[Frontiers in Bioscience E3, 1073-1080, January 1, 2010]

Adrenomedullin and its expression in cancers and bone. A literature Review

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1. ABSTRACT

Adrenomedullin (ADM) was first isolated from pheochromocytoma tissue as a novel vasodilative peptide in 1993. ADM binds to two receptors on plasma membrane which are comprised of Calcitonin Receptor-Like Receptor (CRLR), a member of serpentine receptor superfamily, and Receptor Activity Modifying Protein (RAMP) type 2 or 3. ADM is known to have hypotensive activity. Recently, ADM has been shown to be an almost ubiquitous peptide, synthesized in many mammalian tissues. ADM has potent in vivo angiogenic activity and tumor growth promoting effect in animal models. Many human tumors express ADM. However, only little information exists regarding the expression or the role of ADM in bone and its effect in bone metabolism. It is still not clear whether ADM is involved in pathogenesis and development of osteosarcoma, the most common form of bone tumor. The purpose of this review is to examine the most salient features of adrenomedullin biology, its expression in tumors and its potential implication in the treatment of bone tumors.

2. INTRODUCTION

ADM is a 52-amino-acid peptide originally isolated from human pheochromocytoma cells by Kitamura *et al.* in 1993 (1). The mRNA encoding ADM, however, has been shown to be expressed not only in the adrenal gland but also in various organs and tissues, including vascular endothelial and smooth muscle cells, cardiomyocytes, fibroblasts, neurons and glial cells (2). ADM was initially characterized by its ability to stimulate cAMP production in human platelets, and it exerts a potent and long-lasting vasodilatory effect in rats. Recently, expression and secretion of ADM has been demonstrated in many tumors and biological fluids, and it has been implicated in the modulation of numerous physiological processes (3). Over the past few years, a variety of effects have been attributed to ADM, including the protection of organs from hypertension (4) or infection (5). *In vivo* evidence has suggested that nitric oxide (NO) mediates the effects of ADM in the brain (6), and ADM signaling plays an important role in the regulation of angiogenesis under hypoxic conditions, in the inhibition of

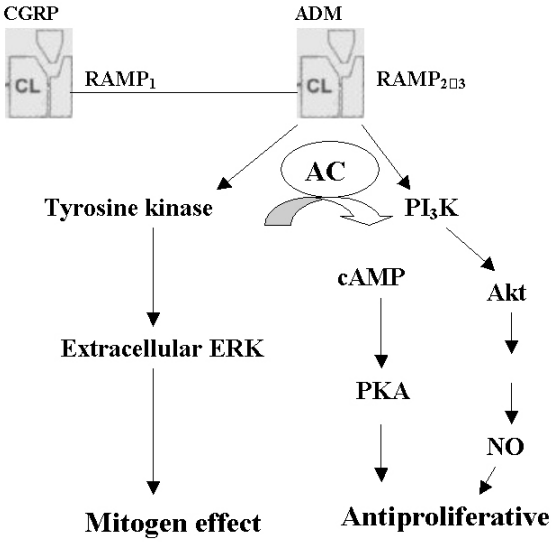

Figure 1. ADM: adrenomedullin; CL: calcitonin receptor; RAMP: receptor activity modifying protein; CGRP: calcitonin gene-related peptide; AC: Adenylate cyclase.

endothelial apoptosis (7) and In the promotion of angiogenesis (8,9). In addition, ADM has protective effects against vascular injury, including oxidative stress (10).

The structure of ADM is homologous to calcitonin gene-related peptide (CGRP), calcitonin and amylin, all of which belong to the same peptide family. The ADM protein contains a 6-amino-acid ring formed by a disulfide bond between residues 16 and 21 (11). ADM is encoded by a gene on chromosome 11 in humans and consists of four exons and three introns. The mature ADM peptide is derived from preproADM, which contains 185 amino acids, and its DNA sequence has been identified in both human and rat tissues (12). Preproadrenomedullin cleavage at the signal peptide between Thr21 and Ala22 yields a truncated propeptide of 164 amino acids that contains ADM. The first pair of basic amino acids, Lys43 and Arg44, is a representative site for proteolytic cleavage and is preceded by Arg41 and Gly42 residue for possible C-terminal amidation, giving a product termed proadrenomedullin N-terminal 20 peptide (PAMP) (13,14). As a novel hypotensive peptide, PAMP was isolated from human plasma, urine, kidney and brain. The transcription of its mRNA is reportedly affected by tumor necrosis factor-α (TNF-α), interleukin-1, interferon-γ, endothelin-1, angiotensin II, vasoactive intestinal peptide and dexamethasone (15,16). Nevertheless, a new study found that PAMP mRNA is increased in ventricular muscle in patients with pulmonary hypertension (17).

