Neuromedin B and its receptor are mitogens in both normal and malignant epithelial cells lining the colon

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NMB is a member of the bombesin-like family of neuropeptides (13), a group of proteins aberrantly expressed by a variety of cancers and of great interest because of their ability to cause tumor cell proliferation (26, 43). As a part of studies evaluating bombesin in cancer, NMB/NMB receptor (NMB-R) mRNA has been detected in cancer cell lines derived from human pancreas (4), prostate (1, 32), breast (15, 18), ovary (33), lung (5, 16, 29), and colon (8, 9, 14). Because NMB has been shown to cause the proliferation of a variety of tumor cell lines, it has commonly been grouped with other bombesin-like peptides and is considered to be an important growth factor in a variety of cancers including those arising in the colon (8, 9, 14).

However, these studies into NMB function are hampered by a lack of specific antibodies to this ligand and its receptor, as well as by the lack of specific NMB-R antagonists. In this paper, we characterize new antibodies to human NMB and the NMB-R. By immunohistochemistry we show that these proteins are normally expressed by epithelial cells lining the human colon, as well as by colorectal cancers. We also show that NMB causes a variety of nonmalignant and malignant human colon cell lines to proliferate, albeit modestly. Overall, these findings indicate that 1) bombesin-like peptides cannot uniformly be classified as cancer-specific growth factors, and 2) that NMB in particular appears to be important in the growth and maintenance of normal human colonic epithelial tissues.

MATERIALS AND METHODS

Materials. Oligotex mRNA Mini Kit was purchased from QIAGEN (Valencia, CA). SuperScript First-Strand Synthesis System for RT-PCR and TRIzol reagent were from Invitrogen (Carlsbad, CA). DNase was from Ambion (Austin, TX). Mammalian protease inhibitor cocktail was purchased from Sigma (St. Louis, MO). Enhanced chemiluminescence (ECL) Plus Western Blotting Detection System and Rainbow colored protein molecular weight markers were from Amersham (Piscataway, NJ). Acrylamide/bis solution (30%) and Precision Plus protein standards were from Bio-Rad (Hercules, CA). BCA Protein Assay kit was from Pierce (Rockford, IL). Goat antimouse IgG horseradish peroxidase (HRP) and protein A agarose were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). NMB was purchased from BACHEM Bioscience (King of Prussia, PA). Anhydrous ethanol (100 and 95%) and xylene were purchased from Pharmaco Products (Brookfield, CT). Tris-buffered saline with Tween 20 (TBST) wash buffer, Target retrieval solution, protein block serum, antibody diluent, EnVision+ HRP, diaminobenzidine (DAB)-Rabbit System, and automated hematoxylin were all from DAKO (Carpinteria, CA). Polyvinylidene difluoride (PVDF) membranes, Auto/Idoline,

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Redassol, Permount, G-418 sulfate, and all other reagents were purchased from Fisher Scientific (Pittsburgh, PA), OPTI-MEM I, trypsin, and all other cell culture reagents were obtained from GIBCO-BRL (Carlsbad, CA). Peptide-N-glycosidase F (PNGase F), SDS/β-mercaptoethanol, and Nonidet P-40 (NP-40) were from Glyko (San Leandro, CA). D-Phe6(bombesin) methyl ester was kindly provided by Dr. David Coy (Tulane University, New Orleans, LA). Antibodies for NMB and NMB-R were synthesized by Zymed Laboratories (San Francisco, CA) and graciously provided by Tularik (San Francisco, CA). 125I-labeled NMB was synthesized commercially by PerkinElmer (Wellesley, MA) without carrier, using 125I in a modification of the iodogen method and purified by reverse-phase HPLC. Balb 3T3 fibroblasts stably transfected to selectively express one of the three known mammalian bombesin receptors [bombesin receptor subtype 3 (BRS3-R)] (23), gastrin-releasing peptide (GRP) receptor (GRP-R) (2), or NMB-R (3)] were graciously provided by Dr. Robert T. Jensen (National Institutes of Health, Bethesda, MD).

