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Norepinephrine upregulates VEGF, IL-8, and IL-6 expression in human melanoma tumor cell lines: implications for stress-related enhancement of tumor progression

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Abstract

Studies suggest that stress can be a co-factor for the initiation and progression of cancer. The catecholamine stress hormone, norepinephrine (NE), may influence tumor progression by modulating the expression of factors implicated in angiogenesis and metastasis. The goal of this study was to examine the influence of NE on the expression of VEGF, IL-8, and IL-6 by the human melanoma cell lines, C8161, 1174MEL, and Me18105. Cells were treated with NE and levels of VEGF, IL-8, and IL-6 were measured using ELISA and real-time PCR. The expression of β -adrenergic receptors (β -ARs) mRNA and protein were also assessed. Finally, immunohistochemistry was utilized to examine the presence of β 1- and β 2-AR in primary and metastatic human melanoma biopsies. We show that NE treatment upregulated production of VEGF, IL-8, and IL-6 in C8161 cells and to a lesser extent 1174MEL and Me18105 cells. The upregulation was associated with induced gene expression. The effect on C8161 cells was mediated by both β 1- and β 2-ARs. Furthermore, 18 of 20 melanoma biopsies examined expressed β 2-AR while 14 of 20 melanoma biopsies expressed β 1-AR. Our data support the hypothesis that NE can stimulate the aggressive potential of melanoma tumor cells, in part, by inducing the production VEGF, IL-8, and IL-6. This line of research further suggests that interventions targeting components of the activated sympathetic-adrenal medullary (SAM) axis, or the utilization of β -AR blocking agents, may represent new strategies for slowing down the progression of malignant disease and improving cancer patients' quality of life.

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Keywords

psychological stress; norepinephrine; melanoma; cytokines; angiogenesis; vascular endothelial growth factor; interleukin (IL)-8; IL-6

1. Introduction

There is evidence that psychological factors can affect the incidence and progression of some cancers (Kruk and Aboul-Enein, 2004; Metcalfe et al., 2007; Reiche et al., 2004; White et al., 2007). Data obtained from studies using animal models support the hypothesis that stress could be a co-factor (Riley, 1975; Saul et al., 2005). Studies in the field of psychoneuroimmunology have shown that psychological stress can affect many aspects of innate and specific cellular immune responses mediated by the endocrine system through bi-directional interactions (Ader, 2007; Rabin, 1999).

Recent studies suggest that stress can also have a direct effect on tumor progression independent of the dysregulation of the immune system. Work from our laboratory and others have shown that vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) are factors that can be modulated by catecholamine hormones. Studies by Sood, Lutgendorf and others have shown that norepinephrine (NE) and epinephrine (E) may influence the progression of ovarian cancer by modulating the expression of MMPs and the angiogenic cytokine, VEGF, in ovarian cancer cells (Lutgendorf et al., 2003; Sood et al., 2006; Thaker et al., 2007). The ability of NE to promote progression of other cancers is supported by our recent work showing that NE enhanced the invasive and pro-angiogenic properties of nasopharyngeal carcinoma (NPC) cells by stimulating the secretion of VEGF, MMP-2, and MMP-9 *in vitro* (Yang et al., 2006). In these two studies it was shown that β -adrenergic receptors (ARs) mediated the production of these proteins. We have further suggested that this property of NE is not restricted to solid tumors through our results showing that NE can upregulate levels of VEGF proteins in culture supernatants of multiple myeloma-derived cell lines (Yang et al., 2008). These observations support the hypothesis that stress-associated activation of the sympathetic-adrenal medullary (SAM) axis can promote tumor progression, in part, by modulating the expression of pro-angiogenic and pro-metastatic factors.

Several clinical studies provide examples of the role that stressors may play as a co-factor for enhancing melanoma tumor progression (Temoshok et al., 1985). Such relationship between behavior and melanoma progression was explored in studies by Fawzy and co-workers (Canada et al., 2005; Fawzy et al., 2003; Fawzy et al., 1993). They found that participation in a psychiatric intervention that enhances effective coping and reduces affective distress had beneficial effects on melanoma progression and was positively correlated with survival (Fawzy et al., 2003; Fawzy et al., 1993).

The hypothesis that psychological stress can affect melanoma progression is further supported by a study using a B16 melanoma mouse tumor model. Mice that were either housed in a crowded condition or in isolation exhibited an increase in tumor growth which was completely abrogated by the oral administration of the β -adrenergic antagonist propranolol (Hasegawa and Saiki, 2002). Although factors involved in the progression of melanoma have been well described, the role(s) these psychological factors play in modulating the growth and spread of tumors is not fully understood.

Tumor angiogenesis is a critical step in the progression of cancer, including melanoma, allowing tumors to grow beyond 1–2 mm in diameter (Gimbrone et al., 1972). The expression of VEGF, interleukin (IL)-8, and IL-6 have been shown to be important for this process (Nilsson

et al., 2005; Tammela et al., 2005; Yuan et al., 2005). Therefore, the goal of this study is to examine the impact of the catecholamine stress hormone NE on the modulation of expression of VEGF, IL-8 and IL-6 in three human melanoma cell lines, C8161, 1174MEL, and Me18105.

