Melanoma Cell Attachment, Invasion, and Integrin Expression is Upregulated by Tumor Necrosis Factor α and Suppressed by α Melanocyte Stimulating Hormone

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We have previously shown α-melanocyte stimulating hormone to protect melanocytes and melanoma cells from the proinflammatory actions of tumor necrosis factor-α. The aim of the study was to extend this work to look into the influence of tumor necrosis factor-α on melanoma cell attachment, invasion, and integrin expression and ask to what extent α-melanocyte stimulating hormone might protect cells from tumor necrosis factor-α stimulation of increased integrin expression. HBL human melanoma cells were studied under resting and stressed conditions using tumor necrosis factor-α as a proinflammatory cytokine. Functional information on the actions of tumor necrosis factor-α on melanoma cells was obtained by examining the strength of attachment of melanoma cells to substrates and the ability of melanoma cells to invade through fibronectin. α3, α4, and β1 integrin expression was detected by Western immunoblotting and the ability of α-melanocyte stimulating hormone to oppose the actions of tumor necrosis factor-α was studied on HBL cell attachment, invasion, and integrin subunit expression. Our results show that tumor necrosis factor-α increases the number of melanoma cells attaching to collagen (types I and IV) and tissue culture polystyrene, increases ability to invade through fibronectin, and upregulates the expression of α3 (28%), α4 (90%), and β1 (65%) integrin subunit expression. In contrast, α-melanocyte stimulating hormone reduced cell attachment, invasion, and integrin expression and opposed the stimulatory effects of tumor necrosis factor-α. In conclusion this study provides further evidence of α-melanocyte stimulating hormone acting to "protect" melanoma cells from proinflammatory cytokine action. Our data support a hypothesis that an inflammatory environment would promote melanoma invasion and that the anti-invasive actions of α-melanocyte stimulating hormone are consistent with its working in an anti-inflammatory capacity.

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The incidence of malignant melanoma has increased worldwide and has doubled over the past decade. Although successful treatment exists if it is detected at an early stage, no single effective choice of treatment exists following tumor "metastasis" and prognosis is associated with an extremely high morbidity and mortality. The use of routine anticancer treatments provides only a marginal benefit for patient prognosis and therefore a further understanding of melanoma progression is necessary in order to develop new therapeutic strategies. The ability of melanocyte cells to transform into melanoma is tightly regulated by adjacent keratinocytes and also by the extracellular matrix (ECM). Detachment of melanoma cells from a primary tumor site, migration, and invasion of the surrounding matrix tissue mark the first stage of metastasis. During initial metastasis melanoma cells are known to express a very different array of surface antigens, in particular the integrin adhesion molecules. This large family of cell surface glycoproteins are reported to contribute to the ability of melanoma cells to migrate, invade, and metastasize (Hynes, 1992; Natali *et al*, 1993; Danen *et al*, 1995).

A number of factors control integrin expression in melanoma cells. In addition to an increase in expression following melanocyte transformation and metastatic progression, integrin expression may also be altered in response to the local cellular environment. Of particular interest is the response to inflammation. Recent work suggests that for some tumors (of which melanoma is one) immune and inflammatory stress can promote metastatic spread (Giavazzi *et al*, 1990; Dekker *et al*, 1994; Link *et al*, 1999). Integrin expression in melanoma cells has been reported to be upregulated in response to proinflammatory cytokines (Creyghton *et al*, 1995) and other stresses, such as sublethal laser radiation (Zhu *et al*, 1999). In this study

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Abbreviations: α-MSH, α melanocyte stimulating hormone; NF-κB, nuclear factor κB; ECL, enhanced chemiluminescence; ECM, extracellular matrix; EDTA, ethylene diamine tetraacetic acid; ICAM-1, intracellular adhesion molecule; PBS, phosphate buffered saline; TNFalfa, tumar necrosis factor alfalfa; UV, ultraviolet.

we present data suggesting that inflammation may regulate the expression of integrin subunits associated with melanoma expression.

Cutaneous melanocytes must be proficient in surviving endogenous and exogenous stresses. Ultraviolet (UV) light from the sun is known to be a causative factor in melanocyte transformation and acute exposure stimulates local release of proinflammatory cytokines, initiating cutaneous inflammation. In addition to the proinflammatory action, UV light also has direct transforming potential by generating intracellular reactive oxygen species, with potential to mutate DNA base pair residues. Research over the last few years has shown that the intrinsic production of prooipomelanocortin peptides in the skin, in particular α-melanocyte stimulating hormone (α-MSH), can inhibit the action of proinflammatory cytokines thereby conveying a cytoprotective role (Lipton *et al*, 1991; 1998; Lipton and Catania, 1997).

α-MSH is a 13 amino acid peptide (the first 13 amino acids of adrenocorticotrophic hormone). It interacts with cells via binding to surface melanocortin-1 receptors. We have previously shown α-MSH to protect melanocytes and melanoma cells from the proinflammatory actions of tumor necrosis factor-α (TNF-α) (Haycock *et al*, 1999a; 1999b). At 24 h α-MSH can be seen to inhibit TNF-α stimulated intercellular adhesion molecule (ICAM-1) upregulation (Hedley *et al*, 1998; Morandini *et al*, 1998). The upregulation of ICAM-1 is a fundamental requirement for the interaction between melanoma cells and infiltrating T lymphocytes and we find that exposure of melanoma cells to α-MSH can attenuate their ability to bind to the surface of T lymphocytes (Hedley *et al*, 2000). Studies on the mechanism of α-MSH attenuation on TNF-α have shown a rapid signaling action within 15 min in melanoma cells and keratinocytes (Haycock *et al*, 2000). α-MSH is effective at inhibiting intracellular peroxide accumulation within 15 min [intracellular peroxide is an activator of nuclear factor κB (NF-κB) transcription factor activity, and hence is an important signaling mediator of inflammation (Meyer *et al*, 1993)]. After 1–2 h α-MSH can be seen to inhibit activation of the transcription factor NF-κB in response to stimulation by TNF-α (Haycock *et al*, 1999a). Throughout all the above studies, the addition of α-MSH typically attenuates the response of cells to TNF-α by approximately 50%.

