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Angiogenic activity of multiple myeloma endothelial cells *in vivo* in the chick embryo chorioallantoic membrane assay is associated to a down-regulation in the expression of endogenous endostatin

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Abstract

We have attempted a fine characterization of the angiogenic response induced by multiple myeloma endothelial cells (MMEC) by using the chick embryo chorioallantoic membrane (CAM) assay and by reverse transcriptase-polymerase chain reaction (RT-PCR). Results showed that in the CAM assay MMEC induced an angiogenic response comparable to that of a well-known angiogenic cytokine, namely fibroblast growth factor-2 (FGF-2), while RT-PCR demonstrated that the expression of endostatin mRNA detected in MM treated CAM was significantly lower respect to control CAM. These data suggest that angiogenic switch in MM may involve loss of an endogenous angiogenesis inhibitor, such as endostatin.

Keywords: angiogenesis • endostatin • chorioallantoic membrane • multiple myeloma

Introduction

Angiogenesis is a constant hallmark of multiple myeloma (MM) progression and has prognostic potential [1]. We have previously isolated endothelial cells (EC) from bone marrow of patients with MM (MMEC) [2]. They show intrinsic angiogenic ability, because they rapidly form a capillary network *in vitro*, and extrinsic ability, because they generate numerous new vessels *in vivo* in the chick embryo chorioallantoic membrane (CAM) [2]. Moreover, MMEC secrete angiogenic cytokines, such as fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF), as well as matrix metalloproteinase-2 and –9 (MMP-2 and MMP-9) and express adhesion molecules facilitating dissemination of these cells [2]. Studies by Rajkumar *et al*. [3], Dominici *et al*. [4], and Laroche *et al*. [5] have confirmed substantial bone marrow angiogenesis in MM.

The bone marrow microenviromnent plays a crucial role in inducing the angiogenic response in MM [6, 7]. Reciprocal positive and negative interactions between plasma cells and bone marrow stromal cells, namely haematopoietic stem cells, fibroblasts, ostoblasts/osteoclasts, EC, EC progenitor cell, T lymphocytes, macrophages and mast cells are mediated by an array of cytokines, receptors and adhesion

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molecules. These cells secrete angiogenic factors, such as interleukin-6 (IL-6), VEGF, FGF-2, hepatocyte growth factor/scatter factor (HGF/SF), transforming growth factor-β (TGF-β) and tumour necrosis factor α (TNF-α) [6, 7].

Here, we have attempted a fine characterization of the angiogenic response induced by MMEC, as compared with monoclonal gammopathy of undetermined significance EC (MGUSEC) in the CAM assay. We investigated the angiogenic response induced by gelatin sponges soaked with the cell suspensions and implanted on the CAM surface from 8 to day 12 of incubation and we studied the effects of MMEC and MGUSEC on the expression of endogenous levels in the CAM of VEGF, FGF-2, angiopoietin-1 (Ang-1), hypoxia-inducible factor-1α (HIF-1α) and endostatin by reverse transcriptase-polymerase chain reaction (RT-PCR).

Materials and methods

Cell cultures

MMEC were obtained from 10 patients with MM and MGSUEC were obtained from 10 patients with MGUS, as previously described [2].

In vivo CAM assay

Fertilized White Leghorn chicken eggs were incubated at 37°C at constant humidity. On day 3 of incubation a square window was opened in the egg shell after removal of 2–3 ml of albumen so as to detach the developing CAM from the shell. The window was sealed with a glass and the eggs were returned to the incubator. Gelatin sponges (Gelfoam, Upjohn Company, Kalamazoo, MI, USA) were cut to a size of 1 mm^3 and placed on top of a growing CAM at day 8 incubation under sterile conditions [8]. The sponges were then adsorbed with 2 μl of cell suspension (18,000 cells per sponge) of MMEC or MGUSEC or with RPMI-1640 medium alone or supplemented with FGF-2 (200 μg/ml) (R & D Systems, Abington, UK), used as negative and positive control, respectively.

The angiogenic response was evaluated 96 hrs after the implants by means of a stereomicroscope connected to an image analyser system (Olympus Italia, Italy). Blood vessels entering the sponges within the focal plane of the CAM were counted by two observers in a double blind fashion at a magnification of 50x. Means ± 1 Standard Deviation (SD) were evaluated for all the parameters and the statistical significance of the differences between the counts was determined by Student's t-test for unpaired data.

