Expression of adrenomedullin 2/intermedin in human adrenal tumors and attached non-neoplastic adrenal tissues

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Abstract

Adrenomedullin 2/intermedin (AM2/IMD) is a new member of calcitonin/calcitonin gene-related peptide family. AM is expressed in various tumors including adrenocortical tumors and modulates tumor growth. The AM2/IMD expression has not been studied, however, in adrenal tumors. The expression of AM2/IMD and AM was therefore studied in human adrenal tumors and attached non-neoplastic adrenal tissues by immunocytochemistry (ICC). Immunoreactive (IR)-AM2/IMD was measured by RIA. Furthermore, the expression of AM2/IMD and its receptor components, calcitonin receptor-like receptor (CRLR), and receptor activity-modifying proteins (RAMPs) 1, 2, and 3 mRNA in these tissues was studied by reverse transcription PCR (RT-PCR). ICC showed that AM2/IMD and AM immunoreactivities were localized in adrenocortical tumors and pheochromocytomas. AM2/IMD and AM immunoreactivities were detected in medulla of attached

non-neoplastic tissues, while the degree of immunoreactivity for AM2/IMD and AM in cortices of attached adrenals was relatively weak or undetectable. RIA detected IR-AM2/IMD in adrenal tumors (0.414 ± 0.12 to 0.786 ± 0.27 pmol/g wet weight, mean $\pm$ S.E.M.) and attached adrenal tissues (0.397 ± 0.052 pmol/g wet weight). Reverse-phase high-performance liquid chromatography showed one broad peak eluted in the similar position to synthetic AM2/IMD with several minor peaks. RT-PCR showed expression of AM2/IMD, CRLR, and RAMP1, RAMP2, and RAMP3 mRNA in tissues of adrenal tumors and attached adrenal glands. In conclusion, AM2/IMD is expressed in human adrenal tumors and attached non-neoplastic adrenal tissues and may play (patho-)physiological roles in normal and neoplastic adrenals as an autocrine/paracrine regulator.

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Introduction

Adrenomedullin 2/intermedin (AM2/IMD) is a novel member of the calcitonin/calcitonin gene-related peptide (CGRP) family, which includes calcitonin, CGRP, amylin, and AM (Chang *et al.* 2004, Roh *et al.* 2004, Takei *et al.* 2004a,b). Two independent research groups first identified this peptide almost simultaneously by genomic searching, naming it AM2 (Takei *et al.* 2004a) and IMD (Roh *et al.* 2004) respectively. CGRP, AM, and AM2/IMD activate calcitonin receptor-like receptor (CRLR)/receptor activity-modifying protein (RAMP) system to transduce their signals; AM2/IMD interacts with CRLR/RAMP1, RAMP2, or RAMP3 non-selectively, whereas

CGRP preferentially interacts with CRLR/RAMP1 and AM with CRLR/RAMP2 or RAMP3 (Roh *et al.* 2004).

It is well known that adrenal medulla and pheochromocytomas express various neuropeptides and vasoactive peptides, such as neuropeptide Y (NPY) and AM, and their receptors (Hinson *et al.* 2000, Spinazzi *et al.* 2005). Moreover, there is accumulating evidence showing production and secretion of these peptides from adrenal cortex and adrenocortical tumors (Takahashi *et al.* 2002). Among these peptides, AM has been thoroughly studied to show the mitogenic effects on adrenocortical tumor cells and the regulatory actions in secretion of adrenocortical steroids and adrenomedullary catecholamines (Hinson *et al.* 2000). Recently, we have reported the presence of immunoreactive (IR)-AM2/

IMD in human tissues including hypothalamus, pituitary, heart, and kidney by RIA and immunocytochemistry (ICC; [Takahashi et al. 2006](#), [Morimoto et al. 2007a](#)). There have been no reports, however, on the expression of AM2/IMD in human adrenal tumors and adrenal glands.

