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Review

Proadrenomedullin-derived peptides as autocrine-paracrine regulators of cell growth

A.S. Belloni, G. Albertin, M.L. Forneris* and G.G. Nussdorfer
Department of Human Anatomy and Physiology, Section of Anatomy, University of Padua, Italy
*Present address: Department of Biochemistry, National University of San Luis, San Luis, Argentina

Summary. Proadrenomedullin (pADM)-derived peptides, adrenomedullin (ADM) and pADM N-terminal 20 peptide (PAMP), are hypotensive peptides, which are expressed, along with their receptors, in several tissues and organs, the function of which they regulate by acting in an autocrine-paracrine manner. Apart from their involvement in the regulation of blood pressure and fluid and electrolyte homeostasis, pADM-derived peptides appear to play a role in the modulation of cell and tissue growth. Evidence has been provided that ADM: 1) favors the remodeling of cardiovascular system under pathological conditions, by exerting an antiapoptotic effect on endothelial cells and an antiproliferogenic and antimigratory action on vascular smooth-muscle cells during neointimal hyperplasia, and by decreasing proliferation and protein synthesis of cardiac myocytes and fibroblasts. These last two effects are mediated by calcitonin gene-related peptide type 1 (CGRP1) receptors coupled to the adenylate cyclase (AC)/protein kinase (PK) A-dependent cascade; 2) inhibits proliferation and enhances apoptosis of kidney mesangial cells, through the modulation of mitogen-activated PK (MAPK) cascades; 3) stimulates proliferation of adrenal zona glomerulosa cells, acting via CGRP1 receptor coupled to the tyrosine kinase-dependent MAPK cascade, thereby possibly being involved in the maintenance and stimulation of adrenal growth; 4) enhances proliferation of skin and mucosa epithelial cells and fibroblasts, by activating CGRP1 receptor coupled to the AC/PKA signaling pathway; and 5) enhances proliferation of several tumor-cell lines through the activation of the AC/PKA cascade, which suggests a potential role for ADM as promoter of neoplastic growth. The growth effects of PAMP have been far less investigated: findings indicate that this peptide, like ADM, enhances adrenal zona glomerulosa-cell proliferation, and, in contrast with ADM, depresses DNA synthesis in some cancer-cell lines. Both pADM-derived peptides are thought to be involved in embryogenesis, such a contention being based on the demonstration of high pADM-gene expression during the crucial phases of organ growth and differentiation.

Key words: Adrenomedullin, Proadrenomedullin N-terminal 20 peptide, Cell proliferation, Apoptosis, Neoplastic growth, Organogenesis

Offprint requests to: Prof. G.G. Nussdorfer, Department of Human Anatomy and Physiology, Section of Anatomy, Via Gabella 65, I-35121 Padova, Italy. Fax: +39-049-827-2319. e-mail: gastone.nusdorfer@unipd.it

Introduction

Adrenomedullin (ADM) is a potent hypotensive peptide, which was originally isolated from extracts of human pheochromocytomas (Kitamura et al., 1993). Subsequently, it was found that ADM is produced by the proteolytic cleavage of a prohormone, the prepro(pp)ADM, that also contains in its NH_2 terminus a unique 20-amino acid residue sequence, exerting a transient hypotensive effect and named pro(p)ADM N-terminal 20 peptide (PAMP) (Kitamura et al., 1994).

In the 7-8 years following their discovery, pADM-derived peptides were shown to be almost ubiquitously expressed in body organs and tissues, and to exert multiple important effects, among which are regulation of blood pressure and fluid and electrolyte homeostasis, and more recently of normal and neoplastic cell growth. Several review articles have been published on ADM and PAMP (Richards et al., 1996; Schell et al., 1996; Nussdorfer et al., 1997; Champion et al., 1999; Samson, 1999; Hinson et al., 2000; Jougasaki and Burnett, 2000; Nussdorfer, 2001). However, none of them have focused specifically on the role of pADM-derived peptides such as cell growth regulators.

After having briefly described the main biological characteristics of ADM and PAMP, we will summarize and discuss findings suggesting that these peptides play important roles in the modulation of cell growth and apoptosis in many normal and pathological tissues.

Biology of pADM-derived peptides

ADM

ADM is a 52- (human) or 50-amino acid peptide (rat) containing a disulfide bridge-formed six-membered ring between adjacent cysteine residues in the 16 and 21 positions, that appears to be essential for its biological activity (Fig. 1). Although originally discovered in human pheochromocytomas and then in adrenal medulla, ADM was subsequently shown to be expressed as mRNA and protein in the cardiovascular system, kidneys, lungs, spleen, digestive apparatus, uterus, ovary, prostate, endocrine glands and brain. The highest expression seems to occur in medullary chromaffin cells, endothelium and vascular smooth muscle cells (VSMC) (Samson, 1999; Hinson et al., 2000).

