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Adrenomedullin Expression in Human Tumor Cell Lines

ITS POTENTIAL ROLE AS AN AUTOCRINE GROWTH FACTOR*

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Although adrenomedullin (AM) previously has been identified in human tumors, its role has remained elusive. Analysis by reverse transcriptase-polymerase chain reaction (RT-PCR) revealed AM mRNA in 18 of 20 human normal tissues representing major organs, and 55 of 58 (95%) malignant cell lines. Western blot and high performance liquid chromatography analysis showed immunoreactive AM species of 18, 14, and 6 kDa that are consistent with the precursor, intermediate product, and active peptide, respectively. Immunohistochemistry and *in situ* RT-PCR performed on paraffin-embedded tumor cell lines of various tissue origins exhibited AM cytoplasmic staining. Neutralizing monoclonal antibody to AM inhibits tumor cell growth in a concentration-dependent manner, an effect that was reversed with the addition of exogenous AM. Responding tumor cells were shown to have approximately 50,000 AM receptors per cell by Scatchard analysis with ^{125}I-AM and expressed AM receptor mRNA by RT-PCR. Our data showed 36 of 48 (75%) tumor cell lines expressed AM receptor mRNA by RT-PCR assessment, all of them also expressed AM. In the presence of AM, cAMP levels were shown to increase in tumor cells. Our collective data demonstrate that AM and AM receptor are expressed in numerous human cancer cell lines of diverse origin and constitute a potential autocrine growth mechanism that could drive neoplastic proliferation.

Adrenomedullin (AM)[1] is a recently identified hypotensive peptide initially isolated from human pheochromocytoma (1). AM and its gene-related peptide, proadrenomedullin N-terminal 20 peptide (PAMP), are the two known bioactive products generated from post-translational enzymatic processing of the 185-amino acid prepro-AM molecule (1–3). Both AM and PAMP are amidated peptides. However, they have been shown to mediate their vasodilatory effects through distinctly different receptor systems (4). AM stimulates adenyl cyclase activity which elevates cAMP levels in smooth muscle cells. It is structurally related to calcitonin gene-related peptide (CGRP), and its vasodilatory effect is inhibited by the CGRP antagonist, $CGRP_{8-37}$ (5–10). Conversely, PAMP has no amino acid sequence homology to AM or CGRP and its biological effects are not blocked by $CGRP_{8-37}$ suggesting the involvement of a separate receptor system (4). Human AM cDNA has been cloned and mRNA expression identified in the adrenal glands, lung, kidney, and heart (2). A high degree of base sequence homology has been found between AM mRNAs isolated from other mammalian species, including rat and pig (11, 12). AM has been also implicated as an important regulator of renal function having natriuretic and diuretic action (13, 14), a potent bronchodilator (15), a regulator of certain central brain actions (16, 17), and a suppressor of aldosterone, adrenocorticotropin and insulin release (18–20). The receptor for AM (AM-R) was recently cloned and sequenced (21); it contains seven transmembrane domains and belongs to the G protein-linked receptor superfamily. Finally, we and others have shown that AM is expressed in a variety of human tumors of both pulmonary and neural lineage including small cell lung cancer, adenocarcinoma, bronchoalveolar carcinoma, squamous cell carcinoma, and lung carcinoids; and ganglioblastoma and neuroblastoma (22, 23). In an attempt to further study the distribution of AM and its receptor in human tumors and determine their role in these malignant disorders, we have used molecular, biochemical, and *in vitro* techniques to analyze a variety of human cancer cell lines of lung, breast, brain, ovary, colon, prostate, and hemopoietic lineages.

MATERIALS AND METHODS

Cell Lines and Normal Tissue—Tumor cell lines evaluated in this study were as follows: small cell lung carcinomas (SCLC, H60, H69c, H82, H146, H187, H209, H345, H446, N417, H510, N592, H735, H774, H889, H1092), non-small cell lung carcinomas (NSCLC, H23, H157, H460, H676, H720, H727, H820, H1264, H1385, H1404, H2087, H2228, A549, UMC11), breast (SK-BR-3, ZR75–1, MCF-7, BT-20, MDA-MD231, BT-474, H2380), colon (H630, SNUC-1), nervous system (T98G (glioblastoma), TC106, CHP100, TC17, PNET, Peii, SY5Y, AS, LAN-1, KCNR-C, KCNR-DRA (neuroblastomas of the peripheral nervous system)), ovarian (NIH:OVCAR-3, SKOV3, OVT2, A2780, CP70), prostate (DU-145), adrenal (H295), chondrosarcoma (SW578), and chronic monocytic leukemia (U937). Additional NSCLC cell lines used to evaluate AM-R that are not listed above are: H520, H726, H835, and H1373. All lung, adrenal, colon, and H2380 breast tumor cell lines were obtained from the National Cancer Institute-Navy Medical Oncology Branch. Nervous system tumors were obtained from the National Cancer Institute, Pediatric Branch. The remaining cell lines came from ATCC, Rockville, MD. Cells were maintained under serum-free/hormone-free conditions in RPMI 1640 without phenol red (Life Technologies, Gaith-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) D14874.

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[1] The abbreviations used are: AM, adrenomedullin; AM-R, adrenomedullin receptor; PAMP, proadrenomedullin N-terminal 20 peptide; CGRP, calcitonin gene-related peptide; MoAb-G6, monoclonal antibody clone G6; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; R_0, RPMI 1640 plus sodium selenite; R_0CM, R0 conditioned cell medium; PBS, phosphate-buffered saline; BSA/PBS, bovine serum albumin in PBS; ABC, avidin-biotin complex; MTT, 3–4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide; bp, base pair(s); Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

ersburg, MD) containing 3×10^{-8} M sodium selenite (R_0) at 37 °C in 5% CO_2 (26).

