The Effects of Adrenomedullin Overexpression in Breast Tumor Cells


Background: Adrenomedullin is a secreted peptide hormone with multiple activities. Several reports have indicated that adrenomedullin may be involved in tumor survival, but this has not been directly shown. Here we evaluate the in vitro and in vivo effects of adrenomedullin overexpression in human breast cancer cells. Methods: The human breast cancer cell lines T47D and MCF7, both of which express low basal levels of adrenomedullin, were stably transfected with an expression construct that contained the coding region of the human adrenomedullin gene or with empty expression vector. Properties of the transfected cells were assessed by proliferation and apoptosis assays, in vitro and in vivo angiogenesis assays, cell migration experiments, and xenograft implants. The effect of synthetic adrenomedullin on human ovarian (ECV) cancer cell motility was also tested. Western blot analysis was used to compare expression levels of several genes whose products are associated with cell growth and regulation of apoptosis. Results: T47D and MCF7 cells transfected with the adrenomedullin construct both expressed high levels of adrenomedullin mRNA and protein. Compared with cells transfected with empty vector, cells that overexpressed adrenomedullin displayed a more pleiotropic morphology, an increased angiogenic potential both in vitro and in vivo, and less apoptosis after serum deprivation. T47D and MCF7 cells did not display measurable motility, but ECV ovarian cancer cells treated with synthetic adrenomedullin were more motile than saline-treated ECV cells. Adrenomedullin-overexpressing T47D cells had higher levels of proteins involved in oncogenic signal transduction pathways (such as Ras, Raf, PKC, and MAPKp49) and lower levels of pro-apoptotic proteins (such as Bax, Bid, and caspase 8) than T47D cells transfected with empty vector. In a preliminary in vivo experiment, three of 10 nude mice injected with adrenomedullin-overexpressing T47D cells developed xenograft tumors, whereas none of the 10 nude mice injected with cells carrying the empty plasmid developed tumors. Conclusions: These results further support the role of adrenomedullin as a survival factor for tumors. Development of physiologically efficient inhibitors of adrenomedullin may prove useful in the clinical management of cancer. [J Natl Cancer Inst 2002;94:1226–37]

Adrenomedullin is a 52-amino-acid peptide that contains an internal disulfide bond and a carboxyl-terminal amide group. It was originally isolated from a pheochromocytoma and was shown to elevate cyclic adenosine monophosphate (AMP) levels.

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See “Notes” following “References.”
in platelets (1). The gene for adrenomedullin encodes a preprohormone that, after post-translational modification, generates two bioactive peptides, adrenomedullin and proadrenomedullin. N-terminal 20 peptide (PAMP). The relative amount of each peptide that is secreted into the medium is regulated by alternative splicing of the pre-messenger RNA (mRNA) (2).

Many functions have been ascribed to adrenomedullin. This peptide can act as a vasodilator (3), a bronchodilator (4), a regulator of hormone secretion (5), a neurotransmitter (6), an antimicrobial agent (7), and a controller of renal function (8).

Several reports also implicate adrenomedullin in some aspects of tumor biology (9). For instance, administering a blocking monoclonal antibody against adrenomedullin resulted in a marked reduction in cancer cell growth in vitro, suggesting that adrenomedullin may function as an autocrine growth factor in cancer cells (10). Adrenomedullin increases thymidine uptake in skin cancer cells, which suggests that this peptide elevates the proliferation index in tumors and may therefore be involved in tumor progression (11). Adrenomedullin has also been shown to inhibit apoptosis in endothelial cells (12) and to induce angiogenesis in a chick chorioallantoic membrane assay (13). In addition, we have recently shown that adrenomedullin expression is strongly induced by hypoxia in a variety of cancer cell lines (14).

All of these characteristics suggest that adrenomedullin may be an important survival factor for tumors, especially when they are at the critical stage of initiating metastatic growth. Colonizing cancer cells are typically exposed to hypoxic environments via their forward migration into avascular areas. Hypothetically, under these conditions, adrenomedullin expression would be enhanced by low oxygen tension, and this peptide would help secure a blood supply by both its angiogenic and its vasodilator capabilities. At the same time, cell growth would be enhanced by the mitogenic activity of adrenomedullin and by its ability to inhibit apoptosis. This hypothesis is further supported by the finding that numerous cancer cell lines and tumor specimens express high levels of adrenomedullin and its receptors compared with normal cells and tissues of the same origin (15).

Recent clinical data indicate that adrenomedullin is overexpressed in cancer patients. For example, patients with colon or lung cancer have higher circulating levels of adrenomedullin than healthy control subjects do (16). Increased expression of adrenomedullin mRNA in ovarian tumors was statistically significantly associated with a poor prognosis (17), and elevated adrenomedullin mRNA was associated with high Gleason scores in prostate cancer (18). Furthermore, intraocular and orbital tumors have been shown to express statistically significantly higher levels of adrenomedullin mRNA than do lesions associated with other eye diseases (19). Patients with Cushing’s syndrome resulting from pituitary adenomas have markedly higher circulating levels of adrenomedullin than do healthy control subjects (20). After surgical removal of the pituitary tumor, adrenomedullin levels decreased to levels found in healthy controls, indicating that the tumor was the main source of adrenomedullin in these patients (20). In patients with leiomyomas, high adrenomedullin expression is associated with increased vascular density (21).