2. Methods

2.1 Cell Lines and Culture Conditions

Three human melanoma cell lines, C8161, 1174MEL, and Me18105, were used in this study. C8161 is a well-characterized human cutaneous malignant melanoma cell line established from an abdominal wall metastasis. These tumor cells metastasize after orthotopic injection into athymic nude mice (Welch et al., 1991). 1174MEL was derived from a lymph node metastasis in a patient who exhibited clinical responses to T cell-based immunotherapy (Restifo et al., 1996). Me18105 was generated from an unspecified metastatic lesion of a melanoma patient (Hicklin et al., 1998). Cells were maintained in complete medium composed of RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), L-glutamine, and 1X antibiotic-antimycotic (Invitrogen Life Technologies, Carlsbad, CA) at 37°C in a humidified atmosphere with 5% CO₂. For NE treatments, serum-free conditions were utilized in order to eliminate the possible influence of factors that can be found in fetal calf serum on the effects of NE on the human melanoma cell lines. Cells were seeded in 6-well plates, with 2×10^5 cells/well in 2 ml of media, and cultured for 1 day in serum-free media in order to allow the cells to adapt to this condition. Cells were treated with NE (Sigma-Aldrich Co., St. Louis, MO) by replacing the media with 2 ml serum-free media containing 0, 0.1, 1, and 10 μ M NE. To assess the effects of NE on levels of VEGF, IL-8, and IL-6, culture supernatants were collected at various time points, centrifuged, and stored at -80°C until assayed by ELISA. Cells were homogenized in TRIzol reagent and stored at -80°C until assayed by real-time PCR.

2.2 Determination of VEGF, IL-8 and IL-6 Protein Concentrations in Culture Supernatants

The concentrations of VEGF, IL-8 and IL-6 were measured using Human Quantikine ELISA Kits (R&D Systems, Minneapolis, MN) following the manufacturer's protocol. The resultant color was read at 450 nm using a Labsystems Multiskan MCC/340 plate reader. The concentrations of the protein of interest in a sample were determined by interpolation from a standard curve.

2.3 Assessment of cell proliferation using [³H]-thymidine incorporation

The C8161, 1174MEL and Me18105 cells were seeded in 200 μ l complete medium in 96-well plates at a density of 7000 cells/well and incubated for 24 hours. The medium was removed and replaced with serum-free medium. The cells were cultured for 1 day in serum-free media, and treated with NE at 0, 0.1, 1, and 10 μ M for 24 hours. Six hours prior to the end of the 24 hour treatment, 0.5 μ Ci [³H]-thymidine (2 Ci/mmol) was added and the cells were incubated at 37°C. After 6 hours of incubation, cells were trypsinized and harvested onto glass fiber filter papers using a PHD Cell Harvester (Cambridge Technology). Each glass fiber filter was placed into a scintillation tube with 4 ml of scintillation cocktail (Bio Safe II, Research Products International Corp.). The amount of [³H]-thymidine incorporated was quantified using a LS60001C liquid scintillation counter (Beckman).

2.4 Real-time PCR Assessment of VEGF, IL-8, and IL-6 Gene Expression

We utilized real-time RT-PCR on NE-treated cell lines in order to determine the effect of NE on VEGF, IL-8, and IL-6 gene expression. Total RNA from cultured cells was isolated using TRIzol reagent following the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA). First strand cDNAs were synthesized using random primers and Superscript II RNase H⁻ reverse transcriptase (Invitrogen Life Technologies). Levels of VEGF, IL-8, and

IL-6 mRNA were analyzed using the Taqman Gene Expression Assays (Assay IDs: VEGF, Hs00173626_m1; IL-8, Hs00174103_m1; IL-6, Hs00174131_m1; Applied Biosystems, Foster City, CA). The housekeeping gene GAPDH was analyzed using the Taqman Human GAPDH Endogenous Control Reagent (Assay ID: 4326317E, Applied Biosystems) and was used as an internal positive control. All TaqMan Gene Expression Assays used in our analyses utilize primer sequences that span across two adjacent exons of the target genes and are thus specific for mRNAs. Levels of each target mRNA were measured with TaqMan fluorogenic probes listed above, and amplified using the 7300 Real-Time PCR system (Applied Biosystems). Reactions were performed with 12.5 μ l TaqMan Universal Master Mix and 1.25 μ l of the specific primer adjusted to a final volume of 25 μ l with water. The cycling conditions were as follows: incubation for 2 min at 50 °C followed by another incubation step at 95 °C for 10 min, afterwards 15 s at 95 °C and 1 min at 60 °C for 40 cycles. The levels of expression of VEGF, IL-8, and IL-6 mRNA in each sample were normalized to the GAPDH mRNA levels. The relative expression of mRNA species was calculated using the comparative C_T method as described by the manufacturer (see User bulletin #2 Applied Biosystems, P/N 4303859, 1997) (Livak and Schmittgen, 2001).