RNA extraction and RT-PCR

Total RNA was extracted from 12 CAM treated with EC obtained from six patients with MGUS and six patients with MM and from six control CAM using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufactures's protocol. The concentration and the purity of the isolated RNA were determined using a spettrofotometric analysis and the integrity of the RNA was verified by means of electroforesis in a formaldehyde agarose gel followed by ethidium bromide staining. The cDNA was then synthetized with 5 μg of total RNA using M-MLV and oligo $(dT)_{18–20}$ as primer (Invitrogen, Carlsbad, CA, USA).

Level of mRNA expression of pro-angiogenic molecules VEGF, FGF-2, Ang-1, HIF-1β and mRNA expression of anti-angiogenic factor endostatin were evaluated by PCR using specific primers and the relative level of the each amplified transcripts were normalized to those of the housekeeping gene β-actin.

PCR reactions were performed in a final volume of 25 μl containing 2 μl of RT products, 10 picomoles of each primer and 1x of RED-Taq polymerase mix (Sigma-Aldrich, Milano, Italy). Preliminary experiments were performed to determine the appropriate conditions (Ta and specific number of cycles) for semi-quantitative RT-PCR and transcript were amplified using different cycle numbers: 25 cycles for β-actin, 30 cycles for VEGF, FGF-2, Ang-1 and for endostatin and 35 cycles for HIF-1β.

PCR products were subjected to electrophoresis in a 1.5% agarose gel and ethidium bromide stained, and then photographed under an ultraviolet transilluminator (Amersham Biosciences). Intensity of bands was quantified as arbitrary optical density units (OD) using the Scion Image Software (based on NIH Image).

Endostatin western blot

Six CAM treated with EC obtained from three patients with MGUS and three patients with MM and three control CAM were homogenized in ice cold buffer containing 250 mM sucrose, 10 mM Tris-HCL, pH 7.4 and protease inhibitors [1μg/ml leupeptin, 1μg/ml pepstatin and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. After homogenization in a Potter apparatus and centrifugation at 1000× *g* for 10 min, the protein content of the supernatant was measured using a Bradford assay (BIO-RAD Laboratories, Hercules, CA, USA). For SDS-PAGE, samples (60 μg/lane) were solubilized in Laemmli buffer, boiled at 90°C for 10 min and runned on a 12% polyacrylamide. The proteins were blotted to a PVDF membrane (Millipore Corporate, Billeira, MA, USA) and immunoblotting was performed using a monoclonal mouse primary antibody against endostatin (Novus Biologicals, Littleton, CO, USA) diluted 1:500 in

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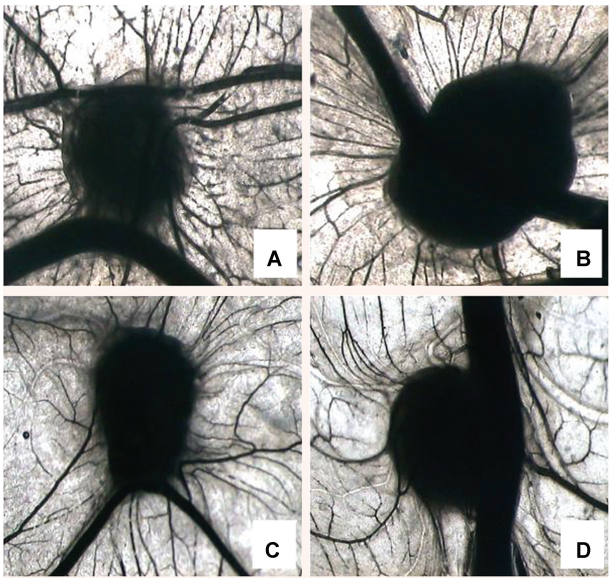

Fig. 1 Angiogenic activity in the chorioallantoic membrane (CAM) assay. Gelatin sponges loaded with FGF-2 (**A**), MMEC (**B**), RPMI-1640 (**C**) and MGUSEC (**D**) were implanted on top of the CAM on day 8. Macroscopic view of the CAM on day 12 shows numerous allantoic vessels converging like spokes toward the sponge in **A** and **B**, while few allantoic vessels are recognizable in **C** and **D**. Original magnification: **A**–**D**, x 50.

TBST. Immunocomplexes were revealed using antimouse horseradish peroxidase–labelled antibody (DAKO Corporation, USA, Sigma Chemical Co., St. Louis, MO) diluted 1:5000 in tris buffered saline tween (TBST). Peroxidase activity was revealed by chemiluminescence (ECL kit, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and blot was immediately exposed to X-ray film. Control experiments were performed using a mouse-anti-β-actin (Abcam, Cambridge, CA, USA) to detect housekeeping protein β-actin to normalize the protein load. Stained bands were scanned and intensity was quantified using the Scion Image System (based on NIH Image).