Results from a wide variety of studies using many different models have suggested that adrenomedullin has a role in tumor growth and metastasis. However, such a role has yet to be demonstrated directly. In this study, we evaluate the in vitro and in vivo effects of adrenomedullin overexpression in human breast tumor cell lines.

**Materials and Methods**

**Cell Culture**

The human breast cancer cell lines T47D and MCF7 were used in this study because they express low basal levels of adrenomedullin (14) and because T47D has a low capacity for growth as a xenograft tumor when cells are injected into the flanks of nude mice (22). In addition, the lung cancer cell line H157 was used as a positive control for the production of adrenomedullin (14), and the ovarian cancer cell line ECV was used for motility assays. All of these cell lines were obtained from the American Tissue Culture Collection (Manassas, VA) and were maintained in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) (Life Technologies, Gaithersburg, MD) at 37 °C in humidified air containing 5% CO2.

**Construction of Expression Plasmid and Production of Stable Transfectants**

The coding region of the human adrenomedullin gene was generated by polymerase chain reaction (PCR) that used cDNA from H157 cells as template and the following oligonucleotide primers: 5'-GGA TCC ATG AAG CTG GTT TCC GTC GCC-3' (sense) and 5'-GAA TTC CTA AAG AAA GTG GGG AGC AC-3' (antisense). After an initial denaturation step at 94 °C for 2 minutes, 30 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 1 minute were performed. The PCR product was digested with restriction endonucleases BamHI and EcoRI (recognition sites underlined) and cloned into the BamHI and EcoRI sites of pCDNA3FLAG so that the FLAG epitope tag was fused in frame with the amino terminus of the adrenomedullin sequence. pCDNA3FLAG is an in-house modified version of pCDNA3 (Invitrogen, Carlsbad, CA) that has had the following linker containing a FLAG epitope cloned into the BamHI/EcoRI site of its multiple cloning site (5'-GAT CAC CAT GGA TTA CAA GGA TGA CGA TGA CAA GGG ATC CAG ATC TGA ATT-3'). The resulting construct, pFLAG-AM, was sequenced to guarantee that the cloned insert was identical to the published adrenomedullin sequence (GenBank accession No. D43639).

T47D and MCF7 cells were transfected with either pFLAG-AM or pCDNA3FLAG by using a calcium phosphate transfection kit (Invitrogen), according to the manufacturer’s instructions, and then were incubated for 48 hours. Stably transfected cells were selected by exposure to 400 μg/mL geneticin (Life Technologies). Individual clones of stable transfectants bearing pFLAG-AM were isolated and screened for adrenomedullin mRNA expression by northern blot analysis, and their levels of adrenomedullin expression were compared with that of H157 cells. In addition, stable transfectants bearing pFLAG-AM were tested for adrenomedullin protein secretion by radiomunnoassay, as previously described (23). The clone from each cell line that expressed the highest level of adrenomedullin mRNA and secreted protein were chosen for further analysis.

**Morphologic Analysis**

Live cells stably transfected with either pFLAG-AM or pCDNA3FLAG were photographed under phase contrast illumination by using a Nikon Diaphot inverted microscope equipped with a 35-mm camera (Nikon, Melville, NY). Cells
were also studied by scanning electron microscopy. Cells were grown on glass coverslips and fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer for 2 hours at 4°C. The cells were postfixed in 1% OsO₄ for 1 hour at 4°C and subjected to critical point drying. Cell morphology was observed in an S-3000N Hitachi scanning electron microscope (Nissei Sangoy America, Schaumburg, IL) operated at 1000 V. Morphologic differences were evaluated by three investigators in a blinded fashion.

**Chick Aortic Ring Angiogenesis Assay**

We used a modified chick aortic ring assay to study the differences in angiogenic potential between the cells transfected with adrenomedullin and cells transfected with the empty plasmid. The modification over the previously published method (24) consisted of the use of tumor cells transfected with either pFLAG-AM or the empty expression vector as a feeder layer and the only source of angiogenic compounds. Briefly, stably transfected tumor cells were resuspended in sterile 2% low-melting-point agarose at a final density of 2.5 × 10⁵ cells/mL, and 1 mL of this solution was dispensed per well into 24-well plates and allowed to solidify at 4°C. Meanwhile, aortic rings (approximately 1 mm in length) were prepared from the five aortic arches that had been conditioned by overnight exposure to the stably transfected cells or 10⁵ cells transfected with either empty plasmid or pFLAG-AM per implant. The tubes were held at 37°C to allow the Matrigel to solidify. Two tubes were inserted into a skin pocket in the back of each anesthetized nude mouse; the pocket was sealed with surgical staples. Mice were 6-week-old females obtained from the National Cancer Institute nude mouse colony (Frederick Cancer Research and Development Center, Frederick, MD). The mice were injected intravenously with 25 mg/mL fluorescein isothiocyanate (FITC)–dextran (100 μL/mouse; Sigma, St. Louis, MO), and 20 minutes later, the tubes were removed from the skin pockets and photographed with an inverted microscope. We also measured the amount of fluorescence trapped in the implants by using an HP Spectrophotometer (Perkin Elmer, Foster City, CA) to evaluate the volume of blood circulating through the newly formed vessels.