In this study we extend work on the potential of α-MSH to antagonize proinflammatory cytokines to investigating the action of α-MSH on α3, α4, and β1 integrin expression in an HBL human melanoma cell line. Melanoma cells were studied under resting and stressed conditions using TNF-α as a proinflammatory cytokine and hydrogen peroxide as a means of inducing oxidative stress. Functional information on the action of α-MSH was investigated by examining attachment and invasion of melanoma attachment of cells exposed to TNF-α or hydrogen peroxide. Data on attachment and invasion was then correlated with changes in the levels of α3, α4, and β1 integrin subunit expression.

MATERIALS AND METHODS

Culture of cutaneous melanoma cells The human cutaneous melanoma cell line (HBL) was established in the laboratory of GG (Ghanem *et al*, 1988). Cells were cultured in Ham's F10 (Gibco, Paisley, Scotland) supplemented with 5% fetal bovine serum, 5% neonatal bovine serum (Sigma, Poole, Dorset, U.K.), 2 mmol per l L-glutamine, penicillin (100 units per ml), and streptomycin sulphate (100 μg per ml). Cells were grown in a humidified atmosphere (5% CO_2/95% air, 37°C) to 60% confluence and then stimulated with α-MSH (10^{-7} M and 10^{-9} M), TNF-α (100 units per ml and 300 units per ml), or H_2O_2 (100 μM) combinations as described previously (Hedley *et al*, 1998; Haycock *et al*, 1999a; 1999b; 2000).

Attachment assay The strength of attachment of melanoma cells to adhere to tissue culture polystyrene, collagen I (10 μg per ml and 200 μg per ml, respectively), and collagen IV (200 μg per ml) was investigated as a functional assay for assessing the relevance of integrin subunit expression. A modified attachment procedure was conducted using HBL melanoma cells based on an assay previously described (Searles *et al*, 1995). Briefly, HBL cells were grown in six- well plates for 24 h and then incubated with TNF-α or hydrogen peroxide in serum-free medium for a further 24 h. Enzyme-free cell dissociation buffer [Sigma, phosphate-buffered saline (PBS) + ethylenediamine tetraacetic acid (EDTA) 0.02% wt/vol] was then added to detach cells by solution phase conjugation of divalent cations (predominantly Ca^{2+} and Mg^{2+}) required for integrin-mediated attachment. A standard physical agitation procedure to assist cellular removal was followed by placing cells (during PBS/EDTA incubation) in culture plates on a motorized shaking table for fixed time periods and at a fixed rotary speed setting (40 oscillations per min). After defined time points (10–50 min), the detached cells (plus PBS/EDTA) were gently removed. The number of melanoma cells remaining attached to the substrate surface was quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; 100 μl, 5 mg per ml for 40 min at 37°C), followed by 100 μl Cellusolve™ in order to elute the colored product formed. Sample optical density was measured at 500 nm (with a reference filter of 630 nm). Control samples consisted of a cell-free sample and test values were subtracted from control values. This provided a convenient measure for assessing the percentage of cells remaining attached to each substrate under investigation. The experiment was repeated on three separate occasions for each time point, using triplicate data from each experiment.

Western immunoblotting for α3, α4, and β1 integrin subunit detection Western immunoblotting for the detection of α3, α4, and β1 integrin subunits on HBL melanoma cells was carried out as described previously (Zhu *et al*, 1999). Briefly, 24 h after incubation with TNF-α (100 and 300 units per ml), α-MSH (10^{-7} M or 10^{-9} M), or hydrogen peroxide (100 μM) in serum-free medium, cells were washed twice with PBS and then lyzed on ice for 10 min (1% vol/vol Triton X-100) in the presence of a buffered protease inhibitor cocktail (10 mM Tris–Cl, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride; pH 7.6). Cell lysates were then centrifuged at 14,000*g* for 15 min at 4°C. Total cellular protein from the centrifuged pellet was measured according to the bicinchoninic acid method (Pierce, Rochford, IL) and the remainder of the extract was incubated at 100°C for 2 min. Ten micrograms of total protein was loaded per gel track and proteins were separated by discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis according to the method of Laemmli (1970) (Mini Protean II dual slab cell, Bio-Rad, Hercules, CA) using a 5% stacking gel and an 8% resolving gel for 1 h (200 V, constant voltage). Proteins were then transferred onto a 0.45 μm PVDF membrane (Bio-Rad) according to the method of Towbin *et al* (1979) (using a Mini Trans-Blot system, Bio-Rad; 100 V, constant voltage). α3, α4, and β1 immunoreactive bands were visualized as follows. Unreacted binding sites were blocked by incubating membranes in commercial dried milk protein solution [5% (wt/vol) in Tris-buffered saline/Tween (TBST): 10 mM Tris–HCl, 0.15 M NaCl, 0.05% (wt/vol) Tween 20, pH 8.0]. Membranes were then incubated with anti-α3, anti-α4, or anti-β1 integrin polyclonal mouse IgG primary antibodies (4 μg per ml in TBST; Upstate Biotechnology, Lake Placid, NY) at 4°C for 18 h, washed with TBST (3 × 10 min) and incubated with horseradish peroxidase conjugated goat antimouse secondary antibody diluted 1:2000 (vol/vol) in TBST for 1 h (Dako, Cambridge, U.K.). Immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) kit according to the manufacturer's instructions (Amersham-Pharmacia, U.K.), with a precise exposure time of 5 min per enhanced chemiluminescence film sheet (Amersham-Pharmacia). Optical density measurements were determined by scanning densitometry (Bio-Rad) and analyzed using Molecular Analyst software (Bio-Rad).