2.1. ADM receptor

ADM and CGRP belong to a family of structurally related peptides that also includes calcitonin (CT) and amylin. ADM and CGRP have common 6-amino-acid ring structures formed by disulfide bridges near the N-terminus and amidated C-termini, both of which are required for their biological activity. The ring structures are not essential for receptor binding. Both peptides are potent vasodilators. ADM acts as a circulating or paracrine hormone and exerts its effects via the G-protein coupled calcitonin-like receptor (CL), a member of the serpentine receptor superfamily, complexed with a receptor accessory modifying protein known as $RAMP_2$ (ADM_1 receptor) or $RAMP_3$ (ADM_2 receptor) (18). RAMP1, 2 and 3 are single transmembrane domain proteins with intracellular C-termini of up to 10 amino acids and extracellular N-termini of approximately 120 amino acids (19, 20). Non-covalent CL receptor/RAMP1 heterodimers at the cell surface constitute CGRP receptors. Co-expression of CL receptors with RAMP2 or 3 results in ADM_1 or ADM_2 receptor subtypes, respectively. CGRP and ADM_1 or ADM_2 receptor subtypes are distinguished by their selectivity for ADM and CGRP agonists and for hADM(22–52), rADM(20–50) and CGRP(8–37) antagonists (Figure 1). Although the sequence identity between both RAMPs is only 30%, ADM_1 and ADM_2 receptors are pharmacologically indistinguishable and are usually co-expressed in the same tissues (21). CL receptor/RAMP1 CGRP receptors and ADM_1 and ADM_2 receptors are predominantly linked to cAMP production. Cells treated with ADM show elevated cAMP in a time-dependent (5–45 min) and dose-dependent (10^{-6}–10^{-14} M) manner (22, 23). Pre-treatment with the ADM receptor antagonist $AM_{22–52}$ partially suppresses the ADM-induced increase in cAMP levels. We have studied the effects of ADM on immortalized human microvascular endothelial cells since endothelial cells are a major source and target of ADM *in vivo*. ADM stimulates nitric oxide synthase (NOS) in ventricular cardiomyocytes (24) and endothelial cells (25) and may also stimulate mitogen-activated protein kinases (MAPKs) in vascular smooth muscle cells (VSMCs) (26) and inhibit MAPK activity in mesangial cells (27). Finally, ADM activates ATP-sensitive K^+-channels in vascular smooth muscle cells independent of the signaling pathways mentioned above (28).

3. ADM AND CELLS

ADM was originally purified from a human adrenal tumor (29). Cuttitta *et al.* extended their initial observation of ADM and L_1 receptors in pulmonary tumors (30, 31) to study the general expression of ADM in human tumor cell lines (32). This opened the possibility of ADM being an autocrine or paracrine growth factor in tumors and even normal cells. ADM has different effects on proliferation depending on cell type and stimulates the proliferation of fibroblasts, keratinocytes, endothelial cells, osteoblasts and many tumor-derived cells. It has been found to be expressed in almost all tumor cells studied to date, suggesting that it may be an important tumor growth factor. In addition, owing to its angiogenic properties, ADM may promote tumor angiogenesis (33). Experimental studies indicate that neutralization of ADM by specific antibodies inhibits tumor growth, whereas ADM overexpression augments tumor growth (34). ADM has been shown to be involved in carcinogenesis and tumor progression by promoting tumor proliferation and angiogenesis and by inhibiting apoptosis. It is thought that inflammatory cytokines and hypoxia-induced expression of ADM by tumor cells drive these processes. ADM increases DNA