Total RNA from the above tumor cell lines was extracted using Trizol (Life Technologies) and following the manufacturers protocol. Poly(A)$^+$ RNA from normal human tissue was acquired through Clontech (Palo Alto, CA); primary epithelial cells were obtained from Clonetics (San Diego, CA).

RT-PCR and Cloning—The oligonucleotide primers were synthesized using a MilliGen/Biosearch 8700 DNA synthesizer (Millipore, Marlborough, MA). Primer sets for human AM detection were as follows: sense, $AM_{250-270}$, 5′-AAGAAGTGGAATAAGTGGGCT-3′; antisense, $AM_{640-660}$, 5′-TGGCTTAGAAGACACCAGAGT-3′; and nested probe antisense, $AM_{541-561}$, 5′-GACGTTGTCCTTGTCCTTATC-3′, with a predicted product of 410 bp. For human AM-R amplification, the following rat primers were selected from the published sequence (21): sense, AM-$R_{476-497}$, 5′-AGCGCCACCAGCACCGAATACG-3′; antisense, AM-$R_{923-946}$, 5′-AGAGGATGGGGTTGGCGACACAGT-3′; antisense probe, AM-$R_{788-811}$, 5′-GGTAGGGCAGCCAGCAGATGACAA-3′, yielding a 471-bp product. Procedures for RT-PCR using these primers have been described previously (22). In brief, reverse transcription was performed using the SuperScript Preamplification System (Life Technologies). A Perkin-Elmer 9600 Thermocycler was used to amplify the samples for 35 cycles with annealing temperatures of 55 and 61 °C, respectively, for the ligand and its receptor. All buffers, enzymes, and nucleotides used were obtained from Applied Biosystems (Perkin-Elmer, Norwalk, CT). PCR products were analyzed electrophoretically using 1% agarose gels, and the ethidium bromide staining was observed under UV light, followed by Southern analysis with ^{32}P-end-labeled probes.

The PCR products were cloned by insertion into the pCRII vector (Invitrogen TA Cloning kit, San Diego, CA), the plasmids purified (Qiagen Plasmid Purification kit, Chatsworth, CA, and Promega Plasmid Clean-up kit, Madison, WI), and nucleotide sequencing was carried out by Sequetech (Mountain View, CA).

Southern Blot Analysis—PCR products were run at 100 volts on a 1% agarose gel and denatured in 1.5 M NaCl, 0.6 M NaOH (30 min × 2), neutralized in 1.5 M NaCl, 2 M Tris (30 min × 2), and blotted onto a 0.2-μm nitrocellulose filter in 20 × SSC by capillary flow transfer overnight. The filter was cross-linked at 80 °C under vacuum then incubated in prehybridization buffer. The antisense nested probe was ^{32}P-end-labeled by standard procedures using T4 polynucleotide kinase. Hybridization with the labeled probe (1×10^6 cpm/ml) was done overnight at 42 °C. Room temperature stringency washing was in 5 × SSC, 0.1% SDS (30 min) and 1 × SSC, 0.1% SDS (30 min). Filters were air dried and autoradiographed on Kodak X-AR5 film.

Antibodies—A previously characterized polyclonal antibody against P072, a fragment of AM (H_2N-TVQKLAHQIYQFTDKDKDNVAPR-SKISPQGY-$CONH_2$), rabbit bleed number 2343 was used for immunohistochemistry and Western blot analysis (22).

A neutralizing monoclonal antibody, designated as MoAb-G6, was developed against the P072 peptide of AM, characterized, and the methodology published (20, 24). MoAb-G6 was affinity purified from ascites using a solid phase immunogen column (20). The antibody selectively binds AM and P072, but it does not cross-react with other peptide amides or structurally related peptides which include CGRP, gastrin releasing peptide, glucagon-like peptide 1, vasoactive intestinal peptide, arginine vasopressin, growth hormone releasing factor, cholecystokinin, amylin, gastrin, oxytocin, calcitonin, α-melanocyte stimulating hormone, pancreatic polypeptide, peptide tyrosine-tyrosine, and *Tabanus atratus* hypotrehalosemic hormone (20). The antibody was tested at serial 2-fold dilutions ranging from 1:100 to 1:204,800.

Western Blotting—Whole cell lysates were generated following a modified protocol that was previously reported (25). In summary, cells were harvested 48 h after their last feeding, washed three times in cold PBS and pelleted by centrifugation (188 × *g* for 10 min at 4 °C). The pellet of intact cells (≈5×10^7 cells) was resuspended in 1 ml of cold PBS containing 1 μM final concentration of each of the following protease inhibitors: Pefabloc (Centerchem Inc., Stamford, CT), bestatin, and phosphoramidon (Sigma). The cell suspension was maintained on ice (4 °C) throughout the extraction procedure, then homogenized, sonicated, clarified by ultracentrifugation (14,000 × *g* at 4 °C), and the final protein concentration determined (BCA kit, Bio-Rad). Cell lysates were diluted in 2 × Tricine sample buffer (with SDS, non-reduced or reduced with β-mercaptoethanol, NOVEX, San Diego, CA) to an approximate protein concentration of 35 μg/50 μl, heated to 95 °C for 3 min, and loaded into the sample well.

Cell lysates were electrophoretically separated on a gradient 10–20% Tricine, SDS-polyacrylamide gel electrophoresis gel (NOVEX), and run at 100 volts for 2 h under reducing (β-mercaptoethanol) and non-reducing conditions. 2 ng of synthetic AM was added to a separate well as a positive control. Transfer blotting was accomplished in the same apparatus equipped with a titanium plate electrode and transferred to a polyvinyldifluoride membrane (Immobilon polyvinyldifluoride, Millipore) at 30 volts for 3 h. The membrane was blocked overnight in 1% BSA/PBS, incubated for 1 h in 1:1,000 dilution of rabbit anti-P072 (bleed number 2343), washed 3 times in PBS, exposed to 1×10^6 cpm of ^{125}I-Protein A for 30 min at 4 °C, washed 6 times in PBS, dried, and autoradiographed overnight at −80 °C on Kodak X-AR5 film. Specificity control consisted of a duplicate membrane incubated in antigen-preabsorbed (10 nmol/ml P072) antiserum.