Fibronectin invasion assay Nunc™ tissue culture inserts (containing a polycarbonate filter with 8 μm diameter pores randomly distributed over the surface) were inverted and 76 μl of human fibronectin (100 μg per ml) was added to the underside of each polycarbonate filter and incubated for 1 h at 37°C. Inserts were reoriented and placed into a 24-well tissue culture plate containing 500 μl of a serum-free invasion assay medium [SFIAM: RPMI-1640 medium supplemented with 20 ng per ml epidermal growth factor, 0.2% (wt/vol) D-glucose solution, 0.1% (wt/vol) bovine serum albumin, 2 mmol per l L-glutamine, penicillin (100 units per ml), and streptomycin sulfate (100 μg per ml)]. A suspension of 1.8×10^5 HBL cells (contained in 500 μl SFIAM) ± TNF-α (300 units per ml), α-MSH (10^{-7} M), or hydrogen peroxide (100 μM) was added to the Nunc inserts. Cells were incubated for 20 h at 37°C in a humidified atmosphere (5% carbon dioxide/95% air). After incubation, HBL cells (plus SFIAM) were collected from the upper and lower compartments of the Nunc chamber and placed into preweighed LP4 perspex tubes. The cells and medium removed was replaced with an equivalent volume of 0.1% (wt/vol) trypsin and 0.02%

(wt/vol) EDTA and remaining adherent cells were incubated for 10 min (37°C). The trypsin/EDTA (plus cells) was removed and placed in sample matched tubes, and a second trypsin/EDTA incubation with the Nunc inserts ensured thorough removal of all cells. After a second trypsin/EDTA incubation, the polycarbonate filter surface (upper chamber) was also gently scraped and any remaining cells were transferred to sample matched tubes. The undersides of each polycarbonate filter as well as the tissue culture plastic of the lower chambers were also scraped and total cell collections were placed in appropriate tubes. Samples were centrifuged (200*g*, 5 min) to separate cells from medium, all supernatant was removed, and tubes were reweighed. In addition, cells in each tube were resuspended in 100 μl medium and counted using a hemocytometer. Noninvading cells were those remaining in the Nunc upper chamber and those attached to the upper surface of the filter. Invading cells were those removed from the underside of the filter, suspended in the medium of the tissue culture wells, and attached to the base of the 24-well chamber (cells collected into the lower tubes). A combined analysis of cell counts plus obtained weight/volume for each cell suspension (weight of tubes containing cell suspensions minus the weight of empty tubes) provided the percentage of the total population of cells that had invaded through the fibronectin monolayer. (This "total housekeeping" approach to counting all cells is an improvement on the assay we first described in Dewhurst *et al* (1997).)

Statistics Statistical comparisons between control and test samples were assessed using the parametric Student's paired *t* test for cell attachment and fibronectin invasion assay experiments. The Mann–Whitney non-parametric *U* test was used to analyze differences in integrin subunit expression by Western immunoblotting.

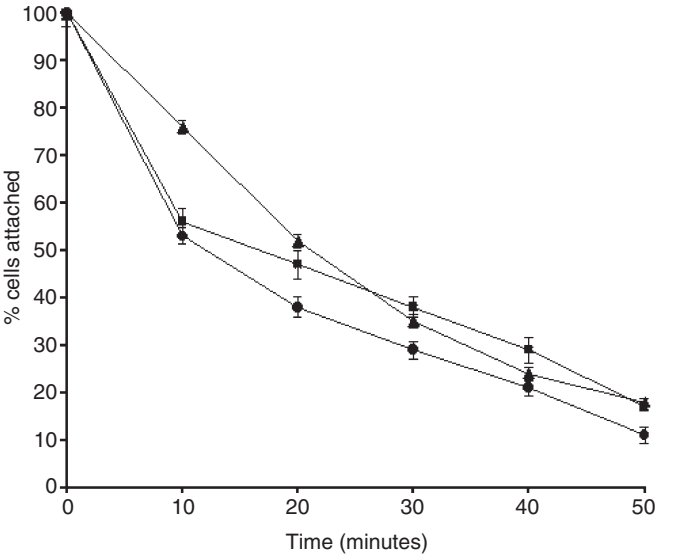

Figure 1. Time course of HBL melanoma cell attached to tissue culture polystyrene in the presence of an enzyme-free PBS/EDTA dissociation buffer. (*a*) Control, no prior stimulation (*filled squares*); (*b*) TNF-α (300 units per ml, 24 h preincubation; *filled triangles*); (*c*) α-MSH (10^{-7} M, 24 h preincubation; *filled circles*).