In order to test the role of mRNA stability in the NE-dependent regulation of VEGF, IL-8, and IL-6 mRNA levels in C8161 cells, the effect of actinomycin D, an inhibitor of *de novo* transcription, on mRNA levels was assessed. C8161 cells were grown in the presence of 5 μ g/ml actinomycin D (Sigma Aldrich, St. Louis, MO) and 10 μ M NE for 1 or 2 hours. Total RNA was isolated and the levels of VEGF, IL-8, and IL-6 mRNA were measured using real-time PCR as above.

2.5 Analysis of β -adrenergic receptor expression by real-time PCR and Western blotting

The expression of ADRB1 and ADRB2 mRNA (encoding for β 1- and β 2-AR, respectively) in C8161, 1174MEL and Me18105 cells were assayed using Taqman Gene Expression Assays (Assay IDs: Hs00265096_s1 and Hs00240532_s1, respectively) as above. The expression of β 1- and β 2-AR proteins was assessed using Western blotting as previously described (Yang et al., 2006).

2.6 Assessment of Signaling Pathways

In order to examine the signaling pathway involved in the NE-dependent effects we treated C8161 cells with a variety of agonists and antagonists. Blocking experiments were first performed with the β -AR antagonist propranolol and the α -AR antagonist phentolamine. Propranolol (1 μ M) or phentolamine (10 μ M) were added to the cell cultures 30 min before adding 10 μ M NE.

Since the cAMP-PKA signaling pathway has been suggested to mediate the effects of NE on ovarian cancer cells to upregulate VEGF (Thaker et al., 2006), a possible role of this pathway for the NE-dependent effects on C8161 cells was tested. C8161 cells were treated with the β -AR agonist isoproterenol, β 1-AR agonist dobutamine, β 2-AR agonist terbutaline, adenylate cyclase agonist forskolin, the selective activator of the cAMP receptor Exchange Protein directly activated by cAMP (EPAC) 8-(4-chlorophenylthio)-2'-O-methyladenosine-3', 5'-cyclic monophosphate (8-CPT), the selective activator of the cAMP-dependent protein kinase (PKA) N6-Benzoyladenine-3', 5'-cyclic monophosphate (6-Bnz-cAMP), and the PKA inhibitors, H-89 and myristoylated protein kinase inhibitor (PKI). C8161 cells were grown in serum-free media alone, 10 μ M NE, 10 μ M isoproterenol, 10 μ M dobutamine, 10 μ M terbutaline, 10 μ M forskolin, 100 μ M 8-CPT, or 100 μ M 6-Bnz-cAMP. To assess the effect of the PKA inhibitors, C8161 cells were first treated with H-89 or PKI for 30 minutes prior to the addition of 10 μ M NE. Cells were collected after a 1 hour incubation to measure IL-8 and IL-6

mRNA and 2 hours for VEGF gene expression analysis. Total RNA was isolated and the levels of VEGF, IL-8, and IL-6 mRNA were measured using real-time PCR as described.

Propranolol and phentolamine were purchased from Fisher Scientific (Waltham, MA); isoproterenol, dobutamine and terbutaline from Sigma (St. Louis, MO); forskolin, 6-Bnz-cAMP, and 8-CPT from Axxora Life Sciences (San Diego, CA); H-89 and myristoylated PKI from Calbiochem (La Jolla, CA).

2.7 Immunohistochemical analysis of β 1- and β 2-AR in melanoma biopsies

Ten biopsies of primary melanoma (nodular, superficial spreading and desmoplastic) and 10 biopsies of metastatic melanoma (lymph node and visceral metastasis) were obtained from the Archives of the Pathology Department and examined for β 2-AR expression as previously described (Yang et al., 2006). Additionally, melanoma biopsies were examined for β 1-AR expression using the V-19 rabbit anti- β 1-AR polyclonal antibody (sc-568) (Santa Cruz Biotechnology, Santa Cruz, CA). A surgical pathologist evaluated each biopsy.

2.8 Statistical Analysis

Experiments were performed in duplicate and repeated at least once. Results were expressed as the mean $\pm$ s.e.m. Changes in VEGF, IL-8, and IL-6 protein levels were evaluated with an analysis of variance (ANOVA) with an LSD post-hoc test using SPSSTM v16.0 (SPSS Science, Chicago, IL). $P \leq 0.05$ was considered significant.

3. Results

3.1 VEGF, IL-8, and IL-6 protein levels in culture supernatants of NE-treated C8161, 1174MEL, and Me18105 cells

We observed a NE dose-dependent and time-dependent increase in VEGF in culture supernatants of C8161 cells with the 6-hour treatment yielding the greatest effect ($F(3,26) = 5.282$, $P < 0.05$). Treatments of C8161 cells with 1 and 10 μ M NE for 6 hours resulted in increases to 176.61 ± 35.70 % (from 59.41 ± 9.21 pg/ml to 112.67 ± 32.86 pg/ml) ($P = 0.001$) and 249.79 ± 26.00 % (from 59.41 ± 9.21 pg/ml to 151.05 ± 30.77 pg/ml) ($P < 0.001$) of control levels, respectively (Fig. 1A).