ADM belongs to a large regulatory peptide family,

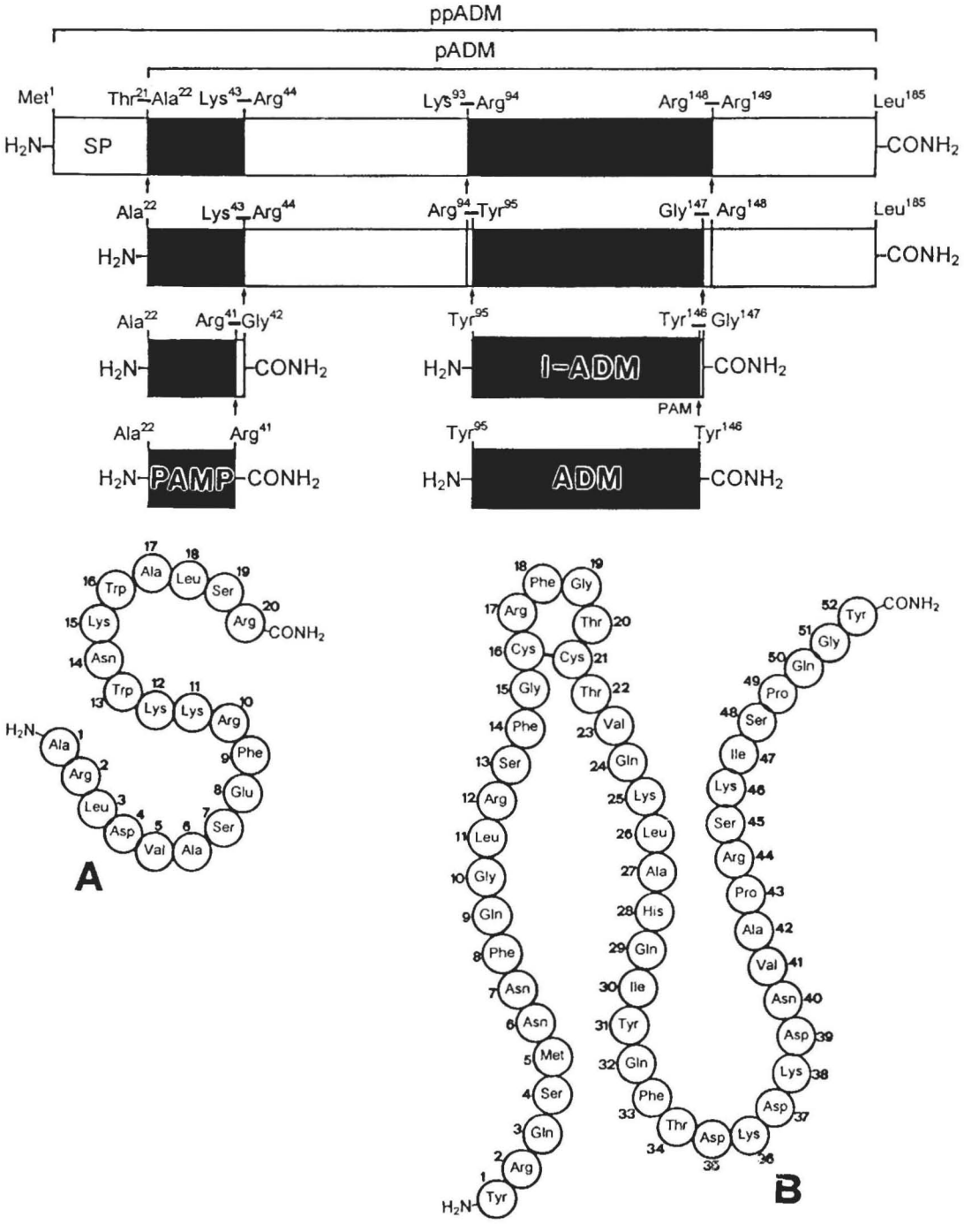

Fig. 1. Schemes illustrating the post-translational processing of ppADM and the complete amino-acid sequence of human PAMP **(A)** and ADM **(B)**. The arrows indicate the sites of the subsequent proteolytic cleavages of the molecules.
I-ADM: immature ADM; PAM: peptidyl-glycine α-amidating monooxigenase; SP: signal peptide.

which includes calcitonin gene-related peptide (CGRP), and the bulk of evidence indicates that it acts through a calcitonin-receptor-like receptor (CRLR), which is expressed in several tissues where ADM is synthesized. CRLR can function as either a CGRP or a selective ADM receptor depending on which member of a family of single transmembrane-domain proteins, called receptor-activity-modifying proteins (RAMPs), is expressed (McLatchie et al., 1998; Foord and Marshall, 1999; Chakravarty et al., 2000): RAMP1 generates CGRP receptors from CRLR, while RAMP2 and RAMP3 produce ADM receptors. Another two subtypes of putative ADM receptors have been identified and called L1 and RDC1, which have high and low affinity for ADM (Hinson et al., 2000). However, these findings have not been reproduced in other laboratories (McLatchie et al., 1998). Evidence indicates that the C-terminal sequence and the six-membered ring structure are both needed for ADM to bind and activate its receptors. Accordingly, ADM(22-52), which lacks N-terminal sequence and the ring structure, acts as a selective antagonist of ADM (Belloni et al., 1998). There is proof that the CGRP type 1 (CGRP-1)-receptor antagonist CGRP(8-37) also acts as an ADM-receptor antagonist in several cell systems (Samson, 1999; Jougasaki and Burnett, 2000; Nussdorfer, 2001).

The main biological actions of ADM concern blood vessels and fluid and electrolyte homeostasis. ADM evokes a sustained hypotension due to the decrease of vascular resistances. Vascular relaxation has been initially exclusively ascribed to the activation of G protein-coupled receptors, whose signaling mechanism involves stimulation of the adenylate cyclase (AC)/protein kinase (PK) A cascade. Subsequent studies demonstrated that ADM-receptor signaling may also involve the activation of phospholipase (PL) C/PKC-dependent pathway and voltage-gated Ca^{2+} channels, leading to an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Further investigations have also provided evidence that ADM may enhance nitric oxide (NO) synthase (NOS), thereby evoking vasodilation through the NO-cyclic GMP (cGMP) pathway. This occurs via a two-fold mechanism: rise in $[Ca^{2+}]_i$ and activation of a G protein-coupled NOS (Samson, 1999; Hinson et al., 2000; Jougasaki and Burnett, 2000).