RESULTS

Effect of TNF-α and α-MSH on HBL melanoma cell attachment HBL melanoma cells were cultured on a standard tissue culture polystyrene surface for 24 h with either TNF-α (300 unit per ml) or α-MSH (10^{-9} M) compared to unstimulated cells. After removal of medium and addition of an enzyme-free divalent cation binding buffer (PBS/EDTA) the percentage of cells remaining attached to the surface was investigated at time points from 0 to 50 min using MTT-ESTA. **Figure 1** illustrates a typical time course experiment and shows that after 10 min of cell dissociation buffer 56% of unstimulated HBL melanoma cells remain attached to the polystyrene surface. This is in contrast to 76% of cells remaining attached after a 24 h incubation with TNF-α (300 units per ml) and 53% of cells remaining attached after incubation with α-MSH (10^{-9} M). **Figure 1** shows that in general terms more cells remain attached following TNF-α stimulation compared to unstimulated cells over a 50 min time course. In contrast, fewer cells remain attached following stimulation with α-MSH. From such time course experiments incubation of cells with dissociation agent for 25 min was chosen as optimal to discriminate between attachment of unstimulated cells and those exposed to α-MSH. **Figure 2**(***a***) summarizes the relative number of melanoma cells remaining attached after a 24 h incubation period with TNF-α, α-MSH, or hydrogen peroxide for cells cultured on tissue culture polystyrene, collagen type I (200 μg per ml or 10 μg per ml) or collagen type IV (200 μg per ml). TNF-α was observed to increase attachment for cells grown on ECM substrates. This increase was statistically significant for cells attached to collagen type IV and collagen type I (at 200 μg per ml and 10 μg per ml) in contrast to cells grown on tissue culture polystyrene. In contrast, preincubation of melanoma cells with α-MSH decreased cell attachment, with α-MSH at 10^{-9} M being slightly more effective than α-MSH at 10^{-7} M. This enhanced response to α-MSH at relatively lower doses has been described previously and is associated with reduced potency at higher doses, typical of a biphasic dose–response curve (Haycock *et al*, 1999a). A significant reduction in cell attachment was noted for all cell culture surfaces, however, when α-MSH was used at 10^{-7} M. Melanoma cells stimulated with an oxidative stress (in the form of exogenous hydrogen peroxide, 100 μM) were observed to significantly increase attachment compared to control unstimulated cells. Significant increases were observed for cells grown on collagen type I (at 200 μg per ml and 10 μg per ml) and on collagen type IV (200 μg per ml), in contrast to cells grown on tissue culture polystyrene. **Figure 2**(***b***) shows that when cells were grown on polystyrene and incubated in the presence of TNF-α (300 units per ml) plus α-MSH (10^{-7} M), α-MSH significantly attenuated the response compared to cells grown in the presence of TNF-α alone.

Effect of TNF-α and α-MSH on HBL melanoma cell invasion through fibronectin The influence of TNF-α and α-MSH on HBL melanoma cell invasion through fibronectin was investigated. Over 20 h unstimulated cells showed an average of $22.7 \pm 2.22\%$ invasion ($n = 3$ experiments). All results are compared to this control invasion, which is expressed as 100% in **Fig 3**. If TNF-α (300 units per ml) was added to the cells during this period then there was a significant increase over the control level of invasion of +27% (see **Table I**). Preincubation of cells with TNF-α for 24 h prior to introducing cells to the fibronectin invasion assay showed a significantly greater increase in invasion over control from +27% to +43%. Maintenance of cells with TNF-α 24 h prior to the assay and a further addition of TNF-α for the 20 h of the assay, however, showed no further increase in invasion (see **Table I**). From this we deduced that the response to TNF-α was largely achieved within the first 24 h. Thus using invasion assay conditions whereby factors are present for 24 h we found that the presence of α-MSH alone (at 10^{-7} M) significantly decreased the level of invasion of these cells by approximately 50% compared to unstimulated HBL cells (**Fig 3**). In the same experiments where TNF-α increased the HBL melanoma invasion by +43% over the control value the addition of α-MSH (10^{-7} M) together with TNF-α (300 units per ml) reduced melanoma cell invasion from +43% over control to 16% below the control value.

Effect of TNF-α and α-MSH on melanoma α3, α4, and β1 integrin expression Using the conditions established for HBL melanoma cell culture from the attachment and invasion studies above we then investigated the level of α3, α4, and β1 integrin expression. This was determined after incubating HBL cells for 24 h with TNF-α (100 and 300 units per ml), hydrogen peroxide (100 μM), α-MSH (10^{-9} M and 10^{-7} M), and α-MSH (10^{-7} M)

