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Gene expression profiling identifies IL-13 receptor α2 chain as a therapeutic target in prostate tumor cells overexpressing adrenomedullin

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Human adrenomedullin (AM) is a 52 amino acid peptide, which shares homology with the calcitonin gene-related peptide. Overexpression of AM in the prostate carcinoma cell line PC-3 results in growth inhibition with a 20% (for human AM) and 35% (for rat AM) increase in doubling time compared to parental or mock-transfected cells. We demonstrate by gene expression profiling that AM overexpression results in the dysregulation of approximately 100 genes. Examples of such genes include many involved in the formation of the cytoskeleton, cell adhesion and the extracellular matrix, as well as regulators of the cell cycle and apoptosis, cytokines and transcription factors. Several genes related to cell growth arrest, such as GADD45, IGF-BP6 and RUNX-3, are upregulated by AM. Interestingly, interleukin-13 receptor α2 (IL-13Rα2) transcripts were significantly increased in clones overexpressing AM, which was confirmed by semiquantitative RT-PCR analysis. In addition, PC-3 cells treated with AM showed an overexpression of IL-13Rα2, which was abolished when cells were preincubated with an anti-AM blocking antibody. When PC-3 cells overexpressing AM and the IL-13Rα2 were treated with the highly specific IL13-PE38 cytotoxin, which binds to this receptor, a concentration-dependent inhibition of protein synthesis was observed. The IC_{50} (concentration of cytotoxin inhibiting protein synthesis by 50%) ranged from 1 to 4 ng/ml. This cytotoxicity was specific as it was neutralized by the excess of IL-13 and confirmed by clonogenic assays. This study describes a novel AM-induced mechanism of tumor sensitization through the upregulation of functional IL-13Rα2 chain, an ideal target for the highly specific recombinant chimeric cytotoxin IL13-PE38.

Key words: cDNA microarrays; prostate cancer; adrenomedullin; IL-13Rα2; gene expression profiling

Prostate carcinoma is the second leading cause of cancer mortality among men in the United States.[1] The 5-year relative survival rate for the patients whose tumors are diagnosed with local or regional disease approaches 100%, but the relative 10- and 15-year survival rates are reduced to 75% and 54%, respectively.[1] Although localized prostate cancer may be successfully treated, 70% of patients eventually progress and develop metastasis. The current nonsurgical therapeutic regimens for treating prostate carcinoma include radio-, hormonal- and chemotherapy, as well as a combination of these regimens, depending on specific circumstances of the patient. The limited success of treating particular forms of prostate cancer requires that additional targets for therapy be identified and tested.

Expression of adrenomedullin, an amidated peptide that was originally isolated from human pheochromocytoma,[2] has been documented in both the human and rat prostate,[3] human prostate carcinoma[4] and prostate cancer cell lines,[4] where it has been suggested that adrenomedullin (AM) may act as an autocrine/paracrine factor. Human AM consists of 52 amino acids (50 amino acids for rat AM) and shares certain homology with the calcitonin gene-related peptide (CGRP). AM appears to be ubiquitously expressed[2,5] and can perform a variety of functions, including vasodilatation, bronchodilation, hormone secretion control, renal homeostasis and regulation of cell growth.[5–7] Some cell types are stimulated to proliferate by AM,[7,8] whereas other cell types are growth-inhibited.[9] The calcitonin receptor-like receptor (CRLR), in conjunction with receptor activity-modifying protein-2 (RAMP-2) and -3 (RAMP-3), functions as AM receptors in numerous cell types.[5] Although elevation of cAMP levels is the most common intracellular event that occurs as a consequence of AM receptor activation,[10] this peptide can also act through calcium[11] and cGMP[12] signaling. AM also interferes with Ca^{++} channels and interacts with cytokines.[13–16]

Consistent with this hypothesis, inflammatory cytokines such as TNF and IL-1 increase AM levels in vascular smooth muscle cells.[17] AM also stimulates IL-6 production in fibroblasts.[18] AM expression and secretion is stimulated by hypoxia, lipopolysaccharide and NO.[17,19,20]

In the context of cancer, AM seems to play a growth-modulatory effect in prostate carcinoma. To investigate the role of AM in prostate carcinoma, we previously generated PC-3 cell line clones, which express high levels of AM. Overexpression of AM in PC-3 cells resulted in cell growth inhibition *in vitro* and *in vivo*.[21] To discover genes potentially responsible for AM action in PC-3 cells, in this study we performed gene expression profiling using cDNA microarrays with approximately 11,000 features. By this analysis, we identified numerous genes whose levels of expression appear to be altered by AM. Here we focused on one gene. Interleukin-13 receptor α2 (IL-13Rα2) is highly upregulated by adrenomedullin. Its upregulation was confirmed by conventional RT-PCR studies. Finally, we demonstrate that upregulated IL-13Rα2 gene results in gene transcription and translation of IL-13Rα2 protein, which is functional. These findings have important implications for understanding the regulation of IL-13Rα2 and for cancer therapy targeted to the IL-13Rα2.

Material and methods

Recombinant proteins and cell culture

Recombinant IL-13 and IL13-PE38 were expressed and purified as previously reported.[22] IL13-PE38 is comprised of IL-13 fused to domain II and III of *Pseudomonas* exotoxin (PE).

We have previously generated and characterized PC-3 clones overexpressing AM or clones carrying the empty pcDNA 3.1 expression vector.[21] PC-3 cells were grown in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS), 1 mM sodium pyruvate, 10 mM HEPES buffer, 2 mM glutamine, 50 U/ml penicillin G sodium and 50 μg/ml streptomycin sulfate in 5% CO_2 atmosphere tissue culture incuba-

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tor. AM-transfected or mock-transfected clones were cultured in the same media containing 150 μg/mL Hygromycin B (Invitrogen) to maintain selection of AM-expressing clones. Since the overexpression of rat AM resulted in more effective growth inhibition than for clones containing human AM,[21] we included both human and rat AM-transfected clones in this study. Clones used in the present work were AM3.2M and AM3.2B (PC-3 clones that overexpress human AM), AM15 and AM17 (clones that overexpress rat AM) and mock-2 (a PC-3 clone carrying the empty vector).

RNA isolation and cDNA microarray analysis

Total RNA was isolated from the clones in culture using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol, with the additional step of DNase treatment. The quality of the total RNA isolated was determined by running aliquots on 1% agarose gel in TAE. The Incyte human UNIGEM v2px set of cDNA clones containing about 11,000 features was arrayed on poly-L-lysine-coated glass slides at the National Cancer Institute Advanced Technology Center. The gene list is available at http://nciarray.nci.nih.gov.

