Norepinephrine Up-regulates the Expression of Vascular Endothelial Growth Factor, Matrix Metalloproteinase (MMP)-2, and MMP-9 in Nasopharyngeal Carcinoma Tumor Cells

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Abstract
Recent studies using ovarian cancer cells have shown that the catecholamine hormones norepinephrine (norepi) and epinephrine (epi) may influence cancer progression by modulating the expression of matrix metalloproteinases (MMP) and vascular endothelial growth factor (VEGF). The purpose of this study is to determine if the stress hormone norepi can influence the expression of MMP-2, MMP-9, and VEGF in nasopharyngeal carcinoma (NPC) tumors by using three NPC tumor cell lines. The NPC cell lines HONE-1, HNE-1, and CNE-1 were treated with norepi. The effects of norepi on MMP-2, MMP-9, and VEGF synthesis were measured by ELISA; functional MMP activity was measured by the invasive potential of the cells using a membrane invasion culture system whereas functional activity of VEGF was analyzed using a human umbilical vein endothelial cell tube formation assay. Norepi treatment increased MMP-2, MMP-9, and VEGF levels in culture supernatants of HONE-1 cells, which could be inhibited by the β-blocker propranolol. Norepi induced the invasiveness of all NPC cell lines in a dose-dependent manner, which was blocked by CMT-3, an MMP inhibitor, and propranolol. Norepi stimulated the release of functional angiogenic VEGF by HONE-1 cells as well. Finally, HONE-1 cells were shown to express β-adrenergic receptors as did seven of seven NPC biopsies examined. The data suggest that catecholamine hormones produced by the sympathetic-adrenal medullary axis may affect NPC tumor progression, in part, through modulation of key angiogenic cytokines.

Introduction
There is evidence that psychological factors can affect the incidence and progression of some cancers (1–3). Data obtained from animal models support the hypothesis that stress could be a cofactor (4, 5). Studies in the field of psychoneuroimmunology have shown that psychological stress can affect many aspects of cellular immune function through the modulation of the endocrine system; these interactions have been shown to be bidirectional (6–8).

Aside from stress-related changes in the immune response, recent studies suggest that stress can affect downstream events that may contribute to tumor progression (e.g., angiogenesis and tumor metastasis refs. 9–11). These processes involve matrix metalloproteinases (MMP) and vascular endothelial growth factor (VEGF). MMPs belong to a family of structurally related calcium- and zinc-dependent endopeptidases that have the ability to digest a broad range of extracellular matrix molecules. These enzymes, which include the collagens, gelatinases, stromelysins, and membrane type (MT)-MMPs, have been implicated in the turnover of the extracellular matrix during tumor development and progression (12–14). VEGF has been well described as a cytokine important for endothelial cell proliferation and the process of angiogenesis essential for tumor development (15).

Recently, works by Sood et al. (9) and Lutgendorf et al. (10, 11) have shown that the catecholamines norepinephrine (norepi) and epinephrine (epi) may influence the progression of ovarian cancer by modulating the expression of MMPs and the angiogenic cytokine VEGF in ovarian cancer cells. Studies in mice support these observations. Using social isolation stress, the mRNA levels of MMP-2, MMP-9, MT1-MMP, and urokinase-type plasminogen activator were higher in tumor and liver tissues of isolated mice than in control mice grouped together (16). As these enzymes have been described to have diverse functions, studies on stress-related effects on MMP expression will help elucidate the relationship between stress and cancer initiation and progression. Whereas MMPs have initially been implicated as having major roles in extracellular matrix degradation, recent work has shown that these enzymes can modulate activities of cytokines and their receptors and the release of sequestered growth factors (17). One cytokine shown to be modulated by MMPs is VEGF. Through this effect on angiogenesis, MMP activity has been further linked to the complex process of tumor development (17).

Nasopharyngeal carcinoma (NPC) is an EBV-associated malignant tumor with the highest incidence observed among the Chinese from Hong Kong and southern China (18). It is a highly invasive and metastatic head and neck cancer characterized by metastasis to the cervical lymph nodes and distant organs (19). MMPs, the gelatinases MMP-2 and MMP-9 in particular, and VEGF have been implicated as contributing to the aggressiveness of highly metastatic NPC tumors (20, 21).