We, therefore, studied the expression of AM2/IMD, along with AM, in human adrenal tumors and attached non-neoplastic adrenal tissues by ICC. We then studied the expression of IR-AM2/IMD in these tissues by RIA with chromatographic characterization. We also studied the expression of AM2/IMD, CRLR, and RAMP1, RAMP2, and RAMP3 mRNA by reverse transcription PCR (RT-PCR).

Materials and Methods

Subjects and tissues

This study was approved by the Ethics Committee of Tohoku University School of Medicine, and informed consent was obtained from each patient before collection of tissue specimens examined in the present study.

Tissues were obtained at surgery from patients with adrenal tumors. Immediately after surgical removal, the tissues were fixed in 4% formalin and embedded into paraffin for ICC or frozen and stored at -80 °C before extraction of peptides and RNA. Adrenal tumor tissues employed for ICC, RIA, and RT-PCR were summarized in [Table 1](#). Adrenocortical carcinomas were histologically diagnosed based upon the criteria proposed by Weiss ([Weiss 1984](#), [Weiss et al. 1989](#)). All surgical specimens, except for adrenocortical carcinomas, contained attached tissues of morphologically normal adrenal glands. Morphologically normal, non-neoplastic, attached adrenal tissues were obtained for RIA from 23 patients with adrenocortical adenomas, cortisol-producing adenomas (*n*=13, two males and eleven females, 32- to 75-year old), and aldosterone-producing adenomas (*n*=10, five males and five females, 40- to 71-year old), and for RT-PCR from 4 patients with aldosterone-producing adenomas (two males and two females, 44- to 67-year old).

Antiserum

The antisera against human AM2/IMD and AM were raised in our laboratory, and their detailed properties were previously reported ([Satoh et al. 1996](#), [Morimoto et al. 2007a](#)). The antiserum against human AM2/IMD was characterized by RIA, yielding cross-reactivities of less than 0.001% with other peptides including human AM, CGRP, calcitonin, and amylin ([Morimoto et al. 2007](#)).

The antiserum against human AM (no. 102-94921) showed cross-reactivities of less than 0.001% with other peptides including human AM2/IMD, CGRP, calcitonin, and amylin ([Satoh et al. 1996](#)).

Immunocytochemistry

ICC was performed by the ABC method using the Vector ABC kit (Vector Laboratories, Burlingame, CA, USA), as reported previously ([Satoh et al. 1996](#)). The antisera against human AM2/IMD and human AM were used at dilutions of 1:4000 and 1:2000 respectively.

In negative controls, AM2/IMD antiserum preabsorbed with synthetic human AM2/IMD₁₋₄₇, AM antiserum preabsorbed with synthetic human AM₁₋₅₂, and normal rabbit serum (at a dilution of 1:4000) were used instead of the AM2/IMD antiserum or AM antiserum. Absorption tests were performed using the antisera incubated with synthetic human AM2/IMD₁₋₄₇ (10 nmol peptide/ml diluted antiserum) or synthetic human AM₁₋₅₂ (10 nmol peptide/ml diluted antiserum) for 20 h at 4 °C before use.

Intensity of immunostaining in the cells was evaluated on a scale of negative, weakly positive, and positive. In our preliminary experiment, the prominent immunoreactivities for AM2/IMD and AM were observed in all benign adrenocortical tumors examined. We defined this intensity as 'positive'. Negative controls using normal rabbit serum instead of appropriate antibodies showed no immunoreactivity. When immunoreactivity was undetected like negative controls using normal rabbit serum, we described it as 'negative'. The immunoreactivity that should be classified between 'positive' and 'negative' was defined as 'weakly positive'.