mRNA amplification by RT-PCR. Cells were lysed using RNA STAT-60. Chloroform was then added, and the solution was centrifuged at 12,000 g for 15 min at 4 °C. The upper aqueous layer was removed, mixed with isoamyl alcohol (0.5 ml per 1 ml of RNA STAT-60 used) and centrifuged at 12,000 g for 10 min at 4 °C. The RNA pellet was washed with 75% ethanol by vortexing and subsequently centrifuged at 7,500 g for 5 min at 4 °C, air-dried, and resuspended in water. mRNA and cDNA were isolated by using the Qiagen and Invitrogen kits as described by manufacturers. Human NMB was amplified from cDNA using forward primer 5′-CGG GGC GCG GAG GC-3′ and reverse primer 5′-GAC GTC TGC CAC GCT TGC TC-3′ (484 bp). Actin, used as a control, was amplified from cDNA using forward primer 5′-ATG GAA GAA GAG ATC-3′ and reverse primer 5′-CTG TCG GCA ATG-3′; and actin-P: 5′-TGC GGG GCG GCT GAG GCT C-3′/H11032; NMB-R: 5′-ATG GAA GAA GAG ATC-3′/H11032; reverse primer 5′-CDC GAC TGT CTC ATG GCC TGG GGC GCG GAG GC-3′ and reverse primer 5′-GCC GAG GCG GAG GGT CTT-3′ (312 bp). Human NMB-R was amplified by using forward primer 5′-CCG ACT CTG CTG GAA AGA A-3′ and reverse primer 5′-GAG TGC CAC GCT TGC TC-3′ (484 bp). Actin, used as a control, was amplified from cDNA using forward primer 5′-ATG GAA GAA GAG ATC GC-3′ and reverse primer 5′-GGA TGC CAC GCT TGG TCT-3′ (245 bp). PCR amplification was carried out for 25 cycles for NMB and actin at 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s. PCR amplification for NMB-R was carried out for 40 cycles at 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s.

Quantitative (real time) PCR. The colon tumor samples for this study were obtained from the National Cancer Institute Cooperative Human Tissue Network. Q-PCR was performed by using a TaqMan 7700 Sequence Detector by Applied Biosystems (Foster City, CA) and specific fluorogenic TaqMan probes. Total RNA was isolated from tumor samples using TRIzol reagent treated with DNase to eliminate genomic DNA. The reverse transcriptase reaction was coupled with quantitative PCR measurement of cDNA copy number in a one-tube format according to the manufacturer’s instructions. NMB and NMB-R expression were normalized against levels found in nonmalignant and malignant intestinal tissues.

TagMan primer sequences used are as follows. NMB-F: 5′-CAA ATA CTT CAG AAA TGA CCA CAA T-3′; NMB-R: 5′-AGG GTC CCA TTC ACC ACC TT-3′; NMB-P: 5′-[6FAM]-CC(AA CAG CGT GGC TTA GAT TGT GCC G)[TAMRA-FAM]-3′; NMB-R-F: 5′-AGG GTC CCA TTC ACC ACC TT-3′; NMB-R-R: 5′-[6-FAM]-CC(AA CAG CGT GGC TTA GAT TGT GCC G)[TAMRA-FAM]-3′; actin-F: 5′-GCC AAG ACC TGT ACC AAG CGA AAA CC-3′; actin-R: 5′-AGG GTC CCA TTC ACC ACC TT-3′; actin-P: 5′-[6FAM]-CC(AA CAG CGT GGC TTA GAT TGT GCC G)[TAMRA-FAM]-3′.

Antibody design. The murine NMB monoclonal antibodies were made against keyhole limpet hemocyanin (KLH) conjugated to the amidated form of NMB. The KLH was conjugated to a cysteine added to the NH₂ terminus of a synthetic amidated NMB peptide, giving a sequence of H₂N-CGCGLWATGHFM-NH₂ that was then raised as the immunogen (Fig. 1A).

NMB-R rabbit polyclonal antisera were made against KLH-conjugated human NMB-R in four separate rabbits. Antisera 1A and 2A were raised against a KLH conjugated cysteine-labeled peptide corresponding to human NMB-R amino acid 367–390 (corresponding to the COOH-terminal region of the receptor, sequence H₂N-CSNAKN-MTVNSVLLNGHSMKQEMMAM-COOH). Antibodies 1B and 2B were raised against a KLH-conjugated cysteine-labeled amidated peptide corresponding to human NMB-R amino acid 244–263 (corresponding to the third intracellular loop region of the receptor, sequence: H₂N-CHNLPGYEYHTKQKMETRRK-NH₂) (Fig. 1B).

