Tumor-Associated Macrophages Promote Angiogenesis and Melanoma Growth via Adrenomedullin in a Paracrine and Autocrine Manner

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Abstract

Purpose: Elevated numbers of tumor-associated macrophages (TAM) in the tumor microenvironment are often correlated with poor prognosis in melanoma. However, the mechanisms by which TAMs modulate melanoma growth are still poorly understood. This study was aimed at examining the function and mechanism of TAM-derived adrenomedullin (ADM) in angiogenesis and melanoma growth.

Experimental Design: We established in vitro and in vivo models to investigate the relationship between TAMs and ADM in melanoma, the role and mechanism of ADM in TAM-induced angiogenesis and melanoma growth. The clinical significance of ADM and its receptors was evaluated using melanoma tissue microarrays.

Results: ADM was expressed by infiltrating TAMs in human melanoma, and its secretion from macrophages was upregulated upon coculture with melanoma cells, or with melanoma cells conditioned media. Meanwhile, TAMs enhanced endothelial cell migration and tubule formation and also increased B16/F10 tumor growth. Neutralizing ADM antibody and ADM receptor antagonist, AMA, attenuated TAM-induced angiogenesis in vitro and melanoma growth in vivo, respectively. Furthermore, ADM promoted angiogenesis and melanoma growth via both the paracrine effect, mediated by the endothelial nitric oxide synthase signaling pathway, and the autocrine effect, which stimulated the polarization of macrophages toward an alternatively activated (M2) phenotype. Finally, immunofluorescence analysis on human melanomas showed that the expression of ADM in TAMs and its receptors was greatly increased compared with adjacent normal skins.

Conclusion: Our study reveals a novel mechanism that TAMs enhance angiogenesis and melanoma growth via ADM and provides potential targets for melanoma therapies.

Introduc
tion

Although melanoma accounts for only approximately 4% of all skin cancers, it is by large the most aggressive and deadliest of skin cancer type, contributing to 80% of skin cancer deaths (1). However, the mechanism underlying the aggressiveness of melanoma is incompletely understood. Accumulating evidences suggest that melanoma growth is influenced by both the host immune response and inflammatory cells within the tumor microenvironment. Among inflammatory cells, macrophages are believed to play the most important role in malignancies and are, therefore, specifically referred to as tumor-associated macrophages (TAM). Although TAMs may display antitumorigenic properties (2, 3), they are primarily regarded as protumorigenic, as indicated by the positive effect of TAMs on tumor angiogenesis and growth (4, 5). The different functions of TAMs may depend on individual activation states (6). Following recruitment, macrophages are "educated" by the tumor microenvironment and exhibit a spectrum of phenotypes, ranging from the classically activated (M1) phenotype to the alternatively activated (M2) phenotype (7). In regressing and nonprogressing tumors, TAMs mainly resemble the M1 type and exhibit antitumor activity. In malignant and advanced tumors, TAMs are biased toward the M2 phenotype that instead favors tumor malignancy (8–10).

Adrenomedullin (ADM), a 52-aa peptide, is a potent vasodilator, belonging to the calcitonin superfamily (11). It is a multifunctional molecule that is involved in
Role of Macrophage-Derived Adrenomedullin in Melanoma

Translational Relevance

This study identifies tumor-associated macrophages (TAM) as the major source of adrenomedullin (ADM) in melanoma. Our results show that TAM-derived ADM can induce the phosphorylation of endothelial nitric oxide synthase in endothelial cells via a paracrine manner and polarize macrophage to an M2 phenotype in an autocrine manner to promote angiogenesis and melanoma growth. ADM and its receptor levels are increased in human melanoma, suggesting their role in melanoma-angiogenesis. This study highlights the significance of ADM, which can be an important link between TAMs and melanoma, and indicates that the suppression of ADM and its receptors is promising for tumor inhibition. It also suggests that the combination of macrophage recruitment inhibition and the "re-education" of macrophage polarization is a feasible anticancer strategy.

Materials and Methods

Cells and culture

All cell lines were obtained from American Type Culture Collection and cultured in culture media containing 10% FBS. Conditioned media (CM) were obtained from serum-free culture media culturing cells for 12 or 24 hours as indicated. Primary peritoneal macrophage (PM) and bone marrow-derived macrophage (BMDM) were isolated and cultured as previously described (26).

Western blotting

Cells were harvested, lysed, applied to SDS-PAGE, and transferred to polyvinylidene difluoride membrane. Membranes were incubated overnight at 4°C with primary antibodies and subsequently incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. Peroxidase activity was visualized with the SuperSignal West Pico Chemiluminescent Substrate from Pierce (Rockford). Each assay was repeated at least 3 times.

