A paracrine loop in the vascular endothelial growth factor pathway triggers tumor angiogenesis and growth in multiple myeloma

Angelo Vacca, Roberto Ria, Domenico Ribatti, Fabrizio Semeraro, Valentin Djonov, Francesco Di Raimondo, Franco Dammacco

Background and Objectives. In tumors, vascular endothelial growth factor-A (VEGF-A) stimulates angiogenesis and vascular permeability by activating the tyrosine kinase receptor-2 (VEGFR-2 or KDR/Flk-1) and-1 (VEGFR-1 or Flt-1).

Design and Methods. The distribution and function of VEGF homologs and their receptors on bone marrow plasma cells, endothelial cells, and other stromal cells (residual stromal cells) were examined in patients with multiple myeloma (MM).

Results. Plasma cells secrete VEGF-A (and VEGF-B, VEGF-C and VEGF-D, albeit marginally) into their conditioned medium (CM). CM VEGF-A stimulates proliferation and chemotaxis in endothelial cells (both being mandatory for angiogenesis) via VEGF receptor-2 (VEGFR-2), and in residual stromal cells via the VEGFR-1. Residual stromal cells secrete VEGF-C and VEGF-D, but little of the other homologs. Their CM VEGF-C and VEGF-D increase in response to plasma cell CM and trigger plasma cell proliferation via VEGFR-3. Proliferation in all cell types parallels VEGFR and extracellular signal-regulated protein kinase-2 (ERK-2) phosphorylation. The homologs and receptors are weakly or inconstantly expressed in patients with monoclonal gammopathies of undetermined significance or vitamin B_{12}/iron deficiency anemias.

Interpretation and Conclusions. This study shows that the VEGF pathway is directly involved in tumor angiogenesis and growth in MM. A paracrine VEGF loop for MM progression is suggested. This, in turn, provides a further indication that the VEGF pathway and its signaling proteins may be appropriate targets in the management of MM.

Key words: angiogenesis, endothelial cells, multiple myeloma, stromal cells, tumor growth, vascular endothelial growth factor.

Haematologica 2003; 88:176-185
http://www.haematologica.org/2003_02/88176.htm

©2003, Ferrata Storti Foundation

From the Dept. of Biomedical Sciences and Human Oncology (AV, RR, FS, FD) and Human Anatomy and Histology, University of Bari Medical School, I-70124 Bari, Italy (DR), Institute of Human Anatomy, University of Berne Medical School, CH-3000 Berne, Switzerland (VD), Dept. of Biomedical Sciences, Section of Hematology, University of Catania Medical School, Ospedale Ferrarotto, I-95124 Catania, Italy (FDR).

Correspondence: Professor Angelo Vacca, M.D., Department of Biomedical Sciences and Human Oncology, Section of Internal Medicine and Clinical Oncology, Policlinico, Piazza Giulio Cesare, 11 I-70124, Bari, Italy. E-mail: avacca@dimo.uniba.it.

Vascular endothelial growth factor-A (VEGF-A), a VEGF homolog, stimulates angiogenesis and vascular permeability by activating the tyrosine kinase receptor-2 (VEGFR-2 or KDR/Flk-1) and -1 (VEGFR-1 or Flt-1), the latter in synergism with placental growth factor.[1,2] Its homolog VEGF-B exerts overlapping activities by activating VEGFR-1.[3] Homologs VEGF-C and VEGF-D are both angiogenic via VEGFR-2 and VEGFR-3 (or Flt-4), and lymphangiogenic (primarily VEGF-D) via VEGFR-3.[4] The VEGF/VEGFR pathway also activates proliferation, chemotaxis and secretion of cytokines, growth factors, and proteolytic enzymes in tumor cells and their associated stromal (or inflammatory) cells.[5,6] For example, melanoma cells secrete VEGF-C which stimulates both angiogenesis and chemotaxis of macrophages via VEGFR-2;[7] breast carcinoma cells secrete VEGF-C which induces lymphangiogenesis via VEGFR-3 and stromal cells secrete VEGF-A which stimulates tumor growth.[8,9]

In multiple myeloma (MM), VEGF-A is produced by plasma cells.[10,11] It acts as an autocrine inducer of growth and chemotaxis via VEGFR-1[12] and is also thought to be an angiogenesis inducer.[10-13] It stimulates stromal cells via VEGFR-2 to secrete interleukin (IL)-6 and thus forms a paracrine loop for growth.[14] The distribution and function of VEGF homologs and their receptors on bone marrow plasma cells, endothelial cells, and other stromal cells are examined in this paper. The results point to the existence of a paracrine loop in the VEGF pathway that supports tumor angiogenesis and growth in MM.