Likewise, IL-8 protein levels in culture supernatants of C8161 cells increased in response to exposure to NE. Upregulation was observed at the 1 hour time point which approached significance ($F(3, 12) = 3.427$, $P = 0.052$). Significant upregulation was also observed after 3, 6, and 24 hours of treatment ($F(3, 28) = 3.613$, $P < 0.05$, $F(3, 31) = 2.984$, $P < 0.05$, and $F(3, 26) = 10.517$, $P < 0.001$, respectively). Peak stimulation was observed after treatment of C8161 cells with 10 μ M NE for 6 hours with IL-8 protein levels increasing to 315.55 ± 64.72 % of control (from 141.46 ± 41.48 pg/ml to 570.18 ± 313.82 pg/ml) ($P < 0.05$) (Fig. 1B).

It is of interest that the greatest change was observed with levels of IL-6 protein. For example, although treatment of C8161 cells with 0.1 μ M NE for 6 hours did not significantly stimulate IL-6 protein levels, treatment with 10 μ M resulted in a robust increase in IL-6 protein level of up to at least 3186.34 ± 1035.33 % of control level (increasing from below the detectable limit of 3.12 pg/ml to 99.41 ± 32.30 pg/ml) ($P < 0.05$) (Fig. 1C).

Overall, the 1174MEL and Me18105 cells exhibited NE-dependent upregulation of VEGF, and IL-8 proteins similar to C8161 cells, albeit to a lesser extent. Treatment of 1174MEL cells with 10 μ M NE for 24 hours resulted in the greatest increase in the level of VEGF up to 186.76 ± 30.22 % of control VEGF levels (increasing from 208.29 ± 64.63 pg/ml to 309.59 ± 52.42 pg/ml) ($P = 0.001$) (Fig. 1D), while IL-8 levels increased up to 345.87 ± 29.70 % of control

levels in the same treatment conditions (increasing from 236.72 ± 88.93 pg/ml to 693.93 ± 214.86 pg/ml) ($P < 0.001$) (Fig. 1E). Me18105 cells did not exhibit as great a response to NE exposure; treatment with $10 \mu\text{M}$ NE for 6 hours resulted in an increase in VEGF levels of up to $194.63 \pm 22.18\%$ of control levels (increasing from 59.29 ± 28.26 pg/ml to 61.69 ± 20.95 pg/ml) ($P < 0.001$) (Fig. 1F). On the other hand, IL-8 levels decreased in culture supernatants in response to NE treatment with levels at $66.50 \pm 6.61\%$ of control after treatment with $10 \mu\text{M}$ NE for 24 hours (81.33 ± 16.98 pg/ml in untreated controls vs. 53.37 ± 10.13 pg/ml in treated cells) ($P < 0.001$) (Fig. 1G). Furthermore, these two cell lines did not produce detectable levels of IL-6 protein in untreated and NE treated culture supernatants.

3.2 The effect of NE on the proliferation of melanoma cells

We subsequently examined whether a NE-dependent effect on cell proliferation is a factor in the observed increase in VEGF, IL-8, and IL-6 protein levels. Using [^3H]-thymidine incorporation, no significant dose-dependent increase in cell proliferation was observed after exposure of the human melanoma cell lines to NE (Fig. 2). The only significant change observed was a decrease in [^3H]-thymidine incorporation after treatment of C8161 and 1174 MEL cells with $10 \mu\text{M}$ NE. These data suggest that a NE-dependent stimulation of proliferation is not a factor in the modulation of the pro-angiogenic protein levels in culture supernatants.

3.3 NE upregulates VEGF, IL-8, and IL-6 gene expression in C8161 cells

To further elucidate the mechanism involved in the modulation of VEGF, IL-8, and IL-6 protein levels in culture supernatants of C8161 cells we examined the effect of exposure to NE on the transcription of these genes. As measured by real-time PCR, we observed that treatment of C8161 cells with NE upregulated the levels of VEGF, IL-8, and IL-6 mRNA with IL-6 mRNA levels being most greatly upregulated. This is consistent with the observed robust increase in IL-6 protein levels after exposure to NE. Treatment of C8161 cells with $10 \mu\text{M}$ NE resulted in a significant increase of VEGF mRNA levels which peaked after 2 hours of treatment (Fig. 3A). In contrast to VEGF and IL-6 mRNA, upregulation of IL-8 mRNA levels were observed with treatment with $0.1 \mu\text{M}$ NE. The induction levels of IL-8 mRNA in C8161 cells peaked after 1 hour of treatment and decreased thereafter (Fig 3B) with maximum levels of IL-8 mRNA observed after treatment of C8161 cells with $1 \mu\text{M}$ NE. Finally, NE was also observed to more efficiently upregulate the level of IL-6 mRNA in cultures of C8161 cells. Whereas the levels of mRNA in cultures exposed to 0, 0.1, and $1 \mu\text{M}$ NE remained low, treatment with $10 \mu\text{M}$ NE resulted in a marked burst in IL-6 mRNA levels peaking after 1 hour of treatment with levels decreasing thereafter (Fig. 3C).

Co-treatment of C8161 cells with $5 \mu\text{g/ml}$ actinomycin D and $10 \mu\text{M}$ NE efficiently inhibited the NE-dependent upregulation of VEGF, IL-8, and IL-6 mRNA levels (Fig. 3D, E, F, respectively). These results suggest that the NE-dependent upregulation of VEGF, IL-8, and IL-6 protein in C8161 cells is mainly due to the stimulation of *de novo* transcriptional activity of these three genes.

