Up-regulation of adrenomedullin gene expression in the regenerating rat adrenal cortex

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Abstract. Adrenomedullin (AM) is an endogenous regulatory peptide that exerts growth-promoting action in several normal and neoplastic tissues, and we investigated whether its gene expression changes during rat adrenal regeneration after enucleation and contra-lateral adrenalectomy. Regenerating adrenals were collected at day 0 (just after enucleation; control rats), 1, 3, 7, 14 and 28 after surgery. The immunocyto-chemical assay of PCNA (proliferating cell nuclear antigen) index confirmed that the early stages of regeneration can be divided into an initial differentiation period (from day 0 to day 3) and a subsequent high proliferative period (days 5 and 7) followed by a decrease in the proliferation activity. Real time-polymerase chain reaction (PCR) demonstrated that AM mRNA expression underwent a marked rise at day 1 of regeneration, attained a maximum level at days 3 and 5, and then declined from day 7, returning to the control value at days 14 and 28. Western blotting showed that AM protein expression was moderately elevated at day 1, was maximal between days 3 and 7, and then decreased at days 14 and 28, although remaining significant over the control value. Taken together, our findings indicate that the increase in the AM gene transcription and translation may be considered one of the early events in the enucleation-induced activation of local adrenocortical stem cells, conceivably favoring both the differentiation and proliferation stages of regeneration. The mechanism underlying this adrenocortical stem cell response does not seem to involve ACTH, because real time-PCR demonstrated that it also occurred in animals whose contra-lateral adrenal glands were spared, and consequently the level of circulating ACTH was in the normal range. It remains to be investigated whether the enucleation-induced relative hypoxia, ensuing from disruption of the vascular bed, and the local release of inflammatory cytokines may be involved in the up-regulation of AM gene expression in regenerating adrenal glands.

Introduction

The mammalian adrenal cortex is composed of three concentric functionally distinct zones, the subcapsular mineralo-corticoid-secreting zona glomerulosa (ZG), the middle gluco-corticoid-secreting zona fasciculata (ZF), and the inner juxta-medullary zona reticularis (ZR), that produces gluco-corticoids and androgens (1,2). Most mitoses occur in the ZG, and most apoptotic cell death in the ZR, which has led to the 'cell migration' theory. According to this theory, new cells arise from stem cells located in the ZG, migrate into the ZF and then reach ZR, where exhausted aged cells die (1). An alternative view localizes adrenocortical stem cells in an undifferentiated zona intermedia between ZG and ZF (3). Under normal conditions, the renewal rate of adrenocortical cells is very low, so that it is conceivable that most stem cells are dormant. Many physiological agonists of adrenal cortex, such as ACTH and angiotensin-II, are able to stimulate adrenocortical growth by enhancing cell division in the ZG (1,3,4).

A useful experimental model of rapid adrenocortical growth, and of subsequent strong activation of local stem cells, is regeneration after enucleation. Following enucleation with the removal of the inner cortex and medulla, remnant parenchymal cells adherent to the gland connective capsule proliferate and restore structural adrenocortical zonation within 4-6 weeks. Optimal regeneration requires contra-lateral adrenalectomy because glucocorticoid secretion from the remaining intact adrenal prevents the increased ACTH secretion, which is essential in driving regeneration (1,4-8). Apart from ACTH, several other regulatory peptides have been reported to favor rat adrenocortical regeneration, and among them adrenomedullin (AM) appears to play a major role (9).

AM is a multi-functional regulatory peptide, originally isolated from human pheochromocytomas, that exerts potent vasorelaxant and hypotensive effects. AM and its receptors are widely expressed in tissues and organs, where AM controls specific and basic functions probably acting in an autocrine-paracrine manner (10-12). Evidence has been provided that AM plays a relevant role in the regulation of

mammalian adrenal cortex physiology. It inhibits Ca^{2+}-dependent agonist-stimulated aldosterone secretion (13) and enhances proliferation of ZG cells both *in vivo* (14) and *in vitro* (15-18); AM and its receptor genes being exclusively expressed in the ZG (16-18). The adrenal growth model during regeneration after enucleation resembles that occurring in embryogenesis (4), where the growth-promoting action of AM is thought to play a relevant role (19). Accordingly, Albertin *et al* (9) showed overexpression of AM mRNA in the regenerating adrenals, as compared to sham-enucleated glands.