Table 1 Adrenal tumor tissues for immunocytochemistry (ICC), RIA, and reverse transcription PCR (RT-PCR) analysis

	ICC		RIA		RT-PCR	
	N	Age (yo)	N	Age (yo)	N	Age (yo)
Aldosterone-producing adenomas	8 (4, 4)	34-68	8 (4, 4)	40-67	4 (3, 1)	52-61
Cortisol-producing adenomas	8 (1, 7)	41-78	13 (2, 11)	32-75	4 (2, 2)	38-60
Non-functioning adenomas	2 (2, 0)	56-65	N/A		1 (1, 0)	56
Adrenocortical carcinomas	17 (6, 11)	18-65	5 (1, 4)	41-69	4 (1, 3)	49-69
Pheochromocytomas	8 (4, 4)	26-68	5 (3, 2)	29-58	7 (5, 2)	29-64
Total	43		31		20	

N, number of cases; yo, year old; N/A, not applicable. Two numbers in the parentheses showed the number of male and female cases respectively.

Peptide extraction and RIA

Tissues were extracted, as reported previously (Morimoto *et al.* 2007). Briefly, the tissue (~750 mg) was boiled in 2 ml of 1 mol/l acetic acid for 10 min. To each sample, 8 ml 50% methanol in 0.5 mol/l acetic acid was added and the tissue was homogenized. The homogenate was centrifuged at 15 000 *g* for 30 min. The supernatant was separated, dried by air, reconstituted in assay buffer (0.1 mol/l phosphate buffer (pH 7.5) containing 0.1% (wt/vol) BSA, 0.2% (vol/vol) Triton X-100, and 0.1% (wt/vol) sodium azide), and assayed.

The RIA of human AM2/IMD was performed as reported previously (Morimoto *et al.* 2007). Human AM2/IMD₁₋₄₇ (Peptide Institute) was used as a standard. [¹²⁵I] AM2/IMD₁₋₄₇ (Phoenix Pharmaceuticals Inc., Belmont, CA, USA) was used as a radioligand.

Chromatographic characterization of tissue extracts was performed by reverse-phase high-performance liquid chromatography (HPLC) using a μ Bondapak C18 column (3.9 × 300 mm, Waters). The tissue extracts were re-extracted with Sep-Pak C18 cartridges (Waters Corp., Milford, MA, USA). The extracts were reconstituted in 0.1% (vol/vol) trifluoroacetic acid (TFA) and loaded onto the column. The HPLC was performed with a linear gradient of acetonitrile containing 0.1% (vol/vol) TFA from 10 to 60% at a flow rate of 1 ml/min·fraction over 50 min. Each fraction (1 ml) was collected, dried by air, reconstituted with assay buffer, and assayed. To prepare AM2/IMD₁₋₄₇ with oxidized methionine, synthetic human AM2/IMD₁₋₄₇ (2 mmol/l) was incubated with 0.3% (vol/vol) H₂O₂ at room temperature for 1 h.

RT-PCR

Total RNA was extracted from tissues by the guanidine thiocyanate–cesium chloride method. Total RNA (4 μ g) was denatured at 65 °C for 5 min and transcribed at 37 °C for 60 min in a reaction mixture (20 μ l) containing 0.5 μ g oligo-dT, 0.5 mmol/l dNTP, and 400 units of Moloney murine leukemia virus reverse transcription (BRL, Gaithersburg, MD, USA). The reaction was stopped by heating at 95 °C for 5 min, diluted with 30 μ l water and stored at –20 °C until PCR analysis. One microliter of the reaction mixture was subjected to PCR. The PCR was performed in a total volume of 20 μ l containing 0.2 mmol/l of each dNTP,

0.25 μ mol/l of each primer, and 0.4 U GoTaq DNA polymerase (Promega Corp).

The sense and anti-sense primers are summarized in Table 2. The primers were designed to contain one intron between the sense and anti-sense primers to exclude a possibility of genomic DNA contaminations. The PCR of AM2/IMD was performed under the following condition; after heating at 95 °C for 2 min, denaturation, annealing, and elongation were carried out at 95 °C for 25 s, 60 °C for 30 s, and 72 °C for 45 s respectively and the reactions were repeated for 35 cycles, followed by 72 °C for 5 min. The PCR of CRLR and RAMP1, RAMP2, and RAMP3 was performed under the following condition; after heating at 94 °C for 2 min, denaturation, annealing, and elongation were carried out at 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 60 s respectively and the reactions were repeated for 30 cycles, followed by 72 °C for 5 min. RNA samples subjected to PCR without reverse transcriptase reactions were used as negative controls. Amplification products were visualized on a 5% polyacrylamide gel stained with ethidium bromide, and viewed on a u.v. box.