ADM directly suppresses angiotensin-II (Ang-II)- and K^+-stimulated aldosterone secretion from adrenal zona glomerulosa (ZG) cells, acting via CGRP1 ADM(22-52)-sensitive receptors, the activation of which is likely to impair Ca^{2+} influx (Nussdorfer, 2001). Moreover, ADM enhances local production of NO, which in turn may concur to the inhibitory effect of ADM (Rebuffat et al., 2001b), inasmuch as NO is known to exert a cGMP-independent aldosterone antisecretagogue action (Kreklau et al., 1999; Hanke et al., 2000). In contrast, ADM stimulates medullary chromaffin cells to release catecholamines (Mazzocchi et al., 1999), which may raise aldosterone secretion acting in a paracrine manner (Nussdorfer, 1996). Also the catecholamine secretagogue effect of ADM occurs via ADM(22-52)-sensitive CGRP1 receptors, which are coupled to the AC-dependent cascade. Findings have been provided that *in vivo* these opposite effects of ADM on ZG may interact with each other in the rat when normal aldosterone secretion has to be restored (Albertin et al., 2000).

Other important peripheral effects of ADM, apart from those concerning cell-growth modulation, have been discovered and recently reviewed (Samson, 1999; Hinson et al., 2000; Nussdorfer, 2001). They can be summarized as follows: 1) inhibition of pituitary ACTH release, the effect becoming relevant when an exceedingly high ACTH secretion has to be counteracted; 2) blunting of hyperosmolality-evoked arginine-vasopressin (AVP) release in the rat; 3) modulation of utero-placental-fetal function, but this effect could be related to ADM action on vascular tone; 4) depression of uterine contractive activity; 5) regulation of insulin secretion from pancreatic islets; 6) modulation of kidney tubular function: ADM enhances Na^+ excretion, without apparently altering GFR, RBF or any other hemodynamic parameter. This effect could be, at least in part, related to the aldosterone antisecretagogue effect of ADM; 7) stimulation of renin release by juxtaglomerular cells; 8) depression of gastric motility and secretion; 9) inhibition of histamine- and acetylcholine-induced bronchoconstriction, which suggests that ADM may play a protective role during acute asthma attacks; and 10) antimicrobial activity: hence, ADM could be involved in the first line of defense of strategic surfaces where it is expressed (skin, and oral, gut and lung mucosae). Many other peripheral effects of ADM may be accounted for by its vascular action.

PAMP

The tissutal expression of PAMP reflects that of ADM, which is obvious due to the origin of the two peptides from a common prohormone, the ppADM (Fig. 1). PAMP binding sites are widely distributed in the body tissues and organs, and are well distinct from those of ADM. They have not yet been fully characterized (Samson, 1999; Nussdorfer, 2001); however, there is evidence that PAMP(12-20) behaves as a selective antagonist of PAMP receptors, thereby suggesting that the N-terminal sequence is essential for PAMP to activate, but not to bind, its receptors (Belloni et al., 1999).

Similarly to ADM, PAMP mainly controls blood vessels and fluid and electrolyte homeostasis. It is generally agreed that the mechanism underlying the transient hypotensive effect of PAMP primarily involves a presynaptic inhibitory action on sympathetic terminals innervating blood vessels. This effect could be mediated by both G protein-signaling mechanisms and impairment of voltage-gated Ca^{2+} channels (Champion et al., 1999; Jougasaki and Burnett, 2000). PAMP, like ADM,

suppresses aldosterone response of ZG cells to $Ca2^{+}$-dependent agonists, but, in contrast to ADM, it inhibits catecholamine release by adrenal medulla. Both effects of PAMP are mediated by PAMP(12-20)-sensitive receptors, whose signaling mechanism is likely to involve the blockade of voltage-gated Ca^{2+} channels of the L-type (Belloni et al., 1999). Like ADM, PAMP inhibits pituitary ACTH release (Nussdorfer, 2001). The biological effects of PAMP have been much less investigated than those of ADM.

ADM and PAMP as autocrine-paracrine regulatory hormones

Both ADM and PAMP circulate in sizeable amounts in the mammalian blood, their basal concentrations being in the pM range. Also in pathological conditions where pulmonary or systemic hypertension and sodium retention have to be counteracted or in the case of heart failure, myocardial infarction and septic shock the plasma concentration of pADM-derived peptides does not exceed 10^{-11} M: hence, it remains well below the threshold for the large part of their at present known biological actions (about 10^{-10} to 10^{-7} M) (Nussdorfer et al., 1997; Samson, 1999; Hinson et al., 2000). However, pADM gene and pADM-derived peptide binding sites are expressed in all the tissues where they exert their modulating effects, and it has been calculated that their local concentrations may easily reach 10^{-8}-10^{-7} M (Nussdorfer, 1996; Nussdorfer et al., 1997). On these grounds, it is currently accepted that pADM-derived peptides act as local autocrine or paracrine factors within the tissues and organs where they are synthesized.

Effects of pADM-derived peptides on cellular growth

Evidence is accumulating that pADM-derived peptides play important roles in the regulation of cell proliferation, differentiation and apoptosis, via the modulation of the mitogen-activated protein kinase (MAPK) cascades.