Design and Methods

Patients and control subjects

Patients who fulfilled the South West Oncology Group (SWOG) diagnostic criteria[15] for MM (n=14) and monoclonal gammopathy of undetermined significance (MGUS) (n=12) were studied at diagnosis. The MM patients (9 M, 5 F) were aged 41-85 years (median = 70.5); 11 had stage IIIA and 3 had stage IIIB[15]; the M-component was IgG in 9, IgA in 3, κ in 2. The MGUS patients (7 M, 5 F) were aged 43-82 years (median = 64.5); 9 had IgG disease and 3 IgA. Control subjects were 12 patients aged 48-82 years (median = 67) with anemia due to iron or vitamin B_{12} deficiencies. The study was approved by the local ethics committee and all patients gave their informed consent.

Cell isolation and preparation of conditioned media (CM)

Plasma cells were obtained by Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) separation from bone marrow aspirates close to the biopsy site, and B-cell enrich-

ment with magnetic microbeads (Dynal, Oslo, Norway) coated with a monoclonal antibody (MoAb) to the plasma cell marker CD38 (Becton Dickinson, Mountain View, CA, USA), as described previously.[16] Cells consisted of >95% tumor plasma cells and their clonally-related cells,[17] as assessed by morphology in May-Grünwald-Giemsa and flow cytometry with the anti-CD38 MoAb (FACSscan, Becton Dickinson), or by immunocytochemical staining with anti-κ or anti-λ polyclonal antibodies (Dako, Glostrup, Denmark) according to the light chain of the M-component. Cells were cultured in serum-free RPMI-1640 medium (SFM) (1×10^7/25 cm^2 flask/6 mL medium) supplemented with 1% glutamine for 24 h in 5% CO_2 humidified atmosphere at 37°C. If the number of harvested plasma cells was lower (as in MGUS or control subjects), cells were cultured in a smaller quantity of medium to preserve the same cell number /medium volume ratio. Cell viability, assessed by trypan blue exclusion was >90%.

The separated mononuclear cell fraction was left to adhere to 25 cm^2 polystyrene flasks in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) and 1% glutamine (complete medium) for 2 h in culture conditions. Adherent cells were stromal cells, i.e., fibroblasts, macrophages, osteoclasts and endothelial cells.[18] To isolate endothelial cells, stromal cells were harvested in trypsin/EDTA solution (0.05/0.02% in phosphate buffered saline [PBS]), washed twice with PBS, suspended at 2×10^7/mL in SFM and incubated for 30 min at 37°C with magnetic microbeads (1×10^7/mL) coated with a rabbit anti-murine IgG antibody (Dynal) and the murine IgG1 MoAb to the endothelial cell marker CD31 (Serotec, UK), in rotation. Microbeads with bound cells were recovered using a side-pull magnetic separation unit, transferred to a 12-well plate or chamber slide (Costar, Cambridge, MA, USA) in 3 mL complete medium/well or slide and left to migrate to the plate/slide surface and grow. The endothelial cell fraction contained >95% $CD31^+$ and factor VIII-related antigen (FVIII-RA, another endothelial marker) cells, as assessed by flow cytometry. Residual stromal cells were cultured as endothelial cells. The trypan blue viability was >90%. Residual stromal cells were cultured in SFM as described for plasma cells to obtain their CM. The two CM were collected, sequentially centrifuged at 1,200 and 12,000 rpm for 10 min respectively, filtered through sterilized 0.22 mm pore-size filters (Costar) and stored at –80°C.

Immunohistochemistry and immunocytochemistry

Blood vessels were detected in 6-mm sections of 4% paraformaldehyde-fixed paraffin-embedded bone marrow biopsies by staining endothelial cells with the anti-FVIII-RA MoAb M616 (Dako) and the anti-VEGFR-2 goat antiserum (R&D Systems Inc., Minneapolis, MN, USA). The very few megakaryocytes, stained by M616 were easily distinguishable by their morphology and size. Angiogenesis was measured as FVIII-RA+ and VEGFR-2+ microvessel areas on 4 to 6×250 fields covering each of two sections per antibody per biopsy by using a planimetric point count method and a computed image analysis described elsewhere.[16] Enriched plasma cells (1 to 2×10^5/slide) were cytospun, fixed in 100% acetone (Merck, Darmstadt, Germany) at –20°C, washed twice in PBS, and stained with anti-VEGF-A and anti-VEGFR-3 rabbit polyclonal antibodies A-20 and C-20 respectively (Santa Cruz Biotechnology Inc., CA, USA). Tissues and cells were red-stained by a three-layer biotin-streptavidin-peroxidase system followed by an aminoethylcarbazole chromogenic solution (Sigma Chemicals Co., St. Louis, MO, USA) and hematoxylin counterstaining as already described.[16]