Statistical analysis

Data are shown as mean $\pm$ S.E.M. unless otherwise stated. Correlations between the scale of AM2/IMD or AM immunoreactivity and the score of Weiss criteria (Weiss 1984, Weiss *et al.* 1989) were examined by χ^2 test with a cross-table for ordinal variables. IR-AM2/IMD concentrations in adrenal tumors and attached adrenal tissues were compared among groups by one-way ANOVA followed by Fisher's protected least significant difference. The statistical analysis was carried out using JMP software version 6.0 (SAS Institute, Cary, NC, USA), and $P < 0.05$ was considered statistically significant.

Results

Immunocytochemistry

Results of ICC for AM2/IMD and AM in adrenal tumors and attached adrenal tissues were summarized in Table 3. All tumors examined, both of cortical and medullary origins, showed positive immunostaining for AM2/IMD and AM. The

Table 2 Primers for reverse transcription PCR (RT-PCR) analysis

Gene	Sense primer	Anti-sense primer	Genbank accession no.
AM2/IMD	CCATGGCCCCGGATCCCCGACG (1–20)	TCAGCCATAGCTGTGGGGGCT (429–449)	AB121034
CRLR	TGCAAAGTGTCAGATTCATTC (1182–1203)	TGTAAGACGCACTACGAAGAGC (1791–1812)	NM005795
RAMP1	GGGGAGACGCTGTGGTG (286–302)	ATGCCCTCAGTGCCTT (556–572)	NM005855
RAMP2	CCATCGAAAAGGATTGGTG (300–319)	CATGGTTGTTGAGAAGCTCGT (603–623)	NM005854
RAMP3	TGCTCTGCGGTGGGTGTC (111–128)	GTGTCGGTGCGTTTGCTG (481–498)	NM005856

AM2/IMD, adrenomedullin 2/intermedin; CRLR, calcitonin receptor-like receptor; RAMP, receptor activity-modifying protein. Numbers in the parentheses showed the nucleotide numbers of each corresponding cDNA.

Table 3 Number of cases showing the immunoreactivities for AM2/IMD and AM

	AM2/IMD			AM		
	Tumor	Attached adrenal		Tumor	Attached adrenal	
		Cortex	Medulla		Cortex	Medulla
Aldosterone-producing adenomas						
Positive	8	0	8	8	0	7
Weakly positive	0	7	0	0	7	1
Negative	0	1	0	0	1	0
Cortisol-producing adenomas						
Positive	8	0	7	8	0	6
Weakly positive	0	6	1	0	7	2
Negative	0	2	0	0	1	0
Non-functioning adenomas						
Positive	2	0	2	2	0	2
Weakly positive	0	2	0	0	2	0
Negative	0	0	0	0	0	0
Adrenocortical carcinomas						
Positive	15	N/A	N/A	15	N/A	N/A
Weakly positive	2	N/A	N/A	2	N/A	N/A
Negative	0	N/A	N/A	0	N/A	N/A
Pheochromocytomas						
Positive	8	0	7	8	0	7
Weakly positive	0	7	1	0	6	1
Negative	0	1	0	0	2	0

AM2/IMD; adrenomedullin2/intermedin, AM; adrenomedullin, N/A; not applicable.

positive ratio of the immunoreactivity for AM2/IMD or AM was not significantly related to either the origins of the tumors (cortical or medullary) or the hormone production (cortisol, aldosterone, or catecholamines). There was no significant difference in the positive ratio of the immunoreactivity for AM2/IMD or AM between benign adrenocortical adenomas (cortisol-producing, aldosterone-producing, and non-functioning adenomas) and adrenocortical carcinomas.