synthesis in a dose-dependent manner via a mechanism involving specific ADM receptors and increased cAMP/PKA (35). Moody *et al.* (36) reported that ADM exerts mitogenic effects on these cells, correlating with increases in cAMP and c-fos expression. In contrast, in some cell types such as vascular smooth muscle cells, mesangial cells, glial cells and glial cell tumors, an inhibitory effect of ADM on growth and intracellular cAMP has been observed in most studies. As with rat VSMCs, ADM has been shown to inhibit serum-stimulated ^{3}H-thymidine uptake, which can be blocked by $CGRP_{8–37}$ (37). These effects indicate inhibition of growth by ADM, which might be expected with increased intracellular cAMP, but some of these results are contradictory to those above. Nevertheless, ADM has also been proposed to be an important factor in embryogenesis and differentiation (38–40).

4. ADM AND TUMORS

ADM has been implicated in the modulation of various physiological functions, ranging from vasorelaxation to cell growth regulation (41, 42). In 1998 ADM was shown to be potently angiogenic using a chick chorioallantoic membrane assay (43), and it has subsequently been found to be pro-tumorigenic by a number of groups using both xenograft studies (44,45) and blocking antibodies (46). We believe that ADM and cancer are related; therefore, here we review the expression of ADM in different human tumors.

4.1. Prostate cancer

Prostate cancer (CaP) is currently the second leading cause of cancer death in men (47). Because androgens stimulate tumor growth, hormone deprivation currently represents the primary treatment for advanced CaP. A recent study (48) showed that while human prostate cancer cell lines PC-3 and DU145 both produce and secrete ADM, it acts as a growth factor for DU145 cells, suggesting the existence of an autocrine loop that could potentially drive neoplastic growth. To investigate this growth factor effect, Rocchi *et al.* used MTT assays to examine the effects of ADM on the growth of prostate cell lines. DU145, PC-3, and LNCaP cells cultured *in vitro* were exposed to 2×10^{-7} M ADM, and the effect on proliferation was followed using an MTT assay. ADM stimulated the proliferation of DU145 by 20% and 25% after 4 and 8 days of treatment, respectively, while both PC-3 and LNCaP cells showed no proliferative response. ADM synthesis and secretion were also observed in the prostate cell line DU145 (49). A preliminary screen for amidated peptides present in cell lines has demonstrated that the ADM mRNA is by far the predominant message encoding two α-amidated peptides, namely, pro-ADM NH_2-terminal 20 peptide (PAMP) and ADM. The data show a marked increase in ADM mRNA levels during xenograft growth for both the PC-3 and DU145 cell lines. The observed elevated response in ADM transcripts expression, a common feature of solid tumors, could be a result of reduced oxygen tension (1% O_2) or exposure to hypoxia mimetics such as desferrioxamine mesylate (DFX) or $CoCl_2$ via a hypoxia-inducible factor-1-dependent mechanism (50).

4.2. Breast cancer

A study by MK Oehler *et al.* (51) showed that most breast cancer patient samples have moderate to strong staining intensity for ADM, whereas only some tumors are ADM-negative. Furthermore, patients with axillary lymph node metastasis were found to have significantly higher ADM peptide expression than patients with no lymphatic metastasis. The probability of metastasis is correlated with the vascular density of primary tumors, and ADM-overexpressing tumors are characterized by increased vascularization.

Enrique Zudaire *et al.* (52) found that ADM is an important regulator of mast cell (MC) function and plays a critical role as an autocrine/paracrine tumor cell survival factor in breast cancer promotion. ADM induces histamine or β-hexosaminidase release from rat and human MCs via a receptor-independent pathway, and human MCs respond to hypoxic insult with elevated ADM mRNA and protein expression. Furthermore, increased angiogenesis might enhance the opportunity for tumor cells to gain access to the lymphatic system and to metastasize. In addition, the angiogenic potential of ADM-overexpressing cells might increase the probability for tumor cells trapped in the lymphatic capillaries to induce neovascularization, giving rise to macroscopic tumor growth.