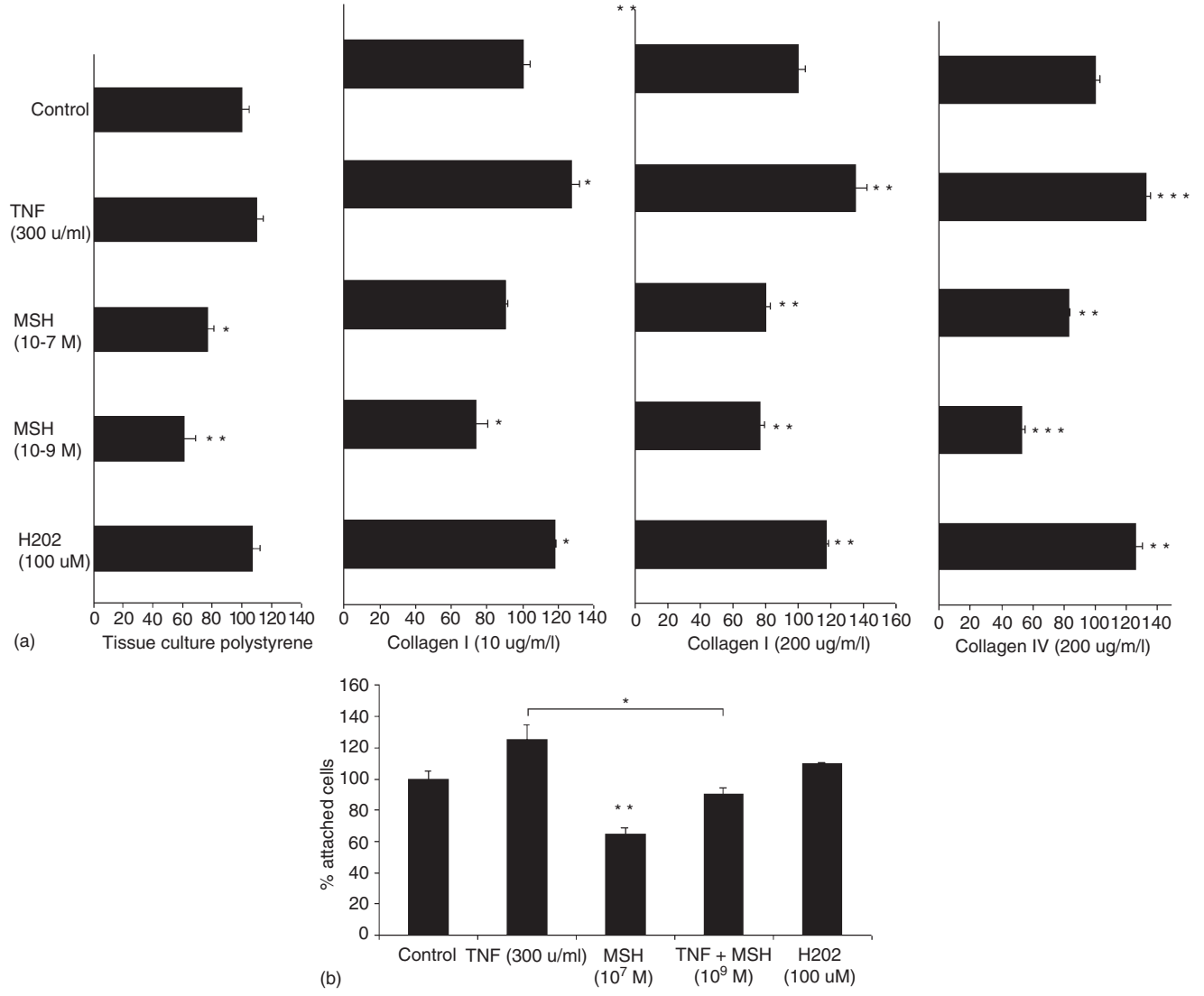

Figure 2. Attachment of HBL melanoma cells to surface substrates after a 25 min challenge with enzyme-free PBS/EDTA dissociation buffer. (*a*) Cells were preincubated for 24 h with TNF-α (300 units per ml), α-MSH (10^{-7} M and 10^{-9} M), and hydrogen peroxide (100 μM). Results are shown for attachment to tissue culture polystyrene, collagen type I (at 10 μg per ml and 200 μg per ml), and collagen type IV (200 μg per ml). Mean ± SEM ($n = 3$). *$p > 0.05$; **$p > 0.01$; ***$p > 0.001$ (compared to control). (*b*) Attachment of HBL cells to polystyrene showing a significant decrease of attachment after 25 min for cells preincubated (for 24 h) with TNF-α (300 units per ml) plus α-MSH (10^{-7} M) compared to TNF-α (300 units per ml) alone. *$p > 0.05$; **$p > 0.01$; ***$p > 0.001$ [α-MSH (10^{-7} M) was compared to control; TNF-α (300 units per ml)+α-MSH (10^{-7} M) was compared to TNF-α (300 units per ml) alone]. Mean ± SEM ($n = 3$).

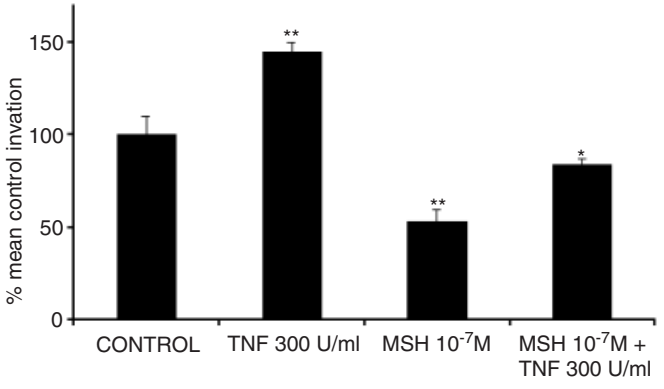

Figure 3. Effect of TNF-α and α-MSH on invasion of HBL melanoma cells through fibronectin. TNF-α (300 units per ml) present for a 20 h time period increased melanoma invasion; α-MSH (10^{-7} M) present for the 20 h of the incubation decreases melanoma invasion. The presence of α-MSH (10^{-7} M) with TNF-α (300 units per ml) opposes the ability of TNF-α (300 units per ml) to increase invasion. Mean ± SEM ($n = 3$).