Immunofluorescence staining

Tumor tissues or cells were stained with indicated primary antibodies and fluorescence-conjugated secondary antibodies as described previously (27).

Trans-well migration and tubule formation assays

Trans-well migration and tubule formation assays were carried out as previously described (27) and experiments were conducted thrice in triplicate.

Animal study

Animal studies were approved by the Institutional Animal Care and Use Committees of Tsinghua University. For each experiment, the mice (Vital River) used were 6- to 8-week-old (7–10 mice per group), and the results were further verified by at least one more reproduction. Cells were counted with a hemocytometer and then subcutaneously injected into C57BL/6 mice (5 × 10⁶ B16/F10 cells for macrophages promoting or AMA-inhibiting tumor growth, or 10⁵ B16/F10 cells for ADM-promoting tumor growth), or nude mice (5 × 10⁵ A375 cells) in 0.1 mL Matrigel solution by 1 mL gauge needle. Tumor volumes were measured with a caliper and calculated by the formula: volume = 0.52 ab² ("a" indicates the long diameter and "b" is the short diameter).

Flow cytometry

After blocking, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD68 antibody and PE-conjugated rat anti-mouse CD206 antibody for 1 hour at 4°C. After washed twice by cold PBS, cells were analyzed by the FACSCalibur flow cytometry system (Becton Dickinson).

Enzyme immunoassay (ELISA)

ADM content was measured by ELISA using the commercial mouse or human ADM kit, following manufacturer's...
instructions (Ground biotechnology diagnosticate). Experiments were conducted thrice in triplicate.

**Human samples**

Tissue microarrays were purchased from Xi’an Aomei, which contained human melanoma tissues (median age 54.9 years, range 25–88, male and female were about fifty-fifty) and adjacent skins. Tissue microarrays were stained with anti-human ADM, CRLR, RAMP2, and RAMP3 antibodies (Santa Cruz) according to the protocol of immunofluorescence staining, and the staining was evaluated by the fluorescence.

**Statistical analysis**

Data are represented as mean and SEM. Statistical analysis of data was carried out using the Student t test or χ² test. P value less than 0.05 was considered as a significant difference.

**Results**

**TAMs contribute to ADM expression and secretion in melanoma**

To investigate the expression and distribution of ADM in melanoma, we analyzed the colocalization of CD68, a macrophage marker, and ADM in human melanoma tissues by immunofluorescence. Most ADM was colocalized with CD68 in melanoma, indicating a correlation between ADM expression and TAMs in melanoma (Fig. 1A). To further determine the source of ADM in melanoma, we detected ADM expression in B16/F10 cells and RAW264.7 macrophages. The expression of ADM was observed in RAW264.7 macrophages, but not in B16/F10 cells (Fig. 1B). We then detected the concentrations of ADM in CM from tumor cells, macrophages, and their cocultured cells by ELISA. Figure 1C shows that ADM secretion from cocultured cells for 24 hours, but not 12 hours, was significantly higher.
than that from individually cultured cells. Furthermore, the ADM secretion from macrophages treated with tumor cells CM was much higher than that from tumor cells treated with macrophages CM, indicating that the increase of ADM secretion was due to TAMs (Fig. 1D). To further confirm the ability of TAMs to secrete ADM, we isolated primary mice PMs and BMDMs, and treated them with B16/F10 CM. Figure 1E shows that ADM secretion from both of them was significantly upregulated upon the B16/F10 CM treatment. These results showed that TAMs are the major source of ADM in melanoma.