Our laboratory has been studying the role that EBV plays in the development of NPC (22). In previous studies, we described the establishment and characterization of an EBV DNA-positive NPC cell line (HONE-1) and an EBV DNA-negative NPC cell line (HNE-1;
In this study, we use these cells to examine the ability of norepi to up-regulate the expression of three factors that have roles in the progression of NPC tumors (i.e., MMP-2, MMP-9, and VEGF). It is known that the ability of norepi to up-regulate MMP-2, MMP-9, and VEGF is mediated by $\beta$-adrenergic receptors ($\beta$-AR). We present data showing that norepi can up-regulate the production of MMP-2, MMP-9, and VEGF by HONE-1 cells. Furthermore, we show that norepi can stimulate the invasive capability of HONE-1 cells through the expression of MMPs. We also show that norepi-treated NPC cells exhibit up-regulated release of functional VEGF. Experiments done to determine if NPC tumor cells express $\beta$-ARs showed that NPC cells express these receptors and that treatment with antagonists to block the binding of norepi to the receptors abrogates the up-regulation of MMP-2 and MMP-9; tumor cells in several NPC biopsies examined also express $\beta$-ARs. The data suggest that norepi, a stress hormone produced after the activation of the sympathetic-adrenal-medullary axis, may play a role in the pathogenesis of NPC.

Materials and Methods

Cell lines and culture conditions. Three different established human NPC cell lines, HONE-1, HNE-1, and CNE-1, were investigated in this study. HONE-1 cells (clone 40) were established in this laboratory and are epithelial tumor cell lines derived from a biopsy specimen of an undifferentiated NPC. The initial characterization of HONE-1 cells has previously been described (23–25). HONE-1 clone 13 and HONE-1 clone 39 cells are clones that were also derived from the parental HONE-1 cells and differ in EBV DNA status. Based on PCR and immunofluorescence analyses for EBV-associated antigens, HONE-1 clone 13 cells are EBV DNA positive whereas clone 39 cells are EBV DNA negative. The EBV DNA–negative HNE-1 cell line was also prepared in this laboratory from an undifferentiated NPC (23). CNE-1 cells were derived from a well-differentiated squamous cell NPC and are EBV DNA negative (26). Cells were maintained in RPMI 1640 supplemented with 15% fetal bovine serum and 0.1% gentamicin sulfate (Gemini Bioproducts, Calabasas, CA). All of the cell lines are routinely screened for Mycoplasma species (GenProbe detection kit, Fisher, Itasca, IL). All cultured cells were maintained at 37°C and experiments were done using 70% to 80% confluent cultures. Cells were seeded in 12-well plates, with 2.5 x $10^5$ per well cultured for 1 day in serum-free medium, and then treated with norepi at 0, 0.1, 1, and 10 $\mu$M/L. In our first set of experiments, supernatants were removed at 1, 3, 6, and 12 hours, centrifuged, and frozen at –80°C until assayed. For blocking experiments, 1 $\mu$M/L propranolol was added to the cell cultures 30 minutes before adding 10 $\mu$M/L norepi. Culture supernatants were collected at 6 hours, centrifuged, and frozen at –80°C until assay.

Determination of MMP-2, MMP-9, and VEGF protein expression. The protein concentrations of total MMP-2 (pro- and active MMP-2), total MMP-9 (pro- and active MMP-9), and VEGF were determined with the use of QuantiKine immunoassays (R&D Systems, Minneapolis, MN) following the protocols of the manufacturer. These assays employ the quantitative sandwich enzyme immunoassay technique. The minimum detectable levels are as follows: MMP-2, 0.03 to 0.40 ng/mL; MMP-9, <0.156 ng/mL; and VEGF, <5.0 pg/mL. The concentrations of active MMP-2 and MMP-9 were determined with the Biotrak Activity Assay System (Amersham Biosciences Corp., Piscataway, NJ) as per protocol of the manufacturer. The MMP experiments were done in triplicate and repeated once.

Real-time PCR assessment of MMP-2, MMP-9, and VEGF gene expression. Semiquantitative real-time reverse transcription-PCR (RT-PCR) was used to assess the expression of MMP-2, MMP-9, and VEGF genes in norepi-treated HONE-1 cells. Total RNA from cultured cells was isolated with TRIzol following the instructions of the manufacturer (Invitrogen Life Technologies, Carlsbad, CA). First-strand cDNAs were synthesized with random primers and Superscript III RNase H– reverse transcriptase (Invitrogen Life Technologies). Levels of MMP-2, MMP-9, and VEGF mRNA were analyzed with the TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA). The housekeeping gene gylceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed with the TaqMan Human GAPDH Endogenous Control Reagent (Applied Biosystems) and was used as an internal positive control. All TaqMan gene expression assays used in our analyses were specific for mRNAs by designing the primer sequences across two adjacent exons of the target genes. Levels of each target mRNA were measured with TaqMan fluorogenic probes listed above and amplified with the 7300 Real-Time PCR system (Applied Biosystems). Reactions were carried out in a 25-$\mu$L volume and each sample was run in duplicate. The PCR thermal cycle conditions used were according to the recommendations of the manufacturer. The levels of expression of MMP-2, MMP-9, and VEGF mRNA in each sample were normalized to the GAPDH mRNA level. The relative expression of mRNA species was calculated using the comparative $C_t$ method (27).