MAPKs are a family of serine/threonine kinases, the best characterized members of which are extracellular (signal) regulated kinases (ERKs), *c-jun* N-terminal kinases (JNKs) and p38s (Campbell et al., 1995; Biesen et al., 1996a,b; Wan and Huang, 1998; English et al., 1999; Fanger, 1999; Garrington and Johnston, 1999; Han and Conn, 1999; Ono and Hann, 2000). MAPK cascade involves a series of cytoplasmic phosphorylations (Fig. 2), where MAPKKKKs activate MAPKKKs, that in turn phosphorylate MAPKKs. The best characterized MAPKKKK is the p21-activated kinase (PAK), and MAPKKKs include Rafs, MAPK/ERKKs1-4 (MEKKs1-4) and apoptosis-stimulating kinase-1 (ASK-1). Rafs activate MEKs 1 and 2, while MEKKs 1-4 and ASK-1 phosphorylate MEKs 3 and 7. There is also the possibility that PAK directly activates MEKs 3 and 6. MEKs 1 and 2 phosphorylate ERKs 1 and 2, MEKs 4 and 7 activate JNKs, MEK 5 phosphorylates ERK 5, and MEKs 3 and 6 and ASK-1 specifically activate p38s. MAPKs then translocate to the nucleus, where they phosphorylate various transcriptional factors, that induce the expression of different proto-oncogenes. ERKs 1 and 2 induce the expression, among others, of the *c-fos* gene, the products of which associate with other related proteins to form the transcriptional factor AP-1, which in turn mediates the transcriptional response of various "late-response" genes leading to cell proliferation and differentiation. JNKs induce the expression of *c-jun* gene, whose products play a role in cell growth, differentiation, survival, apoptosis and cytokine production. *c-Jun* gene may also be activated by ERK5, via the induction of the expression of myocyte enhancing factor 2C (MEF2C). p38s induce MEF2C, thereby activating *c-jun* gene, and phosphorylate a number of PKs, among which MAPK-activated protein kinase-2, diversifying and amplifying their signal. The function of ERK5 is not yet known. Receptor TK is thought to play a pivotal role in the activation of MAPK cascades, because by binding to its agonists it activates Ras, a peptide belonging to a family of low molecular weight GTP-binding proteins, which is able to activate PAK and Rafs. In addition to TK, also G protein-coupled receptors, through the production of PKC and PKA, can activate MAPK cascades via a Ras-independent, not yet known, mechanism. A series of protein phosphatases (PPs), including MAPK phosphatase-1 (MKP-1) and PP2A, may effectively counteract the activity of the MAPK cascades.

In the following subsections, the effects of pADM-derived peptides on the growth of normal, pathological and tumoral tissues will be surveyed, and the possible mechanisms involved will be briefly discussed.

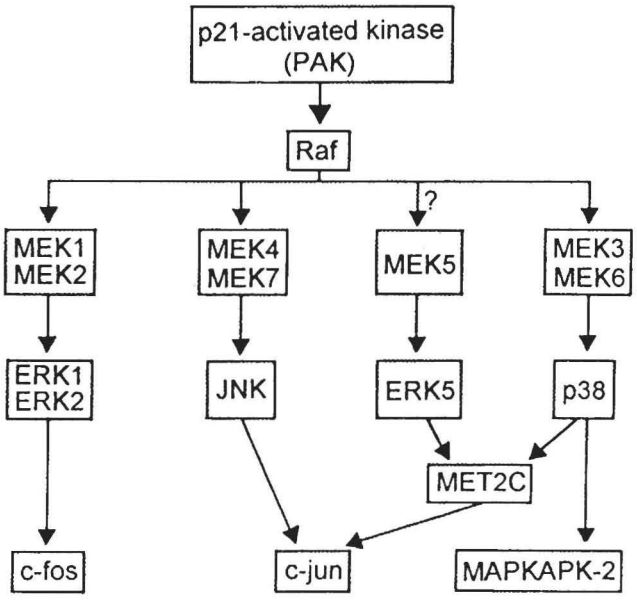

Fig. 2. Simplified scheme illustrating the main pathways of the MAPK cascade. MAPKAPK-2, MAPK activated protein kinase-2.

Blood vessels

Compelling evidence indicates that pADM gene is highly expressed in the blood vessels (Hinson et al., 2000), and that ADM production and secretion are enhanced by inflammatory cytokines in VSMCs (Sugo et al., 1994, 1995). There is also indication that ADM exerts an angiogenic action at least in the chorioallantoid membrane (Zhao et al., 1998). However, there is not a general consensus on the effect of ADM on vascular growth.

Endothelial cells (ECs)

Cultured ECs, obtained from rat arteries (Kato et al., 1997) and human umbilical vein (Sata et al., 2000), were found to display morphological and biochemical signs of apoptosis when rendered quiescent by serum deprivation. ADM concentration-dependently (from 10^{-10} to 10^{-8} M) decreased the percentage of apoptotic cells, without stimulating cell proliferation. This survival effect was abrogated by an anti-ADM antiserum.

ADM enhanced cyclic-AMP (cAMP) production by cultured ECs, but its antiapoptotic effect was not blocked by a cAMP antagonist, thereby suggesting that its mechanism does not involve the AC-dependent cascade. Sata et al. (2000) demonstrated that the survival action of ADM was suppressed by the NOS inhibitor L-NAME, but not by a GC inhibitor, and was mimiked by the NO donor nitroprusside, but not by the cGMP permeable analog 8-bromo-cGMP. On these grounds, they concluded that the antiapoptotic effect of ADM occurs via NOS activation through a cGMP-independent pathway.

VSMCs

Contrasting findings were obtained, showing either stimulating or inhibitory effect of ADM on VSMC growth *in vitro* and *in vivo*, and on neointimal hyperplasia during vascular remodeling.