**TAM-induced angiogenesis and melanoma growth are mediated by ADM**

To assess whether ADM can mediate the function of TAMs in angiogenesis, the migration and tubule formation of human dermal microvascular endothelial cells (HMEC) were examined when treated with B16/F10 or RAW264.7 cells CM or their coculture CM. We found that the coculture CM significantly promoted HMECs migration and tubule formation, which were abrogated by the neutralizing ADM antibody (Fig. 2A and B and Supplementary Fig. S1A and B). To determine the role of macrophages in melanoma growth, RAW264.7 macrophages or B16/F10 CM–treated RAW264.7 macrophages were coinkoculated subcutaneously with B16/F10 cells. RAW264.7 macrophages significantly accelerated tumor growth at the early stage from day 2 to 7, although macrophages themselves were incapable of developing tumors (Fig. 2C). The ability of B16/F10 CM–treated RAW264.7 macrophages in promoting melanoma growth was higher than that of RAW264.7 macrophages at day 4 and 5 (Fig. 2C). However, macrophages did not affect melanoma growth from day 9 to 14 (Supplementary Fig. S2B). These results suggest that exogenous macrophages or B16/F10 CM–treated macrophages only promote melanoma growth at the early stage. To determine the function of ADM and angiogenesis in this process, we harvested tumor tissues at day 7 after implantation and examined the expression of ADM and CD31. When B16/F10 cells were coadministered with RAW264.7 or B16/F10 CM–treated RAW264.7 macrophages, both the expression of ADM (Fig. 2D, left panel) and blood vessel densities (Fig. 2D, right panel) were significantly upregulated. These results showed that ADM is a key mediator in the angiogenesis and growth of melanoma.

![Figure 2](clincancerres.aacrjournals.org)
ADM promotes angiogenesis and melanoma growth via the eNOS pathway

Because eNOS activation in endothelial cells is an important intracellular signaling event in angiogenesis, we examined the function of ADM on the phosphorylation of eNOS in HMECs. ADM (10 nmol/L) induced eNOS phosphorylation as early as 10 minutes and produced a maximal effect at 40 minutes after the treatment (Fig. 3A, top). ADM was able to induce eNOS phosphorylation from the concentration as low as 0.1 nmol/L after 40 minutes of treatment and has a maximal effect at the concentration of 10 nmol/L (Fig. 3A, bottom). Therefore, ADM induces eNOS phosphorylation in both time- and dose-dependent manners. In addition, we detected the effect of ADM on HMECs migration and tubule formation. ADM was found to enhance HMECs migration and tubule formation in a dose-dependent manner (Fig. 3B and C and Supplementary Fig. S3A and B). An eNOS-specific inhibitor, NG-Nitro-l-arginine methyl ester (l-NAME), but not its inactive isomer d-NAME, significantly suppressed these effects of ADM in a dose-dependent manner, although l-NAME itself did not show any effect on these processes (Fig. 3B and C and Supplementary Fig. S3A and B). These results showed that ADM-induced migration and tubule formation of HMECs are mediated by the eNOS signaling pathway in vitro.

Following the in vitro experiments, we further investigated the role of ADM in melanoma growth in vivo. The tumor volume from day 7 to 12 (Fig. 3D, left panel) and the tumor weight on day 12 (Fig. 3D, right panel) were significantly increased by ADM treatment. When coadministered with l-NAME, but not d-NAME, ADM-induced melanoma growth was abrogated, whereas l-NAME itself showed no effect on tumor growth (Fig. 3D). To further confirm the contribution of angiogenesis in this process, we examined CD31 staining in tumor tissues. Compared with the control group, the blood vessel density was much higher in the ADM treatment group, which could be abrogated by the treatment of l-NAME, but not d-NAME (Fig. 3E). Therefore, ADM-induced angiogenesis and melanoma growth are shown to be mediated via the eNOS signaling pathway.

The autocrine effect of ADM contributes to macrophage polarization

In addition to endothelial cells, TAM-derived ADM may also target tumor cells or macrophages themselves in
paracrine and autocrine manners. To confirm this hypothesis, we examined the expression of ADM receptor components in B16/F10 cells and RAW264.7 macrophages. It was shown that CRLR, but not RAMP2 or RAMP3, was expressed in B16/F10 cells (left) and RAW264.7 macrophages (middle), respectively. The colocalization of ADM and its receptor components was detected in RAW264.7 macrophages (right). Scale bars, 20 µm. B, measurement of macrophage phenotypes in different stages of tumor tissues. B16/F10 cells (5 x 10^6) were implanted subcutaneously into mice, and tumors were removed on day 7 (early stage) and on day 14 (late stage). Macrophages were stained with both FITC-labeled CD68 mAb (monoclonal antibody) and PE-labeled CD206 mAb (left). The quantification of CD206^+CD68^+ cells (% of CD68^+ cells; middle) and the percentage of both M1 and M2 macrophage (right) in tumor tissues were analyzed. Scale bars, 100 µm. *** P < 0.001. C, ADM decreases the expression of iNOS and upregulates the expression of Arg-1 in RAW264.7 macrophages. After being treated with ADM or B16/F10 CM for 24 hours, the expression levels of iNOS and Arg-1 were assessed by Western blotting. D, ADM increases the expression of CD206 in RAW264.7 macrophages. RAW264.7 macrophages were stimulated with ADM or B16/F10 CM for 24 hours, stained by both FITC-labeled CD68 mAb and PE-labeled CD206 mAb, and analyzed by flow cytometry. The number in boxed areas indicates the amounts of CD206^+CD68^+ cells (% of CD68^+ cells) in RAW264.7 macrophages. DAPI, 4',6-diamidino-2-phenylindole.