3.4 β 1- and β 2-adrenergic receptors are expressed in human melanoma cell lines

Real-time PCR was utilized to assess the levels of the genes encoding for the β 1- and β 2-AR (ADRB1 and ADRB2, respectively) expressed in C8161, 1174MEL and Me18105 cells. The representative real-time PCR result indicates that the human melanoma cells used in this study express ADRB1 and ADRB2 mRNA with the 1174MEL cells expressing the most ADRB1 mRNA, followed closely by C8161, and Me18105 expressing the lowest amount (Fig. 4A). C8161 cells expressed the most ADRB2 mRNA (Fig. 4B).

The above differences are further buttressed by the observation that the three human melanoma cells differed in the expression of β 1- and β 2-AR proteins as shown by Western blotting.

Differences in the β 1- and β 2-AR expression pattern between C8161, 1174MEL and Me18105 cells were observed. Cell lysates from the three cell lines probed for β 1-AR revealed a band with an apparent molecular weight of $75 \times 10^3 M_r$ (Fig. 4C). However, an additional band migrating at about $85 \times 10^3 M_r$ was observed in 1174MEL and Me18105 cell lysates. On the other hand, C8161, 1174MEL and Me18105 cells exhibited the same pattern of immunoreactive bands when probed for β 2-AR but differed in intensity (Fig. 4D); a band at $47 \times 10^3 M_r$, consistent with the weight of the unglycosylated protein, and bands migrating around 90 to $100 \times 10^3 M_r$, consistent with the size of dimers. Additionally, cells exhibited (at very low abundance) a band at approximately $65 \times 10^3 M_r$ that is consistent with the glycosylated receptor (Salahpour et al., 2003). β 2-AR primarily exists as dimers in C8161 cells. However, it is primarily expressed in the unglycosylated monomeric form in both 1174MEL and Me18105 cells. These bands were not observed in blots incubated with normal rabbit serum (not shown). The significance of the differences in β -AR expression, at the protein and mRNA levels, in C8161 vs. 1174MEL and Me18105 cells is not fully understood.

3.5 Determination of the roles of adrenergic receptors and downstream signaling factors

In an effort to determine whether β -ARs mediate the NE-dependent modulation of VEGF, IL-8, and IL-6 gene expression (Fig. 5A, B, C, respectively) in C8161, 1174MEL, and Me18105 cells, we treated the cells with nonselective AR antagonists. The α -AR antagonist phentolamine efficiently inhibited the NE-dependent stimulation of VEGF, IL-8, and IL-6 gene expression in Me18105 cells. On the other hand, similar treatment of C8161 and 1174MEL cells resulted in an increase in the levels of all three mRNAs. Treatment with the β -AR antagonist propranolol completely abrogated the NE-induced upregulation of VEGF, IL-8, and IL-6 mRNA levels in all three cell lines. These data indicate that the NE-dependent upregulation of VEGF, IL-8, and IL-6 mRNA levels in C8161, 1174MEL, and Me18105 cells is mediated by the β -ARs. Additionally, the above results show that the α -AR mediates the NE-dependent upregulation of these three genes in Me18105 cells.

We assessed whether the cAMP-PKA signaling pathway plays a role in the NE-dependent upregulation of VEGF, IL-8, and IL-6 mRNA levels in C8161 cells (Fig. 5D, E, F, respectively). Cells were treated with the β -AR agonist isoproterenol, the β 1-AR agonist dobutamine, the β 2-AR agonist terbutaline, the adenylate cyclase agonist forskolin, the cAMP analogs, 6-Bnz-cAMP and 8-CPT, and the PKA inhibitors, H-89 and PKI. Treatment with 10 μ M NE or 10 μ M isoproterenol resulted in significant stimulation of VEGF, IL-8, and IL-6 gene expression supporting the hypothesis that ligation of β -ARs is involved in the observed NE-dependent effect. Furthermore, both dobutamine and terbutaline treatments stimulated VEGF, IL-8, and IL-6 gene expression implicating both β 1- and β 2-ARs as mediators of the NE-dependent effect. In addition, although treatments with 10 μ M forskolin, the adenylate cyclase agonist, resulted in higher levels of VEGF, IL-8, and IL-6 mRNA in C8161 cells, exposure of these cells to 100 μ M 8-CPT or 100 μ M 6-Bnz-cAMP did not give the same result. Lastly, we observed the differential effects of the PKA inhibitors, H-89 and PKI, on VEGF, IL-8, and IL-6 mRNA levels in NE-treated C8161 cells. Both inhibitors partially abrogated the effect of NE on VEGF gene expression. On the other hand, H-89 was able to completely inhibit the NE-dependent effect on IL-8 and IL-6 gene expression, while PKI failed to exert the same effect. Further studies will need to be performed to further elucidate the role of the cAMP-PKA pathway or alternative pathways in the modulation of VEGF, IL-8, and IL-6 in C8161 cells.