ADM was reported to induce *c-fos* gene expression in cultured rat VSMCs within 30 min, and to enhance AP-1 DNA binding activity within 120 min (Sato and Autelitano, 1995). Accordingly, Iwasaki et al. (1998, 2001) showed that ADM (from 10^{-9} to 10^{-6} M) raised DNA synthesis and proliferation of cultured rat VSMCs, acting via CGRP(8-37)-sensitive CGRP1 receptors. They also observed that ADM (10^{-7} M) stimulated both TK and ERK1 and 2 activity, and that TK and ERK1 and 2 inhibitors (but not a cAMP antagonist, and PKA or PKC inhibitors) blocked the proliferogenic action of ADM, which allowed them to conclude that ADM stimulates mitogenic activity of cultured rat VSMCs via the activation of the TK-dependent MAPK cascade. In contrast, Kano et al. (1996) reported that ADM (from 10^{-9} to 10^{-7} M) inhibited calf serum-stimulated proliferation of cultured rat VSMCs, an effect abrogated by CGRP(8-37). The soluble cAMP analog dibutyryl-cAMP (db-cAMP) mimiked ADM antiproliferogenic action and ADM raised cAMP production by cultured cells, thereby making it likely that ADM acts via CGRP1 receptor coupled to the AC-dependent cascade. An antiproliferogenic effect of ADM on VSMCs of kidney glomeruli and pulmonary artery has also been observed (Chini et al., 1997; Yoshihara et al., 1998). More recently, two subpopulations of VSMCs were isolated from human pulmonary artery: one expressed and released ADM (ADM^+) and the other did not (ADM^-) (Upton et al., 2001). ADM^+ cells were present in both proximal and distal arteries, while ADM^- cells were restricted to proximal ones. ADM concentration-dependently (from 10^{-9} to 10^{-7} M) raised cAMP production and inhibited PDGF-stimulated (but not basal) DNA synthesis of ADM^-, but not ADM^+ cells. The effect of ADM was mimiked by CGRP, thereby suggesting the involvement of CGRP1 receptors. The presence of ADM-responsive and ADM-unresponsive populations of VSMCs suggests that great caution must be used in interpreting findings concerning the modulatory effects of ADM on vessel growth.

The migration of VSMCs into the intima and the proliferation of migrated cells have been proposed to be a key process of intimal thickening in coronary atherosclerotic lesions and restenosis after angioplasty, a process where both Ang-II and endothelin-1 (ET-1) seem to be involved (Clowes and Schwartz, 1985; Ross, 1986, 1993; McBride et al., 1988; Libby et al., 1992; Clozel et al., 1993; Douglas and Ohlstein, 1993; Glagov, 1994; Dubey et al., 1995; Christen et al., 2001). Investigations suggest the involvement of ADM in neointimal hyperplasia, but again contrasting findings have been obtained.

Shimizu et al. (1999) studied neointimal hyperplasia induced in the rat common carotic artery by baloon injury. The injury increased ADM expression in the tunica media, and the number of S-phase cells after 48 h. A further rise in the number of S-phase cells was observed in the hyperplastic intima at 7 days. These proliferogenic effects were suppressed by the *in vivo* subcutaneous infusion with CGRP(8-37), suggesting that they occur via the ADM-induced activation of CGRP1 receptors. According to Autelitano et al. (1999), the alteration of VSMC glycolytic metabolism mimics ischemic damage. These investigators observed that perturbation of normal glucose metabolism by 2-deoxyglucose enhanced pADM mRNA expression and proliferation of cultured rat aortic VSMCs. However, exogenous ADM effectively counteracted the 2-deoxyglucose-induced proliferation.

In keeping with these last findings, ADM has been found to be a potent antimigration factor for human coronary VSMCs (Horio et al., 1995; Kohno et al., 1997, 1998). VSMCs were cultured and their migration assessed by the Boyden's chamber method. ADM (from 10^{-8} to 10^{-6} M) inhibited Ang-II-induced migration. This effect of ADM was paralleled by an increase in the intracellular cAMP level, and was mimiked by db-cAMP

and the AC activator forskolin, thereby suggesting that it is mediated by the AC-dependent cascade. ADM also blunted the migratory effect of the lysophosphatidylcholine (a prominent component of oxidatively modified LDLs involved in atherosclerotic damage), which conversely was increased by both PDGF and ET-1.

Heart

The bulk of investigations indicate that heart is one of the tissues displaying the highest expression of pADM gene, being basal secretion rate of ADM about 3-fold higher in cardiac fibroblasts than cardiomyocytes (Tomoda et al., 2001). The involvement of ADM in the heart growth and remodeling is well ascertained, but, like in the case of blood vessels, conflicting results have been obtained.

ADM (10^{-7} M) increased *c-fos* gene expression in cultured rat cardiomyocytes and cardiac fibroblasts within 30 min, and enhanced AP-1 DNA binding activity at 120 min in the former but not in the latter (Sato and Autelitano, 1995). IL-1ß and TNF-α are known to favor the progression of cardiac hypertrophy during heart failure (Thaik et al., 1995; Testa et al., 1996; Yokoyama et al., 1997), and ADM could participate in these processess, because both cytokines were found to enhance its expression (as mRNA and protein) in cardiomyocytes and fibroblasts within 24-48 h (Horio et al., 1998). However, this last observation does not clarify whether ADM plays a promoting or inhibitory role on cardiac growth.