Preparation of the cDNA-labeled probes was performed using the Micromax system (NEN Life Science Products, Boston, MA); 20 μg of total RNA from both the reference sample (PC-3 cell line) and the experimental samples (AM3.2M, AM3.2B, AM15, AM17 and mock-2) were reverse-transcribed in the presence of Cyanine 3-dUTP and Cyanine 5-dUTP, respectively, for 1.5 hr at 42°C. The labeled cDNAs were filtered through a Microcon YM-30 column (Millipore, Bedford, MA). The slides were prehybridized in a solution of 5 × SSC, 0.1% SDS and 1% BSA for 1 hr at 42°C. Following the prehybridization, the slides were rinsed in H_2O, isopropanol, and dried by centrifugation at 50*g* for 3 min.

Two μL (10 μg) of COT-1 DNA (Invitrogen), 2 μL (16 μg) of Poly-A (Amersham Pharmacia Biotech, Cambridge, U.K.) and 2 μL (4 μg) of yeast tRNA (Sigma, St. Louis, MO) were added to the cDNA probe solution for a final volume of 10 μL. This was mixed with an equal volume of hybridization solution (50% formamide, 10 × SSC, 0.2% SDS) and placed onto the microarray slide for hybridization at 42°C for 16 hr.

Following hybridization, slides were washed sequentially for 3 min in solutions with increasing stringency: 2 × SSC, 0.1% SDS; 1 × SSC, 0.1% SDS; 0.5 × SSC; and 0.01 × SSC. Immediately after washing, slides were dried by centrifugation as described earlier and scanned using a Genepix 4000Bscanner (Axon Instruments, Foster City, CA).

Three to five arrays for each PC-3 clone were performed. Genes with a 2-fold change in the geometric means of the ratios between the AM-expressing cells or empty vector were filtered and analyzed using hierarchical clustering. Filtered named genes were classified using GeneCards (http://bioinformatics.weizmann.ac.il) and extensive review of the literature.

To discard systematic errors due to dye incorporation, reverse-fluor microarray analysis was performed. In these cases, PC-3 parental cell line cDNA (reference) was labeled with Cy-5, whereas cDNAs from PC-3 clones were labeled with Cy-3. Extensive analysis of reverse-fluor labeling in our laboratory using the same arrays has revealed that dye bias is insignificant.[23]

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA from parental PC-3 cells, mock-2 and AM15, AM17, AM3.2M and AM3.2B clones, isolated with the RNeasy Mini Kit (Qiagen), were used for RT-PCR analysis of the IL-13Rα2 chain and AM receptors. β-actin mRNA amplification from these samples served as an internal control. The optimal RT-PCR conditions for the IL-13Rα2 chain and the primers have been published previously.[24,25] The sequence of the primers and the PCR conditions used for the amplification of AM receptors are listed in Table I. Five hundred ng of total RNA from various cell clones were reverse-transcribed using the RNA PCR kit (Perkin Elmer, Norwalk, CT) as per the manufacturer's instructions. Ten μl of reverse-transcribed products were amplified for 30 cycles using GeneAmp PCR system 9700 (Applied Biosystem, Perkin Elmer). The amplification products were resolved on a 2% agarose gel and stained with ethidium bromide. The band intensities of RT-PCR products for IL-13Rα2 and β-actin were evaluated using a fluorescence densitometer (Molecular Dynamics, Sunnyvale, CA). The relative fluorescence intensity was determined by dividing the intensity of IL-13Rα2 chain mRNA by the density of β-actin band and expressed as the ratio of relative fluorescence units (RFUs).

Real-time RT-PCR analysis

PCR primer sequences for human/rat adrenomedullin and 28s ribosomal RNA (for normalization) used in real-time RT-PCR analysis were as follows: AM sense primer 5′-TACCGCCAGAGCATGAACCA-3′; AM antisense primer 5′-TGCCGTCCTTGTCTTTGTCT-3′; 28S rRNA sense 5′-GGGTGGTAAACTCCATCTAA-3′; 28S rRNA antisense 5′-AGTTCTTTTCAACTTTCCCT-3′. Melting curves and gel analyses were performed to confirm the presence of single amplified species. Quantitative analysis of gene expression was carried out using SYBR PCR Core Reagents (PE Biosystems, Warrington, U.K.) and a Bio-Rad I-Cycler IQ real-time detection system (Bio-Rad, Richmond, CA). The level of gene expression was calculated after normalizing samples to the 28s rRNA level in each sample and is presented as relative units. Samples were analyzed in triplicate.

Expression of IL-13Rα2 after AM neutralization

To determine if neutralization of AM will result in a downregulation of IL-13Rα2, we cultured 80% confluent PC-3 cells in RPMI supplemented with 1% FBS in the presence of human AM (cell-free supernatant obtained from an AM-overexpressing PC-3 cell clone for 48 hr with or without preincubation with blocking anti-AM antibody rabbit serum; 1:100 dilution; a generous gift from Dr. L. Ouafik[26]) for 1 hr, followed by additional 48-hr incubation in the presence of human AM. Controls included the incubation of PC-3 cells with or without AM plus the rabbit IgG isotype antiserum. Total RNA was analyzed for IL-13Rα2 expres-

TABLE I – PRIMER SEQUENCES AND CONDITIONS FOR THE DETECTION OF AM RECEPTORS

Gene name	Genbank no.	Primer	Sequence (5′ → 3′)	Position	Melting temperature
CRLR	U17473	S	TGCTCTGTGAAGGCATTTAC	1,159	63
		AS	CAGAATTGCTTGAACTCTC	1,655	
RCP	AF073792	S	TGGGCAAGAGAACTTGAACA	111	60
		AS	TTCCACGATCAGCTGGATCT	306	
RAMP-1	NM005855	S	GAGACGCTGTGGTGTGACTG	189	61
		AS	TCGGCTACTCTGGACTCCTG	614	
RAMP-2	AJ001015	S	GGACGGTGAAGAACTATGAG	235	60
		AS	ATCATGGCCAGGAGTACATC	517	
RAMP-3	AJ001016	S	TGGAAGTGGTGCAACCTGTC	189	63
		AS	CACGGTGCAGTTGGAGAAGA	347	
β-actin	BC001301	S	TCCATCGTCCACCGCAATG	1,169	55
		AS	TTGTGAACTTTGGGGGATGCTC	1,398	

sion by RT-PCR analysis using the same conditions described above. The amplified RT-PCR products were processed and quantified as described above.

Protein synthesis inhibition assay

The cytotoxic activity of IL13-PE38 was determined by a protein synthesis inhibition assay as previously described.[25,27] Protein synthesis inhibition directly correlates with cell death.[27] Briefly, 10^4 PC-3 clones were cultured in leucine-free medium with or without various concentrations of IL13-PE38 for 20–22 hr at 37°C. For competition studies, cells were preincubated with IL-13 (2 μg/ml) for 40 min at 37°C prior to the addition of IL13-PE38 to the cells. Then, 1 μCi of [^{3}H]-leucine (NEN Research Products, Wilmington, DE) was added to each well and cells were incubated for an additional 4 hr. Cells were harvested and radioactivity was measured by counting the filter mat using a β-plate counter (Wallac, Gaithersburg, MD).