Immunoprecipitation. In all instances, 500 μg of protein was resuspended in RIPA buffer and incubated with 10 μl of NMB-R antibody for 2 h at 4 °C. Protein A-agarose (20 μl) was then added and left on a rocker overnight at 4 °C. The solution was pelleted by centrifugation at 8,000 rpm for 90 s at 4 °C. The supernatant was discarded, and the pellet was washed three times with RIPA buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-10, 0.1% SDS, 0.5% sodium deoxycholate; 1 μM sodium orthovanadate; 5 μM EDTA; 5 μM sodium fluoride). The pellet was reconstituted in 100 μl SDS loading buffer and electrophoresed across a polyacrylamide gel (20% for NMB, 10% for NMB-R) under denaturing and reducing conditions.

Western blot analysis. Just confluent cells were rinsed in PBS and lysed in ice-cold RIPA buffer containing a 1:20 dilution of mammalian protease inhibitor cocktail. Protein concentrations were determined by using the BCA Protein Assay kit as described by the manufacturer. In all instances, 200 μg protein was loaded and electrophoresed across a 10% polyacrylamide gel under denaturing and reducing conditions. The resolved proteins were electrophoretically transferred to PVDF membranes. A Bio-Rad Mini-Protein II Multiscreen (Richmond, CA) was used to test the antibodies at different concentrations. For all reactions, 200 μl of primary antibody was added to each lane for 2 h followed by two 10-min washes with TBST.
Immunoreactive bands were visualized by using a HRP-conjugated goat anti-rabbit IgG and the ECL Plus detection system. Determination of NMB-R glycosylation was performed as recommended by the manufacturer. In all instances, cell membranes were prepared, and 100 μg protein were suspended in 45 μl of 5× reaction buffer (10 mM sodium phosphate, 0.1% sodium azide, pH 7.5), 2.5 μl denaturing solution (2% SDS, 1 M β-mercaptoethanol), and the protein was denatured by heating to 95°C for 5 min. After being cooled to room temperature, 2.5 μl of detergent solution (15% NP-40, 10 mM PNGase F) was added. The mixture was incubated at 37°C for 18 h, and the proteins were separated by SDS-PAGE.

Tumor specimens and histological grading. Colon cancers were randomly selected from the UIC Gastrointestinal Tumor Bank. Differentiation was assessed as previously described (6, 7). Briefly, well-differentiated tumor cells give evidence of formed glands containing malignant columnar cells with small regular nuclei. Moderately differentiated tumors demonstrate well-formed glands but contain cells that are less columnar, or cuboidal, along with reduced cell polarity and dysplastic nuclei. In contrast, poorly differentiated tumors demonstrate a complete absence of gland formation. The UIC Institutional Review Board approved use of these tissues for this study under the stipulation that no linked clinical data could be used as a part of their evaluation and analysis.

Tissue processing and quantitative immunohistochemistry. Tissues were sectioned (4-μm thick) by using a Sakura Accu-Cut SRM 200 Rotary Microtome (Torrance, CA) and then processed for antigen retrieval as follows. Sections were rehydrated in graded alcohols and a running water bath, placed in iodine for 1 min, rinsed in TBST buffer, rinsed twice in Redusol, and rinsed once again in TBST buffer. The slides were then placed in Target retrieval solution at 100°C for 20 min, allowed to cool to room temperature, and rinsed once again in TBST.

Immunohistochemistry was performed by using a two-step indirect immunoperoxidase technique to test for NMB and NMB-R expression, whereas a three-step indirect technique was performed to evaluate GRP and GRP-R expression levels. Briefly, in the two-step method, slides were incubated in a 3% H2O2 solution to quench endogenous peroxidase activity, rinsed with TBST, blocked with Protein Block for 30 min, and rinsed in TBST. Primary antibody (NMB was 1:200 and NMB-R was 1:1,000) was applied at room temperature for 1 h before rinsing with TBST. Labeled polymer rabbit HRP was then added for 30 min, rinsed thoroughly with TBST, followed by incubation with DAB Chromogen for 8 min, and then counterstained for 2 min with hematoxylin. In the three-step method, slides were incubated in a 3% H2O2 solution to quench endogenous peroxidase activity, rinsed with TBST, blocked with protein block for 30 min, and rinsed again in TBST. Primary antibody (GRP, 1:100 and GRP-R, 1:1,500) was applied for 1 h before rinsing with TBST. A biotinylated rabbit IgG was then applied for 10 min, rinsed with TBST, followed by incubation with a streptavidin-HRP for 10 min. Slides were rinsed with TBST followed by DAB chromogen incubation for 5 min and then counterstained for 2 min with hematoxylin. All tissues were then dehydrated in graded alcohols and xylene and placed on coverslips using Permount. For all specimens, control tissues were sectioned (4-μm thick) and processed identically and at the same time, except that primary antibody was not applied. Thus all differences between the experimental and control tissue are ultimately due to DAB identification of the relevant protein.