**Detection of Cell Proliferation by 5-Bromo-2'-Deoxyuridine Incorporation**

Transfected cells were grown overnight at different cell densities in 96-well plates in DMEM that contained or lacked 5% FBS. The following day, proliferation potential was assessed with a 5-bromo-2'-deoxyuridine (BrdU) enzyme-linked immunosorbant assay (Roche Diagnostics, Mannheim, Germany). Briefly, 10 μM BrdU was added to the medium, the cells were incubated for 2.5 hours, and BrdU incorporation into newly formed DNA was quantified.

**Quantification of Cell Survival Under Serum Starvation Conditions**

Transfected cells were seeded in 96-well plates (2 × 10⁴ cells per well) in DMEM lacking FBS. Cells grown in the same medium containing 5% FBS were used as a control. At daily intervals, the number of cells in each well was quantified by using a tetrazolium compound-based cell proliferation assay (Promega, Madison, WI), as previously described (25). To investigate the specific involvement of adrenomedullin in the response of cells to serum starvation, cells carrying the empty plasmid were supplemented with increasing concentrations of synthetic adrenomedullin (Peninsula, San Carlos, CA), and cells transfected with pFLAG-AM were exposed to the Fab fragment of a previously characterized neutralizing anti-adrenomedullin monoclonal antibody, MoAb-G6 (5), for 5 days. The number of cells in each well was then quantified with the proliferation assay.

**Apoptosis Analysis by Flow Cytometry**

T47D cells (2 × 10⁵ cells per well) transfected with empty plasmid or pFLAG-AM were cultured in DMEM with or without 5% FBS for 24 hours. The fluorescent dye JC-1 (Molecular Probes, Eugene, OR) was then added to the medium at 1 μL/mL, and the cells were incubated for 10 minutes at 37°C. JC-1 accumulation in mitochondria is a marker of mitochondrial membrane depolarization and is frequently used as an early marker for apoptosis (26). Cells were then analyzed for JC-1 fluorescence in a Becton Dickinson FACScan cytometer (Mansfield, MA), as described (26).

**Cell Cycle Analysis by Flow Cytometry**

Transfected cells were treated overnight with 10 ng/mL human recombinant tumor necrosis factor-α (TNF-α) (R&D Systems, Minneapolis, MN) or an equal volume of phosphate-buffered saline (PBS) and then stained with propidium iodide, as previously described (27). Cells were analyzed for DNA content by flow cytometry, as described above, and cell cycle analysis was performed with ModFit LT 1.01 software (Becton Dickinson).
Western Blotting

The experiment was performed twice. Imaging, and protein levels from both cell lines were compared. Nescence. The intensity of each spot was quantified by digitaljugate, and the immunoreaction was visualized by chemiluminescence. After washing, the membrane was hybridized for apoptosis. After washing, the membrane was fixed and stained with Hema3 as recommended by the manufacturer (Biochemical Sciences Inc., Swedesboro, NJ). The cells trapped in the porous membrane were photographed through a 25x25x microscope objective, and the number of cells per photographic field was counted in four fields per sample. Basic fibroblast growth factor (bFGF; R&D Systems) was loaded into separate wells at 100 ng/mL and used as a positive control for the induction of migration.

Proteomic Analysis

T47D cells (10⁶ cells per assay) transfected with pFLAG-AM or with empty plasmid were lysed in boiling lysis buffer (10 mM Tris–HCl [pH 7.4], 1 mM sodium orthovanadate, 1% sodium dodecyl sulfate [SDS]) and homogenized by sonication. The protein content of each lysate was quantified by the bicinechonic acid (BCA) assay (Pierce, Rockford, IL) and sent to BD Biosciences (Lexington, KY) for proteomic composition analysis. Briefly, 400 μg of protein from each lysate was loaded into a preparative well that spanned the entire width of a 16 × 16-cm, 5%–15% SDS–polyacrylamide gel, 1 mm thick. The proteins were resolved overnight at constant current and then transferred onto an Immobilon-P nylon membrane (Millipore, Bedford, MA) for 1 hour at 1A in a wet electrophoretic transfer apparatus. Each membrane was incubated in 5% fat-free dry milk in PBS for 1 hour to block nonspecific binding and then was assembled onto a western blotting manifold that isolates 45 channels across the membrane. A different monoclonal antibody was added to each channel and allowed to bind for 1 hour. We selected monoclonal antibodies that recognize proteins whose expression is associated with cell growth and the regulation of apoptosis. After washing, the membrane was hybridized for 30 minutes with a goat anti-mouse horseradish peroxidase conjugate, and the immunoreaction was visualized by chemiluminescence. The intensity of each spot was quantified by digital imaging, and protein levels from both cell lines were compared. The experiment was performed twice.