In the latter study (9) neither the expression of AM as protein nor the early phases (first 3 days) of regeneration were examined. Moreover, sham-enucleated adrenal tissue is not a reliable control because it leads to the over-estimation of AM mRNA up-regulation in regenerating adrenals, as the AM gene is expressed only in the ZG. In fact, AM mRNA content is conceivably under-estimated in the entire adrenal tissue with respect to the regenerating tissue, which is exclusively derived from ZG. Therefore, we investigated the expression of AM as mRNA and protein at the early, middle and late stages of adrenal regeneration using regenerating tissue at time 0 (immediately after enucleation) as a control.

Materials and methods

Animals and experimental design. Adult male Sprague-Dawley rats, weighing 200-220 g, were obtained from Charles-River (Como, Italy). Animals were kept under a 12:12 h light-dark cycle at 23°C and maintained on a standard diet and tap water *ad libitum* until surgery. The experimental protocol was approved by the local Ethics Committee for Animal Studies, and experiments were carried out according to the Italian Law on the protection of laboratory animals. Under ether anesthesia, the left adrenal gland of 63 rats was enucleated, and the right adrenal gland was removed. Surgery was carried out by the dorsal approach without opening the peritoneum (9). Enucleation consisted of an incision to the adrenal capsule and extrusion and complete removal of the inner cortex and medulla; only ZG cells adherent to the capsule remained *in situ*. Rats were given an intramuscular injection of amplital (30 mg/100g body weight) immediately after surgery, housed in individual sterile cages, and maintained on a standard diet and 0.9% NaCl to drink. Animals were decapitated at day 0 (60 min after enucleation; control group), 1, 3, 5, 7, 14 and 28 after surgery (9 rats for each time point), and their regenerating adrenals were promptly removed. Three regenerating adrenals for each group of rats at each time point were fixed in Bouin's solution for 24 h, embedded in paraffin and sectioned at a 6-μm thickness for immunocytochemistry. Other specimens were frozen and stored at -80°C for reverse transcription (RT)-polymerase chain reaction (PCR) and Western blot analysis (3 regenerating adrenals for each time point for each technique).

Nine rats underwent left adrenal enucleation as described above, but the right gland was not excised. They were decapitated at day 0, 3 and 5 after surgery (three rats for each time point), and their regenerating adrenals were removed and used for RT-PCR.
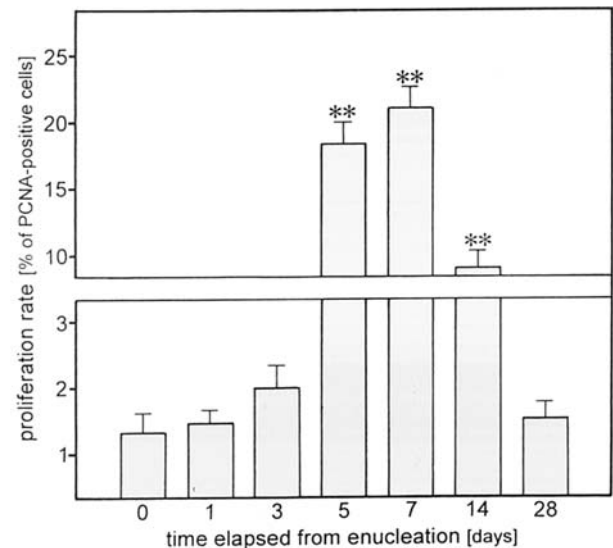

Figure 1. Proliferation rate of regenerating rat adrenal cortex in relation to the time elapsed from enucleation and contra-lateral adrenalectomy. Bars are the means ± SEM (n=3). $^{**}P<0.01$ compared to the controls (time 0).
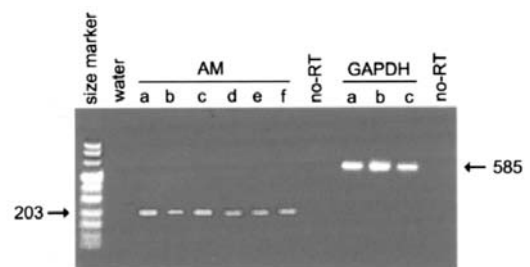