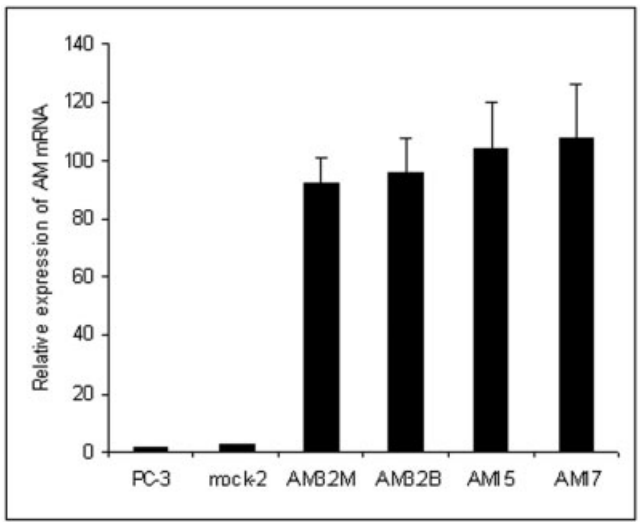

FIGURE 1 – Relative expression of AM in PC-3 parental cells and PC-3 clones stably transfected with human AM (AM3.2M and AM3.2B), rat AM (AM15, AM17), or empty vector (mock-2) as measured by quantitative real-time RT-PCR. Values were normalized to the expression of 28s rRNA. The expression of AM mRNA in clones transfected with the AM gene is approximately 100-fold higher than that compared to the parental and mock-transfected cells.

Colony formation assay

In vitro cytotoxic activity of IL13-PE38 on PC-3 clones was also evaluated by a colony formation assay.[28] Cells were harvested from culture, washed and resuspended in complete medium. Cells were plated in quadruplicate in 100 cm^2 Petri dishes (Falcon; Becton Dickinson, Lakeridge, NJ) and cultured overnight. The number of cells per plate was selected so that more than 100 colonies were obtained in the control group. The cultures were then incubated with IL13-PE38 (0–1,000 ng/ml) for 10 days at 37°C in a humidified CO_2 incubator. After removing the medium, the colonies were washed with PBS and stained with 0.025% crystal violet in 25% ethyl alcohol. Colonies with 50 or more cells were scored. The number of colonies observed in IL13-PE38-treated cultures was expressed as a percentage of the number of colonies formed in untreated control cultures.

Results

Inhibition of cell growth by AM

Quantitative analysis of AM mRNA levels (normalized to 28s rRNA levels) was performed by real-time RT-PCR to confirm the overexpression of AM in the stably transfected PC-3 cell clones. Significantly higher levels (approximately 100-fold increase) of AM mRNA were found in AM-transfected cell clones compared to the parental cell line or the clone containing the empty vector (mock-2) (Fig. 1). Protein levels of AM in the conditioned media of these clones assessed by radioimmunoassay demonstrated AM levels of 16 pg/mL in the PC-3 parental cell line, whereas clones overexpressing AM had approximately 10-fold higher levels.[21]

We have compared the average doubling time for all 4 clones that overexpress human and rat adrenomedullin with that of the parental cell line and the mock-transfected clone. The doubling time of parental PC-3 cells was determined to be 18 ± 2.1 hr, whereas that of PC-3 cells overexpressing human AM was 22 ± 1.6 hr and 28 ± 2.9 hr for cells overexpressing rat AM. Our results demonstrate that AM can significantly inhibit the proliferation of PC-3 cells.

Expression of AM receptors

The AM receptor complex CRLR/receptor component protein (RCP)/RAMP was analyzed in PC-3 cells and the clones that were transfected with AM by RT-PCR. Parental PC-3, mock-transfected and AM-overexpressing cells expressed CRLR, RCP and RAMP-2 (Fig. 2). No expression of RAMP-1 and RAMP-3 was detected. The expression of CRLR and RAMP-2 seemed to be higher in the cell clones overexpressing AM. For RAMP-2, a band of the

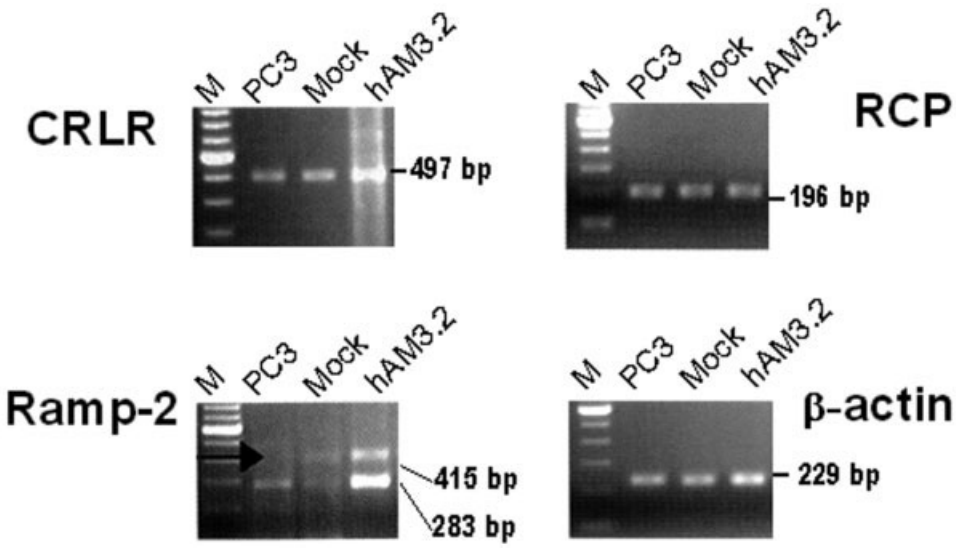

FIGURE 2 – Expression of AM receptors in PC-3 parental cells and PC-3-transfected cells. CRLR, RCP and RAMP-2 are expressed in these cells. RAMP-1 and RAMP-3 are not expressed. For RAMP-2, the expected band (283 bp) plus another band that might correspond to an alternative spliced form (415 bp) were identified.

expected size (283 bp) plus another band of 415 bp that could correspond to an alternative spliced form[29] were identified. Bands were sequenced to ensure their identities.