Motility Assays

We used 96-well ChemoTx microplates (NeuroProbe Inc., Gaithersburg, MD) to study cell motility. The filter membrane separating the upper and lower chambers of each well was coated with 10 μg/mL fibronectin. Synthetic adrenomedullin was diluted in DMEM at various concentrations and loaded into the lower chambers, and 2 × 10⁵ ECV cells were loaded into the upper chambers. After a 4-hour incubation at 37 °C, the membrane was fixed and stained with Hema3 as recommended by the manufacturer (Biochemical Sciences Inc., Swedesboro, NJ). The cells trapped in the porous membrane were photographed through a 25x25x microscope objective, and the number of cells per photographic field was counted in four fields per sample. Basic fibroblast growth factor (bFGF; R&D Systems) was loaded into separate wells at 100 ng/mL and used as a positive control for the induction of migration.

Xenografts

T47D cells transfected with pFLAG-AM or with empty plasmid were injected into the flanks of athymic (nude) mice (1 × 10⁷ cells/mouse). The mice (10 animals per cell line) were checked daily for tumor formation by palpation, and after tumors were detected, tumor volume was estimated by measuring the size of the tumor in three dimensions twice a week. This experiment was conducted in a blind fashion under an approved animal protocol.

Statistical Analysis

When appropriate, data from cells treated in different ways were compared by using a two-tailed Student’s t test (SPSS version 10; SPSS Inc., Chicago, IL). All statistical tests were two-sided, and P values less than .05 were considered statistically significant.

RESULTS

The tumor cell lines T47D and MCF7 were stably transfected with pFLAG-AM, a construct encoding human adrenomedullin, and with the empty plasmid. One clone from each cell line that expressed high levels of adrenomedullin by northern blot analysis (Fig. 1, A) and by radioimmunoassay (Fig. 1, B) was chosen for further analysis. The adrenomedullin mRNA transcript in the transfected cells was smaller than that in the control cells because only the open reading frame of the gene was transfected into them (Fig. 1, A). Results were similar for both breast cancer cell lines, and the following statements are applicable to both cell lines unless indicated otherwise.

Morphologic Changes Associated With Adrenomedullin Overexpression

We observed that the clones that overexpressed adrenomedullin had a different appearance than the parental (i.e., untransfected) cells, especially for those clones derived from T47D cells. Both parental cells and cells transfected with the empty plasmid had a typical epithelial morphology, characterized by large flat cells with a smooth surface and very few cellular processes (Fig. 2, A–C). By contrast, cells that overexpressed adrenomedullin were smaller and more rounded than the parental cells and had numerous long projections (Fig. 2, D–F). When we analyzed the adrenomedullin-overexpressing cells by scanning electron microscopy, we observed that the cell surface had numerous microvilli (Fig. 2, E and F).

Angiogenic Potential of Transfected Cells

Synthetic adrenomedullin has been shown to induce angiogenesis in a chorioallantoic membrane assay (13). Here we used a modified aortic ring assay for angiogenesis to test whether tumor cells that overexpress adrenomedullin were able to induce sprouting of newly formed blood vessels. In this assay, soluble factors secreted by agarose-embedded tumor cells transfected with pFLAG-AM or with empty plasmid were tested for their ability to induce angiogenesis in chicken aortic rings. As shown in Fig. 3, A–D, T47D cells transfected with the empty plasmid induced minimal growth of microvessels from the chick aortic rings (mean area covered by microvessels = 7685 μm²; 95% confidence interval [CI] = 3468 to 11902 μm²), whereas tumor cells that overexpressed adrenomedullin induced a prominent sprouting of vascular structures from chicken aortic rings (mean
incubated in serum-free medium for 24 hours. * indicates a statistically significant difference in the mean amount of secreted adrenomedullin between cells transfected with the adrenomedullin construct and those transfected with the empty plasmid (P \textless .001). Next, cells that overexpressed adrenomedullin or that contained the empty vector were subjected to serum deprivation, suggesting that overexpression of adrenomedullin might be associated with a slight growth disadvantage in the presence of serum, those that overexpressed adrenomedullin showed a statistically significant difference in the amount of newly formed DNA between cells that overexpressed adrenomedullin and those that contained empty plasmid-transfected cells (P = .035) or conditioned medium collected from those cells (P = .038) had statistically significantly more FITC fluorescence than implants that contained only Matrigel, whereas implants that contained cells transfected with the empty plasmid or their conditioned medium were indistinguishable from the Matrigel control (Fig. 3, G). There was also a statistically significant difference in the amount of FITC fluorescence between implants that contained adrenomedullin-overexpressing cells and those that contained empty plasmid-transfected cells (P = .010). In both assays, synthetic adrenomedullin was also able to induce angiogenesis at concentrations of 10 nM or higher (results not shown).

**Impact of Adrenomedullin Overexpression on DNA Synthesis as Measured by BrdU Incorporation**

To investigate whether overexpression of adrenomedullin could influence DNA synthesis, an indirect measure of cell proliferation, we studied BrdU incorporation in the stably transfected T47D cells. For cells cultured in the presence of serum, we detected no differences in the amount of newly formed DNA between cells that overexpressed adrenomedullin and those that contained the empty vector (Fig. 4, A), indicating that adrenomedullin per se did not act as a growth factor under these conditions. By contrast, when the cells were grown in the absence of serum, those that overexpressed adrenomedullin showed a statistically significantly higher incorporation of BrdU than those transfected with the empty vector (P<.001) (Fig. 4, A). Therefore, adrenomedullin overexpression was associated with a stimulation of DNA synthesis in cells exposed to serum starvation.