Results and discussion

The ability of MMEC and MGUSEC to induce an angiogenic response *in vivo* was assessed with the CAM-gelatin sponge assay. Vessels entering the sponge were recognized macroscopically and counted (Table 1 and Fig. 1). CAM implanted with the medium containing FGF-2 or MMEC gave significantly higher vessel counts and numerous allantoic vessels converging like spokes toward the sponges were recognizable (Table 1 and Fig. 1). By contrast, when the sponges were loaded with the medium alone and with MGUSEC, physiologic angiogenesis was observed in the form of few allantoic vessels partly around and partly converging toward the sponge (Table 1 and Fig. 1).

Table 1 Angiogenic response assessed by the chick embryo chorioallantoic membrane (CAM)-gelatin sponge assay

Sponge loaded with	No of blood vessels at the sponge-CAM boundary
RPMI-1640	8±2
FGF-2	27±3*
MGUSEC	9±3
MMEC	25±4*

*P<0.001 *versus* RPMI-1640 and MGUSEC

In order to evaluate the angiogenic effects of MMEC and MGUSEC, mRNA expression of pro-angiogenic factors including VEGF, FGF2, Ang-1, HIF-1α and mRNA expression of anti-angiogenic molecule endostatin were measured in treated chicken CAM by RT-PCR. The expression level of each transcript was compared to those detected in control CAM.

As shown in Fig. 2, treatment of CAM with MMEC and MGUSEC did not produce significant difference in the expression of pro-angiogenic factors mRNA in comparison with control CAM treated with medium alone. Moreover, no statistical difference was found in VEGF, FGF-2, Ang-1, HIF-1α and endostatin mRNA content between MM and MGUS treated CAM (P<0.05). In contrast, the expression of endostatin mRNA detected in both MGUS and MM treated CAM was significantly lower respect to control CAM (MGUS OD: 120.1±13 and MM OD: 48.3±19.6 *versus* CTRL OD: 205.84 ± 19.6; P<0.05).

Western blot analyses were also performed to study whether the expression of endostatin changes in MMEC and MGUSEC treated CAM respect to control. As shown in Fig. 3, according to RT-PCR quantification, endostatin was significantly lower also at the protein level in both MGUSEC and MMEC treated

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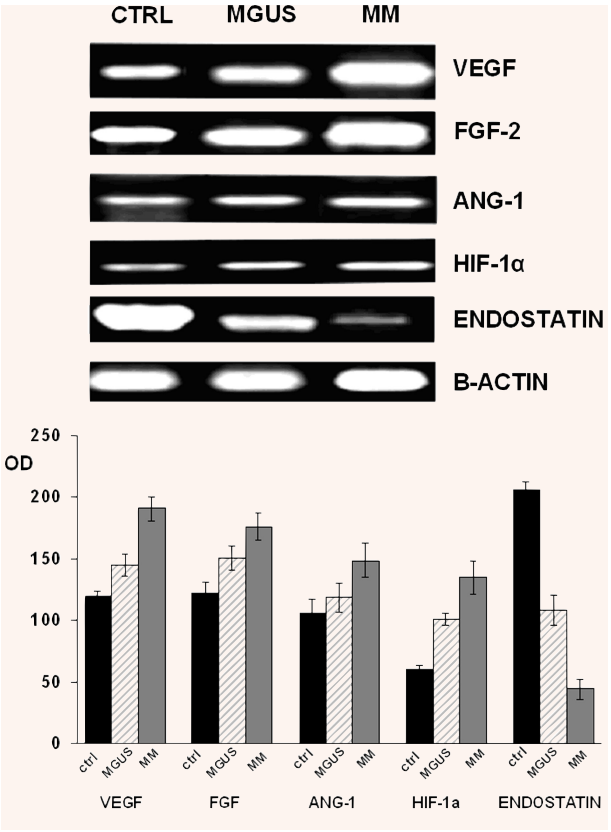

Fig. 2 Expression levels of mRNA coding for VEGF, FGF-2, Ang-1, HIF1α and endostatin evaluated by semi-quantitative RT-PCR. Transcript levels from the CAM assay are referred to EC obtained from 6 MM and 6 MMGUS patients and the error band represents the standard deviation of six experiments.