The membrane invasion culture system assay. The membrane invasion culture system chamber was used to measure the in vitro invasiveness of the HONE-1 cells to examine the norepi-dependent stimulation of MMP function (9, 28). Briefly, a polycarbonate membrane with 10-mm pores (Osmonics, Livermore, CA) was uniformly coated with a defined basement membrane matrix consisting of human laminin, type IV collagen, and gelatin and used as the intervening barrier to invasion. Both upper and lower wells of the chamber were filled with serum-free RPMI medium containing 1 x 10^5 Mito + (Collaborative Biomedical, Bedford, MA). Single-cell tumor cell suspensions, prepared after trypsinization, were seeded into the upper wells at a concentration of 1 x 10^5 cells per well. After a 24-hour incubation in a humidified incubator at 37°C in 5% CO2, cells that had invaded through the basement membrane were collected, stained, and counted by light microscopy (28). Invasiveness was calculated as the percentage of cells that had successfully invaded through the matrix-coated membrane to the lower wells compared with the total number of cells seeded into the upper wells and corrected for cell proliferation. As a control, HONE-1 cells were treated with CMT-3 (0.5 uM/L), a chemically modified tetracycline with broad-spectrum MMP inhibitor activity (Colla-Genex Pharmaceuticals, Newton, PA; ref. 9).

In vitro human umbilical vascular endothelial cell tube formation assay. To assess the angiogenic activity of VEGF produced by HONE-1 cells stimulated with norepi, HONE-1 cells were grown in either serum-free medium alone or in medium containing 10 $\mu$M/L norepi. Treated and untreated HONE-1 cells were grown at 37°C in 5% CO2 for 1 hour. Supernatants were collected and stored at –80°C until use. All supernatants were concentrated 10-fold using a YM-10 Microcon centrifugal filter device (Millipore Corp., Bedford, MA). Human umbilical vein endothelial cells (HUVEC; BioWhittaker, Inc., Walkersville, ME) were cultured in these supernatants on growth factor–depleted Matrigel (Discovery Labware, Bedford, MA) distributed in a 96-well plate (60 $\mu$L/well) and allowed to solidify at 37°C for 1 hour. HUVECs (passage 3) were serum starved in M200 minimal medium (Cascade Biologicals, Portland, OR) for 8 hours at 37°C. All controls and samples were resuspended in M200 medium and had 1.0 x 10^5 HUVECs per well. All components [M200 medium alone, M200 medium + 10 ng/mL recombinant human VEGF, the cell supernatants alone or with 2 $\mu$g/mL anti-human VEGF neutralizing antibody (R&D Systems)] were rotated at 4°C for at least 1 hour before addition to HUVECs. All conditions were added to the polymerized Matrigel and incubated at 37°C for 10 hours. Then, tube formation between HUVECs was observed and digital pictures were captured with an Olympus inverted microscope. Quantification of angiogenic activity was measured by counting branch points from tubes formed between discrete endothelial cells in a blinded manner. Total branch points in three high-power fields (using a 20 x objective lens) were counted per well.

Analysis of $\beta$-AR expression by RT-PCR and Western blotting. The expression of $\beta$-ARs in HONE-1 cells was assessed by RT-PCR and Western blotting because it is to these receptors that norepi has been shown to primarily bind. The RT-PCR analysis of $\beta$-ARs in HONE-1 cells was as previously described for ovarian cell lines (10). For the Western blot analysis of $\beta$-AR expression, cell lysates were prepared from 5 x 10^5 HONE-1 cells in teniposide.
200-µL buffer containing 100 mmol/L Tris-HCl (pH 7.6), 0.5% Triton X-100, 2 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 1 µL of protease inhibitor cocktail. PMSF and protease inhibitor cocktail were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Proteins were electrothermally separated in 10% polyacrylamide gels, transferred to Hybond-ECL membranes (Amersham Biosciences), and probed with 1.0 µg/mL of either the V-19 rabbit anti-ß1-AR polyclonal antibody (sc-568) or the H-73 rabbit anti-ß2-AR polyclonal antibody (sc-9042) overnight at 4°C. As negative controls, blots were incubated in 1.0 µg/mL of normal rabbit immunoglobulin G (IgG; sc-2027). Antibodies sc-568, sc-9042, and sc-2027 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Blots were then incubated in 1:1,000 dilution of anti-rabbit IgG-peroxidase conjugate (Calbiochem, San Diego, CA) for 1 hour at room temperature. Positive reactivities were visualized with the Phototope-HRP Western Detection System (Cell Signaling Technology, Inc., Danvers, MA).