The specificities of the AM2/IMD and AM immunostaining were confirmed by the absorption tests in all types of the tumors. The representative findings of the absorption tests in non-functioning adenomas were shown in Fig. 1: immunoreactivity for AM2/IMD (Fig. 1A) was completely abolished by the absorption with synthetic AM2/IMD (Fig. 1C), but not AM (Fig. 1B). Similarly, immunoreactivity for AM (Fig. 1D) was completely abolished by the absorption with AM (Fig. 1E), but not AM2/IMD (Fig. 1F).

In aldosterone-producing adenomas, AM2/IMD and AM were diffusely immunolocalized in the tumor cells, which were mainly composed of clear cells (Fig. 2A and B respectively). In attached adrenals, immunoreactivity for AM2/IMD was weak (seven cases) or undetectable (one case) in cortical tissues, whereas positive immunostaining was clearly seen in medulla (Fig. 2D). Similarly, AM immunoreactivity was weak (seven cases) or undetectable (one case) in cortical tissues, whereas positive immunostaining was clearly seen in medulla (Fig. 2E). In the seven cases with positive immunostaining for AM2/IMD and AM in attached cortices, immunoreactivities for AM2/IMD and AM were focally detected in the subcapsular cortices

(arrowheads in Fig. 2D and E respectively). Unlike the attached cortices of aldosterone-producing adenomas, those of cortisol-producing adenomas (data now shown), non-functioning adenomas (data not shown), and pheochromocytomas (Fig. 2M and N) did not show such remarkable immunoreactivities for AM2/IMD and AM in their subcapsular cortices. Negative controls using normal rabbit serum showed no immunoreactivities both in the tumor cells (Fig. 2C) and in the attached non-neoplastic tissues (Fig. 2F).

Similarly to aldosterone-producing adenomas, AM2/IMD and AM were immunolocalized in cortisol-producing adenomas and non-functioning adenomas (data not shown).

Figure 1 (A and D) Immunocytochemistry for adrenomedullin 2/intermedin (AM2/IMD) and adrenomedullin (AM), (B and E) absorption tests using AM2/IMD and AM antiserum preabsorbed with AM, and (C and F) absorption tests using AM2/IMD and AM antiserum preabsorbed with AM2/IMD respectively in non-functioning adenoma. Bars, 100 μ m.

Figure 2 Immunocytochemistry for adrenomedullin 2/intermedin (left panel: A, D, G, J and M) and adrenomedullin (middle panel: B, E, H, K and N), and negative controls using non-immune rabbit serum (right panel: C, F, I, L and O) in (A–C) aldosterone-producing adenoma, (D–F) attached adrenal tissue of aldosterone-producing adenoma, (G–I) adrenocortical carcinoma, (J–L) pheochromocytoma, and (M–O) attached adrenal tissue of pheochromocytoma respectively. Cp, capsule; Cx, cortex; Md, medulla. The arrowheads indicate subcapsular cortex, attached to aldosterone-producing adenoma. Bars, 100 µm in A through L and 200 µm in M, N, and O.

In attached adrenal tissues of the tumors, immunoreactivities for AM2/IMD and AM were weak or undetectable in cortical tissues, whereas AM2/IMD and AM immunoreactivities were detected in medulla similarly to the tumors (Table 3).

In adrenocortical carcinomas, AM2/IMD and AM were immunolocalized in the tumor cells of all 17 cases (Fig. 2G and H respectively). No significant correlations were found between the scale of AM2/IMD or AM immunoreactivity and the score of Weiss criteria ($P=0.2744$ in both analyses). Negative controls using normal rabbit serum showed no immunoreactivity in the tumor cells of adrenocortical carcinomas (Fig. 2I).