Chromogen abundance was quantified by quantitative immunohistochemistry as previously described (24, 25). To do this, images were acquired by using a SPOT RT Digital Scanning Camera (Diagnostic Instruments, Sterling Heights, MI) attached to a Nikon E600 microscope (Stamford, CT). In all cases, files were saved in tagged-image file format. The amount of chromogen present is represented by all information contained in the experimental image file minus that contained in the control image file. Chromogen quantity is expressed in the valueless units of energy units per pixel as previously described (24, 25).

Cell proliferation. In all instances, 50,000 cells were plated in defined medium in 12-well plates and cultured under standard conditions for 24 h. The cells were then washed with PBS and cultured for another 5 days in OPTI-MEM alone (i.e., serum-free) or in the presence of 1 nM NMB, 1 μM NMB, 1 μM GRP, 1 μM n-Phe6 bombesin (methyl ester), a GRP-R specific antagonist (10, 11, 22), or antibody anti-NMB4 to NMB at a concentration of 1:100. In all instances, fresh ligand or antibody was added daily to replace that which may have been internalized or destroyed by endogenous enzymatic activity. At the indicated time points, cells were detached by exposure to 500 μl of 0.25% trypsin/1 mM EDTA (purchased as a premade solution from Invitrogen) for 2 min. Total numbers of detached cells were determined by using a Beckman-Coulter Z2 counter (Miami, FL).

RESULTS

Expression of NMB and NMB-R mRNA. Few studies exist studying NMB/NMB-R expression in human colonic tissues. We therefore evaluated NMB/NMB-R expression by RT-PCR in the human colon cancer cell lines Caco-2 and HT-29 as well as in the nonmalignant human colonic epithelial cell line NCM-460 (27). To confirm the specificity of our gene-specific primers, we also evaluated mRNA obtained from murine Balb 3T3 fibroblasts stably transfected to ectopically express BR3-R (23), GRP-R (2), or NMB-R (3). RNA isolated from each cell line was incubated with reverse transcriptase followed by PCR amplification. Negative controls included the absence of template as well as reverse transcriptase. Primers specific for NMB-R mRNA only detected message in NMB-R transfected cells and did not detect related message for GRP-R or BR3-R in Balb 3T3 fibroblasts transfected to express only those bombesin receptor subtypes, thereby confirming the specificity of our primers. In Caco-2, HT-29, and NCM-460 cells, NMB (312 bp) and NMB-R (484 bp) PCR products of the expected size were obtained (Fig. 2).

We then quantified the amount of NMB/NMB-R mRNA present in normal colonic epithelium and in colon cancer (Fig. 3). To do this we obtained freshly resected human tissues from the NCI Cooperative Human Tissue Network. Both NMB and NMB-R mRNA expression could be detected in normal colonic epithelial tissues, with the amount of both being approximately similar (Fig. 3). However, wide variations in the amount of NMB and NMB-R mRNA expression could be detected in colon cancers from a variety of individuals (e.g., compare patient 7 vs. patient 36, Fig. 3). Interestingly, the amount of NMR mRNA and NMR-R mRNA was not necessarily the same from each tumor.

Expression of NMB protein. To determine NMB protein expression levels, we evaluated mouse monoclonal antibodies directed against human NMB. Four clones that tested positive in ELISA immunoprecipitated 125I-labeled NMB, and blocked binding of 125I-labeled NMB to NMB-R (data not shown), were tested for their ability to recognize endogenously expressed NMB in Caco-2 cells.