3.6 Melanoma biopsy specimens express the β 1- and β 2-AR

In order to determine the clinical relevance of our results, we examined all three histological types of primary melanoma tumors including nodular, superficial-spreading and desmoplastic. We also examined lymph node and visceral metastases. All three types of primary melanoma

tumors exhibited strong β 2-AR immunoreactivity in the tumor cells (9/10 cases) (Table 1). The staining was homogeneous and present both cytoplasmically as well as on the plasma membrane (Fig. 6). The metastases gave a similarly strong, homogeneous pattern of staining for both lymph nodes as well as visceral metastases (9/10 cases; not shown).

This staining was not apparent in negative control slides wherein no primary or secondary antibodies were applied (not shown). As a follow up, we examined melanoma biopsies for β 1-AR expression and observed 6/10 of primary melanoma cases and 8/10 of metastatic melanoma cases were positive for the receptor (Table 1).

4. Discussion

In this study, we examined the effect of NE on the expression of factors important in melanoma tumor progression. We found that exposure of the human melanoma tumor cell line, C8161, to NE resulted in the induction of VEGF, IL-8, and IL-6 protein levels. This NE-dependent effect was not as strong in the 1174MEL and Me18105 cell lines as compared to the C8161 cell line. We show that the upregulation of the levels of these proteins in culture supernatants is not due to a stimulation of cell proliferation. In contrast to the observed difference in the NE-dependent modulation of protein levels in the three cell lines, we show that NE efficiently upregulated VEGF, IL-8, and IL-6 mRNA levels in all three cell lines. We further show that the expression of the three genes in C8161 cells is mainly due to increased *de novo* transcription. We show that C8161, 1174MEL and Me18105 cells expressed different levels of the β 1- and β 2-AR proteins and the mRNA that encode them (the ADRB1 and ADRB2, respectively). It is currently not known what accounts for the difference in the transcriptional and translational effects of NE on these three proteins. Evidence supporting the role of β -ARs in the NE-dependent effect is provided by the results showing that propranolol completely inhibited the NE-dependent upregulation of gene expression in all three cell lines, and that the agonists dobutamine and terbutaline stimulated VEGF, IL-8, and IL-6 gene expression in C8161 cells.

The activation of the hypothalamic-pituitary-adrenal (HPA) and the SAM axes that results in an increase of stress hormone levels have been shown to induce immune dysregulation significant enough to produce health outcomes, such as the slowing of wound healing (Glaser and Kiecolt-Glaser, 2005; Kiecolt-Glaser et al., 1995; Mercado et al., 2002). Stress-associated immune dysregulation can upregulate the production of proinflammatory cytokines, such as IL-1, TNF- α , and IL-6 (Glaser and Kiecolt-Glaser, 2005; Gomez-Merino et al., 2005; Johnson et al., 2005; Kiecolt-Glaser et al., 2005; Segerstrom and Miller, 2004). Inflammation has been linked to a significant percentage of cancers suggesting another alternative whereby stress could be a co-factor for modulating the growth and progression of tumor cells. Stress-induced changes in the cellular immune response results in decreased antigen-specific T-cell and natural killer (NK) cell responses and have implications for the immune response to immunogenic tumors (Glaser and Kiecolt-Glaser, 2005; Padgett and Glaser, 2003). Studies have also implicated stress-related effects on immune function as having an influence on tumor progression (Ben-Eliyahu, 2003; Goldfarb and Ben-Eliyahu, 2007; Lamkin et al., 2008; Reiche et al., 2004; Saul et al., 2005). The data obtained in this study support the hypothesis of an alternative pathway linking stress with cancer progression via effects on other biological processes involving the SAM axis (Antoni et al., 2006; Reiche et al., 2004; Saul et al., 2005; Thaker et al., 2007).

Work by Entschladen and others have indicated a role for catecholamines in tumor progression (Entschladen et al., 2004). For example, catecholamines have been shown to promote the migration of breast and colon cancer cells *in vitro* (Drell et al., 2003; Masur et al., 2001) and the metastasis of human PC-3 prostate cancer cells in nude mice (Palm et al., 2006), effects that could be inhibited by the addition of β -blockers. The data from this study suggest that NE

may further enhance tumor progression by stimulating the process of angiogenesis. The processes promoted by the NE-dependent upregulation of VEGF, IL-8, and IL-6 expression are yet to be fully understood. However, these factors have been implicated in several processes associated with melanoma tumor progression including the angiogenic process (Mahabeleshwar and Byzova, 2007). For example, it has been shown that VEGF released by melanoma cells is an important mediator of neo-vascularization and has been shown to be a marker for melanoma progression (Löffek et al., 2006; Osella-Abate et al., 2002). In addition, serum levels of IL-6 have been associated with advanced stages of the disease, the ability to discriminate progressive from non-progressive disease, and have been used as a serum marker for monitoring response to chemotherapy and survival. Furthermore, IL-8 and IL-6 are released in the culture supernatants of highly invasive melanoma cells (Bar-Eli, 1999; Payne and Cornelius, 2002). Both these factors are involved in angiogenesis by either directly stimulating the proliferation and differentiation of endothelial cells or by inducing the production of additional factors in other cells (Bar-Eli, 1999).