Horio et al. (1999) showed that ADM did not affect basal protein synthesis in cultured adult rat cardiomyocytes, but concentration-dependently (from 10^{-7} to 10^{-6} M) enhanced ET-1-stimulated one. Conversely, ADM inhibited DNA and collagen synthesis in cultured cardiac fibroblasts under both basal and Ang-II-stimulated conditions. Quite different results were obtained by Tsuruda et al. (1998, 1999): ADM concentration-dependently suppressed Ang-II-induced proliferation and protein synthesis in both cultured heart myocytes and fibroblasts obtained from neonatal rats. The inhibitory effect of ADM occurred via CGRP1 receptors, because it was blocked by CGRP(8-37). Of interest, CGRP(8-37) and an anti-ADM monoclonal antibody were found to increase *per se* Ang-II (10^{-6} M)-enhanced de novo protein synthesis in cultured cardiomyocytes (Tsuruda et al., 1998), suggesting that endogenous ADM modulates cardiac growth. All the above mentioned investigators agree that cardiac effects of ADM involve the activation of the AC-dependent cascade, inasmuch as ADM raised cAMP production and db-cAMP mimiked ADM effects.

In keeping with an inhibitory action of ADM on heart growth are the findings obtained *in vivo* in Dahl salt-sensitive and DOCA salt-hypertensive rats (Dobrzynski et al., 2000; Zhang et al., 2000). It was found that adenovirus-mediated pADM-gene delivery via a single intravenous injection decreased systolic blood pressure, left ventricular mass, the diameter of cardiomyocytes and interstitial fibrosis in the heart, and raised cardiac cAMP levels. However, it must be stressed that beneficial cardiac effects of ADM could be dependent on its antihypertensive action.

Kidney mesangial cells (MCs)

Several investigations consistently showed that cultured rat MCs express and secrete ADM, and display a sizeable cAMP response to ADM (Chini et al., 1995, 1997; Segawa et al., 1996; Michibata et al., 1998; Parameswaran et al., 1999, 2000a). All these investigators demonstrated that ADM (from 10^{-10} to 10^{-7} M) inhibited either basal or PDGF-enhanced proliferation of cultured MCs.

The bulk of evidence strongly suggests that ADM modulates MAPK cascade in MCs. Chini et al. (1997) observed that ADM (10^{-9} M) hampered PDGF-induced MAPK activation. Further insight was provided by Parameswaran et al. (1999, 2000a,b), who showed that ADM not only decreased [^{3}H]thymidine uptake, but also increased apoptosis in cultured MCs. ADM (10^{-7} M) inhibited ERK1 and 2, and stimulated JNK and p38 activity. The IP3-kinase inhibitor Wortmannin annulled the p38-stimulating effect of ADM, and blunted apoptosis promoting effect of ADM, without affecting the antiproliferogenic one. Togawa et al. (1997) demonstrated that ADM (10^{-7} M) induced the expression in cultured MCs of MKP-1, which has been previously found to depress proliferation of these cells (Sun et al., 1993; Sugimoto et al., 1996). Accordingly, Parameswaran et al. (2000a,b) observed that the PP2A inhibitor okadaic acid abolished ADM-induced lowering in DNA synthesis, apoptosis and inhibition of ERK1 and 2 activity. Taken together, these findings make it likely that the mechanism underlying the antiproliferogenic action of ADM on MCs involves the stimulation of protein phosphatases blunting MAPK activity. Parameswaran et al. (2000b) showed that PP2A activation was also involved in the resensitization of cAMP response to ADM.

To summarize, the above surveyed findings allow us to draw the following tentative conclusions: 1) the ADM antiproliferogenic action on MCs is exclusively mediated by the ERK1 and 2 inhibition ensuing from the activation of PP2A and perhaps MPK-1; 2) the ADM apoptotic action involves both ERK1 and 2 inhibition and p38 stimulation; and 3) stimulation of JNK plays a minor role, and the involvement of the AC-dependent cascade in the ADM effects on MCs is still a not yet addressed issue.

Adrenal cortex

Although pADM gene does not seem to be expressed in adrenocortical cells, ADM is produced in adrenal medullary cells (Nussdorfer, 2001). Due to the

very strict interlacement between adrenocortical and medullary tissues occurring in adrenal glands (Nussdorfer, 1996), ADM locally produced by chromaffin cells may easily affect parenchymal cortical tissue.

The effects of ADM on the proliferative activity of adrenocortical cells have been investigated in the rat both *in vivo* and *in vitro*. Using the perfusion technique of the *in situ* left adrenal gland, Andreis et al. (2000) showed that ADM concentration-dependently (from 10^{-10} to 10^{-8} M) increased mitotic index and DNA synthesis in the ZG (but not zona fasciculata/reticularis), the effect being abrogated by both CGRP(8-37) and ADM(22-52). The ZG proliferogenic effect of ADM (10^{-8} M) was abolished by either the TK selective inhibitor tyrphostin-23 or the MEK1 and MEK1 and 2 inhibitors PD-98059 and U0126, while it was unaffected by selective inhibitors of the AC/PKA-, PLC/PKC- and PLA2/cyclooxygenase or lipoxygenase-cascades. ADM (10-8 M) stimulated TK and ERK1 and 2 activities in dispersed ZG cells, and the effect was annulled by either CGRP(8-37) and ADM(22-52) or preincubation for 30 min with tyrphostin-23. Similar results were obtained using cultured ZG cells (Semplicini et al., 2001). Preliminary results indicate that also PAMP enhanced proliferation of cultured rat ZG cells through the activation of the TK-ERK1- and 2-dependent cascade (Rebuffat et al., 2001a).