Figure 2. Ethidium bromide-stained 2% agarose gel showing cDNA amplified with rat AM and GAPDH-specific primers from RNA of regenerating adrenal cortex of control rats which underwent enucleation with (a,b,c) and without contra-lateral adrenalectomy (d,e,f). Primer sequences included AM sense-9-5', 5'-GCAGAACAACTCCAGCCTTT-3' and AM antisense-211-3', 5'-GCGAACCCAATAACATCAGG-3' (203 bp); GAPDH sense-181-5', 5'-CCCTTCATTGACCTCAACTA-3' and GAPDH antisense-765-3', 5'-GCCAGTGAGCTTCCCGTTCA-3' (585 bp). The PCR program was 35 cycles at 95°C for 60 sec, 57°C for 45 sec and 72°C for 90 sec. The first lane was loaded with 200 ng of Marker VIII (Boehringer, Mannheim, Germany). No amplification of PCR mixture without prior RT of RNA is shown as the negative control.

Proliferating cell nuclear antigen (PCNA) immunostaining. The PCNA immunostaining technique was used to estimate the number of regenerating adrenal cells entering the S phase of their cycle (20). PCNA-positive cells were identified using the LSAB2 kit purchased from Dako (Glostrup, Denmark). Peroxidase-labeled antibodies were detected with Sigma Fast 3',3'-diaminobenzidine 0.7 mg tablets (Sigma-Aldrich Corp., St. Louis, MO). Negative controls were carried out by omitting the primary antibody. Nuclei were counterstained with hematoxylin, and the PCNA index (% of PCNA-positive cells) was calculated at x400 by counting 2,000 cells in each regenerating adrenal tissue.

RT-PCR. Total RNA was extracted from the regenerating adrenal cortexes and reverse transcribed to cDNA (21-24).
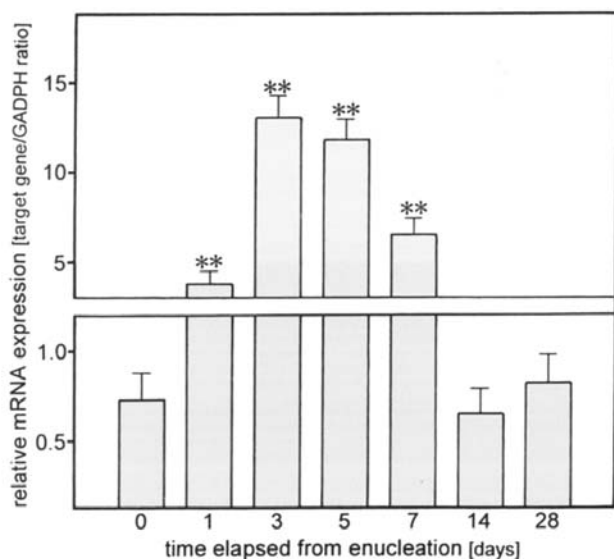
Figure 3. Semi-quantitative real time-PCR assay of AM mRNA expression in the regenerating rat adrenal cortex in relation to the time elapsed from enucleation and contra-lateral adrenalectomy. Bars are the means ± SEM (n=3). **P<0.01 compared to the controls (time 0).

Figure 5. Western blot analysis of AM in exemplary protein extracts from the regenerating rat adrenal cortex at days 0 (a), 1 (b) and 5 (c) from enucleation and contra-lateral adrenalectomy. AM protein is recognized as a single band of ~6 kDa, and the specificity of detection is demonstrated by the absence of immunodetection when the antibody was preabsorbed with 10^{-7} M AM. Molecular mass standards are shown in lane 1.
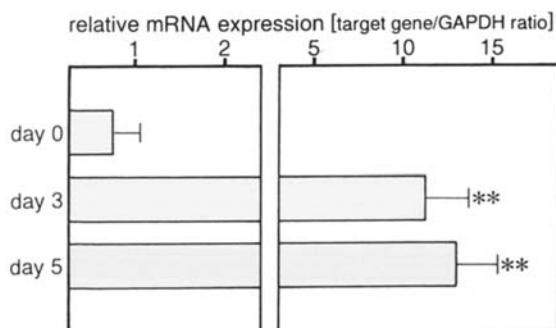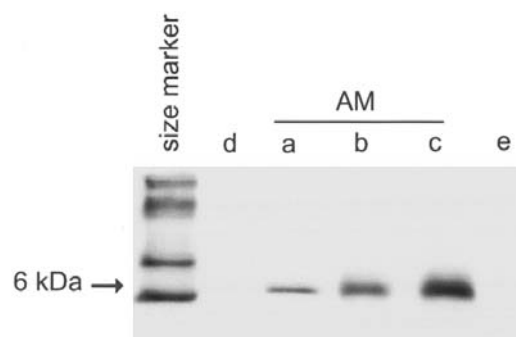