Since the recent studies by Sood and others suggest that the NE-dependent effects on the growth of ovarian tumor cells in nude mice are mediated through the tumor cell cAMP-PKA signaling pathway (Thaker et al., 2006), we focused on this signaling pathway in our study. Our results show that isoproterenol is a potent inducer of VEGF, IL-8 and IL-6 gene expression in C8161 cells supporting the role of β -ARs in this pathway. In addition, our results show that the adenylate cyclase agonist forskolin stimulated the expression of the VEGF, IL-8 and IL-6 genes while the cAMP analogs 8-CPT and 6-Bnz-cAMP did not efficiently stimulate the expression of the three genes. Furthermore, our observation that H-89 effectively inhibited the NE-dependent upregulation of IL-8 and IL-6 mRNA levels in C8161 cells while PKI did not can be explained by the fact that H-89 has inhibitory effects on the activities of protein kinase G (PKG) and PKC as well (Chijiwa et al., 1990; Hidaka and Kobayashi, 1992). These observations suggest that the cAMP-PKA signaling pathway may play a limited role in mediating these effects and further elucidation of the signaling pathway involved has yet to be addressed.

It is of interest that we are seeing differences in the expression of pro-angiogenic proteins in cells derived from different kinds of tumors in spite of the fact that the tumor cells express β -ARs and that these proteins can be induced by NE in each study (Yang et al., 2008; Yang et al., 2006). Although NPC, multiple myeloma-derived, and melanoma cell lines all express immunoreactive bands of the same relative molecular weights in Western blots detecting β 1- and β 2-ARs, the abundance of the specific bands differed among these types of cancers. We still do not know how these differences play a part in their varying responses to NE. Our data add to the growing literature showing that the modulatory effects of psychological factors may have important clinical relevance and may lead to new approaches for treating cancer.

Consistent with our NPC study, we also observed the expression of β 2-AR on tumor cells in a majority of melanoma biopsies supporting the possible clinical relevance of our *in vitro* results. It is important to note that in this study we examined different classes of melanoma, i.e., primary melanoma (nodular, superficial spreading and desmoplastic) and metastatic melanoma (lymph node and visceral metastasis). We found that these classes of melanoma express the β 1-AR as well. That a majority of the melanoma tumors we examined expressed β -ARs supports our hypothesis that melanoma tumor cells at various stages of the disease have the potential to respond to NE.

Derived from neural crest stem cells, human malignant melanoma is one of the most deadly of human neoplasms, exhibiting aggressive metastatic behavior even when its primary tumor is very small (Safarians et al., 1996). Whereas most human cancers metastasize when they reach a size of 1 cm diameter, melanomas can metastasize when they are only 1 mm in size

demonstrating a 1000 fold increase in inherent metastatic potential (Barsky et al., 1997). Our traditional methods of staging and predicting distal metastasis and melanoma recurrence are based on measurements of tumor thickness in the skin and presence of tumor in regional lymph nodes but these prognostic markers fall short in the majority of cases where the likelihood of tumor recurrence and metastasis remains indeterminate (Balch et al., 2001). Although a number of genetic abnormalities have been identified in human melanomas including significant alterations involving the BRAF pathway (Haluska et al., 2007), to date no molecular subclassification scheme has improved upon the prognostic information imparted by thickness alone (Palmieri et al., 2007). Since it can be assumed that the overall tumor burden at presentation is low in the majority of cases, it is likely that host rather than tumoral factors alone regulate recurrence or cure. Stress may be one important host cofactor. Certainly the presence of adrenergic receptors and signaling in human melanoma cell lines and cases of human melanoma support this hypothesis.

Our data support the hypothesis that the stress hormone, NE, can stimulate the aggressive potential of melanoma tumor cells by inducing the release of proteins, including VEGF, IL-8, and IL-6. The role(s) that psychological factors may play in tumor progression is not fully understood but the connection between the HPA and SAM axes that have been described by our laboratory and others may add to our understanding of the role of psychological stressors on the progression of some tumors. The fact that these three factors are upregulated in C8161, a cell line known for its highly metastatic phenotype, and not to the same extent in 1174MEL and Me18105, supports the hypothesis that different tumor cell populations may respond differently to the release of stress hormones and that stress can be a co-factor for the progression of a highly metastatic cancer. It is not known whether 1174MEL and Me18105 produce metastatic tumors in a nude mouse. To our knowledge, this is the first report to suggest that stress-related activation of the SAM axis may have a role in human melanoma tumor progression.

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Figure 1. NE differentially modulates the levels of VEGF, IL-8, and IL-6 protein in culture supernatants of C8161 (A, B, and C), 1174MEL (D and E), and Me18105 (F and G) cells after treatment with NE. Levels of protein in culture supernatants were measured after treatment with 0, 0.1, 1, and 10 μM NE for 1, 3, 6, and 24 hours. Values are presented as percent of untreated control levels. ND, not detectable. Error bars represent the mean $\pm$ s.e.m. *, $P \leq 0.05$; **, $P \leq 0.001$.

Figure 2.