Changes in gene expression profiles associated with AM overexpression in PC-3 cells

In order to identify changes in gene expression, which could be associated with the inhibition of PC-3 cell growth by AM, we performed cDNA microarray experiments. This analysis identified approximately 100 genes whose expression was altered by AM using selection criteria of at least a 2-fold change in expression in at least 3 of the arrays. Hierarchical cluster analysis revealed that both clones overexpressing human AM had very similar gene expression patterns. Clone AM15 overexpressing rat AM exhibited a very similar expression profile to that of the clones expressing

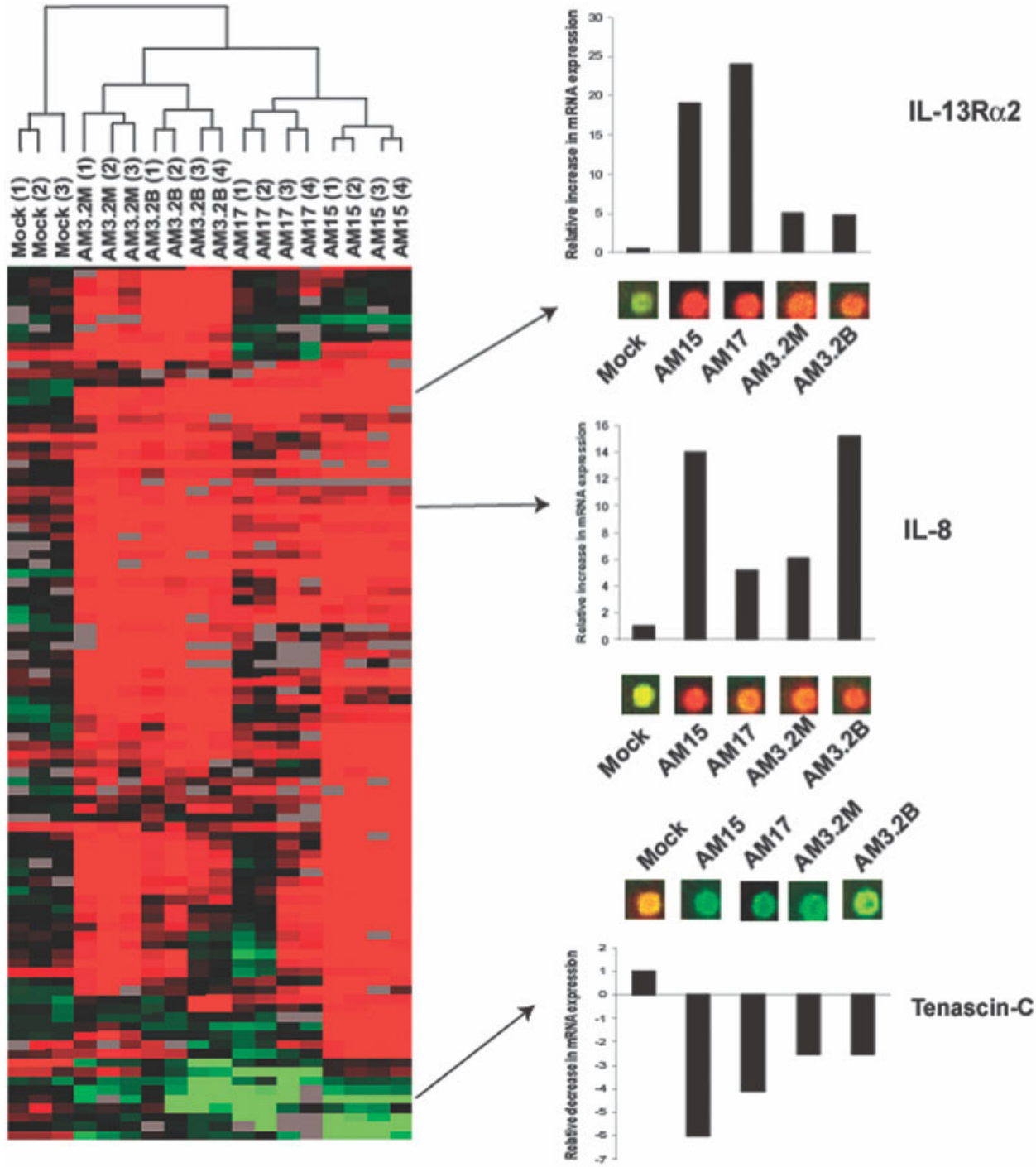

FIGURE 3 – Hierarchical clustering of gene expression patterns in PC-3 clones overexpressing AM or carrying the empty vector pcDNA3.1 (mock-transfected). PC-3 clones overexpressing human AM (AM3.2M and AM3.2B) demonstrated similar gene expression profiles and clustered together. Clones overexpressing rat AM (AM15 and AM17) clustered separately from cells expressing human AM, although gene expression profiles were similar (especially for clone AM15). IL-13Rα2 and IL-8 are examples of upregulated genes and tenascin-C of a downregulated gene in all of the cell lines. Few changes were observed in the empty vector-transfected clone.

human AM, while the second rat cell line clone AM17 did not show as extensive an overlap (Fig. 3).

Examples of genes upregulated in all the PC-3 clones overexpressing AM were the cytokine-related genes *interleukin-13Rα2* and *interleukin-8*. An example of a downregulated gene was the adhesion-related gene *tenascin-C* (Fig. 3). In contrast to the clones overexpressing AM, only 6 genes exhibiting a 2-fold change in expression were found in the clone carrying the empty vector compared to the parental PC-3 cell line.

Sixty-one genes were found to be consistently dysregulated in all of the AM-overexpressing PC-3 clones compared to the parental cell line. These genes were classified according to their presumed functions (Table II), including genes involved in extracellular matrix composition, cell cycle/apoptosis, tumor-related cytokines, cytoskeleton, adhesion proteins and transcription factors. Interestingly, AM upregulated the expression of genes associated with cell growth arrest, including *GADD45*, *IGF-BP6*, *STK-17*, *5′ nucleotidase* and TNF-related proteins. These results are coincident with our observations that cell proliferation is reduced in PC-3 cells overexpressing AM compared to parental PC-3 cells.

Overexpression of AM in PC-3 cells also resulted in dysregulation of expression of extracellular matrix-related proteins (Table II). Some of the upregulated genes belong to the urokinase-type plasminogen activator superfamily. Overexpression of AM in PC-3 cells notably upregulated 2 of the Runt family members of transcription factors, *RUNX-1* and *RUNX-3*. These transcription factors play an important role in cell growth arrest. These data are in keeping with our results in PC-3 cells and suggest that AM-mediated cell growth inhibition might be related to the upregulation of *RUNX-3*.

Six genes were found dysregulated (2-fold increase or decrease) in mock-transfected PC-3 clones compared to the PC-3 parental cell line. These genes were *midkine* (clone ID 940845), *aldehyde dehydrogenase 1-A3* (clone ID 2884153), *milk fat globule-EGF factor 8* (clone ID 1319020), *interferon-induced transmembrane-3* (clone ID 2949427), *zygin-1* (clone ID 2844322) and *tropomyosin-2* (clone ID 3176845). The alteration in expression of these genes probably results from the introduction of the pCDNA3.1 vector into the PC-3 cells. However, since we only studied one such clone, other random factors could be involved.