Table I. The effect of TNF-α (300 units per ml) on HBL melanoma cell invasion through a fibronectin monolayer *in vitro*

	Percentage invasion compared with control (mean ± SEM, $n = 3$)	
Control (no additions)	100 ± 9.8	
TNF-α (300 units per ml) present for 20 h of assay	127 ± 3.88	$p = 0.03$
Preincubation with TNF-α (300 units per ml) for 24 h	143 ± 7.04	$p = 0.01$
Preincubation with TNF-α (300 units per ml) for 24 h plus presence in assay for 20 h	143 ± 9.58	$p = 0.02$

in combination with TNF-α (300 units per ml). When cells were incubated for a 24 h time period with TNF-α (at 300 units per ml) a significant upregulation of all integrin subunits studied was observed (α3, α4, and β1). An example of Western

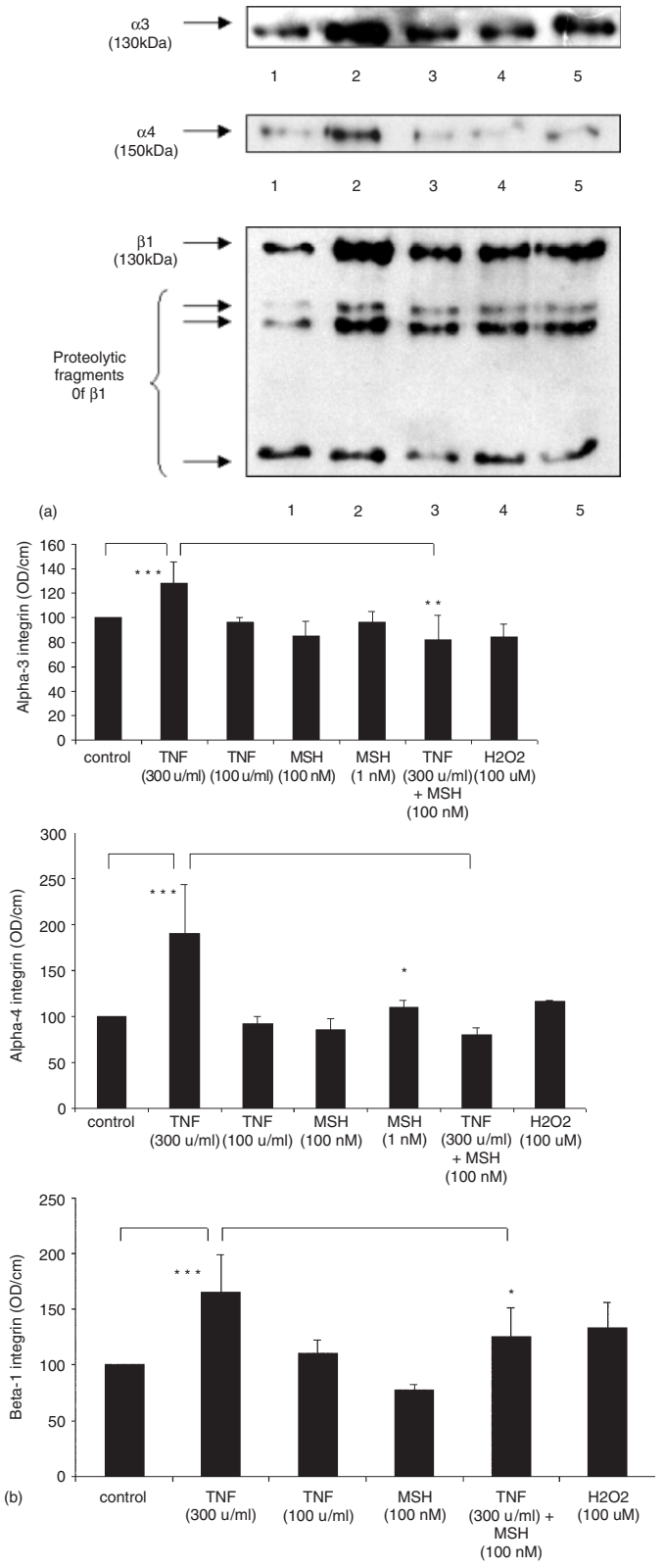

Figure 4. (*a*) **The effect of TNF-α, hydrogen peroxide, α-MSH, and TNF-α+α-MSH on relative levels of integrin α3, α4, and β1 subunits in HBL human melanoma cells detected by Western immunoblotting.** (*a*) *Lane (1)* Unstimulated control HBL cells; *lane (2)* TNF-α (300 units per ml, 24 h); *lane (3)* α-MSH (10^{-7} M, 24 h); *lane (4)* TNF-α (300 units per ml, 24 h)+α-MSH (10^{-7} M, 24 h); *lane (5)* hydrogen peroxide (100 μM, 24 h). (*b*) Scanning densitometry data taken from Western immunoblots identifying changes in levels of α3, α4, and β1 integrin subunits. Single gel runs were scanned and then combined after normalizing data. (10 μg total protein loaded per gel track). Mean ± SEM ($n = 5$). *$p > 0.05$; **$p > 0.01$; ***$p > 0.001$.