NMB shares 7 of its 10 amino acids with its closely related member in the bombesin family GRP. This degree of overlap resulted in the generation of a NMB antibody uniquely recognizing NMB. Hence we set out to determine whether we could create a NMB antibody in concert with that which we have already synthesized to recognize GRP (Fig.
1A), which would allow us to distinguish between these two proteins.

All four NMB monoclonal antibodies (α-NMB1, α-NMB2, α-NMB3, and α-NMB4) were tested at concentrations of 1:100, 1:200 (Fig. 4), and 1:400 (not shown). Antibodies α-NMB1 and α-NMB2 at concentrations as high as 1:100 did not identify any specific protein by immunoprecipitation (Fig. 4). However, α-NMB3 at a concentration of 1:100, and α-NMB4 at the same concentrations as well as at a concentration of 1:200, identified two proteins whose molecular masses were ∼1.2 and ∼1.5 kDa (Fig. 4). In contrast, our previously characterized polyclonal antibody to GRP (6) identified a single band at ∼2.5 kDa (predicted 2.8 kDa) (Fig. 4). The predicted molecular mass of NMB is 1.1 kDa, suggesting that our two different bombesin antibodies are capable of distinguishing between immunoprecipitated NMB and GRP.

Because antibody α-NMB4 worked best for immunoprecipitation, we used this antibody for immunohistochemistry. Because we previously demonstrated that although GRP was not detectable in normal colonic mucosa but was detectable in colon cancer (6), we used this tissue type as substrate for evaluating our NMB antibody. Preparations were incubated with 1 μM or 10 μM NMB antibody to examine whether this could be the case, Caco-2 cell membranes (100 μg total protein) were incubated with 10 μM NMB antibody at 37°C for 18 h (Fig. 5A, B, C). Immunoprecipitation was then performed to provide greater insight as to which band likely represented the NMB-R. After immunoprecipitation, all antibodies identified a single band of ∼80 kDa (Fig. 5D). Because the predicted molecular mass is 43 kDa, this suggested the possibility that the NMB-R is posttranslationally modified by glycosylation. To evaluate whether this could be the case, Caco-2 cell membranes (100 μg total protein) were incubated with 10 μM of the non-specific deglycosylating agent PNGase F as previously described (2, 20). Incubation of membranes at 37°C for 18 h yielded multiple additional bands at 65, 47, and 43 kDa (Fig. 5E), confirming that the NMB-R is glycosylated.

To further assess NMB-R antibody specificity, cell lines stably transfected to ectopically express only human NMB-R, GRP-R, or BRS3-R were evaluated. Total proteins from these cell lines were exposed to NMB-R antibody 2B (1:1,000) or to GRP-R antibody (1:1,500). After exposure to NMB-R antibody 2B, a single band was detected at ∼40 kDa in the NMB-R transfected cell line but not in GRP-R or BRS3-R expressing cells (Fig. 7). In contradistinction, GRP-R antibody identified three separate bands in protein extracts from GRP-R express-
Fig. 4. NMB and GRP antibody characterization by immunoprecipitation. NMB antibodies were designed and generated as described in MATERIALS AND METHODS and applied against total protein isolated from the human colon cancer cell line Caco-2 separated by SDS-PAGE. Both the ~1.2- and ~1.5-kDa NMB isoforms are readily detected for antibody α-NMB4 at dilutions of 1:100 and 1:200, whereas the other antibodies do not reliably detect NMB. Immunoprecipitation of our previously characterized antibody to GRP (6) is used at a concentration of 1:75 to identify a specific single band at ~2.5 kDa and is shown for comparative purposes.

**Immunohistochemistry.** We then used the most specific and sensitive antibodies for NMB (α-NMB4) and NMB-R (2B), along with antibodies directed against GRP and GRP-R, to evaluate for the expression of these proteins in normal and malignant epithelial cells lining the colon.

We found that unlike GRP/GRP-R, which are only expressed by malignant colon cancer cells (Ref. 6 and Figs. 8 and 9), NMB/NMB-R are expressed in normal colonic epithelial cells (Figs. 8–10) as well as in colon cancers of varying stages of tumor cell differentiation (Figs. 8 and 9). Interestingly, most NMB expression in normal colonic epithelial cells was found to be in the middle third of each crypt (Fig. 10A), an area that contains the bulk of the proliferating cells within this structure. In contrast, NMB-R appeared to be equally expressed along the entire length of the crypt (Fig. 10B). Finally, and unlike GRP/GRP-R, nuclear immunopositivity for both NMB and NMB-R was detected in normal and malignant cells (Figs. 8 and 9).