Overexpression of adrenomedullin enhances expression of IL-13Rα2 mRNA in PC-3 cells

AM-overexpressing cells demonstrated increased mRNA expression for IL-13Rα2, a high-affinity specific receptor for IL-13. IL-13 is a predominantly Th-2 cell-derived pleiotropic immune regulatory cytokine.[30] To confirm IL-13Rα2 gene upregulation as determined by microarray analysis, RT-PCR analysis was performed for IL-13Rα2 in the various PC-3-derived cell lines. These results confirmed that, indeed, IL-13Rα2 was increased in the AM-overexpressing clones

TABLE II – CLASSIFICATION OF GENES DIFFERENTIALLY EXPRESSED IN PC-3 CLONES OVER-EXPRESSING AM COMPARED TO THE PARENTAL CELL LINE

Category	Clone ID	Name	Mean ratio			
			hAM3.2B	hAM3.2M	rAM15	rAM17
Extracellular matrix-related proteins						
	557012	**SERPIN-B2 (PAI-2)**	27.7 ± 9.1	40.2 ± 12	95.7 ± 33	8.7 ± 1.9
	927392	**SERPIN-B8**	4.0 ± 0.6	6.2 ± 0.8	2.8 ± 0.1	2.5 ± 0.7
	1674454	**Connective tissue growth factor**	3.0 ± 0.4	1.9 ± 0.4	2.9 ± 0.1	1.2 ± 0.1
	1453450	**Tenascin-C (cytotactin)**	−2.5 ± 0.1	−2.5 ± 0.1	−10 ± 0.1	−4.1 ± 0.1
	2056158	**Plasminogen activator, urokinase**	4.7 ± 0.7	5.6 ± 0.8	5.9 ± 0.4	4.1 ± 0.8
	5183574	**Syndecan-4**	1.7 ± 0.2	3.6 ± 0.5	2.8 ± 0.1	1.0 ± 0.2
Cytokines involved in tumor growth						
	3360476	**Interleukin-13Rα2**	4.0 ± 0.5	3.8 ± 0.9	19.6 ± 4.9	24.1 ± 3.5
	2785701	**Interleukin-8**	5.3 ± 0.8	16.9 ± 1.4	13.7 ± 0.6	3.9 ± 1.4
Cytoskeleton						
	1649959	**Keratin-7**	2.0 ± 0.1	2.9 ± 0.2	2.96 ± 0.3	1.5 ± 0.2
	1435374	**Keratin-18**	2.3 ± 0.3	2.5 ± 0.4	2.5 ± 0.4	1.33 ± 0.06
Cell cycle/Growth-related factors/Apoptosis						
	2352645	**Amphiregulin**	2.7 ± 0.3	6.2 ± 0.7	7.9 ± 0.4	1.33 ± 0.06
	443631	**Epiregulin**	2.6 ± 0.3	3.6 ± 0.5	5.0 ± 0.5	1.2 ± 0.1
	3660752	**GRO-1**	3.7 ± 0.9	3.7 ± 0.7	2.7 ± 0.4	3.4 ± 0.4
	1702350	**Growth arrest GADD45A**	2.9 ± 0.7	2.0 ± 0.2	3.8 ± 0.2	1.6 ± 0.1
	1968126	**Growth arrest IGF-BP6**	2.2 ± 0.3	2.0 ± 0.2	2.9 ± 0.2	2.1 ± 0.3
	2416415	**Apoptosis-inducing STK-17**	3.4 ± 0.3	3.1 ± 0.1	2.4 ± 0.1	2.0 ± 0.3
	2555673	**Bcl-2-related protein A1**	8.1 ± 1.9	10 ± 0.8	4.8 ± 0.2	3.2 ± 0.5
	2205246	**TNF-Receptor, member 6**	2.3 ± 0.4	2.0 ± 0.3	2.6 ± 0.3	2.5 ± 0.4
	1878791	**TNF-alpha induced protein 3**	4.3 ± 0.7	14.8 ± 3.8	6.8 ± 0.7	4.2 ± 0.6
	1718651	**5′ Nucleotidase (CD-73)**	3.3 ± 0.3	3.5 ± 0.6	3.8 ± 0.3	6.3 ± 0.4
Transcription factors						
	2229852	**RUNX-1**	2.6 ± 0.2	3.7 ± 0.4	2.4 ± 0.3	1.7 ± 0.2
	885297	**RUNX-3**	2.7 ± 0.2	3.6 ± 0.6	3.4 ± 0.1	1.6 ± 0.06
	570512	**Activating transcription F1**	1.5 ± 0.1	2.8 ± 0.1	4.4 ± 0.5	2.0 ± 0.4
Cell adhesion						
	2844322	**Cell adhesion molecule FEZ-1**	1.4 ± 0.1	3.0 ± 0.3	7.9 ± 0.9	2.0 ± 0.3
Others						
	1213932	**Metallothionein-1E**	1.5 ± 0.3	3.7 ± 0.5	6.4 ± 0.5	4.0 ± 0.2
	2513883	**Metallothionein-1L**	1.6 ± 0.5	4.7 ± 1.1	4.0 ± 0.1	2.07 ± 0.9
	1817434	**Transketolase**	−2.0 ± 0.3	−1.6 ± 0.7	−1.6 ± 0.4	−2.5 ± 0.06
	1846463	**Phosphate transporter, member 1**	3.6 ± 0.3	3.4 ± 0.5	3.8 ± 0.3	2.0 ± 0.1
	1998510	**MGC11034**	−2.0 ± 0.5	−5.2 ± 0.3	−2.5 ± 0.3	−4.8 ± 0.1
	382416	**Cerebellar-degeneration antigen-1**	−1.8 ± 0.5	−3.3 ± 0.3	−2.2 ± 0.3	−1.4 ± 0.1

(Fig. 4*a*). The fluorodensitometric results of RT-PCR products for AM and for the β-actin control were expressed as the ratio of RFUs of the ethidium bromide-stained bands. The 2 human AM-expressing PC-3 clones had 2.5 and 3.4 times more IL-13Rα2 as compared to the parental PC-3 or empty-vector PC-3 cells. However, rat AM-expressing cells appeared to produce slightly higher levels of IL-13Rα2, with a relative ratio of 4 (Fig. 4*b*). These results show a distinct pattern of enhanced expression of IL-13Rα2 gene mRNA in AM-overexpress-