immunoblot data is illustrated in **Fig 4**(***a***) (all adjacent gel tracks contain 10 μg protein). In contrast, when HBL cells were stimulated with TNF-α at 100 units per ml for 24 h, no observable increase in integrin expression was noted. **Fig 4**(***b***) shows scanning densitometric data of five independent experiments where the 130 kDa immunoreactive band for α3 and β1 integrin subunits and the 150 kDa band for the α4 subunit were quantified for relative volume and cross-sectional area in comparison to adjacent bands in a single gel run and relative data were normalized and combined to give averaged values. A 28% average increase was observed for α3 integrin, a 90% average increase was observed for α4 integrin, and a 65% average increase for β1 integrin was observed compared to unstimulated control samples ($n = 5$). Limited proteolysis of the β1 integrin subunit was also noted (as shown in **Fig 4*a***), consistent with previous studies (Zhu *et al*, 1999). α-MSH (10^{-9} M and 10^{-7} M) stimulation alone did not significantly alter α3, α4, and β1 integrin expression compared to unstimulated control samples (**Fig 4*a***, ***b***). When α-MSH (10^{-7} M) was combined with TNF-α (300 units per ml), however, we found a significantly decreased expression of α3 (46%, $p < 0.01$), α4 (110%, $p < 0.05$), and β1 (40%, $p < 0.05$) integrin subunits compared to corresponding integrin levels for samples stimulated with TNF-α (300 units per ml) alone (**Fig 4*b***).

DISCUSSION

Integrins play a major role in regulating cellular growth, differentiation, and death by regulating the interaction between cell and ECM (Hynes, 1992; Stuiver and O'Toole, 1995). Several studies of human cutaneous melanoma have also demonstrated that expression and function of surface integrin adhesion molecules in melanoma cell lines correlate with invasive or metastatic potential (Edward, 1995). Furthermore, a considerable amount of evidence from experimental studies *in vitro* and *in vivo* indicates that integrins have a role in melanoma tumorigenesis, invasion, angiogenesis, and metastasis (Seftor *et al*, 1999). In this study an HBL human melanoma cell line was chosen to investigate whether a correlation exists between melanoma cell behavior and integrin expression in response to proinflammatory cytokine action, oxidative stress, and the anti-inflammatory peptide α-MSH. To this end we deliberately studied cells under stressed and nonstressed conditions prior to looking at the actions of α-MSH. Melanoma cell behavior was studied as an ability to attach to different matrix proteins and an ability to invade through a fibronectin monolayer. Data were then correlated using the above conditions on changes in levels of expression of the α3, α4, and β1 integrin subunits.

Previous evidence has suggested that inflammatory cytokines have potential to upregulate integrin expression in melanoma cells (Dekker *et al*, 1994). Interleukin-1α and TNF-α are reported to increase α4, α5, and α6 integrin expression in melanoma cells (Dekker *et al*, 1994). The upregulation of integrin expression has also been found to correlate with enhanced migration of the cells on a fibronectin substrate (Dekker *et al*, 1994). In light of the data suggesting that integrins can be upregulated by inflammatory stress and literature suggesting that immune and inflammatory stresses can promote metastatic spread (Bogden *et al*, 1997; Hofer *et al*, 1998) we therefore investigated the ability of TNF-α and hydrogen peroxide (as an experimental model for oxidative stress) to upregulate α3, α4, and β1 integrin expression in a human HBL melanoma cell line, which we had previously documented as being responsive to α-MSH (Ghanem *et al*, 1988; Haycock *et al*, 1999a; Hedley *et al* 2000). We report on a strong positive correlation between enhanced melanoma cell attachment, invasion, and upregulation of integrin adhesion molecules in the presence of the proinflammatory cytokine TNF-α. α-MSH alone was observed to minimally decrease melanoma attachment, invasion, and integrin expression. α-MSH strongly inhibited the action of TNF-α on increasing melanoma attachment, invasion, and

expression of all integrin subunits studied, however. We therefore present preliminary *in vitro* evidence to support the hypothesis that a proinflammatory environment may promote melanoma cell adhesion and invasion. In addition, the ability of α-MSH to attenuate the response of melanoma cells to TNF-α is consistent with α-MSH acting as an anti-inflammatory peptide (Lipton and Catania, 1997), and in this capacity may serve to oppose metastatic spread.

The HBL human melanoma cell line used in this study has been investigated extensively *in vitro* in examining the role of α-MSH as an anti-inflammatory. It is known to express a relatively high number of MSH receptors (1000–3000 sites per cell), as determined by radioligand competitive binding, and has a wild-type melanocortin-1 receptor sequence. We have previously shown that α-MSH can antagonize TNF-α stimulated ICAM-1 upregulation, NF-κB transcription factor activation, glutathione peroxidase action, and intracellular peroxide species generation. This study extends previous work through to melanoma invasion, integrin expression, and attachment.

During melanoma cell invasion cells move through the ECM by a complex series of events that involves adhesion to ECM proteins and also proteolysis of matrix to facilitate migration. A key interaction between the melanoma cell and a given matrix protein is facilitated by a family of adhesive integrin proteins. Integrins comprise an α and β subunit and by binding specifically to a wide range of matrix proteins they physically link the ECM via the cellular membrane to intracellular cytoskeletal proteins (predominantly actin microfilaments). ECM are composed of several macromolecules including fibronectin, laminin, collagens, and proteoglycans (Ruoslahti and Pierschbacher, 1987). The binding of integrins to the ECM initiates assembly of actin microfilaments and the accumulation of numerous different proteins, including signaling molecules, integrins, and components of the cytoskeleton, into focal adhesion structures. A number of these proteins, including focal adhesion kinase, paxillin, and tensin, are tyrosine phosphorylated upon an integrin–matrix interaction and the β1 integrin subunit is reported to have a centrally important role in these processes (Burridge *et al*, 1988; 1992; Bockholt and Burridge, 1993). At present over 20 different α and β combinations have been reported, together with many diverse functions including location of cells to a native position in a tissue, involvement in the immune response, lymphocyte homing, platelet aggregation, embryologic development, and wound healing but also the metastatic spread of certain malignancies including melanoma (Stuiver and O'Toole, 1995).