To more precisely determine the amount of NMB/NMB-R expressed by normal and malignant epithelial cells lining the colon, we performed quantitative immunohistochemistry as previously described (24, 25). In normal colonic epithelium there was ~1.8-fold more NMB expression in the proliferative zone of the crypt compared with the crypt base (Fig. 11). In contrast, we did not detect evidence of GRP expression by normal colonic epithelial cells (Fig. 8). Because our NMB
antibody does not distinguish between this NMB and GRP during immunohistochemistry (Fig. 5), and because we have previously demonstrated that colon cancers express GRP (6), we cannot differentiate colon cancer NMB/GRP expression. In contrast, our NMB-R and GRP-R antibodies do differentiate between these two proteins. By quantitative immunohistochemistry, we detected similar amounts of NMB-R in the base as in the proliferative zone of crypts located in normal colonic epithelium (Figs. 10 and 11). NMB-R expression increased modestly (~1.4-fold) but significantly \((P < 0.05)\) in moderately and well-differentiated colon cancer cells compared with nonmalignant tissues, whereas levels in poorly differentiated tumor cells were similar to that observed in normal colonic epithelial tissues (Fig. 11).

**Cell proliferation.** Because bombesin and bombesin-like peptides are commonly viewed to act as mitogens in the context of cancer, we then evaluated the contribution of NMB to the proliferation of nonmalignant (NCM-460) and malignant (Caco-2 and HT-29) colon cancer cell lines. After 5 days in serum-free medium, NCM-460, Caco-2, and HT-29 cells increased \(1.6 \pm 0.1\)-fold, \(1.4 \pm 0.2\)-fold, and \(1.3 \pm 0.2\), respectively (Fig. 12). Addition of 1 nM and 1 \(\mu\)M NMB universally increased the proliferation of these three cell lines and did so after 5 days in culture to a far greater degree than was observed with 1 nM or 1 \(\mu\)M GRP. Indeed, 1 \(\mu\)M GRP increased proliferation of Caco-2 and HT-29 cells to a similar extent as 1 nM NMB, and NCM-460 cells, which we have previously shown do not express GRP-R, were unresponsive to GRP at any concentration tested. These data show that NMB is more effective than GRP at stimulating proliferation in these three cell lines but that neither peptide produces a particularly robust response.

**DISCUSSION**

In this paper, we show for the first time that NMB and its receptor are expressed by normal and malignant epithelial cells lining the human colon. Importantly, and in contrast to other bombesin-like peptides such as GRP, we found that normal colonic epithelial tissues express both NMB and its receptor. In both normal and malignant human colonic cell lines, we show that NMB acts as a growth factor, and as such may be important in normal colonocyte proliferation. This indicates that bombesin-like peptides cannot be considered as acting uniformly as cancer-specific growth factors. Rather, these findings indicate that selected members of the bombesin family, such as NMB, are likely involved in the normal physiological function of colonic epithelial cells. As such, the presence of NMB and NMB-R in colon cancer may be without prognostic or therapeutic significance.

Few studies have been performed investigating the expression of NMB/NMB-R in normal and malignant gastrointestinal tissues. Binding studies (37) identified the presence of specific NMB-R-prefering receptors in rat esophageal muscularis mu-
cosae. A more recent paper (9) described NMB mRNA to be present in the overwhelming majority of normal human colonic mucosal epithelial tissues evaluated. The few studies of human colon cancers and colon cancer cell lines, typically using RT-PCR or in situ hybridization to look for specific mRNA species, have found that message for NMB-R is typically not present, whereas that for NMB is (8, 9, 14). Yet this is not only in contrast to our own observations in which both message and protein for NMB and NMB-R are present in normal and cancerous colonic tissues. It also stands in contrast to that which has been observed in other tumor types.

With the use of a variety of techniques ranging from radioimmunoassay to RT-PCR, NMB and its receptor have been found to be widely present in cell lines derived from breast (15, 18), ovary (33), prostate (1, 32), pancreas (4), small cell (5, 16), and nonsmall cell lung cancers (29, 30). In these cancerous cell lines, NMB is thought to act in an autocrine manner as a modest mitogen. This raises the question as to the accuracy of the previous, albeit few, studies failing to demonstrate NMB/NMB-R coexpression in colonic tissues.