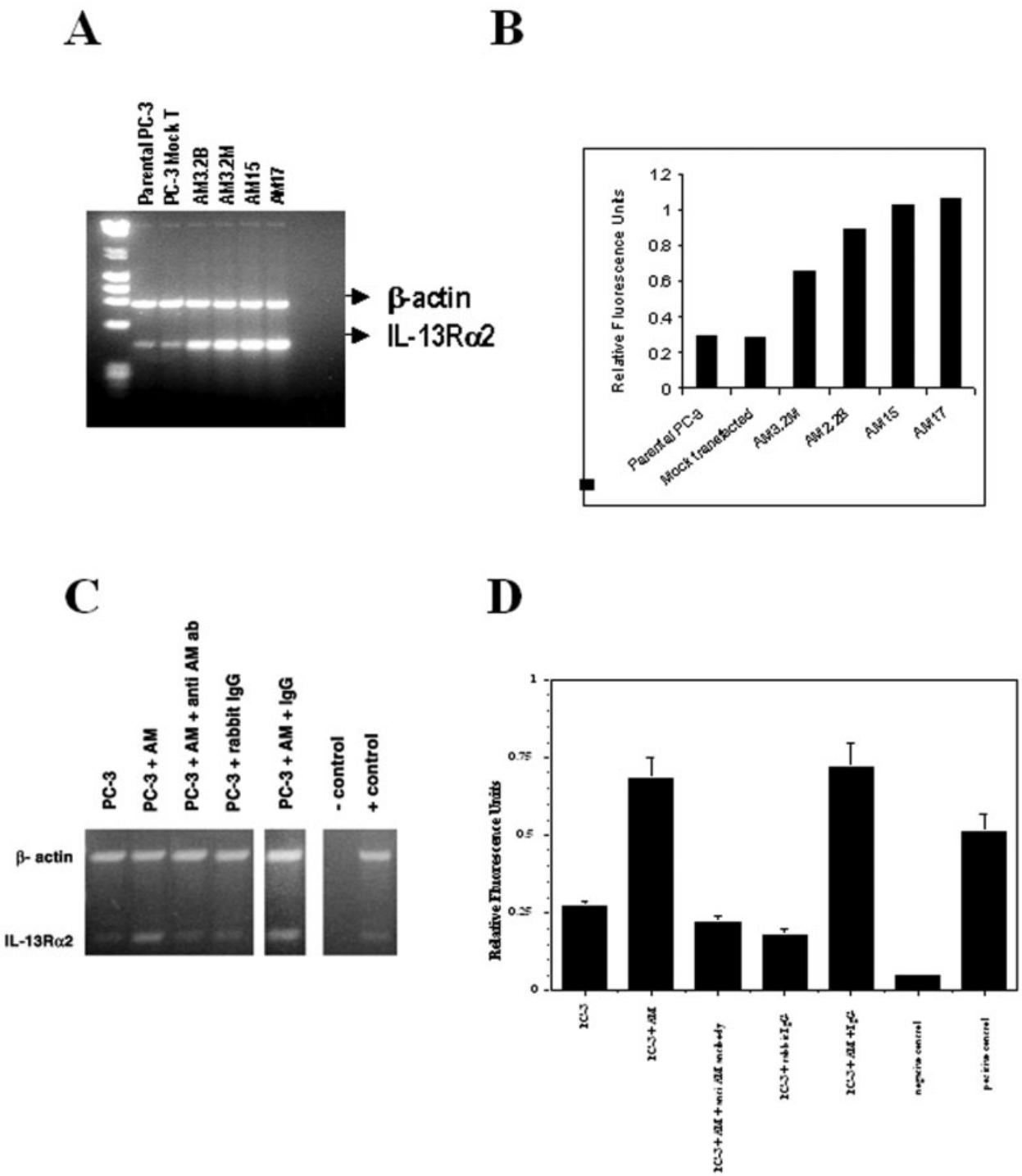

FIGURE 4 – Relative expression of IL-13Rα2 mRNA. (*a*) RT-PCR demonstrates low expression of IL-13Rα2 in the parental cell line and cells carrying the empty vector. In contrast, clones overexpressing AM show higher expression of IL-13Rα2. (*b*) Fluorodensitometric analysis of IL-13Rα2 PCR products with respect to β-actin products confirms the increase in expression in AM-transfected PC-3 clones. (*c*) Treatment of PC-3 cells with AM results in an upregulation of IL-13Rα2. Neutralization of AM by a blocking antibody produces a downregulation of IL-13Rα2. The nonspecific isotype control antibody does not cause such an effect. (*d*) Quantitative analysis of IL-13Rα2 with respect to β-actin products (relative fluorescence units) in AM-neutralized cultures indicates that blocking antibody also downregulates IL-13Rα2 expression.

ing prostate tumor cells that is not dependent on the species of origin of AM. Our results are intriguing as adrenomedullin and IL-13Rα2 genes are apparently nonrelated genes and have seemingly independent biologic functions.

We next tested whether neutralization of AM resulted in a downregulation of IL-13Rα2. PC-3 cells cultured in the presence of AM (from the supernatants of AM-overexpressing PC-3 clones) showed an increased expression of IL-13Rα2 compared to PC-3 cultured with supernatants of parental cells (Fig. 4*c*). Addition of the AM peptide showed similar results (not shown). When PC-3 cells were preincubated with anti-AM blocking antibody and then cultured in the presence of AM, no increase in IL-13Rα2 was found. Preincubation of PC-3 cells with control rabbit IgG and subsequent addition of AM also showed an increase in IL-13Rα2 mRNA (Fig. 4*c*). The quantification of the bands confirmed these results (Fig. 4*d*). A significant increase in the RFUs for IL-13Rα2 was found in PC-3 cells treated with AM and AM plus IgG. When the anti-AM blocking antibody was added, a significant decrease in the RFUs for IL-13Rα2 reaching basal levels was found (Fig. 4*d*).