**Immunohistochemical analysis of ß2-AR in NPC biopsies.** Seven paraffin-embedded NPC biopsies were obtained from the tumor archives of the Pathology Department. The biopsies were cut at 4 µm and placed on positively charged slides. Tissue section slides were placed in a 60°C oven for 1 hour, cooled, and deparaffinized with xylene and rehydrated through washes with a series of graded ethanol solutions to water. All slides were quenched for 5 minutes in a 3% H2O2 solution in methanol to block endogenous peroxidase.

The detection system used a labeled streptavidin-biotin complex (Dako, Carpinteria, CA). Consecutive applications of the following were done: primary antibody for the primary antibody species, an enzyme-conjugated streptavidin for signal amplification, and a substrate chromogen, 3'-amino-diaminobenzidine, for visualization and tissue location of the antigen. Slides were then counterstained in Richard Allen hematoxylin (Fisher Scientific International, Inc., Hampton, NH) and dehydrated through graded ethanol. Normal mouse IgG was used as negative control of the first antibody. A surgical pathologist evaluated each biopsy.

EBV in situ hybridization was done with the oligonucleotide INFORM EBER Probe (Ventana Medical Systems, Inc., Tuscon, AZ) on the BenchMark XT automated system according to the recommended protocol of the manufacturer.

**Statistical analysis.** Continuous variables were compared by either Student’s t test or ANOVA and categorical variables by χ² test. If appropriate, nonparametric tests (Mann-Whitney test) were used to compare differences.

The basal level of MMP-2, MMP-9, and VEGF expression for two different cell-lines, HONE-1 clone 13 cells and HONE-1 clone 39 cells, is defined by a zero dose of norepi at the following time points: 1, 3, 6, and 12 hours. Two-way ANOVA was used to determine whether the main effects of time and replication were significant. If the main effect of replication was not significant, it was dropped from the model. Each time point was tested against the 1-hour time point to determine whether the difference was statistically different from zero. The Holmes method was used to adjust for multiple comparisons. Linear, quadratic, and cubic trends for the time points were tested with the partial F test. Additionally, the main effect of cell line was entered into the model to see if the difference between the two cell lines was significant. MMP-2, MMP-9, and VEGF expression was checked for normality, which is a necessary condition for ANOVA. If the expression was not normally distributed, a nonparametric Kruskal-Wallis method was used to determine whether the main effects of time and duplication were significant.

Two-way ANOVA was used to determine VEGF-mediated changes in HUVEC tube formation, with treatment as the dependent variable. P < 0.05 was considered significant.

**Results**

Norepi-mediated up-regulation of expression of MMP-2, MMP-9, and VEGF in HONE-1 cells. As already discussed, the synthesis of MMPs and VEGF by tumor cells has been implicated in the aggressiveness of NPC tumors (20). We examined the effects of norepi on the secretion of MMP-2, MMP-9, and VEGF in two clones of HONE-1 cells, clone 13 (EBV DNA positive) and clone 39 (EBV DNA negative). The ability of norepi to stimulate the release of the three proteins was not related to the presence of EBV DNA. There was a dose-dependent increase in MMP-2, MMP-9, and VEGF in HONE-1 clone 39 (Fig. 1). For example, treatment of HONE-1 clone 39 cells with 10 µmol/L norepi resulted in a maximum increase in MMP-2 levels of 226.30 ± 10.56% above control after 12 hours and a maximum change of 332.35 ± 20.38% above control MMP-9 levels after treatment with 10 µmol/L norepi for 12 hours. Norepi 10 µmol/L induced a 35% to 52% increase in active MMP-2 and a 39% to 62% increase in active

![Figure 1](https://example.com/figure1.png)

Figure 1. MMP-2 (A), MMP-9 (B), and VEGF (C) concentrations in medium from HONE-1 clone 39 cells after treatment with norepi. Levels of protein in culture supernatants were measured after treatment with 0.1, 1, and 10 µmol/L norepi for 1, 3, 6, and 12 hours. Points, percent change from untreated HONE-1 cells; bars, SE.
MMP-9 levels in both clones of the HONE-1 cell lines 6 hours after treatment (data not shown). Finally, treatment of HONE-1 clone 39 cells resulted in the levels of VEGF in the culture supernatants peaking after 3 hours of treatment regardless of the norepi concentration (172.08 ± 9.988, 282.18 ± 35.29%, and 261.52 ± 40.37% enhancement with 0.1, 1, and 10 μmol/L norepi, respectively). Similar results were observed for HONE-1 clone 13 cells (not shown). Our data show that both HONE-1 clone 13 and clone 39 cells exhibited norepi-dependent increase in MMP-2, MMP-9, and VEGF release despite differences in EBV DNA status.