**Effects of Adrenomedullin Overexpression on Apoptosis**

We observed that when T47D cells were cultured in the presence of serum, those that overexpressed adrenomedullin grew at a slightly reduced rate by 5 and 6 days in culture when compared with those that carried the empty plasmid (Fig. 4, B). This finding suggested that overexpression of adrenomedullin might be associated with a slight growth disadvantage in the presence of serum. Next, cells that overexpressed adrenomedullin or that contained the empty vector were subjected to serum deprivation, and the number of viable cells was followed daily by a prolif-
eration assay. We observed a time-dependent reduction in number of cells that carried the empty plasmid that was compatible with the induction of apoptotic cell death (Fig. 4, C). By contrast, the cells that overexpressed adrenomedullin maintained a relatively constant population size during the 6 days of the assay (Fig. 4, C). Addition of synthetic adrenomedullin to cells that contained the empty plasmid and that were cultured in serum-free medium resulted in a dose-dependent increase in cell viability (Fig. 4, D). Exposure of adrenomedullin-overexpressing cells to a neutralizing anti-adrenomedullin monoclonal antibody induced dramatic cell demise (Fig. 4, E).

To confirm that the reduction in population size was consistent with the induction of apoptosis, we analyzed the same cells by flow cytometry after exposing them to JC-1, a mitochondrial potential-sensitive fluorescent dye that detects the disruptions in mitochondrial transmembrane potential that are a common early feature of cells undergoing apoptosis. Cells grown in the presence of serum accumulated in a single peak of green fluorescence regardless of their adrenomedullin expression status (Fig. 5, A and C). After serum starvation, cells containing the empty vector (as well as untransfected cells) accumulated in a second peak of fluorescence at a higher fluorescence emission, which indicated that mitochondrial membrane depolarization, an early event in apoptosis, had occurred in those cells (Fig. 5, B). By contrast, after serum starvation, cells that overexpressed adrenomedullin accumulated in a much smaller peak of highly fluorescent cells (Fig. 5, D). We conclude that, under conditions of serum starvation, tumor cells that overexpress adrenomedullin undergo less apoptosis than tumor cells that express endogenous levels of adrenomedullin.

Effects of Adrenomedullin Overexpression on Tumor Cell Motility

An increase in cell motility is generally associated with a more malignant phenotype in cancer cells (28). We therefore studied the motility of T47D and MCF7 cells and found that they did not have any noticeable capability for migrating through a fibronectin-coated filter membrane, either before or after transfection with pFLAG-AM. Therefore, to study whether adrenomedullin plays a role in this important cancer characteristic, we used an ovarian cell line well known for its migrating ability. Addition of synthetic adrenomedullin to ECV ovarian tumor cells resulted in a dose-dependent increase in the number of cells that traversed the membrane (Table 2). The increase in motility was modest but statistically significant, even though it did not reach the levels induced by bFGF as the positive control, sug-
suggesting that adrenomedullin may also play a role in inducing tumor cell migration.

**Changes in Proteomic Profiles After Adrenomedullin Overexpression**

To understand the molecular basis for the physiologic changes associated with adrenomedullin overexpression, we performed a proteomic comparison of our stably transfected cells looking specifically at a group of proteins whose expression is associated with mitogenic and apoptosis pathways. Analysis of the proteomic profiles for transfected T47D cells showed clear differences between cells that overexpressed adrenomedullin and those that carried the empty plasmid. Table 3 contains data for the proteins whose levels differed the most between cells.
transfected with pFLAG-AM and those transfected with empty plasmid. The levels of some proteins—including the anti-apoptotic molecule Stat3, those encoded by the Ras and Raf oncogenes, the morphology-changing protein gelsolin, MAPKp49 (a member of the mitogen-activated intracellular cascade), and poly[ADP-ribose]polymerase (PARP) (a molecule that disables DNA repair)—were higher in cells that overexpressed adrenomedullin than in cells that carried the empty plasmid. Conversely, levels of pro-apoptotic molecules (i.e., Bax, Bid, caspase 6, caspase 7, caspase 8, MEKK3, and TRADD), the cell cycle regulator cyclin D2, and the cell adhesion molecule Fak were lower in cells that overexpressed adrenomedullin than in cells that carried the empty plasmid. These changes in protein levels are compatible with the physiologic changes, such as apoptosis reduction, growth activation, increase in motility, and morphological changes, that we observed in association with adrenomedullin overexpression.

To confirm these observations, we analyzed cell extracts from transfected cells by western blotting that used antibodies against two of the molecules whose levels differed widely between the two types of transfectants. As shown in Fig. 5, E, cells transfected with the adrenomedullin plasmid had higher levels of
the anti-apoptotic protein Stat3 and lower levels of the pro-apoptotic protein caspase 8 than did cells transfected with the empty plasmid.