When analyzing plasma ADM levels and clinicopathological features of breast cancer patients, a significant positive correlation between tumor size and plasma ADM levels has been observed. These results suggest that the source of circulating plasma ADM in these patients is indeed breast malignancies. Active tumor growth, hypoxia and the associated ADM overexpression might be responsible for increased production and release of the peptide. In addition to such properties, ADM might have an adaptive function in tumors by increasing intratumoral blood flow due to its well known vasodilatory properties.

Nevertheless, ADM expression in breast cancer does not correlate with menopausal status, tumor extent, distant metastasis status, histological tumor type, grading or expression of the estrogen receptor (ER) or progesterone receptor (PR). A significant association between histological grading and ADM has been found, however, in the analysis of ovarian malignancies (53). Until now, much research has shown a wide range of ADM concentrations in healthy women (54). Whether this observation is tumor type-specific or whether the study could not detect an association because of the limited numbers of cases is unclear.

4.3. Other cancers

In summary, ADM is a hypoxia-induced peptide under control of the hypoxia-inducible transcription factor-1 (HIF-1), as are several other angiogenic factors such as vascular endothelial growth factor (VEGF). Thus, circulating levels of ADM have been shown to be elevated in various disease states, including solid tumors such as gastrointestinal malignancies. Since the ADM peptide is rapidly secreted once it is produced in cells, it is conceivable that ADM peptide in the blood stream of tumor

patients reflects ADM secretion from the malignancies.

Still, no data are available concerning ADM peptide expression in bone malignancies or circulating ADM peptide in bone tumor patients.

5. ADM AND BONE

Expression of ADM and its receptor have not been detected in the cardiovascular system and fluid homeostasis but have also been observed in osteoblasts during later stages of rodent embryogenesis and in maturing chrondrocytes of fetal mice (55,56). Further research (57) has demonstrated that ADM acts on osteoblasts to increase cell growth comparable to known osteoblast growth factors such as transforming growth factor-beta (TGF-β). Secondly, ADM increases protein synthesis *in vitro* and the area of mineralized and unmineralized bone *in vivo*. ADM expression is correlated with many different tumor types and is often high in tumor cells and patient plasma samples. Unfortunately, its effects on bone tumor cells or in arthritis are not yet known. This has important clinical implications; thus, if we can clarify the expression and effect mechanism of ADM and its receptor in bone, we will be able to use ADM as a novel method to treat some diseases that do not currently have effective therapies.

5.1. ADM and arthritis

ADM acts as an endogenous immunomodulatory factor with predominant anti-inflammatory effects. ADM and its receptors are detected in several immune cells, including macrophages/monocytes and T cells, and their expression levels increase under inflammatory conditions (58–60).

Elevated ADM levels have been found in plasma, joint fluid and synovial tissue in arthritis (61–62). In addition, a recent study evaluates the therapeutic effects of ADM using an experimental model of rheumatoid arthritis with antigen-induced arthritis (63). Using ovalbumin injected into the joint space of pre-immunized rabbits as the experimental group, ADM was injected into the contralateral knee joint space, and the degree of joint swelling and histological change was compared. ADM reduced edematous changes and infiltration of inflammatory cells in the synovial tissues and significantly reduced TNF-α mRNA expression in a dose-dependent manner.

ADM ameliorates the inflammatory response and may be useful as a treatment for rheumatoid arthritis. A possible mechanism for this effect involves reducing the expression of Th1-driven autoimmune and inflammatory responses. Moreover, ADM decreases the generation and/or activation of efficient $CD4^+$–$CD25^+$ regulatory T cells in arthritis with the capacity to suppress autoreactive responses and restore immune tolerance (64). As a result, ADM reduces the frequency of arthritis, ameliorates its symptoms and prevents joint damage.

5.2. ADM and bone metabolism

Bone growth and maintenance are highly regulated processes. Throughout life, bone constantly undergoes remodeling, maintaining a balance between bone formation by osteoblasts and bone resorption by osteoclasts. This balance depends on the coordinated activities of many systemic hormones and locally acting factors in the bone microenvironment. Recently, studies have found that ADM is expressed at high levels in osteoblasts and chondrocytes in mouse and rat embryos (65) and is also expressed in primary neonatal rat osteoblasts (66) and human chondrocytes cultured from articular cartilage explants.