The colonic epithelium is exquisitely sensitive to hypoxic stress such that human tissues are not infrequently injured.
during surgical resection. Most studies have focused on the presence of mRNA, molecules that are themselves fragile and suffer the consequences of tissue necrosis. In contrast, we herein studied mRNA expression in colons obtained from the NCI Cooperative Human Tissue Network, an organization specifically designed to harvest sensitive tissues with maximum care. Even so, we found the range in both NMB and NMB-R mRNA expression to be extraordinarily wide (Fig. 3).

Yet the main strength of our study is the identification of NMB and NMB-R proteins by virtue of our having designed antibodies and antisera to probe for these proteins. Using these antibodies, we clearly demonstrate that both proteins are co-expressed in normal colonic epithelial cells, as well as in colonic adenocarcinomas (Figs. 8–10).

Whereas our findings clearly indicate that NMB can act as a growth factor in the colon (Fig. 12), the magnitude of this growth effect is not large and begs the question as to whether this peptide hormone could have other actions not identified by this study. Intriguing and underappreciated studies by Traynor and O’Grady (34, 35) suggest that NMB and other bombesin-like peptides may play an important role in modulating colonic ion secretion. Alternatively, the ability of the NMB-R when activated to induce focal adhesion kinase phosphorylation in an actin-dependent manner (36) suggests the possibility that NMB

Fig. 9. Expression of NMB-R (top) and GRP-R (bottom) in human colon cancer and adjacent nonmalignant tissue. NMB-R was detected using antibody 2B as described in MATERIALS AND METHODS. NMB-R is expressed in normal colonic epithelial cells (A), as well as well-differentiated tumor cells (B), moderately differentiated tumor cells (C), and poorly differentiated tumor cells (D). In contrast, there is no evidence of GRP-R expression in normal colonic epithelial cells. Panoramic images demonstrate tissue magnification at ×100, while the corresponding insets for A–D show magnification at ×1,000.
is critical to regulating cell shape and growth, distinct from its ability to cause cell proliferation.

In this respect, NMB/NMB-R may be similar to what we have argued to be the case for other members of the bombesin family, such as GRP and its receptor (reviewed in Ref. 19). Like NMB, GRP has been shown to act as a modest mitogen in many colon cancer cell lines (6). GRP/GRP-R are not expressed by normal colonic epithelial cells in adults but are aberrantly upregulated in well-differentiated but not poorly differentiated colon cancer cells (6), whereas colon cancers arising in GRPR−/− mice progressively dedifferentiate over time (7). When present, GRP/GRP-R appear to mediate their morphogenic effects by inducing the phosphorylation of focal adhesion kinase (17).

Although we show that NMB/NMB-R mRNA expression is increased in colon cancers (Fig. 3) and that NMB-R protein expression is slightly higher in well, as opposed to poorly differentiated, colon cancer cells (Fig. 11), we also show that in contrast to GRP/GRP-R, NMB/NMB-R are expressed by nonmalignant epithelial cells lining the colon. Although this observation in and of itself does not exclude a possible role for NMB/NMB-R in regulating epithelial cell differentiation, the expression of these proteins in normal colonocytes indicates that NMB/NMB-R function is not unique to cancerous cells. Indeed, the finding that NMB/NMB-R expression is highest in colonic epithelial cells lining the crypt proliferating zone (Fig. 10) suggests that these proteins may be involved in the normal maintenance of colonocyte numbers. However, to definitively determine the role of NMB/NMB-R in normal and malignant colonic epithelial cell function will require the study of animals genetically incapable of expressing either or both proteins. Importantly, no alteration in the normal intestinal phenotype has been reported for mice lacking NMB-R (39).

In summary, we have demonstrated that NMB and NMB-R are present in normal and malignant colonic epithelial cells. The expression of these varies considerably at the level of mRNA, whereas their expression at the level of proteins is remarkably similar, regardless of the tissue
histology. NMB can act as a growth factor in both normal and cancerous cells lines, although the magnitude of its growth effect is modest. Other functions for NMB and its receptor, besides growth regulation, are likely and remain to be elucidated.

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