As the presence or absence of the EBV DNA in HONE-1 cells did not influence the ability of the cells to respond to norepi treatment, we then examined whether there was a significant difference in the basal expression of MMP-2, MMP-9, and VEGF between HONE-1 clone 13 and HONE-1 clone 39 cells. HONE-1 clone 39 cells exhibited an overall higher basal level of MMP-2 in the culture supernatants at all four time points (P < 0.001) compared with HONE-1 clone 13. On the other hand, HONE-1 clone 13 cells produced higher basal levels of VEGF in the culture supernatants at all time points compared with HONE-1 clone 39 (P < 0.001). There was no significant difference between the basal levels of MMP-9 in the culture supernatants of the two HONE-1 clones (P = 0.65). Further investigation is needed to explore the significance of these observations.

**Norepi does not modulate MMP-2, MMP-9, and VEGF gene expression in HONE-1 cells.** To examine whether the up-regulation of expression of MMP-2, MMP-9, and VEGF was a result of norepi-dependent transcriptional regulation, we cultured HONE-1 clone 39 cells in the presence of 1 μmol/L norepi for 0.5, 1, 2, 3, and 4 hours. This concentration was used because it resulted in the most robust effect on MMP-2, MMP-9, and VEGF protein expression in the previous experiments. We did not observe significant up-regulation of gene expression after norepi treatment (not shown). Together, these data suggest that the increase in levels of MMP-2, MMP-9, and VEGF proteins is not a result of transcriptional changes. This is consistent with observations by others that MMP expression can be regulated posttranscriptionally, including release from intracellular stores (29, 30).

**Norepi-induced up-regulation of MMP-2, MMP-9, and VEGF is accompanied by an increase in the cell invasive properties of HONE-1 cells in vitro.** To determine if the norepi-induced production of MMP-2, MMP-9, and VEGF resulted in functional proteins, we measured the ability of norepi-treated and untreated NPC tumor cells to penetrate a defined basement membrane matrix barrier. HONE-1 clone 39 cells were treated with 0.1, 1, and 10 μmol/L norepi and assayed 24 hours later for invasiveness using the membrane invasion culture system assay (31). Norepi induced a dose-dependent increase in invasive capacity that peaked with almost a 4-fold enhancement in the penetration of cells treated with 10 μmol/L norepi (P < 0.001; Fig. 2A). Similar effects were observed with two other NPC-derived cell lines, HNE-1 and CNE-1, with 10 μmol/L norepi producing 3-fold increases in invasive capacity in both CNE-1 (P = 0.01) and HNE-1 (P = 0.009) cells (Fig. 2A). To examine whether this effect on invasion is unique to norepi, we tested the effect of epi on the invasive capacity of HONE-1 cells. Treatment of HONE-1 cells with 10 μmol/L epi enhanced their invasiveness by ~ 3.5-fold (P = 0.03; Fig. 2B). These observations suggest that both norepi and epi up-regulate the invasiveness of HONE-1 clone 39 cells.

As MMPs have been implicated as playing a key role in the digestion of the components of the basement membrane during metastasis, we tested the ability of the broad-spectrum MMP inhibitor CMT-3 to inhibit the norepi-mediated effects on the invasiveness of HONE-1 cells. Treatment of HONE-1 clone 39 cells with 5 μmol/L CMT-3 completely blocked the 1 μmol/L norepi-stimulated invasion (Fig. 2C). These results suggest that MMPs play a critical role in mediating the effects of norepi on the ability of HONE-1 tumor cells to invade the basement membrane matrix in the membrane invasion culture system assay.

