP27 expression to a similar extent in all three samples compared with untreated cells (not shown). Similar results were observed for PIG1 cells (not shown). As shown in Fig 4, it was observed that adenoviral overexpression of either HSP27 or HSP70 did not ade-