Influence of Adrenomedullin Overexpression on In Vivo Tumorigenesis

Results obtained in the previous experiments suggested that cells that overexpress adrenomedullin might be more tumorigenic than their low adrenomedullin-producing counterparts (i.e., cells transfected with empty plasmid) in vivo. To test this hypothesis, we injected T47D cells stably transfected with pFLAG-AM or empty plasmid into nude mice and followed the development of xenograft tumors. None of the 10 mice injected with T47D cells containing the empty plasmid developed tumors during the 4 months the mice were followed. This finding is in agreement with previous studies that showed that T47D is a cell line with low tumorigenic potential (22). By contrast, three of the 10 mice injected with T47D cells that overexpressed adrenomedullin produced tumors 8–10 weeks after injection, sug-
Table 1. Influence of adrenomedullin overexpression on cell cycle changes induced by TNF-α*

<table>
<thead>
<tr>
<th>Cell cycle stage</th>
<th>Untransfected MCF7</th>
<th>MCF7 + empty plasmid</th>
<th>MCF7 + adrenomedullin construct</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated TNF-α-treated</td>
<td>P</td>
<td>Untreated TNF-α-treated</td>
</tr>
<tr>
<td>G0/G1</td>
<td>69 (65 to 73)</td>
<td>0.043</td>
<td>71 (67 to 75)</td>
</tr>
<tr>
<td>G2/M</td>
<td>14 (12 to 16)</td>
<td>.057</td>
<td>9 (8.4 to 9.6)</td>
</tr>
<tr>
<td>S</td>
<td>16 (13 to 19)</td>
<td>.045</td>
<td>20 (18 to 22)</td>
</tr>
<tr>
<td>G0/G1</td>
<td>2 (1.2 to 2.8)</td>
<td>.832</td>
<td>2 (1.5 to 2.5)</td>
</tr>
<tr>
<td>Apoptosis†</td>
<td>0.87 (0.81 to 0.93)</td>
<td>&lt;.001</td>
<td>0.40 (0.36 to 0.44)</td>
</tr>
</tbody>
</table>

<.001 = confidence interval.
*Cells were treated overnight with either phosphate-buffered saline (Untreated) or with 10 ng/mL tumor necrosis factor-α (TNF-α) and then stained with propidium iodide and subjected to flow cytometry to determine cell cycle stage as a function of DNA content. Cell cycle stages are expressed as the percentage of the total number of cells analyzed (10,000 cells per measurement). The software used to calculate the percentages did not take into consideration the sub-G1/G0 fraction; therefore, the percentages in the first four rows of each column add to approximately 100%. The percentages in the fifth row (apoptotic cells) were calculated by counting four different fields. Basic fibroblast growth factor (bFGF) was added by counting four different fields. Basic fibroblast growth factor (bFGF) was added by counting four different fields.
†The sub-G1/G0 fraction of cells was considered to consist of apoptotic cells.

Table 2. Influence of synthetic adrenomedullin on cell motility of human ovarian cancer ECV cells*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of cells/field (95% CI)</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>18 (11 to 25)</td>
<td>.2666</td>
</tr>
<tr>
<td>0.1 nM adrenomedullin</td>
<td>27 (16 to 38)</td>
<td>.0004</td>
</tr>
<tr>
<td>1 nM adrenomedullin</td>
<td>62 (54 to 70)</td>
<td>.0002</td>
</tr>
<tr>
<td>10 nM adrenomedullin</td>
<td>89 (76 to 102)</td>
<td>.0002</td>
</tr>
<tr>
<td>100 nM adrenomedullin</td>
<td>71 (52 to 90)</td>
<td>.0043</td>
</tr>
<tr>
<td>100 ng/mL bFGF</td>
<td>183 (151 to 215)</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

*Mean number of cells and the 95% confidence intervals (CI) were calculated by counting four different fields. Basic fibroblast growth factor (bFGF) was added as a positive control for migration induction.
†Difference from the untreated control cells (Student’s t test).

Table 3. Comparison of specific protein levels between T47D cells overexpressing adrenomedullin and T47D cells transfected with the empty plasmid*