Figure 4. Semi-quantitative real time-PCR assay of AM mRNA expression in the regenerating rat adrenal cortex in relation to the time elapsed from enucleation without contra-lateral adrenalectomy. Bars are the means ± SEM (n=3). *P<0.05 and **P<0.01 compared to the controls (time 0).
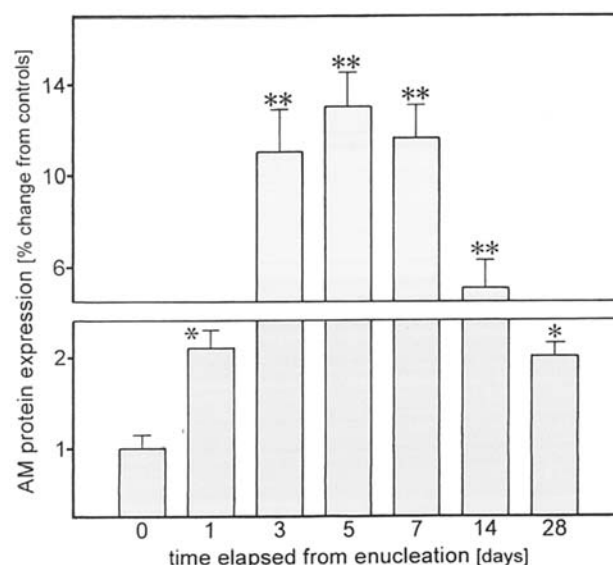

Figure 6. Quantitative Western blot analysis of AM protein expression in the regenerating rat adrenal cortex in relation to the time elapsed from enucleation and contra-lateral adrenalectomy. Bars are the means ± SEM (n=3). *P<0.05 and **P<0.01 compared to the controls (time 0).

Conventional PCR was carried out as previously described (9,25,26) using the primers indicated in the legend of Fig. 2. To rule out the possibility of amplifying genomic DNA, one PCR was performed without prior RT of the RNA. Detection of the PCR amplification product was carried out by size fractionation on 2% agarose gel electrophoresis. The specificity of the PCR was verified by sequencing analysis (27). Semi-quantitative real time-PCR was performed as previously detailed (28-31) in a Bio-Rad I-Cycler iQ Detection System (Bio-Rad Laboratories, Milan, Italy). Briefly, reactions were carried out in 25 μl of final volume solution containing 800-nM-specific primers (Fig. 2 legend), 12.5 μl iQ Sybr-Green Supermix (Bio-Rad Laboratories) and 2 μl of RT-reaction solution. The PCR program included a denaturation step at 95˚C for 3 min, 35 cycles of two amplification steps (95˚C for 15 sec and annealing at 60˚C for 30 sec), and melting curve at 60-90˚C with a heating rate of 0.5˚C/10 sec. The specificity of amplification was tested at

the end of each run by real time melting analysis using the I-Cycler iQ software 3.0. All samples were amplified in duplicate, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene; its mRNA expression equated to 1.

Western blotting. Proteins were extracted from regenerating adrenals in sample buffer containing protease inhibitors, and 100 μg of proteins was subjected to SDS-PAGE under reducing conditions as detailed previously (32,33). Samples were resolved in a standard gel apparatus and then transferred to nitrocellulose membranes (Sigma-Aldrich Corp.). Membranes were blocked for 60 min at 37˚C in Blotto A with 0.05% Tween-20, and then incubated for 60 min at 37˚C with the primary goat polyclonal anti-AM antibody (Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:1,000 in Blotto A. After washing in Tris-buffered saline, membranes

were incubated for 60 min at room temperature with Cruz Marker-compatible peroxidase-conjugated anti-goat IgG rabbit secondary antibodies (1:2,000 dilution). Negative controls were obtained by using a primary antiserum preabsorbed with 10^{-7} M rat AM (Phoenix Pharmaceuticals, Belmont, CA). Immunoreactive bands were detected by ECL Advanced Western Blotting Detection kit (Amersham, Aylesbury, UK). Blots were scanned and quantified with a Bio-Rad Chemiluminescence Molecular Imaging system (Bio-Rad Laboratoires) and expressed as percent change from the control (taken equal to 1).

