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Research Article

Angiopoietin-2 in Bone Marrow milieu promotes Multiple Myeloma-associated angiogenesis

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ABSTRACT

Angiopoietin-2 (Ang-2) is involved in angiogenesis in both solid and hematological malignancies. In Multiple Myeloma (MM), serum Ang-2 correlates with disease progression and response to therapy. To address the patho-physiologic role of Ang-2 in MM associated angiogenesis, we used sera from patients with active MM, which contained significantly higher levels of the molecule, compared to those from patients with smoldering MM and Monoclonal Gammopathy of Undetermined Significance. MM Bone Marrow (BM) sera with high Ang-2 concentration specifically contributed to endothelial cell (EC) activation, while Ang-1 containing sera maintained EC stabilization. The functional dichotomy of Ang-1 and Ang-2 was confirmed by the triggering of distinctive signaling pathways down-stream the common Tie-2 receptor, i.e., the Akt or the ERK- phosphorylation pathway. Notably, Ang-2 but not VEGF serum levels correlated with BM micro-vessel density, further underscoring the key role of Ang-2 in angiogenesis. Western Blot, RT-PCR and immunocytochemistry identified MMEC as the major source of Ang-2, at variance with MM cells and $CD14^+$ BM monocytes. These data suggest that Ang-2 produced in the BM milieu may contribute to MM angiogenesis and suggest that the molecule can be further exploited both as angiogenesis biomarker and as a potential therapeutic target.

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Introduction

Multiple Myeloma (MM) is a plasma cell tumor, which grows almost exclusively inside the Bone Marrow (BM). Tight interactions between MM cells and BM microenvironment are central to MM progression, as they allow the delivery of pro-survival signals and confer chemo-resistance to neoplastic cells [19,22,5]. MM evolution is accompanied by microenvironmental changes [20], including neo-angiogenesis [23].

The formation of new blood vessels is regulated by a finely balanced equilibrium between pro- and anti-angiogenic factors which operate in concert under physiological conditions. Within a

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tumor microenvironment, the excessive and dysregulated expression of angiogenic cytokines and chemokines derails this equilibrium and generates abnormally increased microvessel density (MVD) and leaky, mal-functioning vessels ([6,37,4,30]). The transition from Monoclonal Gammopathy of Undetermined Significance (MGUS) to active MM (AMM) is characterized by the "angiogenic switch" [47], sustained by the over-production of angiogenic cyto-chemokines produced by MM cells and upregulated by MM cell interaction with the surrounding microenvironment [38,11,39]. Given the multifactorial complexity of angiogenesis, the identification of key molecules involved in the MM-associated angiogenic switch could have prognostic implications and be instrumental for the development of targeted therapies. As several prototypic angiogenic molecules, including VEGF and FGF, are equally expressed by MM cells isolated from MGUS, smoldering MM (smMM) and AMM [25], emerging molecules with angiogenic properties deserve to be considered. In particular, the essential role of Angiopoietins (Angs) in the pathophysiologic regulation of angiogenesis has been recognized in solid tumors and in hematological malignancies as well [21,26,27,17].

Ang-1 and-2, the main members of the Angs family, compete in binding to the common receptor Tie-2 on EC exerting opposite effects: Ang-1 acts as an agonist, inducing Tie-2 phosphorylation and down-stream Akt-pathway activation, thus resulting in vessel maturation and integrity maintenance; Ang-2 is considered an antagonist in that it induces vessel destabilization [14,15]. However, the role of Ang-2 appears to be more complex, as it is able to promote either vessel growth or regression depending on the microenvironmental cytokine context [44]. The dichotomy of Ang-1 and Ang-2 is reflected by the modality of their production: Ang-1 is constitutively released by stabilizing mural cells, while Ang-2 is stored in EC, released upon activation and then acts via an autocrine/intracrine fashion [13,21]. Ang-2 has also been reported to be produced by tumor B cells in chronic lymphocytic leukemia [26,27].