Dot Blot Evaluation—HPLC fractions were screened for AM immunoreactivity using a modified solid phase assay technique previously described (24). Samples (150 μl/well) from consecutive fractions were added in numerical order to a 96-well polyvinylchloride microtiter plate (Dynatech Labs, Chantilly, VA), sealed with a plastic adhesive coverslip (Dynatech), and frozen overnight at −80 °C. The plastic seal was perforated over each well with an 18-gauge needle, frozen plates were placed inside the collection drum of a 12EL freezemobile unit (Virtis Company, Gardiner, NY) and freeze dried. Without removing the coverslip, the residual powder was resuspended in 50 μl of PBS using a fine pipette tip and mixed on a mini-orbital shaker (Bellco, Vineland, NJ) for 3 h at room temperature. The supernatant was aspirated, the well coated with 1% BSA/PBS for 1 h at room temperature, washed twice with PBS, and AM immunoreactivity detected with rabbit anti-P072 (bleed number 2343, 1:1000 dilution, 50 μl/well) followed by adding ^{125}I-Protein A (50,000 cpm/well). Wells were cut out via hot wire technique and bound radioactivity was measured on a 1277 Gammamaster instrument (Wallac, Gaithersburg, MD).

HPLC Characterization—The lung carcinoid cell line, H720, was acclimated to grow in RPMI 1640 under a serum-free/hormone-free environment (R_0) and the resulting conditioned medium (R_0CM) was subjected to RP-HPLC fractionation (26, 27). Protease inhibitors were added, as described for whole cell lysates, to consecutive 1-liter harvests of R_0CM and stored at 4 °C until further processed. Pooled R_0CM (10 liters) was freeze-dried (Freezemobile 12EL, Virtis), reconstituted to 500 ml with distilled water, centrifuged, and filter sterilized (0.45 μm) to remove particulate matter. The resulting filtrate was loaded onto a semipreparative C18 column (DeltaPak, Millipore, 30 × 300 mm) using an auxillary rotary pump (Ranin, Woburn, MA), with a flow rate of 15 ml/min. Column retentate was selectively eluted over 150 min using a 5–60% acetonitrile gradient containing 0.1% trifluoroacetic acid and monitored at 210 and 280 nm (Beckman System Gold HPLC, San Ramon, CA). Twelve ml/min fractions were collected, freeze-dried, and stored at −80 °C until further analysis. Stored fractions were resuspended in 2 × Tricine sample buffer and subjected to Western blot analysis as described previously.

Immunohistochemistry—Cell pellets from tumor cell lines grown in R_0 were fixed in either 4% paraformaldehyde or Bouin's for 2 h, embedded in 1% low melting point agarose, and further embedded in paraffin. Sections were stained using the avidin-biotin complex (ABC) method. Briefly, after an overnight incubation with rabbit anti-human P072 antibody (1:1000), the cells were incubated with biotinylated goat anti-rabbit immunoglobulin (1:200, Vectastain, Burlingame, CA) and then with avidin-biotin peroxidase complex (1:100, Vectastain). Preincubation of the antiserum with 10 nmol/ml of human P072 was used as the absorption control. The bound antibodies were visualized using diaminobenzidine (Sigma) and H_2O_2. Sections were lightly counterstained with hematoxylin.

In Situ RT-PCR—Analysis was performed on cell lines using a direct method as described previously (28). In brief, sections were mounted on silanated slides, dewaxed, permeabilized with proteinase K (10 μg/ml, 15 min at 37 °C), and reverse transcription performed (Superscript II, Life Technologies). PCR was completed after 20 cycles in an Omnislide thermocycler (Hybaid, Holbrook, NY). Composition of the PCR mixture was similar to the solution used for standard PCR with the addition of digoxigenin-11-dUTP to label the products. Digoxigenin-tagged amplicons were visualized with a digoxigenin detection kit (Boehringer Mannheim). Omission of the RT step or of the specific primers in the PCR mixture were used as negative controls.

Growth Assays—MTT techniques are described elsewhere (29). In brief, a single cell suspension of 2×10^5 cells/ml (50 μl/well) was seeded into 96-well polyvinylchloride plates and an appropriate concentration of MoAb-G6 and AM was added in a volume of 50 μl. The assay was performed in TIS medium (RMPI 1640 plus 10 μg/ml transferrin, 10 μg/ml insulin, and 3×10^{-8} M sodium selenite). After 5 days growth at 37 °C, 5% CO_2, in a humid incubator, the dye and solubilization solutions were added from the Promega Proliferation Assay (Madison, WI),

which was a variation of the MTT assay (30). The Bio-Rad Microplate Manager plate reader and software was used to determine the change in number of viable cells from dye reduction measured by absorbance at 570 nm.

Receptor Binding—Receptor binding analysis was performed as described previously (31). Briefly, cells (5×10^4) were placed in 24-well plates coated with fibronectin (20 μg/well). When a monolayer was formed, the cells were washed 4 times in TIS buffer followed by incubation with receptor binding medium (TIS plus 1% BSA and 1 mg/ml bacitracin). The cells were incubated with ^{125}I-AM (Phoenix Pharmaceuticals) for 30 min at 37 °C. After washing the cells 4 times in receptor binding buffer at 4 °C, they were dissolved in 0.2 N NaOH and counted on a γ-counter.