In pheochromocytomas, the tumor cells of all eight cases were positively immunostained for AM2/IMD and AM, and representative findings of spindle shape-like tumor cells immunostained for AM2/IMD and AM were shown in Fig. 2J and K respectively. In attached adrenals, immunoreactivities for AM2/IMD (Fig. 2M) and AM (Fig. 2N) were weakly observed or undetectable in cortices, whereas those

for AM2/IMD and AM were detected in the attached medulla with similar degrees to pheochromocytomas (Fig. 2M and N respectively). Negative controls using normal rabbit serum showed no immunoreactivity in both the tumor cells and the attached tissues of pheochromocytomas (Fig. 2L and O respectively).

RIA

IR-AM2/IMD was detected in the tumor tissues of cortisol-producing adenomas (0.572 ± 0.087 pmol/g wet weight, $n=13$), aldosterone-producing adenomas (0.786 ± 0.27 pmol/g wet weight, $n=8$), adrenocortical carcinomas (0.493 ± 0.14 pmol/g wet weight, $n=5$), and pheochromocytomas (0.414 ± 0.12 pmol/g wet weight, $n=5$; Fig. 3). The IR-AM2/IMD was also present in attached adrenal tissues containing cortex and medulla (0.397 ± 0.052 pmol/g wet weight, $n=23$; Fig. 3). No significant differences in the tissue concentrations of IR-AM2/IMD were identified among these tumors and attached adrenal glands ($P=0.1631$).

The IR-AM2/IMD in adrenocortical carcinoma (Fig. 4A), pheochromocytoma (Fig. 4B), and attached non-neoplastic adrenal tissues (Fig. 4C) was characterized by reverse-phase HPLC. Reverse-phase HPLC showed that a large amount (more than 65%) of the IR-AM2/IMD in the tissues was eluted in the similar position to synthetic AM2/IMD, forming a broad peak with multiple shoulders in the 30th–

Figure 3 Immunoreactive-adrenomedullin 2/intermedin concentrations in cortisol-producing adenomas, aldosterone-producing adenomas, adrenocortical carcinomas, pheochromocytomas, and attached non-neoplastic adrenal tissues. CPA, cortisol-producing adenomas; APA, aldosterone-producing adenomas; ACC, adrenocortical carcinomas; Pheo, pheochromocytomas; Attached, attached non-neoplastic adrenal tissues.

Figure 4 Reverse-phase HPLC of immunoreactive-adrenomedullin 2/intermedin (AM2/IMD) in (A) adrenocortical carcinoma, (B) pheochromocytoma, and (C) attached non-neoplastic adrenal tissues. The arrows indicate the elution position of AM2/IMD₁₋₄₇. Dotted lines show a gradient of acetonitrile. ACN, acetonitrile.

38th fractions (30–38 min; Fig. 4). A peak in the position of 24th fraction (24 min) was commonly seen in the extracts of all three tissues. Furthermore, one or two smaller peaks were eluted after the position of synthetic AM2/IMD. Oxidized AM2/IMD was eluted in the same fraction as that of authentic AM2/IMD, and synthetic human AM was eluted at the 32nd fraction (32 min; data not shown).

RT-PCR

RT-PCR analysis showed the expression of AM2/IMD mRNA in tissues of all adrenal tumors examined: cortisol-producing adenomas ($n=4$), aldosterone-producing adenomas ($n=4$), non-functioning adenomas ($n=1$), adrenocortical carcinomas ($n=4$), pheochromocytomas ($n=7$), and attached adrenal glands ($n=4$; Fig. 5). In the analysis of AM2/IMD mRNA expression (the first lane in Fig. 5), two RT-PCR products were detected (431 and 449 bp) due to the insertion/deletion polymorphism of AM2/IMD gene (NCBI refSNP ID rs3840963). No apparent relationship was found between the appearance of these genotypes and the phenotypes of the tissues, i.e. hormonal function, benign or malignant potential, and neoplastic or non-neoplastic tissues. The expression of CRLR and RAMP1, RAMP2, and RAMP3 mRNA was also identified in all the adrenal tumors and attached adrenal tissues, listed above (Fig. 5). Negative controls without reverse transcriptase reactions gave no bands (data not shown).