ADM was colocalized with all ADM receptor components in RAW264.7 macrophages (Fig. 4A, right panel). These results strongly suggested an autocrine loop of ADM in TAMs. To assess whether the tumor microenvironment facilitates this autocrine effect, we utilized B16/F10 CM to treat RAW264.7 macrophages and detected the expression of ADM receptor components by Western blotting. We found that B16/F10 CM dramatically increased the expression of all ADM receptor components in RAW264.7 macrophages (Supplementary Fig. S4). These results showed that TAM-derived ADM not only stimulates ADM secretion from RAW264.7 macrophages (Fig. 1D) but also upregulates its receptors expression, thus effectively enhancing the autocrine effect.
Macrophages have functional plasticity with the ability to change their functional profiles in response to different tumor microenvironments (28). Compared with M1, M2 macrophages produce lower amount of inducible NOS (iNOS), but higher amount of arginase 1 (Arg-1), and express M2-specific surface markers such as CD206 (29). We examined the expression of CD68 and ADM at different stages of melanoma and found that they were dramatically upregulated in the late stage relative to their levels in the early stage of tumors (Supplementary Fig. S6). Moreover, confocal microscopy studies showed that the percentage of CD206+ cells out of CD68+ cells was much higher at the late stage compared with that at the early stage of tumors (Fig. 4B, left and middle panels). Most TAMs were identified as M1 macrophages (defined as CD68+ CD206−) at the early stage and M2 macrophages (defined as CD68− CD206+) at the late stage of melanoma, respectively (Fig. 4B, right panel). These results showed that macrophage phenotype can be shifted from M1 to M2 type during the tumor growth, which may be resulted by the enhancement of ADM expression. To further clarify this point, RAW264.7 macrophages were treated with ADM or B16/F10 CM, and expression levels of iNOS, Arg-1, and CD206 were detected by Western blotting or flow cytometric analysis, respectively. We found that ADM or B16/F10 CM significantly suppressed iNOS expression, whereas it enhanced the expression of Arg-1 and CD206 in RAW264.7 macrophages (Fig. 4C and D). These results showed that ADM can polarize RAW264.7 macrophages from M1 to M2 type.

The autocrine effect of ADM promotes melanoma growth in vivo

To investigate the autocrine effect of ADM on melanoma growth in vivo, we examined the effect of the ADM receptor antagonist, AMA, on tumor growth. One week after B16/F10 or A375 cells implantation, intraperitoneal injection of AMA proceeded for 5 or 8 days. We found that the tumor growth was significantly inhibited by AMA in both B16/F10 mouse and A375 human melanoma models (Fig. 5A). It was found that ADM expression levels in melanoma tissues were positively stained for ADM (49 of 60), RAMP2 (43 of 59), and RAMP3 (45 of 58), whereas the expression levels of them were much lower in control tissues (Fig. 6 and Supplementary Table S1). The expression of ADM and its receptors is intimately associated with human melanomagenesis

It was found that ADM expression levels in melanoma tissue (Supplementary Fig. S6) and plasma (data not shown) are increased with mouse melanoma growth. To assess the relationships of the expression of ADM and its receptors to human melanoma, we stained tissue microarrays containing samples of clinical melanoma nodules. Interestingly, we found that most of human melanoma tissues were positively stained for ADM (49 of 59), CRLR (46 of 60), RAMP2 (43 of 59), and RAMP3 (45 of 58), whereas the expression levels of them were much lower in control tissues (Fig. 6 and Supplementary Table S1). ADM and its receptors levels are unrelated to the
clinicopathologic status of age and gender (Supplementary Table S2). These results illustrated that the expression of ADM and its receptors are associated with melanomagenesis in melanoma patients.

Discussion

It is now widely accepted that TAMs play a critical role in regulating angiogenesis and melanoma growth. Clinical studies show a positive correlation between TAM density and melanoma prognosis (30). Animal tumor models suggest that TAMs depletion inhibits angiogenesis and tumor growth (31–33), whereas increased number of TAMs exhibits the opposite effect (34). Despite these functional studies, the precise mechanism by which TAMs facilitate angiogenesis and melanoma growth remains incompletely understood. In this study, we unravel a novel mechanism which shows that ADM is a key regulator for the involvement of TAMs in melanoma growth via both paracrine and autocrine manners. Other factors in the tumor microenvironment may act as the downstream of ADM or interact with ADM to regulate the function and activity of TAMs.