Receptor Quantification—The binding of ^{125}I-AM was investigated as a function of radiolabeled peptide concentration. MCF-7 cells (0.5×10^6) in 24-well plates were incubated with increasing concentrations of ^{125}I-AM and the amount bound determined in the absence or presence of 1 μM AM. The difference between the two represents specific binding B. The amount of specifically bound AM (B) was divided by the free radiolabeled peptide (F) and plotted as described (32). The data was best fit with a straight line indicating a single class of sites.

cAMP Assays—Cyclic AMP was assayed by RIA using a kit obtained from DuPont NEN. Cells in 24-well plates were resuspened in TIS medium containing 1% BSA, 1 mg/ml bacitracin, and 100 μM isobutylmethylxanthine. AM was added ranging from 0.1 pM to 10 μM and after 5 min the reaction was terminated by adding an equal volume of ethanol. The supernatants were tested for cAMP using the RIA kit following the manufacturer's instructions. AM at 30 nM was used to increase cAMP. 1 μg/ml of MoAb-G6, IgAκ, and IgAλ was added to the cells with or without 30 nM AM. The mean value ± S.D. of four determinations was calculated using MCF-7.

Statistical Analysis—MTT assay data were statistically evaluated by analysis of covariance using mixed linear models (SAS, PROC MIXED/ Statistical Analysis System, Cary, NC, 1996). The calculations utilized the relative OD (optical density) attained by dividing the cell-treated mean OD with the non-treated control cell mean OD to obtain the percentage of cell growth. Cell lines, MoAb-G6 and AM concentrations were set as main effects, background was retained as a covariate, and the replicates within wells used as the random term.

RESULTS

Human AM/AM-R mRNA Expression in Normal and Malignant Cells—RT-PCR was used to evaluate AM ligand and receptor mRNA in a variety of cancer cell lines of diverse origin and normal human tissues (Fig. 1, *A* and *B*). The resulting 410- and 471-bp RT-PCR products for AM and AM-R mRNA were confirmed by Southern blotting with antisense nested probes. The cloned PCR products for normal human adrenal gland and H720 were further verified as authentic fragments of the AM and AM-R message by nucleotide sequencing in either direction using primers at the SP6 and T7 promoter regions. The majority of neoplastic cell lines tested (55/58, 95%) were shown to express AM message. These cell lines originated from a variety of tissues: the adrenal gland, bone marrow, breast, cartilage, colon, lung, nervous system, ovary, and prostate. AM mRNA was not found in the following cancer cell lines and normal human tissues: H187 (SCLC), H23 (adenocarcinoma), H460 (large cell carcinoma), thyroid, and thymus. A larger band, around 600 bp, was seen upon Southern blotting in some cancer cell lines and normal tissues, and may represent alternate splicing of AM. To check whether this additional band is genomic DNA, we ran PCR from RNA and cDNA from the same cell line and tissue. In the RNA samples, we do not get any band on ethidium bromide staining or Southern blotting (data not shown). The fact that certain tissues were negative by Northern blot evaluation does not preclude its expression of the mRNA, as demonstrated by RT-PCR. For example, the brain showed AM expression by RT-PCR, but not by Northern analysis (2). Although less extensively studied, the data observed for AM-R mRNA suggest that this molecule is also widely expressed. We demonstrate localization of receptor message in several normal tissue and in 75% (36/48) of the neoplastic cell lines examined (Fig. 1*B*). Our data demonstrates AM-R mRNA, by RT-PCR, in the following normal human tissues: brain, heart, lung, and adrenal gland (Fig. 1*B*). Tumor cell lines that did not express AM-R are H187, H345, H510, H520, N592, H726, H835, H889, A549, H1373, H2087, and SNUC-1.

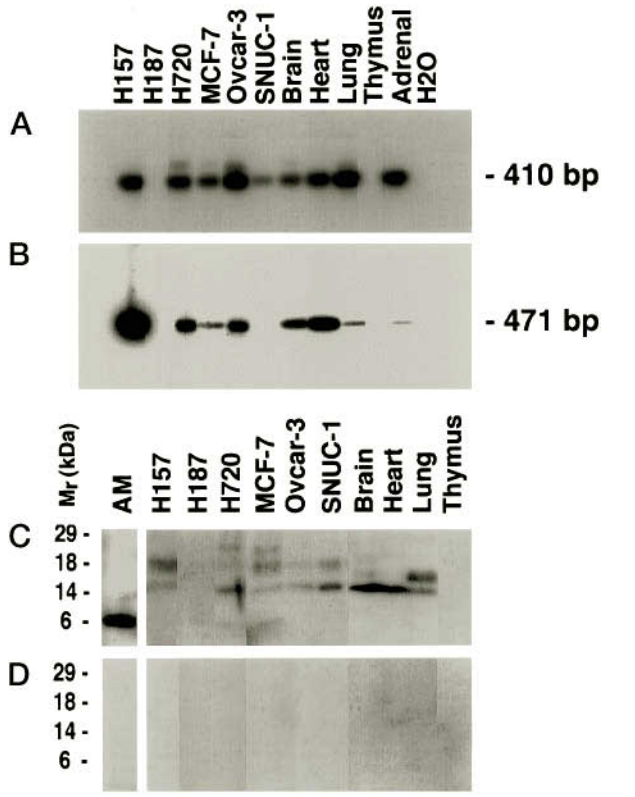

FIG. 1. **Representative sample of human tumor cell lines and normal human tissues screened for AM and AM-R.** Southern blot analysis demonstrates the predicted 410-bp product for AM (*A*) and a 471-bp product for AM-R mRNA (*B*) after RT-PCR amplification. *C*, Western blot analysis of unreduced cell extracts shows immunoreactive species of 18, 14, and 6 kDa when using a rabbit antiserum to AM (1:1000). In addition, there is a 22-kDa immunoreactive entity that may be attributed to post-translational processing. Synthetic AM control is 2 ng. *D,* the absorption control was negative.

Production and Secretion of AM Peptide by Human Tumor Cell Lines—Select cancer cell lines, as shown in Fig. 1, were adapted to grow under R_0 conditions and the resulting whole cell lysates from such lines were examined for AM immunoreactivity by Western blot analysis using a previously characterized rabbit antiserum (22). Fig. 1*C* illustrates the electrophoretic profile of the AM-like peptides identified.