Representative [3H]-thymidine incorporation assessing the effect of NE on cell proliferation. Cell proliferation was measured using [3H]-thymidine incorporation (see Materials and Methods) after C8161, 1174MEL, and Me18105 cells were treated with 0, 0.1, 1, and 10 μ M NE for 24 hours. Six hours prior to the end of the 24 hour treatment, 10 μ l of [3H]-thymidine (2 Ci/mmol) was added to medium. The labeling reaction was terminated and quantified using a liquid scintillation counter. Data are each expressed as mean $\pm$ s.e.m.; *, $P \leq 0.05$; **, $P \leq 0.001$.

Figure 3.

The NE-dependent upregulation of VEGF, IL-8, and IL-6 mRNA levels in C8161 cells is mainly through the stimulation of transcription and not through increased mRNA stability. Representative real-time PCR assay measuring VEGF (A), IL-8 (B), and IL-6 (C) mRNA levels in C8161 cells after treatment with NE. Levels of mRNA in C8161 cells were measured using real-time PCR after treatment with 0, 0.1, 1, and 10 μ M NE for 1, 2, 3, and 4 hours. Peak expression of VEGF mRNA was observed at 2 hours while highest levels of IL-8 and IL-6 mRNA levels were seen after 1 hour of treatment.

Representative real-time PCR assay measuring VEGF (D), IL-8 (E), and IL-6 (F) mRNA levels in C8161 cells after treatment with 5 µg/ml actinomycin D (Act D), 10 µM NE (NE), or 10 µM NE plus 5 µg/ml actinomycin D (NE + Act D) for 2 hours (A) or 1 hour (B and C). Values are presented as percent of untreated control levels. Error bars represent the mean ± s.e.m; *, P ≤ 0.05; **, P ≤ 0.001.

Figure 4.

Expression of $\beta 1$ -AR and $\beta 2$ -AR in C8161, 1174MEL and Me18105 melanoma cells. Representative real-time PCR assay measuring ADRB1 (A) and ADRB2 (B) mRNA levels in C8161, 1174MEL and Me18105 cells.

Western blot analysis of $\beta 1$ -AR and $\beta 2$ -AR protein expression in C8161, 1174MEL and Me18105 melanoma cells. (C) Lysates from C8161, 1174MEL and Me18105 cells probed for $\beta 1$ -AR revealed a band with an apparent molecular weight of $75 \times 10^3 M_r$ ($\rightarrow$); 1174MEL and Me18105 cell lysates exhibit an additional band at $85 \times 10^3 M_r$ ($\leftarrow$).

(D) Several bands were observed when cell lysates were probed for $\beta 2$ -AR. A single band with molecular weight of about $47 \times 10^3 M_r$ was expressed in the three cell lines ($\leftarrow$) that is consistent with the unglycosylated protein. The band at about $65 \times 10^3 M_r$ (*) is the glycosylated receptor, while the bands at about 90 to $100 \times 10^3 M_r$ are dimers (H). These bands were not observed in blots incubated with normal rabbit serum (not shown).

Figure 5.

Assessment of the role of β -adrenergic receptors and the cAMP-PKA signaling pathway. Effect of β -blocker (propranolol) and α -blocker (phentolamine) on the NE-dependent modulation of VEGF, IL-8, and IL-6 mRNA in C8161, 1174MEL, and Me18105 cells. Representative real-time PCR assay measuring VEGF (A), IL-8 (B), and IL-6 (C) mRNA levels in C8161, 1174MEL, and Me18105 cells after treatment with 10 μ M NE (NE), 10 μ M phentolamine plus 10 μ M NE (NE + Phen), or 1 μ M propranolol plus 10 μ M NE (NE + Prop) for 2 hours (A) or 1 hour (B and C) when peak gene expression were observed (See Fig. 3A–C).

Representative real-time PCR assay measuring VEGF (D), IL-8 (E), and IL-6 (F) mRNA levels in C8161 cells after treatment with 10 μ M NE (NE), 10 μ M isoproterenol (Iso), 10 μ M dobutamine (Dob), 10 μ M terbutaline (Ter), 10 μ M forskolin (Fsk), 100 μ M 6-Bnz-cAMP (6-Bnz-cAMP), 100 μ M 8-CPT (8-CPT), 10 μ M H-89 plus 10 μ M NE (NE + H89), or 10 μ M myristoylated PKI plus 10 μ M NE (NE + PKI) for 2 hours (A) or 1 hour (B and C). Values are presented as percent of untreated control levels. Error bars represent the mean $\pm$ s.e.m; *, $P \leq 0.05$; **, $P \leq 0.001$.

Figure 6.

A case of primary superficial-spreading melanoma illustrates intense β 2-AR cytoplasmic/membrane immunoreactivity within the balls of melanoma cells (arrows) occupying the dermal/epidermal junction. The overlying epidermis and underlying dermis are non-immunoreactive. This staining was not apparent in negative control slides wherein no primary or secondary antibodies were applied (not shown). Bar = 200 μ m.

Table 1

The presence of β 1- and β 2-AR in tumor cells within melanoma tumor biopsies.

PRIMARY MELANOMA	TYPE	β 1-AR (positive/total)	β 2-AR (positive/total)
	Nodular	2/3	3/3
	Superficial-spreading	2/4	3/4
	Desmoplastic	2/3	3/3
METASTATIC MELANOMA	SITE		
	Lymph node	4/5	4/5
	Visceral	4/5	5/5