**Norepi stimulates the release of functional VEGF by HONE-1 cells.** We used the HUVEC tube formation assay, an *in vitro* method to measure angiogenic activity, to assess the ability of norepi to stimulate the release of functional VEGF by HONE-1 clone 39 cells. We found a significant norepi-dependent stimulation of angiogenic activity after 1 hour of treatment compared with supernatants from HONE-1 clone 39 cells grown in serum-free medium alone (0 versus 4.0 ± 0.6 tubes formed, respectively; *P* = 0.002; Fig. 3A and B). Furthermore, incubation of HUVECs with cell supernatants from 10 μmol/L norepi–treated HONE-1 cells to which 2 μg/mL of an anti-VEGF IgG neutralizing antibody were added effectively reduced the angiogenic activity 1.7-fold (4.0 ± 0.6 versus 2.3 ± 0.3; *P* = 0.06), supporting the idea that the major angiogenic factor in the supernatants is indeed related to the presence of VEGF. The observation that the addition of anti-VEGF neutralizing antibody does not completely eliminate the angiogenic activity suggests that there may be other angiogenic factors in these culture supernatants that are unrelated to VEGF or that anti-VEGF antibodies will not neutralize. These results implicate norepi as an inducer of the release of biologically active VEGF by HONE-1 cells.
Expression of $\beta$-ARs in HONE-1 cells. We next determined if the mechanism of action of norepi on HONE-1 cell invasiveness involves $\beta$-ARs. RT-PCR analysis showed that both clones of HONE-1 cells tested constitutively express the transcripts for both $\beta_1$-AR and $\beta_2$-AR (Fig. 4A). Western blot analysis of $\beta_1$-AR and $\beta_2$-AR expression in lysates of HONE-1 clone 39 cells further showed that these cells express both $\beta$-ARs (Fig. 4B). Cell lysates probed for $\beta_1$-AR revealed a band with an apparent molecular weight of 75 kDa. Cell lysates probed for $\beta_2$-AR revealed several immunoreactive bands: two bands with molecular weights of $\approx 47$ to 50 kDa consistent with the weight of the unglycosylated protein, a band at $\approx 65$ kDa consistent with the expected weight for the glycosylated receptor, and two to three bands at $\approx 90$ to 100 kDa possibly resulting from dimerization (32). These bands were not observed in blots incubated with normal rabbit serum (data not shown).

In an effort to further determine whether these $\beta$-ARs mediate norepi effects on HONE-1 clone 39 cells, we treated cells with the broad-spectrum $\beta$-blocker propranolol (1 $\mu$mol/L) 30 minutes before adding 10 $\mu$mol/L norepi (10). Propranolol completely abrogated the norepi-induced up-regulation of MMP-2 and MMP-9 release, suggesting that the action of norepi is through these receptors (Fig. 4C and D). Additionally, we examined whether these receptors mediated the effects of norepi on the invasiveness of HONE-1. Treatment of cells with propranolol immediately following their introduction into the membrane invasion culture system chamber and 30 minutes before the addition of 10 $\mu$mol/L norepi blocked the norepi-mediated stimulation of invasion, but propranolol alone had no effect on invasive ability (Fig. 2C).

NPC tumor cells in biopsy specimens express $\beta$-AR. Based on the functional effects of norepi on invasion and angiogenic processes, we next asked whether $\beta$-ARs are also expressed on human NPC tumors. All NPC tumors expressed $\beta_2$-AR on the membranes in a large number of epithelial cells (Fig. 5A). Seven NPC paraffin-embedded biopsies were obtained from the tumor archives of The Ohio State University Department of Pathology. Three of these seven biopsies were shown to be EBV DNA positive either at the mRNA or protein level (data not shown). The histopathologies of these tumor biopsies are provided in Table 1: two of the tumors were nonkeratinizing (type 2), four were undifferentiated (type 3), and one was intermediate between types 2 and 3.

Discussion

In this study, we found that exposure of NPC tumor cell lines to a key stress hormone, norepi, resulted in increased invasion and greater production of factors responsible for invasion and angiogenic responses. We also provide preliminary data that epi may also produce similar results.

$\beta_1$-AR and $\beta_2$-AR mRNAs and proteins were expressed in HONE-1 cells. Evidence supporting the role of these receptors in the norepi-dependent effect is provided by our results showing that propranolol inhibited the norepi-dependent up-regulation of MMP-2 and MMP-9 release. Importantly, we also found that the NPC tumor cells in seven different NPC biopsies examined express $\beta$-AR, suggesting the results obtained with NPC-derived tumor cell lines are relevant to NPC tumors in vivo and the role that increased
levels of catecholamines might play in the growth and spread of NPC tumor cells. These interactions were not related to the EBV DNA status of the HONE-1 cells. To our knowledge, this is the first report to show that stress-related activation of the sympathetic-adrenal-medullary axis may have a role in NPC progression.