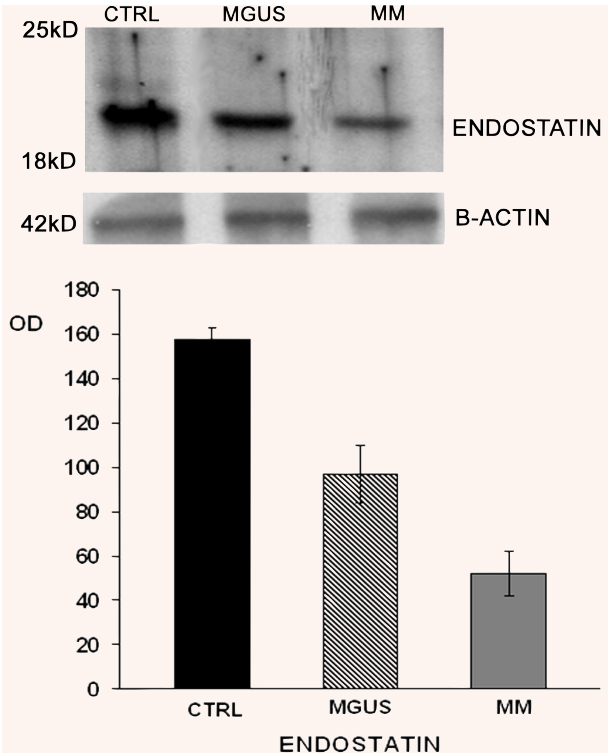

Fig. 3 Immunoblotting analysis and quantification after Western blot analysis of endostatin expression in CAM treated with EC obtained from 3 MM and 3 MGUS patients. The error band represents the standard deviation of three experiments.

CAM respect to control CAM (MGUS OD: 97.14±10 and MM OD: 52.3±13.6 *versus* CTRL OD: 158.71±5.52; $P<0.05$).

Several angiogenic cytokines are involved in MM progression. MM plasma cells secrete VEGF, whereas both VEGFR-1 and VEGFR-2 are markedly elevated in the bone marrow stromal cells, suggesting that a paracrine growth pathway mediated by VEGF-activated stromal cells, secretion of IL-6 on their part, and subsequent activation of plasma cells may occur [1]. In active MM, we found secretion of the VEGF-A isoform by plasma cells, overexpression of VEGFR-2 by bone marrow microvessels and isolated EC and of VEGFR-1 by the other stromal cells, secretion of VEGF-C and VEGF-D by stromal cells, and expression of their cognate receptor VEGFR-3 by plasma cells, which suggests another paracrine loop of angiogenesis and tumour growth in MM [9]. Parallel studies by Kumar *et al.* [10] found secretion of VEGF by several MM cell lines and fresh patients' bone marrow plasma cells, as well as expression of VEGFR-1 and VEGFR-2, which suggests multiple VEGF-mediated autocrine pathways of tumour growth and paracrine stimulation of angiogenesis.

When we studied the angiogenic potential of plasma cell suspension obtained from patients with active MM and implanted them onto the CAM, we demonstrated a vasoproliferative response, significantly higher than that induced by cell suspension obtained from patients either with non-active MM or MGUS [11].

HGF/SF is yet another angiogenic factor identified in the culture medium of both human MM cell lines [12] and freshly isolated plasma cells [13]. Still other factors may be responsible for bone marrow angiogenesis in MM: the plasma cell secretion products, such as TGF-β, IL-1β, IL-6 and IL-8 [14]; granulocyte-colony stimulating factor (G-CSF),

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granulocyte macrophage-colony stimulating factor (GM-CSF) and TNF-α [15], secreted by bone marrow microenvironment cells recruited and activated by plasma cells. Since human MM cell lines produce Ang-1 that up-regulates the Tie-2 cognate receptor in bone marrow EC, involvement of the Ang-1/Tie-2 loop in MM neovascularization has been demonstrated [16].

Conversely, MMEC secrete angiogenic cytokines, such as FGF-2, VEGF and Ang-1 as well as matrix metalloproteinase-2 and -9 (MMP-2 and MMP-9) and express adhesion molecules facilitating dissemination of these cells [1]. Other studies, however, showed that the expression levels of VEGF, FGF-2, and their receptors were similar among plasma cells from MGUS and MM, suggesting that increasing angiogenesis from MGUS to MM is, at least in part, explained by increased tumour burden rather than increased expression of VEGF/VEGFR-2 by plasma cells [17].

Another possibility is that the inhibition of angiogenesis in MGUS is lost with progression, hence that the switch from MGUS to MM may involve a loss of an anti-angiogenic activity [17]. Our data are in accord with this statement, because the expression of endostatin mRNA detected in both MGUS and MM treated CAM was significantly lower respect to control CAM.

The phenotypic switch to angiogenesis in tumour growth involves more than simple up-regulation of angiogenic activity and is thought to be the result of a net balance of positive and negative regulators [18], and our study suggests that angiogenic switch in MM, investigated by using an *in vivo* experimental model such as the chick CAM, may involve loss of an endogenous angiogenesis inhibitor, namely endostatin.

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