Discussion

The present study is the first study showing the expression of AM2/IMD in human adrenal tumors of both cortical and medullary origins and attached non-neoplastic adrenal tissues. We also demonstrated the expression of CRLR and RAMP1, RAMP2, and RAMP3 mRNA, which are components of the receptor system for AM2/IMD, in these tissues. These results have raised a possibility that AM2/IMD secreted from neoplastic and normal adrenal cells may act as an auto-crine/paracrine factor.

It is well known that adrenal medulla and pheochromocytomas produce and secrete various neuropeptides and vasoactive peptides, such as AM, NPY, enkephalin, and somatostatin (Lundberg *et al.* 1979, Adrian *et al.* 1983, Hinson *et al.* 2000, Spinazzi *et al.* 2005). On the other hand, the knowledge on the pathophysiological roles of adrenocortical regulatory peptides had been limited. There is, however, accumulating evidence that adrenal cortex and adrenocortical tumors secrete certain types of peptides, such as AM, endothelin-1, urocortins, and urotensin II (UUI), which may regulate the growth of adrenocortical cells and the secretion of adrenocortical steroid hormones (Takahashi *et al.* 1998, 2002, Thomson *et al.* 2001, Albertin *et al.* 2005, 2006, Fukuda *et al.* 2005). Recently, we have reported the expression of UUI and UUI receptor (UT-R) in human adrenal tumors and attached non-neoplastic adrenal tissues by ICC (Morimoto

Figure 5 Reverse transcription PCR of adrenomedullin 2/intermedin, calcitonin receptor-like receptor, receptor activity-modifying proteins 1, 2, and 3 mRNA in cortisol-producing adenomas, aldosterone-producing adenomas, non-functioning adenoma, adrenocortical carcinomas, pheochromocytomas, and attached non-neoplastic adrenal tissues. CPA, cortisol-producing adenomas; APA, aldosterone-producing adenomas; ACC, adrenocortical carcinomas; Pheo, pheochromocytomas; Attached, attached non-neoplastic adrenal tissues; M, molecular marker of 100 bp ladder; bp, base pairs.

et al. 2008). In particular, UII immunoreactivity was stronger in the adrenocortical tumors than in non-neoplastic cortices of attached adrenals, suggesting that UII may be related to the regulation of tumor growth in adrenocortical tumors (Morimoto *et al.* 2008). Similarly to UII, AM was reported to have mitogenic action on adrenocortical cells (Andreis *et al.* 2000, 2002, Belloni *et al.* 2001, Nussdorfer 2001, Semplicini *et al.* 2001, Malendowicz *et al.* 2003, Rossi *et al.* 2003) and adrenocortical tumor cells (Albertin *et al.* 2005). AM2/IMD may have a similar mitogenic action because AM2/IMD shares its receptor systems with AM (Roh *et al.* 2004).

The present study has demonstrated that the degree of immunoreactivity for AM2/IMD and AM was stronger in adrenocortical tumors than in attached normal cortices. These findings raised a possibility that both AM2/IMD and AM may be related to the tumor growth of adrenocortical tumors as autocrine/paracrine factors, like UII. Furthermore, we also showed stronger immunoreactivities for AM2/IMD and AM in the subcapsular outer cortices of attached adrenal tissues of aldosterone-producing adenomas, but not other types of adrenal adenomas. It was reported that AM was expressed in the zona glomerulosa cells in rat and human adrenals (Kapas *et al.* 1998, Kapas & Hinson 2002, Rebuffat *et al.* 2002a,b). The present study first showed the expression of AM2/IMD in the human adrenal cortex.