AM induction of IL-13Rα2 results in functionally active protein

In order to determine whether the IL-13Rα2 mRNA induced by AM was translated into protein and whether IL-13Rα2 protein was functionally active, we performed a cytotoxicity assay using a highly specific chimeric fusion protein that consisted of IL-13 and a truncated form of *Pseudomonas* exotoxin (IL13-PE38 or IL-13 cytotoxin). In this assay, the IL-13 cytotoxin binds to IL-13 receptor sites on the cell surface of the tumor cells and is internalized by endocytosis.[18] The endocytosed protein is processed in the cytosol and then blocks protein synthesis leading to cell death. Cytotoxic activity of IL13-PE38 on adrenomedullin-transfected PC-3 cell lines was performed using a protein synthesis inhibition assay, which correlates with the amount of functional IL-13Rα2 receptors present on the plasma membrane. We have previously documented that the activity of this cytotoxin is highly specific, killing a variety of human tumor cells that overexpress IL-13 receptors.[25,30,31] The PM-RCC cell line was used as a positive control, since this cell type has been shown to express high levels of IL-13Rα2 protein and is extremely sensitive to the IL13-PE38 toxin (IC_{50}, 0.1 ng/ml; data not shown).[27] Two human and 2 rat AM-expressing PC-3 clones showed an IC_{50} (concentration of IL-13 toxin causing 50% inhibition of protein synthesis) of 1–4 ng/ml (Fig. 5). Parental and mock-transfected PC-3 cells, which expressed low levels of IL-13Rα2, were minimally sensitive to IL13-PE38 and never reached an IC_{50} even when a concentration of 1,000 ng/ml of cytotoxin was used. The cytotoxic effect was more pronounced in the 2 PC-3 clones expressing rat AM (AM15 and AM17), with an IC_{50} of ≥1.1 ng/ml, than for the cells overexpressing human AM. The IC_{50} for the rat AM cells was similar to that for the PM-RCC-positive control cell line (data not shown). The specificity of IL-13 toxin-mediated cytotoxicity was further confirmed by neutralization assays in the presence of excess of IL-13. In all the AM-overexpressing PC-3 clones, IL-13 was able to neutralize cytotoxic activity, which indicated the specificity of the cytotoxin (Fig. 5).

The relative cytotoxic sensitivities to IL13-PE38 of the human and rat AM-expressing PC-3 clones correlated to the differences in their rates of cell proliferation. The stronger inhibitory effect observed for PC-3 clones overexpressing rat AM is most likely due to structural differences between rat and human AM.

Reduced colony formation of AM-overexpressing PC-3 cells after treatment with IL13-PE38

In vitro assays were performed to determine whether the constitutively high AM-expressing PC-3 cells were less able to form colonies without any treatment or in response to exposure to IL13-PE38 than PC-3 cells, which did not express high levels of AM. Table III shows that PC-3 cells expressing either human or rat AM made significantly less number of colonies compared to PC-3 or PC-3 mock-cells. In addition, in the presence of IL13-PE38, AM-expressing cells made significantly fewer colonies compared to wild-type PC-3 or mock-transfected PC-3 cells. These results suggest that AM-expressing cells

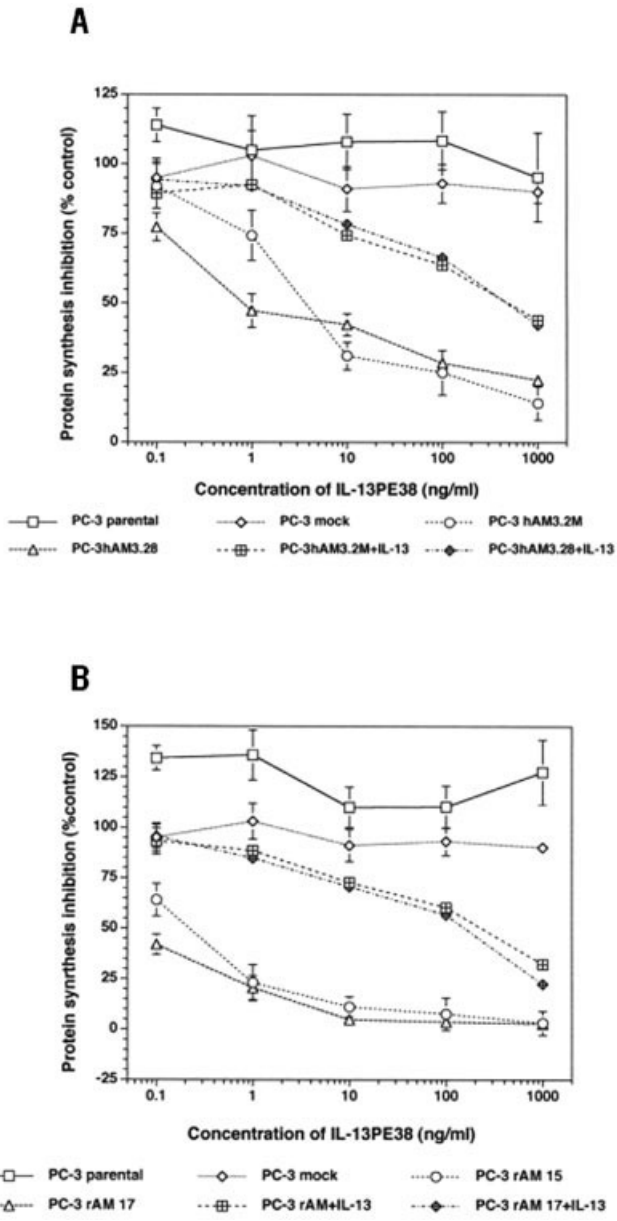

Figure 5 – IL13-PE38 cytotoxic assay. Clones overexpressing either human (*a*) or rat (*b*) AM have impaired protein synthesis following treatment with the IL-13 cytotoxin (IC_{50} ranging from 1 to 4 ng/mL). The strongest inhibition is observed for PC-3 clones that overexpress rat AM, with IC_{50} values similar to those for the positive control PM-RCC cells.

are highly sensitive to the IL-13 cytotoxin. The range of sensitivity (IC_{50}) was determined to be 4–5.5 ng/ml of IL13-PE38 in human AM-expressing PC-3 cells and 0.9 and 1.1 ng/ml in rat AM-expressing PC-3 cells. These results correlate well with the IC_{50} values obtained by the protein synthesis inhibition assay described above. Parental PC-3 and the mock-transfected PC-3 cell line failed to achieve IC_{50} at the highest concentration of IL-13 cytotoxin. These studies indicate that IL-13Rα2 chain induced by AM is functional, making the cells extremely sensitive to IL-13 cytotoxin.

Discussion

Adrenomedullin is a widely expressed peptide that exerts a variety of biologic functions, including vasodilatation, bronchodilation, control of hormone secretion, renal homeostasis and the regulation of cell

TABLE III – *IN VITRO* INHIBITION OF COLONY FORMATION IN AM TRANSFECTED PC-3 CELL LINES BY THE TOXIN IL13-PE38

IL13-PE38 (ng/ml)	Colonies (% control)[1]					
	PC-3	PC-3 Mock	PC-3 hAM3.2M	PC-3 hAM3.2B	PC-3 rAM15	PC-3 rAM17
0.1	96.0 ± 3.2	92 ± 4.6	74.0 ± 4.8	68.0 ± 1.6	70.3 ± 2.1	68.0 ± 5.2
1.0	95.2 ± 4.8	94.2 ± 3.2	61.2 ± 2.8	56.2 ± 2.3	48.2 ± 3.1	51.6 ± 3.6
10.0	98.2 ± 5.6	95.2 ± 4.0	36.1 ± 2.4	31.3 ± 0.9	22.4 ± 1.2	19.2 ± 1.7
100.0	84.6 ± 6	82.0 ± 7.0	10.8 ± 0.6	9.2 ± 1.6	6.5 ± 1.7	12.0 ± 1.9
1000.0	70.2 ± 8.6	78.2 ± 6.2	0.7 ± 0.3	3.6 ± 0.8	2.9 ± 0.5	9.3 ± 2.2
IC_{50} (ng/ml)	>1000	>1000	4	5.5	0.9	1.12