There are studies that suggest that stress-related activation of the sympathetic-adrenal-medullary axis may have a role in the progression of ovarian tumors in that norepi can stimulate the production of VEGF by ovarian tumor cell lines (10). Using the SKOV3 ovarian tumor cell line, MMP-2 and MT1-MMP were shown to be important for angiogenesis as well as extracellular matrix remodeling (14). It is of interest that stress has been linked to the expression of MMP and VEGF expression in ovarian tumor cell lines, including SKOV3. The tumor cells express h-ARs, and when treated with epi and norepi, an increase in the production of MMP-2, MMP-9, and VEGF was observed (9, 10, 14). In a recent report, Thaker et al. (33) induced ovarian tumors in nude mice. Stress, in the form of restraint and social isolation, was used to activate the sympathetic-adrenal-medullary axis and induce catecholamine hormones (e.g., norepi) levels. Tumors in stressed mice showed an increase in vascularization and increases in VEGF, MMP-2, and MMP-9 levels were found in tumor tissue; enhanced tumor growth was observed in the stress mice. These data support the clinical significance of increases in sympathetic-adrenal-medullary stress hormones as they relate to tumor progression. The activation of the hypothalamic-pituitary-adrenal and the sympathetic-adrenal-medullary axes and the subsequent increases of stress hormone levels have been shown to induce immune dysregulation significant enough to produce health outcomes, such as the slowing of wound healing (8, 34, 35). The data obtained in this study and the previous studies by Sood et al. (9), Lutgendorf et al. (10), and Thaker et al. (33) provide evidence for an alternative pathway that links stress with cancer progression via effects on other biological processes.

Stress-associated immune dysregulation can up-regulate the production of proinflammatory cytokines, such as interleukin (IL)-1, tumor necrosis factor α, and IL-6 (8). Inflammation has been linked to a significant percentage of cancers, suggesting another pathway whereby stress could be a cofactor for modulating the growth of tumor cells (7, 8). Several studies have implicated stress-related effects on immune function as having an influence on tumor progression (1, 5, 7). The results obtained in a recent study by Thaker et al. (33) and in this study continue to support the stress-cancer connection mediated by catecholamines.

The coexpression and possible interaction between MMPs and VEGF have been described in several human cancers. For example,
high levels of VEGF and MMPs have been described in patients with ovarian cancer. MMP-2 and MMP-9 have been shown to induce the release of VEGF in two ovarian tumor cell lines, OVCA3 and SKOV3, suggesting that an interplay between VEGF and the gelatinases may be a factor in progression of ovarian tumors (36, 37). In addition, it has been shown that levels of MMP-9 and VEGF are positively correlated in head and neck squamous cell carcinoma (38) and expression of these is important for neovascularization in the tumors (39). The gene expression level of VEGF has been shown to be positively correlated with those of MT1-MMP, MMP-2, and MMP-9 in human glioblastomas (40). The levels of MT1-MMP and VEGF proteins were also colocalized within tumor cells and endothelial cells. As MT1-MMP is a major factor in the activation of MMP-2, this observation further implicates MMPs in the control of expression and bioavailability of VEGF and strengthens the link between MMPs, VEGF, and angiogenesis. The fact that norepi up-regulates the expression of VEGF, a growth factor also shown to induce MMP expression (41), suggests that the up-regulation of MMP-2 and MMP-9 observed in this study and others may be an effect mediated by VEGF in HONE-1 cells. The fact that we did not detect evidence for the up-regulation of gelatinase gene expression while showing increased levels of secreted protein is consistent with observations that MMP expression can be regulated posttranscriptionally, including release from intracellular stores (29, 30).

The data suggest that the norepi effects on HONE-1 cells involve the ligation of β2-ARs and are mediated downstream by factors within the β-AR signaling pathway. Treatment of HONE-1 cells with propranolol before treatment with norepi abrogated the norepi-induced up-regulation of MMP-2 and MMP-9, as well as their invasive capabilities, implicating β-AR as a mediator for this effect. The participation of these receptors and the signaling pathway involved in the stimulation of VEGF secretion are of great interest and are currently under investigation.

NPC is a highly invasive and metastatic head and neck cancer that has the ability to metastasize to distant organs (19). MMPs, the gelatinases MMP-2 and MMP-9 in particular, have been implicated in contributing to the aggressiveness of highly metastatic forms of NPC tumors (20, 42, 43). Our results suggest that norepi may have a role in modulating the expression of MMPs in NPC. Although our studies did not examine the effect of norepi on the expression of other MMPs,zymographic analysis of culture supernatants from untreated and norepi-treated HONE-1 clone 13 and clone 39 cells suggests that MMP-2 and MMP-9 are the predominant MMPs secreted by these cells (data not shown). It is likely that MMP-2 and MMP-9 expression in NPC is mainly involved in the degradation of extracellular matrix components for these tumors to metastasize. However, as MMPs have been implicated in other processes during tumor progression, including angiogenesis (14), these enzymes may well be very important factors during NPC development. Future studies include the examination of the possible interaction between MMP-2, MMP-9, and VEGF in HONE-1 cells.