Andreis *et al.* previously reported that AM attenuated aldosterone secretion from dispersed human adrenocortical cells and aldosterone-producing adenomas in Ca^{2+} -dependent manner (Andreis *et al.* 1997, 1998, Forneris *et al.* 2001, Rebuffat *et al.* 2002b). AM2/IMD and AM expressed at outer cortices of the attached adrenals may attenuate the overproduction of

aldosterone from aldosterone-producing adenomas. On the other hand, aldosterone enhanced AM secretion from human aortic vascular smooth muscle cells (Uemura *et al.* 2002). AM was reported to promote proliferation and inhibit apoptosis of cultured human adrenocortical cells of zona glomerulosa (Rebuffat *et al.* 2002b). Further studies are required to clarify whether AM2/IMD and AM expressed at outer cortices of the attached adrenals might contribute to the regulation of these pathophysiological processes, such as the hyperplastic changes of zona glomerulosa cells, the so-called 'paradoxical hyperplasia', seen in aldosterone-producing adenomas (Sasano *et al.* 2004).

Taylor *et al.* (2005) previously reported that the concentration of IR-AM2/IMD in rat adrenal tissues was below the level of detection by RIA, in contrast to our present report on human adrenal tissues. This discrepancy may be partly attributed to much smaller weights of rat adrenal tissues. Our previous study reported that IR-AM was not detected in 90% cases of adrenocortical tumors by RIA, in contrast to relatively high expression of AM mRNA by northern blotting (Takahashi *et al.* 1998). In the present study, however, all adrenocortical tumors showed detectable levels of IR-AM2/IMD by RIA. On the other hand, IR-AM was not detected in the same cases of adrenocortical tumors by RIA (data not shown). This difference between IR-AM2/IMD and IR-AM contents in the tissues might be explained by the property of AM2/IMD that might be stored in the tumor cells, in contrast to AM (Takahashi *et al.* 1998).

The AM2/IMD₁₇₋₄₇ antiserum used in the present study was raised in a rabbit by injecting the C-terminal fragment of AM2/IMD, AM2/IMD₁₇₋₄₇. Roh *et al.* (2004) showed that AM2/IMD₈₋₁₆ was essential for the bioactivity of AM2/

IMD, whereas AM2/IMD_{17–47} fragment had no biological effects but was functional antagonist of AM2/IMD action. The AM2/IMD antibody used in the present study therefore recognizes the C-terminal portion of AM2/IMD, which may have receptor-binding ability but has no biological activity. HPLC-coupled RIA, however, confirmed that RIA using this antiserum could detect mainly the whole molecule of AM2/IMD_{1–47}. Chromatographic studies in tissue extracts of adrenocortical carcinomas, pheochromocytomas, and attached adrenal glands showed IR-AM2/IMD eluted in the position of authentic AM2/IMD, indicating the presence of authentic AM2/IMD in these tissues. On the other hand, the IR-AM2/IMD eluted between the 30th and 38th fractions appears to be multicomponent, and therefore, we could not deny a possibility that other molecular forms of AM2/IMD are present in these fractions. Furthermore, HPLC showed multiple peaks; a peak eluted at the 24th fraction and other minor peaks. These multiple peaks may represent other naturally occurring molecular forms of AM2/IMD, and artifacts generated during the extraction procedures and/or degradation processes during the tissue harvest, for example acid hydrolysis of Asp-Pro (40–41) bond by the extraction procedure using 1 mol/l acetic acid.

In conclusion, we demonstrated that AM2/IMD was expressed in adrenal tumors of both cortical and medullary origins and attached non-neoplastic adrenal tissues. AM2/IMD expression was stronger in tumor cells of adrenocortical tumors than in non-neoplastic portion of attached adrenal cortices. We also showed the expression of AM2/IMD, CRLR, and RAMP1, RAMP2, and RAMP3 mRNA in the adrenal tumors and attached adrenal tissues. These findings suggest that AM2/IMD, as well as AM, may have autocrine/paracrine regulatory roles in adrenal tumors and attached non-neoplastic adrenal tissues, such as tumor growth.

Declaration of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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