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Fold increase</th>
<th>Fold decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelsolin</td>
<td>Morphologic changes</td>
<td>—‡</td>
<td>—‡</td>
</tr>
<tr>
<td>MAPKp49</td>
<td>Mitogen cascade</td>
<td>1.7 (1.3 to 2.1)</td>
<td>2.7 (2.3 to 3.0)</td>
</tr>
<tr>
<td>PARP</td>
<td>Disables DNA repair</td>
<td>1.5 (1.4 to 1.6)</td>
<td>1.5 (1.4 to 1.6)</td>
</tr>
<tr>
<td>PKC</td>
<td>Cell cycle control</td>
<td>3.5 (3.3 to 3.7)</td>
<td>1.2 (1.0 to 1.4)</td>
</tr>
<tr>
<td>Raf</td>
<td>Oncogene</td>
<td>17.1 (13.9 to 20.3)</td>
<td>1.2 (1.0 to 1.4)</td>
</tr>
<tr>
<td>Ras</td>
<td>Oncogene</td>
<td>1.9 (1.8 to 2.0)</td>
<td>1.9 (1.8 to 2.0)</td>
</tr>
<tr>
<td>Stat3</td>
<td>Anti-apoptotic</td>
<td>—‡</td>
<td>—‡</td>
</tr>
<tr>
<td>Bax</td>
<td>Pro-apoptotic</td>
<td>10.8 (9.4 to 12.2)</td>
<td>10.8 (9.4 to 12.2)</td>
</tr>
<tr>
<td>Bid</td>
<td>Pro-apoptotic</td>
<td>4.0 (3.2 to 4.8)</td>
<td>4.0 (3.2 to 4.8)</td>
</tr>
<tr>
<td>Caspase 6</td>
<td>Execution caspase</td>
<td>—‡</td>
<td>—‡</td>
</tr>
<tr>
<td>Caspase 7</td>
<td>Execution caspase</td>
<td>1.6 (1.4 to 1.8)</td>
<td>1.6 (1.4 to 1.8)</td>
</tr>
<tr>
<td>Caspase 8</td>
<td>Initiation caspase</td>
<td>10.9 (8.2 to 13.6)</td>
<td>10.9 (8.2 to 13.6)</td>
</tr>
<tr>
<td>Cyclin D2</td>
<td>G1 to S transition</td>
<td>4.7 (2.8 to 6.6)</td>
<td>4.7 (2.8 to 6.6)</td>
</tr>
<tr>
<td>Fak</td>
<td>Cell adhesion</td>
<td>2.7 (2.3 to 3.2)</td>
<td>2.7 (2.3 to 3.2)</td>
</tr>
<tr>
<td>MEKK3</td>
<td>Stress pathway</td>
<td>—‡</td>
<td>—‡</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNF-α death pathway</td>
<td>7.2 (5.8 to 8.6)</td>
<td>7.2 (5.8 to 8.6)</td>
</tr>
</tbody>
</table>

*CI = confidence interval; MAPK = mitogen activated protein kinase; PARP = poly(ADP-ribose) polymerase; PKC = protein kinase C; Fak = focal adhesion kinase; MEKK = MAPK/ERK regulated kinase kinase kinase; TRADD = TNF receptor-associated death domain protein; TNF = tumor necrosis factor.
†Fold increase or decrease is the intensity of a particular spot in the adrenomedullin overexpressing cells as compared with the intensity of the corresponding spot in cells transfected with the empty plasmid.
‡The indicated protein was below the detection limit of the assay in one of the samples (in the empty plasmid cells when an increase was reported and in the adrenomedullin overexpressing cells when a decrease was reported), and therefore a numerical comparison is not possible.

**DISCUSSION**

In a recent article, Hanahan and Weinberg (28) describe a series of characteristics inherent to tumor cells. These include growth factor production, insensitivity to growth inhibition signals, ability to evade apoptosis, sustained angiogenesis, tissue invasion capability, and unlimited replicative potential. We have shown here that most of these characteristics were enhanced in two breast cancer cell lines that overexpressed adrenomedullin. Cells that overexpressed adrenomedullin displayed morphologic changes, a higher potential for inducing angiogenesis, increased resistance to apoptosis, higher rate of incorporating nucleotides into newly formed DNA, increased growth rate under stress, and increased tumorigenicity in vivo compared with cells that expressed endogenous levels of adrenomedullin. These phenomena were accompanied by increases in the levels of several anti-apoptotic molecules and oncogene products and a decrease in the levels of several pro-apoptotic proteins. In the light of Hanahan and Weinberg’s criteria, these observations suggest that adrenomedullin induces a more carcinogenic behavior in tumor cells that express high levels of the peptide.

Morphologic changes in response to adrenomedullin expression have not been previously reported. However, morphologic changes similar to those described here have been observed in neural tumor cells upon activation of certain oncogenes (29,30), and such changes also were associated with a more aggressive phenotype in vivo. The presence of projections on cells that overexpress adrenomedullin suggests that these cells have a more dynamic plasma membrane than cells that express low levels of adrenomedullin. Both the elevation in gelsolin, a protein that participates in the cytoskeletal restructuring that underlies morphologic changes, and the reduction in cell adhesion molecules such as Fak are consistent with the observed changes in morphology. All of these data, together with the fact that adrenomedullin increases tumor cell motility, suggest that tumor...
cells that express high levels of adrenomedullin may be more likely to be involved in metastasis.

As for the change in angiogenesis, angiogenic potential is one of the main features of tumor growth. Without this capability, the tumor would starve early in its biologic history, and no clinical manifestation would occur. Cancer cells synthesize and secrete many angiogenic molecules, such as vascular endothelial growth factor, bFGF, and other growth factors that induce the proliferation of blood vessels into the growing tumor (31). Therapies that target angiogenesis have been found to successfully reduce tumor growth in animals, and several such therapies are currently in clinical trials (32). On the basis of results from our study as well as those from a previous report (13), we conclude that adrenomedullin is an angiogenic factor that is secreted by tumor cells.