A significant increase of circulating Ang-2 and a reduced Ang-1/Ang-2 ratio have been reported to correlate with disease severity in MM [24,43,33]. However, the precise role of Angs in MM-associated angiogenesis is poorly known. The cellular source of Ang-2 in MM is likewise a matter of debate, the contribution of MM cells to Ang-2 production being still controversial [18,45].

Our study aimed at identifying the mechanisms of Ang-2 in MM triggered angiogenesis and investigating its cellular source within MM microenvironment. The results warrant Ang2 validation as a bio-marker of ongoing angiogenesis and suggest the possibility that it might be a putative therapeutic target.

Materials and methods

Patients and sera collection

Bone Marrow (BM) and peripheral blood (PB) samples were obtained from patients with MM (active, $n=29$; smoldering, $n=11$) and MGUS ($n=9$), recruited at the Department of Hematology, Ospedale San Raffaele, Milan. Informed consent was obtained in accordance with the Declaration of Helsinki and the approval for use of primary samples was obtained from the Institutional Review Board of the San Raffaele Scientific Institute. Peripheral blood of 9 healthy controls (HD) was also collected. BM biopsy samples of MM patients ($n=6$) were analyzed for microvessel density (MVD) quantification. Briefly, Bouin's-fixed, paraffin-embedded sections were cut, stained with Haematoxylin-Eosin and evaluated for morphology and plasma cell ratio assessment. Additional sections were stained with anti-CD34 MoAb (QBEnd/10, Novocastra, Newcastle upon Tyne, UK). All CD34+ vessels were evaluated as previously reported [36].

Cell isolation and culture

Human umbilical vein endothelial cells (HUVEC) were isolated from human cord by collagenase treatment as described [1] and propagated in complete medium (CM), containing 20% heat-inactivated fetal bovine serum (FBS), 40 μg/ml ECGS (Sigma Aldrich) and 90 μg/ml heparin. All experiments were carried out with HUVEC at passage 2–4. Multiple Myeloma EC (MMEC) were obtained as described [46] and maintained in CM. BM mononuclear cells were obtained by density gradient centrifugation (Ficoll–Hypaque, Pharmacia, Piscataway, NJ, USA), as previously described [31]. MM plasma cells were isolated by immunomagnetic positive selection of BM derived CD138+ cells (EasySep™, Stemcell Technologies).

Reagents and antibodies

Recombinant (r) Ang-1 and rAng-2 (both from R&D System), were used at 400 ng/ml; $rVEGF_{165}$ (from Thermo Scientific, Pierce Biotechnology) was used at 50 ng/ml, based on previous titration curves. Anti-phospho-Akt and anti-phospho-Tie2 were from R&D Systems; blocking polyclonal anti-Ang2 (C-19), monoclonal anti-Ang-2 (F-1), monoclonal anti-phospho-Erk 1/2 (E-4) and polyclonal anti-Tie-2 receptor (C-20) sc-324 were from Santa Cruz. The specificity of both polyclonal and monoclonal anti-Ang-2 antibodies was tested in WB analysis and shown in Fig. S1A; the neutralizing activity of the polyclonal Ab C-19, used at 10 μg/ml as in [26,27] and assessed in a permeability assay, is shown in Fig. S1B. Alexa Fluor 488- and 594-labeled anti-mouse IgG were from Molecular Probes. DAPI (2 nM) for nuclear staining was from Sigma. Cultrex BME from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells was purchased from Tema Ricerca (Bologna, Italy).

Cytokine and chemokine determination in sera samples

Ang-1, Ang-2 levels in sera samples from MM patients and Healthy Donors (HD) and VEGF were quantified using specific Quantikine solid-phase-enzyme-linked immunosorbent assays (ELISA) (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

EC permeability assay

The experiment was performed as described previously [1]. Briefly, HUVECs (5×10^4/well) were seeded on Transwell inserts (0.4 μm pore size; Costar) and then exposed to medium with 20% of serum samples. TNF-α 4 ng/ml served as positive control. Alternatively, cells were exposed to 400 ng/ml rAng-1 and 400 ng/ml rAng-2. After ON incubation, Dextran-FITC (SIGMA) was added to the upper wells and the lower compartments were filled with EBM-2.

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