Molecular mass species of 18, 14, and 6 kDa were identified in tumor cell lysates and presumably represent AM precursor, processed intermediates, and the authentic peptide, respectively. There is also a 22-kDa immunoreactive species in two cancer cell lines, H720 and MCF-7. The specificity of our immune-detection assay was confirmed by an antibody absorption control, which eliminated the specific bands (Fig. 1*D*). To further corroborate the expression of authentic AM by tumor cells, we analyzed HPLC fractions of R_0CM from the lung carcinoid cell line NCI-H720. Column retentate contained AM-like immunoreactivity having an elution time consistent with the synthetic peptide (≈89 min) (Figs. 2, *A* and *B*). In addition, immunoblot analysis of consecutive HPLC fractions within the 88–92-min region revealed a major 6-kDa immunoreactive band, while the 124–129-min fractions expressed both the 18- and 14-kDa entity (Fig. 2*C*). Additional immunoreactive peaks were identified at 26.4, 53.6, 135.6, and 141.0 min, but they were not further characterized (Fig. 2). The protein extracts

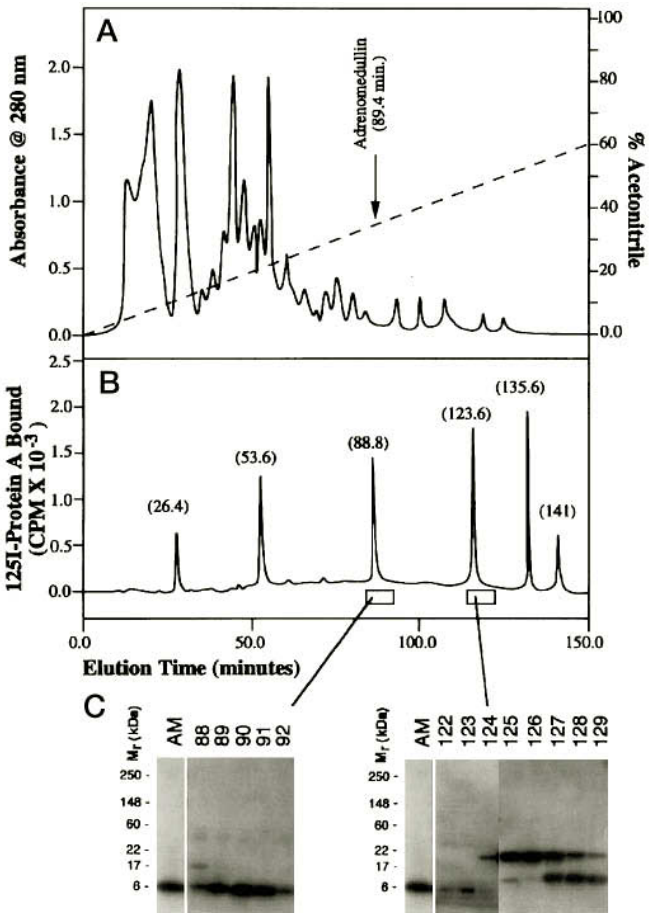

FIG. 2. **HPLC profile, dot blot evaluation, and Western blot analysis of H720 conditioned medium.** *A*, fractionation of 10 liters of H720 CM (concentrated to 500 ml before injection) compared with the elution time of synthetic AM at 89.4 min (*arrow*). AM immunoreactivity occurs at approximately 88.8 min in the H720 CM as shown by dot blot (*B*) and Western blot (*C*) analysis. The latter demonstrates that the fractions at 88–92 min contain the 6-kDa entity, and those at 124–129 min contain the 14- and 18-kDa entities. The amount of synthetic AM is 2 ng.

were run under reduced conditions and no difference in the molecular weight of immunoreactive peaks was noted (data not shown). The immune recognition site is at the C-terminal region of AM and is therefore unaffected by reduction with β-mercaptoethanol. In addition, the larger molecular mass species (22 kDa) is not the result of aggregate formation between precursor molecules at intra-Cys-Cys linkage, but assumed to be a result of varying post-translational modification.

AM Regulates Human Tumor Cell Proliferation—Results obtained by immunohistochemical and *in situ* RT-PCR examination of paraffin-embedded R_0-adapted cell lines were consistent with previously reported data on normal lung and pathological lung specimens (22). Analysis of human tumor cell lines showed AM expression by immunohistochemical and *in situ* RT-PCR, as shown in Fig. 3. It is interesting to note that AM expression in SCLC H774 demonstrates the highest intensity of staining in the outer cell layers (proliferative zones) of individual colonies, a finding that could implicate AM in growth regulation (Fig. 3*A*). Consistent with this idea was the fact that AM had been shown to elevate cAMP, a signal transduction pathway known to modulate cellular growth (6). To further investigate this suspected phenomenon, we used MTT assay techniques to examine the effects of AM on the growth of several diverse tumor cell lines (lung, colon, breast, and ovary). Exogenous addition of AM (with a final concentration range between 0.1 and 100 μM) to R_0-grown cell cultures was ineffective in stimulating growth, although there was some nonspecific toxicity at the higher range. Since our test cell lines were known to produce authentic AM peptide, we assumed that this inability to stimulate growth with extrinsic ligand could possibly mean that the cells had already achieved maximal proliferative effects using intrinsic factor.