According to the cell migration theory (Nussdorfer, 1986), ZG in mammals is the cambium layer involved in adrenocortical cell renewal, which suggests that ADM may enhance and maintain the growth of the entire gland. In light of the well demonstrated inhibitory action of ADM and PAMP on Ca^{2+}-dependent agonist-stimulated aldosterone secretion, the above surveyed findings stress the complex role played by pADM-derived peptides in the regulation of adrenocortical physiology. They raise the possibility that these peptides may act as aldosterone secretion negative modulators in adult growth-quiescent adrenals and as growth promoter in immature glands. In this connection, it appears worth mentioning that pADM-induced TK-MAPK stimulation is independent of the activation of both AC/PKA and PLC/PKC cascades that are both involved in the aldosterone secretagogue action of many agonists, including ACTH, Ang-II and ET-1 (Nussdorfer et al., 1999).

Skin and mucosae

Epithelial cells

Normal human skin cell lines were found to express pADM gene and to possess ADM binding sites, which were not displaced by either ADM(22-52) or CGRP(8-37). ADM stimulated DNA synthesis in these cell lines (Martinez et al., 1997a). Similar results were reported in a human oral keratinocyte cell line by Kapas et al. (1997). They also observed that the proliferogenic effect of ADM was concentration-dependent (from 10^{-11} to 10^{-8} M), and attenuated by an AC inhibitor, but not by PKC or TK inhibitors, thereby suggesting the involvement of the AC/PKA cascade.

Fibroblasts

ADM concentration-dependently (from 10^{-10} to 10^{-10} M) enhanced DNA synthesis and proliferation in cultured Swiss 3T3 cells (Whiters et al., 1996; Isumi et al., 1998), a cell model currently used to identify extra- and intracellular mitogenic signals (Rozengurt, 1986). Both groups of investigators observed that ADM also raised cAMP production and that ADM proliferogenic effect was blocked by a PKA inhibitor. Rat-2 fibroblast cell line was found to secrete ADM and, like Swiss 3T3 cells, to possess ADM binding sites and to display a CGRP(8-37)-suppressable cAMP response to ADM (from 10^{-10} to 10^{-6} M) (Coppock et al., 1999). However, ADM blunted either basal or PDGF-stimulated MAPK activity, a finding which does not accord well with a possible mitogenic action of this peptide.

Isumi et al. (1998, 1999) demonstrated that Swiss 3T3 produced ADM and that ADM production is variously affected by inflammatory cytokines: TNF-α and IL-1ß increased, and TGF-ß1 and INF-γ decreased it. In turn, ADM suppressed IL-1ß-induced TNF-α production and gene expression in this cell line, the effect being blocked by either CGRP(8-37) or a PKA inhibitor. These investigators concluded that ADM secreted by fibroblasts may play a role as local regulator of mesenchymal-cell functions in inflammatory or wounded regions.

Tumors

Evidence indicates that ADM is expressed, as mRNA and protein, in several tumors, including small and non-small cell lung carcinomas (Martinez et al., 1995), astrocytomas and glioblastomas (Takahashi et al., 1997), pheochromocytomas (Kitamura et al., 1993; Hinson et al., 2000), aldosteronomas, non-functioning adrenal adenomas, cortisol-secreting adenomas and adrenal carcinomas (Takahashi et al., 1998), ectopic ACTH-secreting tumors (Murakami et al., 1998) and human prostate adenocarcinomas (Rocchi et al., 2001). These last investigators also detected the expression of peptidyl-glycine α-amidating monooxigenase (PAM), the enzyme converting inactive glycine-extended ADM to mature ADM form (Kitamura et al., 1998) (Fig. 1). Interestingly, they observed that the expression of both pADM and PAM was about 3-fold higher in prostate carcinomas than in benign prostate hyperplasia, which could suggest the involvement of ADM in the stimulation of malignant mitotic activity.

ADM expression and ADM receptors were detected in a great number of tumor cell lines derived from lung large-cell, small-cell, squamous and bronchioloalveolar carcinomas; breast carcinomas; colon adenocarcinomas;

glioblastomas; neuroblastomas; ovarian adenocarcinomas; prostate carcinomas; adrenocortical carcinomas (H-295 and SW-13); chondrosarcomas; chronic monocytic leukemia; and skin melanomas (Miller et al., 1996; Martinez et al., 1997a,b; Kubo et al., 1998; Takahashi et al., 1997, 1998, 2000a,b; Rocchi et al., 2001). The expression and secretion of ADM were modulated by inflammatory cytokines, but the effect depended on the type of cell line: IL-1ß and INF-γ increased and TNF-α decreased them in the glioblastoma cell line T986 (Takahashi et al., 1997); and IL-1ß and TNF-α stimulated them in the adrenocortical carcinoma cell line SW-13, INF-γ being ineffective (Takahashi et al., 2000a). ADM was found to raise DNA synthesis in skin melanoma, human teratocarcinoma PA1 and prostate carcinoma DU-945 (Martinez et al., 1997a; Moody et al., 2000; Rocchi et al., 2001), and to enhance *c-fos* mRNA in both C6 glioma and teratocarcinoma cell lines (Moody et al., 1997, 2000). Evidence has also been provided that ADM increased cAMP production in several carcinoma-cell lines (Miller et al., 1996; Martinez et al., 1997a; Moody et al., 1997; Takahashi et al., 1997), a finding suggesting that the proliferogenic effect of this peptide involves the activation of the AC-dependent cascade. Contrasting results were obtained by Ando et al. (1997), who reported that ADM ($5x10^{-9}$ or $5x10^{-8}$ M) inhibited proliferation and DNA synthesis in human neuroblastoma TGW cells, an effect which became manifest within 48 h and was suppressed by both CGRP(8-37) and ADM(22-52).