Accumulating studies suggest that macrophages in the tumor microenvironment can be switched into 2 distinct phenotypes, namely M1 and M2 (8), whereas the identity of these macrophages during melanoma growth remains poorly clarified. Our present study shows that not only the TAM density is increased but also its phenotype is switched from M1 to M2 during the melanoma growth. These results provide a new insight into the dynamic nature of TAMs during the tumor growth and support that ADM can function as a key factor in this process. As mentioned above, ADM secretion from macrophages is correlated with inflammation and hypoxia. The link between inflammation and melanoma (35), or hypoxia and melanoma (36) is well established. The distribution of ADM in human melanoma, the low level of constitutive ADM expression in B16/F10 cells, and the ability of TAMs to secrete ADM support our hypothesis that TAMs are the primary source of ADM in melanoma. It was reported that TAMs can enhance tumor angiogenesis by upregulating hypoxia-induced angiogenic factors, such as VEGF (5). In this study, we provide evidences that ADM is required and sufficient for TAM-induced angiogenesis and melanoma growth. Collectively, these observations suggest that TAM-derived ADM is a key factor for TAM-induced angiogenesis and melanoma growth. ADM, therefore, represents an important link between TAMs and melanoma growth.

ADM receptors are expressed in endothelial cells and can be upregulated under the hypoxic condition (37). Thus, TAM-derived ADM is able to interact with its receptors on endothelial cells to accelerate angiogenesis in a paracrine manner. Our observations show that ADM can stimulate angiogenesis, which is consistent with previous reports (38, 39). However, most previous studies have specifically focused on the exogenous ADM. This study shows that ADM from TAMs can also stimulate angiogenesis via the paracrine loop. Although it has been reported that ADM promotes angiogenesis in vitro through the activation of some signaling pathways (38), the nature of TAM-derived ADM and its mechanism involved in angiogenesis and tumor growth merit further studies. The activation of eNOS in endothelial cells is an important intracellular signaling event for angiogenesis (40–42). VEGF, similar to ADM, is a
proangiogenic factor, which enhances angiogenesis through the activation of eNOS signaling pathway (43). In this study, we provide evidences that ADM-induced angiogenesis and melanoma growth are modulated by the eNOS signaling pathway. These results can help us find new approaches to manipulate the activation of ADM and provide new targets for melanoma therapeutics.

The autocrine effect of ADM is often observed in angiogenesis and tumor growth (27, 44–46). We report here that all ADM receptor components are present on macrophages and colocalized with ADM. Thus, TAM-derived ADM can influence macrophages themselves in an autocrine manner. Inhibition of ADM receptors expression or activities can impair the tumor angiogenesis and growth (20–22, 47). Interestingly, we show that the expression of all ADM receptor components can be upregulated by the B16/F10 CM, suggesting that the autocrine effect of ADM on TAMs is facilitated by the tumor microenvironment. It was reported that mediators of inflammation are critical constituents of the tumor microenvironment (35, 48, 49), which strongly influence the polarization of TAMs (7, 8). This concept is supported by our study showing that the type of macrophage is switched from M1 to M2 during the tumor growth. ADM is an inflammation-related polypeptide, and its expression can be upregulated by hypoxia (25) and during the melanoma growth. It has recently been reported that there are more M2-like TAMs enriched in hypoxic areas within tumors, which have a superior proangiogenic activity in vivo (50). In this study, we report that both the B16/F10 CM and ADM induce the switch of RAW264.7 macrophages to an M2-like type. These findings support the hypothesis that ADM is involved in macrophage polarization. Importantly, the autocrine effect of ADM in macrophages polarization contributes to melanoma growth in vivo. To our knowledge, this is the first study to illustrate the autocrine loop of ADM on macrophage polarization and melanoma growth.

In sum, our findings show a previously unknown connection among TAMs, ADM, and melanoma growth and provide a new insight into the mechanism of the involvement of TAMs in melanoma. More importantly, our present study has several therapeutic implications, including that it provides a basis for future cancer therapies via combining both the inhibition of macrophages recruitment and the “re-education” of macrophages polarization. It suggests that the suppression of ADM and its receptors is promising for cancer therapy and also provides the evidence to support the eNOS blockade strategy for interfering ADM signal in the treatment of cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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