It is generally accepted that integrin expression is upregulated as melanoma cells acquire a more metastatic phenotype (Schadendorf *et al*, 1993; Marshall *et al*, 1998). In this study, however, we specifically investigated the α3, α4, and β1 integrin subunits. α3 integrin, in particular, is associated with an increased motility of melanoma cells (Yoshinaga *et al*, 1993). The expression of α4 integrin is generally associated on the one hand with retention of the cells at a primary tumor site, but after entering the circulation α4 integrin expression is thought to promote melanoma cell accumulation of disseminated cells in distant tissues (Holzmann *et al*, 1998). These same integrins (α3, α4) can also confer invasive and metastatic properties on human melanoma cells when injected into nude mice (Johnson, 1999), and an increase is associated with poor clinical prognosis in melanoma patients. β1 integrin was investigated because it is well accepted as a centrally important integrin subunit for general cell attachment to ECM proteins. It is also suggested, however, that the β1 integrin subunit is involved in melanoma cell migration and migration-associated matrix reorganization (Friedl *et al*, 1998). Using blocking anti-integrin antibodies and tumor cell and migration-associated matrix reorganization, it has been shown that adhesion and migration is β1 dependent and that migrating cells use focal adhesions of integrin proteins coclustered with cytoskeletal components at contact points with collagen fibers (Friedl *et al*, 1998).

In addition to an alteration of integrin expression upon melanocyte transformation and melanoma progression, we have previously reported that α3, α4, and β1 integrin (and also focal adhesion kinase) can be upregulated in response to Nd:YAG laser therapy, used clinically in the removal and depigmentation of naevi (Zhu *et al*, 1997). Laser usage is typically at 532 nm and, if the applied energy is sufficient, generation of intracellular reactive oxygen species will result with the potential activation of oxidative stress responding transcription factors (e.g., NF-κB and c-jun/fos) and a consequent upregulation of gene expression (Baeuerle and Henkel, 1994). The exposure dose of laser energy of UV light is usually of a sublethal level (Zhu *et al*, 1997); however, if an inflammatory response is observed it is reasonable to suggest that the dose delivered will have been sufficient to initiate proinflammatory transcription factor activation. This is of paramount importance for the clinical use of 532 nm Nd:YAG laser therapy, as any sublethal dosage applied to naevi cells could have potentially serious consequences to the surviving population of laser-treated cells.

Oxidative stress (as hydrogen peroxide) was investigated as it has previously been reported that α-MSH can inhibit both proinflammatory cytokines and oxidative stress. It is well accepted that intracellular oxidative stress arises after TNF-α binds to its receptor (Schulze-Osthoff *et al*, 1993). A number of similar actions have also been documented between the action of TNF-α and oxidative stress (e.g., activation of NF-κB). We therefore decided to investigate whether any similar observations were noted between the ability of TNF-α to increase melanoma attachment and integrin expression and hydrogen peroxide, and if so whether α-MSH had the potential to inhibit. The data showed that oxidative stress was not as effective as proinflammatory TNF-α at effecting attachment or integrin expression. A small significant increase was observed for melanoma attachment when cells were cultured on ECM proteins (collagen types I and IV) compared to tissue culture polystyrene. No significant increases were observed, however, for changes in integrin expression. The data therefore suggest that inflammatory stress is a more important factor than oxidative stress in this study.

Our data are consistent with previous reports by ourselves and others that α-MSH is able to antagonize proinflammatory cytokine action. For example, it is reported that α-MSH can oppose a TNF-α stimulated upregulation of ICAM-1 in human melanocytes and melanoma cells (Hedley *et al*, 1998; Morandini *et al*, 1998). It is also reported that α-MSH can inhibit the TNF-α stimulated activation of the NF-κB transcription factor in human melanocytes, melanoma cells (Haycock *et al*, 1999a; 1999b), and keratinocytes (Moustafa *et al*, 2002), and can inhibit TNF-α and peroxide activation of glutathione peroxidase and intracellular peroxide accumulation in melanoma cells and keratinocytes (Haycock *et al*, 2000). Furthermore, it is also known to inhibit the ability of activated T lymphocytes to bind to the surface of melanoma cells (Hedley *et al*, 2000). The above studies are supportive of a broad action and potent anti-inflammatory role for the α-MSH peptide, originally reported by and reviewed in Lipton and Catania (1997). An extension to the anti-inflammatory action of α-MSH on melanoma biology is reported in this study on an ability to reduce invasion of HBL melanoma cells through fibronectin, correlated with a reduction in inflammatory cytokine upregulation of integrin expression and melanoma cell adhesion. Thus an emerging hypothesis is consistent with α-MSH protecting melanoma cells from proinflammatory stress by reducing the ability of these stimuli to upregulate adhesion molecule (including integrin) expression. In the context of melanoma invasion such actions of α-MSH would be consistent with reducing the ability of melanoma cells to migrate or invade through ECM proteins, and hence a role for α-MSH as an antimetastatic factor is suggested.

In summary this study provides further evidence for α-MSH acting to protect melanoma cells from proinflammatory stress. We demonstrate that melanoma invasion, cell attachment, and integrin expression are upregulated by the proinflammatory

cytokine TNF-α and are opposed by α-MSH, consistent with inflammation promoting metastasis and α-MSH acting in an anti-inflammatory capacity to oppose metastatic spread.

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