[1]Results are expressed as percentage of colonies formed by the treated cells compared to untreated control cells.

growth.[5-7] Depending on the particular cell type, AM may promote or inhibit cell growth.[8,9] Although AM is expressed in normal prostate epithelium and prostate cancer, its function in the prostate remains unknown. In order to investigate its potential functional role in the prostate, we previously generated stably transfected PC-3 cells that overexpress AM, which led to cell growth arrest and decreased rates of proliferation in PC-3 cells.[21] We have shown that PC-3 cells and clones with overexpression of AM express receptors for AM. Both AM and CGRP act through the same receptor known as CRLR.[32] CRLR coupled to the RAMP-2 or -3 binds predominantly to AM, whereas coupling to RAMP-1 leads to CGRP binding.[32] RCP is required for the transduction of the signal.[33] Receptors for AM have also been found in other human tumor cell lines.[6] Secretion of the AM peptide and expression of the AM receptors in PC-3 cells suggest an autocrine role of AM in the growth inhibition and upregulation of IL-13Rα2.

We have demonstrated by cDNA microarray analysis in the present study that the overexpression of AM in PC-3 cells alters the pattern of expression of 100 genes (approximately) involved in cell cycle arrest and apoptosis, the formation of the cytoskeleton, cell adhesion and the extracellular matrix, cytokines and transcription factors. *GADD45*, *IGF-BP6* and *RUNX-3* are genes upregulated by AM, which are related to cell growth arrest. The growth arrest and DNA damage-inducible gene (*GADD45*) is a p53-regulated gene involved in DNA repair, which has been found to be upregulated in cells subjected to stress.[34] UV irradiation and overexpression of FGF_2 lead to the upregulation of gene *GADD45*.[35] The ectopic overexpression of *GADD45* results in cell growth arrest and sensitization of cells to undergo apoptosis.[35] *RUNX-3* (which is overexpressed by AM) is another gene related to cell cycle regulation. Lack of this gene causes gastric epithelial hyperplasia and a reduced rate of apoptosis.[36] In addition, RUNX-$3^{-/-}$ epithelial cells are less sensitive to TGF-β-mediated apoptosis and gastric cancer cells overexpressing RUNX-3 are significantly less tumorigenic.[36]

Another gene upregulated by AM in PC-3 cells is the *urokinase-type plasminogen activator* (*u-PA*), which participates in the degradation of the extracellular matrix through the generation of plasmin and mediates intracellular signaling through cAMP.[37] u-PA is also involved in proteolytic activation of growth factors, including TGF-β and FGF_2.[37] The *serin proteinase inhibitor B2* (*SERPIN-B2*, also called *plasminogen activator inhibitor-2*, or PAI-2), which was also found upregulated by AM in our study, modulates the effect of u-PA.[38] PAI-2 inhibits cell migration and can modulate apoptosis.[38] The dysregulation of the u-PA system in PC-3 cells due to the overexpression of AM suggests that this peptide can induce changes in motility, invasiveness and cellular signal transduction. These interesting issues will be addressed in future studies.

IL-13Rα2 is a member of a cytokine receptor superfamily that is internalized after binding to its ligand without mediating signal transduction.[25] IL-13Rα2 strongly increased its expression (approximately 4–25 times) in PC-3 cells transfected with the AM gene. Since earlier studies showed that tumor cells expressing IL-13Rα2 can be dramatically sensitized to the cytotoxic effects of IL-13 cytotoxin, thus leading to cell death through apoptosis,[39] we decided to investigate this gene. Our earlier studies demonstrated that tumor cells with low levels of IL-13Rα2 become very sensitive to the cytotoxic effects of IL-13 cytotoxin after transient or stable transfection of this receptor chain *in vitro* and *in vivo*.[25] First, we have validated by RT-PCR the result obtained by microarray analysis. Second, we demonstrated that there is not only an upregulation of IL-13Rα2 mRNA as a consequence of AM overexpression, but also an upregulation of the protein, which makes the cells highly sensitive to IL-13 cytotoxin. Third, we have shown that upregulation of IL-13Rα2 mediated by AM can be blocked by an anti-AM-specific antibody. The sensitization of PC-3 cells to the cytotoxin IL13-PE38 is quite remarkable in some of the clones with high expression of AM. Thus, the IC_{50} values of inhibition for the clones AM15 and AM17 are closer to those obtained for the highly sensitive cell PM-RCC (1–4 ng/mL), which was used in our assay as a positive control. This is a striking and novel finding, since no association between AM and the IL-13Rα2 signaling pathway leading to toxin sensitization had previously been identified. The molecular mechanism involved in the regulation of AM and IL-13Rα2 expression remains undetermined but is under current investigation. It is possible that AM enhances the half-life of mRNA and/or protein and may interact with IL-13Rα2 promoter to enhance its expression.

The fusion of toxins to IL-13 is a promising therapeutic approach for treating tumors and is currently in clinical trials for brain tumors.[40] The results presented in this study suggest that novel strategies utilizing adrenomedullin signaling may be developed to increase the expression of IL-13Rα2, thereby sensitizing tumors to IL-13Rα2-based therapies. Such approaches might include the direct delivery of AM using gene or protein systems to increase expression of IL-13Rα2 followed by the targeting of IL-13-based cytotoxins to the tumor cells.

Kawakami *et al*.[25] reported that the chemokine IL-8 was upregulated in MDA-MD-231 and PANC-1 cells as a result of IL-13Rα2 overexpression. Interestingly, our microarray results revealed that, in addition to the overexpression of IL-13Rα2 in AM-overexpressing cells, there is a concomitant increase in IL-8. IL-8 has been implicated in tumor growth and metastases through its role in regulating angiogenesis.[41] AM has been shown to stimulate tumor angiogenesis through induction of HIF-1.[20] In addition, AM acts as a proangiogenic factor *in vivo* in breast cancer[42] and endometrial tumors.[43] Therefore, it will be of interest to examine interaction between AM, IL-8 and IL-13Rα2 in relation to tumor angiogenesis.

In summary, numerous changes in gene expression associated with the upregulation of adrenomedullin in prostate cancer cells have been identified. This approach has provided additional insight into genetic consequences of adrenomedullin function. We show a novel AM-induced mechanism of tumor sensitization through the upregulation of functional IL-13Rα2 chain, which is a target for the specific recombinant chimeric cytotoxin IL13-PE38.

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