To verify this hypothesis, we used MoAb-G6 to block the biological activity of endogenous AM. During the characterization of MoAb-G6 we demonstrated that it blocked AM's biological functions and did not cross-react with other known tyrosine amide peptides or with the structurally related CGRP and amylin (20). We used the MTT assay to evaluate MoAb-G6 for its effect on the growth of 5 human tumor cell lines (NCI-H157, NCI-H720, MCF-7, NIH:OVCAR-3, and SNUC-1), and a dose-dependent suppression was observed in 4 of them (Table I). This growth suppression in the above cancer cell lines, after the addition of MoAB-G6, was observed in as low a dose as 1–10 μg/ml. At the highest concentration of MoAb-G6 used (100 μg/ml), a consistent growth suppression was observed among the different cancer cell lines examined with the exception of SNUC-1 (Table I). In the colon cancer cell line, SNUC-1, AM-R expression was undetectable by RT-PCR (Fig. 1*B*), which may be the reason why MoAb-G6 had no effect on its growth. Representative data for MCF-7 are depicted in Fig. 4*A*, which shows that an isotypic control mouse myeloma protein (TEPC 15, IgAκ, Sigma) was ineffective in blocking growth over the same dose range. MoAb-G6 induced inhibition of tumor cell growth was abolished by exogenous addition of AM, with maximal recovery at 10 μM, thus verifying the specificity of growth suppression by the neutralizing antibody (Table I, Fig. 4*B*). Four of 5 tumor cell lines tested showed statistically significant dose-response effects due to the addition of MoAb-G6 and reversal of the inhibition with AM (Table I). The main effects for statistical evaluation included differences between the cell lines, and each of the concentrations for MoAb-G6 and AM.

AM Receptors Are Present in Human Tumor Cell Lines and AM Increases Intracellular cAMP—In addition to the RT-PCR analysis of the AM-R mRNA (Fig. 1*B*), we used cAMP response to synthetic AM and ^{125}I-AM binding to demonstrate the presence of functional AM receptors in responding tumor cell lines. Several cancer lines demonstrated selective binding of ^{125}I-AM, which was not competitively blocked by the synthetic homolog P072 or the gene-related peptide PAMP, as shown by representative data for breast cancer cell line MCF-7 (Fig. 4*C*). The data show that specific binding is inhibited by unlabeled AM in a dose-dependent manner with an IC_{50} of 10 nM. The number of receptors per cell is approximately 50,000 for MCF-7 with a K_d of 4 nM, as determined by Scatchard analysis with ^{125}I-AM. As illustrated for MCF-7, AM binding to this receptor induced a rapid increase in cellular cAMP over a dose range of 10 pM to 1 μM (Fig. 4*D*). In contrast, P072 and PAMP had no effect on cAMP (Fig. 4*D*). We have also observed that the addition of MoAb-G6 (1–10 μg/ml) to the cell line, MCF-7, inhibits the AM-mediated increase in intracellular cAMP. cAMP levels in the presence of 30 nM AM were 22.0 ± 4.2 fmol ($p < 0.01$). Addition of 1 μg/ml MoAb-G6 in the presence of 30 nM AM, decreased levels of cAMP to 15.7 ± 2.2 fmol ($p < 0.05$). At 10 μg/ml MoAb-G6 plus 30 nM AM, cAMP further decreases to 13.8 ± 2.2 fmol ($p < 0.01$). Conversely, isotypic controls (IgAκ, IgAλ) over the same dose range did not alter cAMP levels. Baseline cAMP levels for MCF-7 were 8.7 ± 0.6 ($p < 0.01$).

DISCUSSION

In this study we presented strong evidence for the existence of an autocrine growth loop involving AM and AM-R in human tumor cell lines of very different origins. These cells express

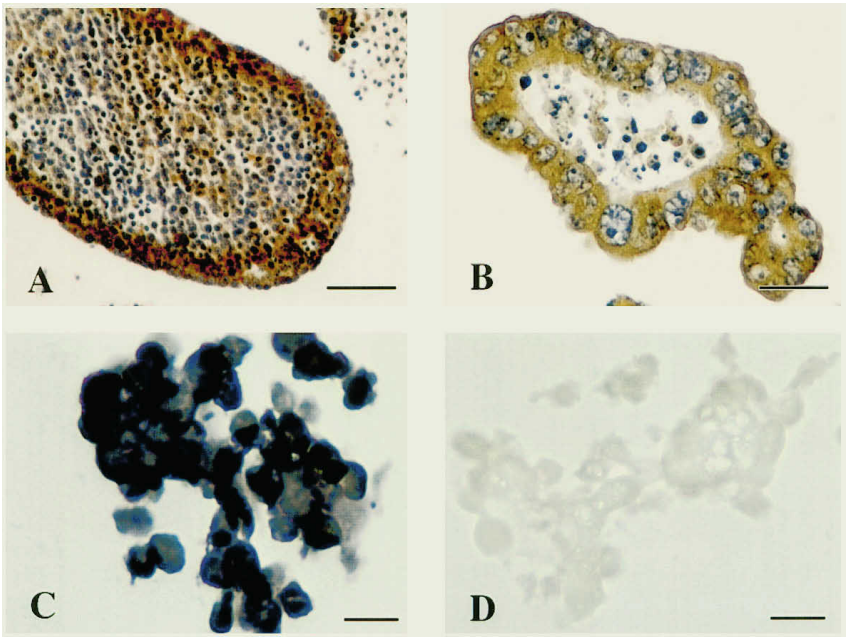

FIG. 3. **Immunohistochemical and *in situ* RT-PCR analysis of human cancer cell lines for AM.** *A*, immunohistochemical analysis for AM in SCLC H774 and (*B*) ovarian carcinoma cell line NIH: OVCAR-3. Note the peripheral distribution of AM immunoreactivity in H774 colonies. Magnification for *A*, 160 × (*bar* = 50 μm) and *B*, 320 × (*bar* = 25 μm). *C, in situ* RT-PCR for AM mRNA in carcinoid cell line H720; and *D*, negative control in a serial section where primers were substituted by water in the PCR mixture. Magnification for *C* and *D*: 600 × (*bar* = 10 μm).

TABLE I

Inhibitory effects caused by MoAb-G6 and recovery with the addition of AM

We demonstrate that addition of MoAb-G6 causes growth inhibition in 4 out of 5 tumor cell lines. Furthermore, we demonstrate specificity by reversing the effects of MoAb-G6, in a dose dependent manner, with the addition of synthetic AM. Colon cancer cell line, SNUC-1, did not respond to MoAb-G6. Values are the mean of 24 wells in three different experiments and their standard deviations. For *p* values, the data was statistically evaluated by analysis of covariance using mixed linear models.