The presence of the EBV genome in NPC tumor cells affects NPC metastasis and angiogenesis through the induction of the expression of prometastatic and proangiogenic factors by certain EBV proteins (44). For example, EBV latent membrane protein-1 (LMP-1) induces VEGF, MMP-1, and MMP-9 expression (44) and the transactivator BZLF-1 induces expression of MMP-1 and MMP-9 (44). However, the nature of the relationship between LMP-1 and the expression of these genes in HONE-1 cells is not known. We have observed that the LMP-1 gene is present in HONE-1 clone 13 cells by microarray analysis and real-time Q-PCR at the time these experiments were done (data not shown). Further investigations are needed to elucidate the role of LMP-1 in the norepi-mediated changes in MMP-2, MMP-9, and VEGF expression in HONE-1 clone 13 cells. Our observation that norepi up-regulates the levels of MMP-2, MMP-9, and VEGF shows that there are other pathways used in the regulation of the expression of these tumor markers that do not involve EBV-encoded LMP-1. For example, it has been shown that nuclear factor κB (NF-κB) is involved in the up-regulation of expression of several prometastatic and proangiogenic genes, including MMP-9 and VEGF (45). Although LMP-1 has also been shown to activate the NF-κB signaling pathway (46), our observations are consistent with those of Thornburg et al. (47) that activation of NF-κB p50 homodimers in NPC occurs independently of LMP-1 expression. It has been shown that norepi can activate NF-κB when it binds to β-AR (48).

The clinical relevance of our in vitro results is supported by the data showing the expression of β2-AR on tumor cells of 7/7 type 2 and type 3 NPC biopsies. That all seven NPC tumors we examined showed the presence of β2-AR in plasma membranes supports our hypothesis that NPC tumor cells have the potential to respond to norepi, and perhaps epi as well. The expression of β2-AR in these NPC biopsies is consistent with their undifferentiated morphology because it has been shown that β2-AR is expressed by keratinocytes in human epidermis and that its expression is down-regulated during differentiation (49). Additional clinical relevance of this line of research is provided by a study using the B16 melanoma mouse tumor model. Mice that were

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Table 1. The presence of β2-AR on NPC tumor cells in undifferentiated NPC biopsies

<table>
<thead>
<tr>
<th>Diagnosis/comments</th>
<th>Morphology</th>
<th>Type</th>
<th>EBV</th>
<th>β2-AR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Lymphoepithelioma</td>
<td>Undifferentiated</td>
<td>3</td>
<td>NT</td>
<td>1+</td>
</tr>
<tr>
<td>2 Not indicated</td>
<td>Undifferentiated</td>
<td>3</td>
<td>+ (IHC)</td>
<td>1+</td>
</tr>
<tr>
<td>3 Carcinoma</td>
<td>Undifferentiated</td>
<td>3</td>
<td>NT</td>
<td>2+</td>
</tr>
<tr>
<td>4 Nonkeratinized</td>
<td>Undifferentiated</td>
<td>2</td>
<td>+ (IS)</td>
<td>2+</td>
</tr>
<tr>
<td>5 Not indicated</td>
<td>Undifferentiated</td>
<td>3</td>
<td>+ (IS)</td>
<td>2+</td>
</tr>
<tr>
<td>6 Acute and chronic inflammation</td>
<td>Undifferentiated</td>
<td>2</td>
<td>NT</td>
<td>2+</td>
</tr>
<tr>
<td>7 Lymphoid hyperplasia</td>
<td>Undifferentiated</td>
<td>2-3</td>
<td>NT</td>
<td>3+</td>
</tr>
</tbody>
</table>

Abbreviations: NT, not tested; IHC, immunohistochemistry; IS, in situ hybridization.
either housed in a crowded condition or housed in isolation both showed an increase in tumor growth. The oral administration of the β-adrenergic antagonist propranolol completely abrogated the stress enhancement of tumor growth (50). Thaker et al.’s study (33) supports these findings.

To our knowledge, there has not been any previous study to indicate a relationship between psychological stress and NPC tumor formation and development. This article presents findings to suggest that at least two catecholamines may contribute to the development and progression of this aggressive tumor.

Acknowledgments

Received 7/7/2006 revised 8/18/2006 accepted 8/28/2006

Grant support: National Cancer Institute (NCI) grant CA100243. The Gilbert and Kathryn Mitchell Endowment and The Ohio State University Comprehensive Cancer Center Core Grant CA16058 (N.R. Glaser), NCI grants CA11079301 and CA10929801 (A.K. Sood), and National Heart, Lung, and Blood Institute Grant HL007176 (C.B. Marsh).

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We thank Ryan Roberts and Elise Donovan for their technical assistance, and D. Dittmer and C. Hilscher for expert real-time quantitative PCR and for communicating primer sequences before publication.

References

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