ADM belongs to the calcitonin family, which is a group of peptide hormones consisting of calcitonin, CGRP, amylin and ADM-2 (intermedin). These all share structural similarities and produce a rapid reduction in serum calcium levels. ADM stimulates the proliferation of primary rat osteoblasts at near physiological concentrations of 10^{-11} M and 10^{-12} M (67). It also significantly increases the osteoid area and mineralized bone area.

Osteoporosis is a common systemic skeletal disorder characterized by reduced bone mass, increased bone turnover and micro-architectural deterioration of bone tissue, leading to bone fragility and increased risk of fracture (68). It is closely related to bone resorption, which is dependent on numerous processes but involves a key role for osteoclasts. Osteoclasts are sometimes activated via mechanisms dependent upon prior osteoblastic stimulation. Osteoblasts possess surface receptors for parathyroid hormone (PTH), parathyroid hormone-related protein (PTHrP), ADM and CGRP. *In vitro* experiments have shown that ADM is a potent mitogen for fetal rat osteoblasts (69). This effect is dependent on the presence of the IGF-1 receptor and is blocked by AMY antagonists.

5.3. ADM and osteosarcoma

Osteosarcoma is a kind of malignant tumor originating from mesenchymal tissue with an incidence that is second highest among primary bone tumors. It is characterized by the generation of spindle stromal cells of bone-like tissue (70, 71). Since the standard treatment before 1970 was osteotomy, the majority of patients died of lung cancer metastasis within 2 years, and the annual survival rate was only 10–20% (72). In recent years, however, with the extensive application of neoadjuvant chemotherapy and limb salvage surgery, the annual survival rate and quality of life of patients with osteosarcoma have been greatly improved (73, 74). Still, the general prognosis of osteosarcoma has not significantly improved, and a change in surgical or chemical treatment is unlikely to alter disease-related poor prognosis in the near future (75). A marker for osteosarcoma must be discovered to determine its biological characteristics and prognosis, and an effective molecular target must be identified for clinical treatment.

The main reason for increased expression of ADM in osteosarcoma is thought to be hypoxia (76–77). The initiation and development of a tumor rely on angiogenesis, the formation of capillary vessels that provide the tumor with a structure into which nutrients enter and wastes exit; in other words, the generation and secretion of angiectasia and angiogenesis-related substances may be required for tumor survival (78). A solid tumor with a high

degree of malignancy exceeds the load of its blood vessels due to its active growth; therefore, the central region of the tumor is under hypoxic conditions. Hypoxia may increase ADM by activating hypoxia-inducible factor (HIF-1), given that ADM has the ability to expand blood vessels and alleviate tumor hypoxia (79). Ouafik (80) showed that externally supportive access to ADM can stimulate the *in vitro* growth of malignant glioma cells, and the use of ADM specific polyclonal antibodies can block the binding of ADM to its cell receptors, causing a 33% reduction in the *in vitro* growth of malignant glioma U87 cells (P <0.001). Anti-ADM antibodies can be injected into the tumor, with mice allogeneic vaccination resulting in a 70% weight reduction in subcutaneous U87 xenografts (P <0.001) after 21 days of treatment.

6. CONCLUSION

ADM has been shown to mediate multiple cell responses, including the regulation of cardiovascular tone, bronchodilation, natriuretic action, antimicrobial activity, inhibition of hormone release, growth regulation, apoptosis survival and the induction of angiogenesis. Additionally, it has been shown that many human tumor cell lines express high levels of ADM mRNA and that ADM is released into the culture medium. Inhibiting tumor angiogenesis is an effective method to treat malignant tumors. As the result of an in-depth study on ADM and the occurrence and development of tumor vessels, ADM is expected to become a new target for anti-angiogenesis therapy. All of these findings support the idea that ADM can be used as an effective autocrine/paracrine growth factor and that the *in vivo* growth of tumors can be inhibited by blocking the activity of ADM generated by tumor cells. ADM will be a useful predictive and prognostic marker in human cancers.

7. ACKNOWLEDGEMENT

Special thanks go to Wei Jiang for support in proofreading the paper.

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Key Words: Adrenomedullin, Angiogenic, Calcitonin, Receptor-like receptor, Cancer, Osteosarcoma, Review

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