Tumor cell line	Tumor type	% Growth ± S.D.			*p* value
		MoAb-G6 (100 μg/ml)	*p* value	MoAb-G6 + AM (10 μM)	
H157	Adenosquamous	68.6% ± 7.4	<0.0001	91.8% ± 8.4	<0.0001
H720	Lung carcinoid	64.3% ± 18.3	<0.0184	100.0% ± 14.0	<0.05
MCF-7	Breast adeno-CA[a]	44.7% ± 4.3	<0.0001	89.7% ± 5.8	<0.0001
OVCAR-3	Ovarian adeno-CA	64.3% ± 9.9	<0.0001	98.0% ± 13.5	<0.0001
SNUC-1	Colon adeno-CA	96.9% ± 4.5	NS[b]	100.0% ± 10.2	NS

[a] CA, carcinoma.
[b] NS, not significant.

mRNA for both the ligand and the receptor, they produce the peptide as shown by Western blot and immunohistochemistry, proliferation assays reveal that tumor growth can be significantly suppressed by a monoclonal antibody which blocks the biological activity of AM, and this inhibition can be reversed by the addition of external peptide. In the past, it has been demonstrated that for growth stimulation to occur a minimal receptor occupancy threshold (around 10%) is required; this has been described for other peptides such as gastrin-releasing peptide (33). This could explain why addition of external AM did not have any effect on growth but we still could demonstrate binding and cAMP increases. AM growth effects on the cells is demonstrated only when MoAb-G6 is used to block intrinsic AM. This set of characteristics, together with the binding assays and cAMP induction experiments, clearly implicates the existence of a newly defined autocrine loop mechanism which could potentially drive neoplastic growth as has been described for other peptides (24, 34). It seems that autocrine action is a common phenomenon that regulates most tumor cells and includes diverse peptides and/or proteins. In addition, AM may have an adaptive value for tumors by increasing the blood flow within the tumor bed through its well known vasodilatory function (5–10) or by suppressing T-lymphocyte differentiation and cytotoxic function through cAMP elevation (35, 36), thereby enabling the cancers to circumvent immune surveillance. This could explain why some cancer cell lines express AM even if they do not express AM-R.

The Western blot analysis corroborates the immunohistochemical and *in situ* RT-PCR data and demonstrates the expression of several different molecular mass AM immunoreactive species in a variety of human tumor cell lines. These included: 1) an 18-kDa entity which is presumed to be the AM precursor molecule based on the predicted size as calculated from cDNA data (2); 2) a single processed intermediate of 14 kDa; and 3) a 6-kDa species which electrophoretically migrates at the same molecular mass as the synthetic AM standard. The above immunoreactive entities remained unchanged when reduced with β-mercaptoethanol, thereby ruling out peptide dimerization. In addition, a 22-kDa species has been identified in two tumor cell line extracts (H720 and MCF-7) which is thought to represent a post-translationally modified molecule consistent with glycosylation, mucylation, phosphorylation, or any combination thereof. Absolute confirmation of the identity of these AM immunoreactive species will be accomplished by future amino acid sequencing studies. It is interesting to note that not all of the tumor cell line extracts showed the presence of the 6-kDa entity, which presumably represents the fully processed peptide. It is possible that most of the cell lines release the processed peptide into the conditioned medium quickly after processing rather than storing it in the cell, or that it is rapidly degraded enzymatically.

One of the obvious questions that arises from these studies is whether the peptide's ability to elevate intracellular cAMP correlates with its growth promoting effects. The role of cAMP