Statistical analysis. The results were expressed as the means ± SEM (n=3), and the statistical significance of the differences among experimental groups was estimated using ANOVA followed by the Duncan's multiple range test.

Results

The PCNA index of regenerating adrenocortical cells did not undergo significant changes until 3 days after enucleation. It reached a maximum (15-fold increase) at 5 and 7 days post surgery and then declined, returning to the control level at day 28 (Fig. 1).

Conventional RT-PCR detected the expression of AM mRNA in the control regenerating adrenal cortex (time 0) (Fig. 2). Real time-PCR showed that AM gene expression was markedly increased at day 1 after surgery (~3-fold), attained a maximum level at days 3 and 5 (~8-fold), and then decreased at day 7 (4.5-fold increase). At days 14 and 28, it was not significantly different from the controls (Fig. 3). In rats with intact right adrenals, AM mRNA expression in the regenerating glands displayed 6.5- and 7.5-fold increases at days 3 and 5 after surgery, respectively (Fig. 4).

Western blotting demonstrated a single 6-kDa AM protein band in the regenerating adrenals (Fig. 5). AM protein expression underwent a moderate rise at day 1 after enucleation (~2-fold), was maximal at days 3, 5 and 7 (10- to 12-fold increases), and then decreased at days 14 and 28, although remaining significant over the control value (Fig. 6).

Discussion

The growth of the adrenal cortex during the early stages of regeneration can be divided into two phases: an initial differentiation period (from day 0 to day 3) and a subsequent high proliferation period (from day 4 to day 7). From day 8, post-enucleation proliferative activity starts to decrease (7). Our present PCNA-assay findings are in complete agreement with this contention.

Coupled real time-PCR and Western blot analysis clearly showed that the early stages of adrenal regeneration were associated with up-regulation of the AM gene transcription and translation. At day 1, AM expression, as both mRNA and protein, was markedly enhanced, and the increase reached a maximum at days 3 and 5 for mRNA and days 3 and 7 for protein. Hence, up-regulation of AM gene expression may be considered one of the early events in the enucleation-induced activation of local adrenocortical stem cells, conceivably favoring both the differentiation and proliferation stages of

regeneration. This contention is in keeping with the well-known growth promoting action of AM on ZG cells (14-18), as well as with its reported stimulating effect on clonal growth of cord blood-derived mesenchymal stem cells (34).

At present, only hypotheses can be advanced on the mechanism(s) involved in the AM gene up-regulation during adrenal regeneration. Although ACTH, via cyclic-AMP, has been reported to enhance AM release from skin keratinocytes (35), the increase in its blood levels during the early stages of regeneration seems to indicate a role of minor relevance. In fact, a marked increase in AM mRNA occurred at days 3 and 5 of regeneration even in rats whose contra-lateral adrenals were preserved, and the levels of circulating ACTH were in the normal range [RIA assay - ACTH plasma concentration (pg/ml ± SEM) 3 days after surgery: sham enucleation, 70±12; enucleation and contra-lateral adrenalectomy, 143±22 (P<0.05); and enucleation without contra-lateral adrenal-ectomy, 75±15]. During the early stages of regeneration, local stem cells adherent to the capsule are conceivably exposed to relative hypoxia due to the mechanical disruption of the adrenal vascular bed, and hypoxia, via HIF, is known to up-regulate AM gene expression (36-39). Moreover, enucleation surely induces an initial inflammatory response in the regenerating adrenal tissue, and inflammatory cytokines have been shown to enhance AM gene expression through NO-dependent and NO-independent pathways (39,40).

Be that as it may, the increased local production of AM may probably play a relevant role during the early stages of adrenal regeneration. Many lines of evidence demonstrate that AM exerts a potent *in vivo* and *in vitro* pro-angiogenic action (41-43) that may partly ensue from its direct effect on vascular endothelial cells, and partly from its stimulating effect on local VEGF production (9,44,45). Hence, AM may not only increase regenerating adrenocortical cell growth, but also stimulates neovascularization to restore a normal adrenal vascular bed.

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