We also observed increased resistance to apoptosis in adrenomedullin-overexpressing cells. Nontumor cells undergo programmed cell death when they are not able to properly repair DNA damage. Tumor cells, on the other hand, keep proliferating regardless of the integrity of their DNA (33). If a cancer cell is still able to turn on its apoptotic machinery, it is relatively easy to target it with the appropriate therapies, but when the cell loses this regulatory mechanism, it becomes much more resistant to chemotherapeutic drugs. Results from this study and a study by Kato et al. (12) suggest that adrenomedullin is an anti-apoptotic factor and, therefore, that cancer cells that express high levels of adrenomedullin may be more virulent than cancer cells that express low levels of adrenomedullin. Most tumor cells produce at least some adrenomedullin and express specific receptors for this peptide (9,34). The presence of the adrenomedullin receptor on a cell that produces adrenomedullin would result in an autocrine loop that might increase cell survival. However, it is important to point out that adrenomedullin from sources other than the tumor cells themselves (i.e., paracrine sources, such as fibroblasts, blood vessels, immune cells, that surround the tumor bed) could also influence the apoptotic behavior of tumor cells. This possibility was confirmed by our demonstration that the addition of synthetic adrenomedullin to T47D cells that contained the empty plasmid was associated with a dose-dependent increase in cell viability. We are gradually beginning to understand the importance of nontumor cells in the development of cancer (28,35,36), but more attention is needed in understanding how it relates to adrenomedullin production.

Our observation that adrenomedullin-overexpressing cells behave differently, depending on whether they are grown in the presence or absence of serum, is interesting because there has been some controversy in the literature on whether adrenomedullin induces or inhibits cell growth. For example, Kano et al. (37) reported that adrenomedullin inhibits the growth of rat vascular smooth muscle cells, but 2 years later, Iwasaki et al. (38) proposed that adrenomedullin was a stimulatory growth factor for the same cell type. When these and other reports (9) are carefully reviewed, it becomes apparent that when adrenomedullin has been proposed to act as a growth factor, the experiments leading to that conclusion were performed in the absence of serum. We found that, in the presence of serum, cells that overexpressed adrenomedullin grew slightly slower than parental cells or cells transfected with the empty plasmid—a possibility that was also suggested by the results of the cell cycle analysis—but the reason for this behavior is not known. By contrast, under stress conditions, such as serum deprivation or exposure to TNF-α, cells that overexpressed adrenomedullin had a survival advantage. We propose, on the basis of the differential behavior of cells overexpressing adrenomedullin in serum-free and serum-containing medium, that, under stress conditions, adrenomedullin functions as an anti-apoptotic molecule rather than as a growth factor.

The change we observed in cell cycle dynamics reflects another important difference between cells expressing various levels of adrenomedullin. Normal cells will not progress into S phase until certain regulatory molecules are produced and/or activated. Cancer cells are not bound by this restriction and usually proceed through the G1/S checkpoint much faster than normal cells. In this study, we have shown that tumor cells that overexpress adrenomedullin are less sensitive than cells that express endogenous levels of adrenomedullin to signals that block the transition from the G1 phase to the S phase and induce apoptosis. One of the key regulators of this checkpoint is cyclin D2; in our proteomic analysis, the level of this molecule was lower in cells overexpressing adrenomedullin than in cells expressing endogenous levels of adrenomedullin. Thus, reduction of cyclin D2 levels may be one of the mechanisms through which high levels of adrenomedullin allow cells to escape from cell cycle regulation.

The effects of adrenomedullin overexpression on tumor cells included changes in the levels of several anti-apoptotic proteins, oncogene products, and pro-apoptotic proteins. The level of Stat3, a molecule that has been shown to counteract apoptosis (39), was elevated in the adrenomedullin-overexpressing cells, whereas the levels of several pro-apoptotic proteins (e.g., Bax, Bid, and several caspases) were reduced compared with the levels of those proteins in cells carrying the empty plasmid. Similarly, levels of the products of the proto-oncogenes Ras and Raf were elevated, as were some components of the signal transduction pathways for growth factors (e.g., PKC and MAPKp49) in adrenomedullin-overexpressing cells as compared with cells carrying empty plasmid. In agreement with these observations, a recent study (40) on endometrial cancer cells has shown that adrenomedullin elevates Bcl-2 levels and thus prevents cell death caused by hypoxia, further supporting the role of adrenomedullin as a survival factor for tumor cells.

Finally, our preliminary results obtained with nude mice injected with T47D cells transfected with either the adrenomedullin construct or the empty plasmid indicate that overexpression of adrenomedullin may have an impact on tumor growth in vivo. However, because the number of tumors observed during this experiment was small, no meaningful statistical analysis could be performed. Future confirmation of these data will be necessary to support our hypothesis about the effect of the impact of the overexpression of adrenomedullin in vivo. All the results presented here, together with the fact that hypoxia induces high levels of adrenomedullin expression through the transcription factor hypoxia-inducible factor-1 (14), suggest that adrenomedullin is an efficient survival factor for tumor cells. If so, adrenomedullin may make an excellent biologic target for developing intervention strategies against human malignancies.

Note added in proof. During the revision process of this manuscript, a report was published on the tumorigenic potential of adrenomedullin in endometrial tumor cells, further strengthening the role of adrenomedullin in carcinogenesis (41).
References


(21) We gratefully acknowledge the expert technical support of Alexandra Rivera in the in vivo angiogenesis assay. Manuscript received August 16, 2001; revised June 12, 2002; accepted July 11, 2002.

Notes

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