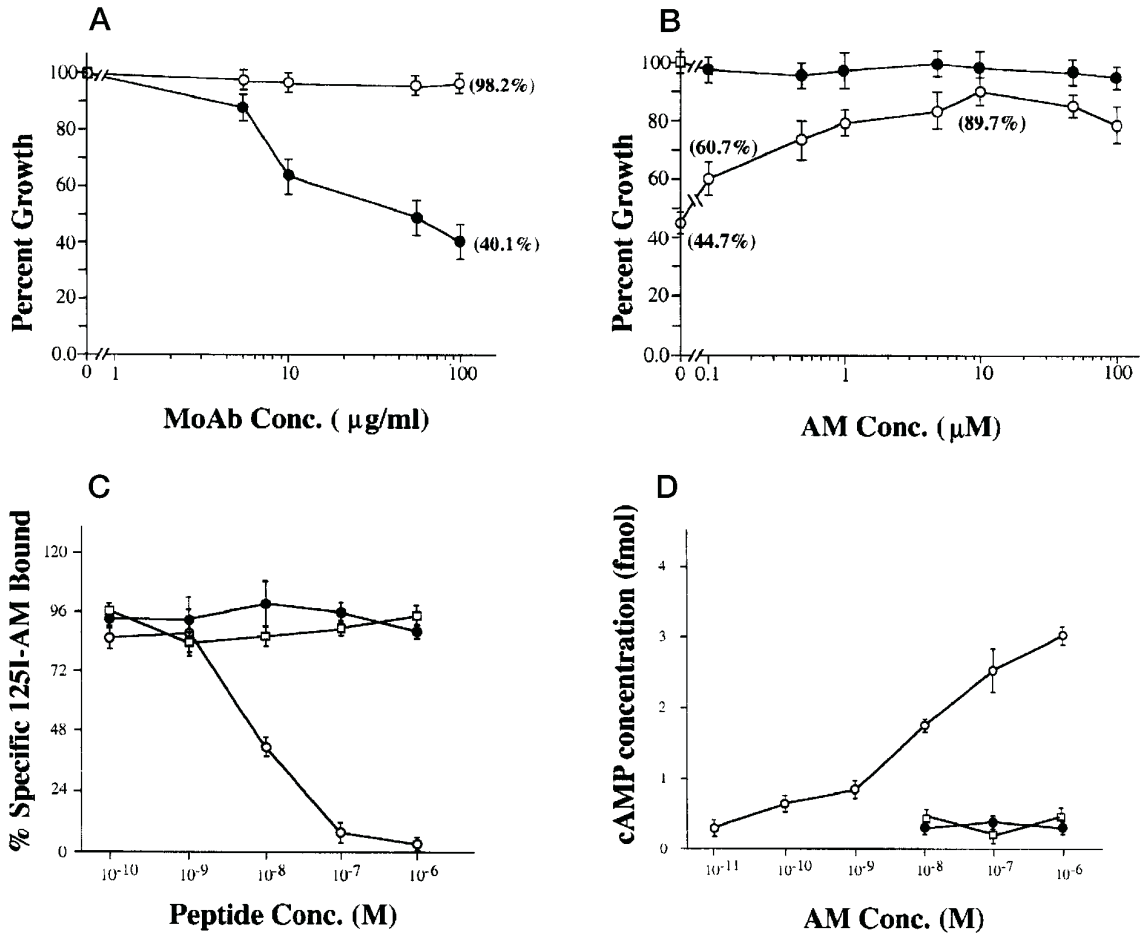

FIG. 4. **Growth effects of AM.** A representative human tumor cell line, MCF-7, was used to show the growth effects, receptor binding, and cAMP variation by AM under serum-free, hormone-free conditions. *A*, inhibitory effects of MoAb-G6 (●) compared with no effect from its mouse myeloma isotypic control, IgAκ (○). *B*, effects of MoAb-G6 were overcome by the addition of synthetic AM (○) compared with the addition of AM alone (●). *C*, specific receptor binding is measurable by competition with synthetic AM (○) while it is negligible for PAMP (●) or P072 (□). *D*, cyclic AMP is increased with the addition of synthetic AM (○) in a dose-dependent manner, but not with PAMP (●) and P072 (□).

as a growth regulator has been previously established in a variety of human tumor cell lines (31, 37–40). This secondary signal transducer has been reported to have contradictory effects on proliferation in different tumor cell systems (37, 38, 40). This dual function has been shown to depend on the relative amounts of two distinct cAMP-dependent protein kinase A isoforms; RI associated with cell growth/transformation and RII correlating with growth inhibition/differentiation (38, 41). Studies to evaluate AM and cAMP-protein kinase A interactions have already been started in normal systems of the rat and mouse. AM has been shown to induce cell cycle progression from G_0 to G_1, elevate c-*fos* mRNA expression, and increase AP-1 DNA binding activity in rat smooth muscle cells, an action which is blocked by the protein kinase A inhibitor H-89 (42, 43). AM has recently been demonstrated to be a mitogen for Swiss 3T3 cells, elevating cAMP in a dose dependent fashion and mediating a protein kinase A response (44). Conversely, AM has also been reported to suppress the growth of rat mesangial cells via the cAMP pathway (45). This diametric relationship of AM and growth regulation in different cell systems appears to be directly involved with the protein kinase pathway and probably relates to RI/RII activity (38).

Several substances that regulate the expression of AM have been identified. These include enhancing factors such as interleukin-1α and -β, tumor necrosis factor α and β, lipopolysaccharide, certain adrenocortical steroids, angiotensin II, endothelin-1, bradykinin, substance P, and adrenaline; and suppressor factors such as forskolin, 8-bromo-cAMP, thrombin, vasoactive intestinal polypeptide, and interferon-γ (46–49). Many of these factors help activate the immune system during an inflammatory response. A recent review on the causes and prevention of cancer by Ames *et al.* (50) made two interesting points: 1) increased cell division gives rise to increased risk of cancer, which can be driven by increased levels of particular hormones; and 2) chronic infection or inflammation contribute to one-third of the world's cancers. Given that interleukin-1α/β, tumor necrosis factor α/-β, and lipopolysaccharide are agents of immune inflammation that are known to increase the expression of AM and that AM can mediate trophic effects on tumor cell lines, these findings indirectly implicate AM as a potential risk factor for malignant conversion. Since most of the tumor cell lines we examined expressed this peptide, it may represent a generic target for intervention strategies to disrupt neoplastic transformation.

We are now actively pursuing alternative methods to block the growth promoting effects of AM and AM-R on tumor cell proliferation. Studies with antisense oligonucleotides to the initiation site of AM ligand and receptor messages have been initiated and will be evaluated by MTT growth assays. This approach has been previously used to inhibit insulin-like growth factor II effects on cervical cancer cells which are mediated through autocrine and paracrine growth regulation with much success (51). In addition, new antibodies (polyclonal and monoclonal) to hydrophilic regions of the AM receptor are being

generated which potentially could induce steric interference with ligand and receptor interaction, an avenue we have previously used to disrupt insulin-like growth factor I and insulin-like growth factor I receptor binding in lung tumor cell lines (52). Finally, peptide antagonists to AM should also be considered based on the investigative route taken to inhibit GRP regulation in small cell lung cancer (33). Any or all of the proposed studies may prove to be an appropriate strategy for intervening in AM regulated growth of human cancer cells in an *in vitro* and *in vivo* setting.

In summary, our data demonstrate that AM is expressed in a large variety of human tumor cell lines and that it can function as an autocrine growth factor capable of driving a self-perpetuating state in malignant disorders. Responding tumor cell lines were shown to express AM receptors and showed peptide-mediated increases in intracellular cAMP. These findings, together with reports that AM is found in tumor tissue from pathological specimens (22, 23), point toward the need for additional investigative studies to determine the precise role of AM in carcinogenesis. In addition, given the implications of AM on cell growth, it will be interesting to evaluate the relationships of this peptide with other sites of rapid cellular proliferation such as embryogenesis, wound repair, and epithelial turnover.

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