The investigations on the effect of PAMP on tumor cells are very scarce. Earlier studies showed that PAMP (10^{-9} or 10^{-6} M), like ADM, inhibited proliferation and DNA synthesis in human neuroblastoma TGW cells (Ando et al., 1997). The effect was blocked by pertuxis toxin (PTX) and ω-conotoxin, thereby making it likely that PAMP acted through the inhibition of the N-type Ca^{2+} channels via a PTX-sensitive G protein-coupled receptor. Subsequent studies demonstrated that PAMP (10^{-6} M) was able to inhibit DNA synthesis in teratocarcinoma cells, as well as to hinder ADM (10^{-9} M)-induced cAMP production and c-fos gene expression (Moody et al., 2000). Since PAMP did not displace ADM binding, it may be conceived that it hampers the mechanism involved in the ADM proliferogenic action on this cell line.

Possible role of pADM-derived peptides in embryogenesis

Compelling, although indirect, evidence suggests an important role for pADM-derived peptides in the regulation of cell growth and differentiation during embryogenesis, where they could interact with growth factors, among which TGF-ß.

The expression of ADM, as mRNA and protein, has been extensively investigated in the mouse and rat embryos by Montuenga et al. (1997). In the mouse, ADM was first expressed in the heart at embryo day (ED) 8, peaked at ED10 and then plateaued. ADM expression peaked at ED11 in arteries; at ED12 in the skin; at ED13 in the skeletal muscles, chondrocytes, kidney metanephric duct and adrenal gland; and at ED14 in hypertrophic cartilage and osteoblasts. The highest expression was detected in the heart and hypertrophic cartilage. PAMP expression was lower, but followed that of ADM. Similar results were obtained in the rat embryo. TGF-ß1 expression overlapped that of ADM (Montuenga et al., 1998). Immunocytochemistry suggested that ADM may also be involved in the development of rat pancreas (Martinez et al., 1998): ADM-ir first appeared in the cap-like pancreatic anlage of the duodenal wall at ED11.5, coinciding with the appearance of glucagon. At ED14 ADM-ir was co-localized in a low number of a-cells and in a high number of ß-cells of the primitive islets, where somatostatin- and pancreatic polypeptide (PP)-cells were not yet present. At postnatal day 1, ADM-ir was present in 100% of α-cells, somatostatin-cells and PP-cells, and in a low number of ß-cells. In young adult rats, ADM was present only in PP-cells. In light of the above reviewed findings, these investigators concluded that ADM (and perhaps PAMP) is intensely expressed in tissues and organs where strong mesenchymal-epithelial interactions take place, like heart and kidney, thereby supporting the view that ADM controls cell proliferation and migration. Moreover, they stressed that ADM expression correlates with the onset and progression of differentiation processes, e.g. formation of cartilage templates and pancreatic islets.

ADM was also highly expressed in murine and rat trophoblastic giant cells, which are derived from the conceptus and are directly in contact with maternal tissues at the implantation site (Montuenga et al., 1997; Yotsumoto et al., 1998). According to Montuenga et al. (1997), this finding might indicate the involvement of ADM in the proliferation and invasive activity of embryo trophoblast. However, Yotsumoto et al. (1998) observed that ADM expression fell dramatically in trophoblastic giant cells at ED10.5, coinciding with the completion of mature chorioallantoic placenta, and that these cells did not possess ADM receptors, suggesting that the peptide acts on the maternal tissues rather than on the embryonic ones. Due to the vasodilatory and angiogenic effect of ADM (Zhao et al., 1998), these investigators proposed the involvement of ADM in the establishment of an efficient embryo-maternal circulation.

Concluding remarks

The preceding sections of this survey have shown that in the few years that have lapsed since the discovery of pADM-derived peptides, a great number of studies have accumulated, suggesting that these peptides, and especially ADM, play potentially important roles in the autocrine-paracrine regulation of cell growth under physiopathological and pathological conditions.

To summarize, it seems quite well demonstrated that ADM: 1) favors the remodeling of cardiovascular system; 2) inhibits the growth of glomerular MCs; 3) enhances adrenocortical growth; 4) stimulates proliferation of epithelial cells and fibroblasts; and 5) acts as growth promoter in several neoplastic tissues and cell lines. Both ADM and PAMP are thought to play regulatory roles during embryogenesis, a contention based on the finding that pADM-gene expression peaks during the crucial phases of organ growth and differentiation.

However, the relevance of the role of pADM system (pADM-derived peptides and their receptors) in the modulation of the above mentioned processes remains to be demonstrated. The largest part of investigations have been carried out on cultured cells, and its is well known that the growth behavior *in vitro* does not reflect the *in vivo* one. *In vivo* studies are few, and always examined the effects of the administration of exogenous pADM-derived peptides. Hence, the role of endogenous pADM system was not assessed.

In vitro and *in vivo* studies aimed at evaluating the effects of the prolonged exposure to the presently available selective antagonists of ADM and PAMP and of the delivery of antisense oligonucleotides blocking transcription e.g. of PAMP and CRLC genes could allow us to ascertain whether the disruption of endogenous pADM system affects cell growth during adult and fetal life. They could also ascertain whether the hyperactivity of the endogenous pADM system may be, at least in part, involved in triggering malignant neoplastic growth.

These kind of investigations, along with the continuous development of new, potent, and selective antagonists of pADM-derived peptide receptors, will not only increase our knowledge on the basic mechanisms controlling normal and pathological cell proliferation, but also, and more importantly, will open novel perspectives in the therapy of many diseases linked to dysregulation of tissue growth.

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