In situ hybridization for VEGFR mRNA

This was performed as described elsewhere.[19] Deparaffinized biopsy sections, cytospun plasma cells, and endothelial cells or residual stromal cells grown on the chamber slides were hybridized overnight at 50°C with 5 µg/mL of three 5'-biotin-labeled oligonucleotides (Genenco Life Science, Florence, Italy), the first of 90 bases complementary to the sequence 1451-1540 of the VEGFR-1 mRNA,[20] the second of 90 bases complementary to the sequence 3981-4071 of the VEGFR-2 mRNA,[21] and the third of 80 bases complementary to the sequence 3831-3911 of the VEGFR-3 mRNA.[22] The hybridization signal was revealed by alkaline phosphatase activity on a Western-blue-stabilized substrate (Promega Co., Madison, WI, USA).

Antibodies and Western blot

Rabbit antisera to VEGFR-1 and the phosphorylated form of the mitogen-activated protein (MAP) extracellular signal-regulated protein kinase-2 (ERK-2, Santa Cruz Biotechnology Inc., CA, USA), those to VEGF-A, VEGFR-2 and VEGFR-3 just described, goat antisera to VEGF-C and VEGF-D (R&D Systems Inc.), and anti-rabbit or anti-goat horseradish peroxidase-labeled antisera (Chemicon International Inc., Temecula, CA, USA) were used for the Western blotting, which was performed as described elsewhere.[23] Briefly, 50 mg proteins of total cell extracts and 10 mg proteins of the CM were measured with the Bradford method (Bio-Rad Laboratories, Richmond, CA, USA) and subjected to 8% sodium dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Gels were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (NEN™, Life Science, Boston, MA, USA), which was incubated for 1 h in 5% non-fat dry milk in tris(hydroxymethyl)-aminomethane buffer saline (TBS) with

© Ferrata Storti Foundation

0.1% Tween 20, washed in the same buffer, and incubated with the primary and secondary antibodies for 1 h at room temperature. After washing in TBS-Tween, the membrane was incubated with enhanced chemiluminescence (NEN™ Life Science), and the signal revealed by exposure to Kodak Biomax film (Eastman Kodak Company, Rochester, NY, USA). The intensity of bands was expressed as arbitrary optical density (OD) units by reading with Fluorad (Bio-Rad Laboratories).

Enzyme-linked immunosorbent assay (ELISA) for VEGF homologs

Proteins (100 μg/100 μL) of plasma cell, endothelial cell, and residual-stromal cell CM were measured with the Bradford method and tested in triplicate for VEGF-A, VEGF-B and VEGF-D levels by applying a sandwich ELISA (Quantikine Human VEGF-A, VEGF-B and VEGF-D, R&D Systems Inc.), according to the manufacturer's instructions. VEGF-C levels were quantified by the same ELISA: 96-well plates were coated with a goat MoAb to VEGF-C (R&D Systems Inc.) and sequentially loaded with the CM proteins, the horseradish peroxidase-labeled anti-VEGF-C antibody and a tetramethylbenzidine chromogenic solution (R&D Systems Inc.). The colorimetric reaction was read with a Benchmark Microplate Reader (Bio-Rad Laboratories) at 450 nm with λ correction at 540 nm, and referred to a calibration curve set up with known concentrations of VEGF-C (Sigma Chemical Co.) versus those of bovine serum albumin (BSA) as control.

Cell proliferation and chemotaxis assays

The proliferation of enriched plasma cells was determined in each MM patient by using ^{3}[H]thymidine incorporation in short-term cultures.[24] Two $\times 10^5$ cells/mL in complete culture medium (RPMI-1640 medium with added 10% FCS and 1% glutamine - positive control) or in SFM alone (negative control) or in SFM supplemented 1:1 (vol:vol) with residual-stromal cell CM, or with 1 μg/mL of VEGF-C or VEGF-D (Sigma Chemical Co.) were pulse-labeled with 7.4 kBq/well [^{3}H]thymidine (specific activity 70 GBq/mMol; Amersham, Bucks, UK) for 8 h before harvesting. After 72 h cells were harvested automatically, transferred onto filter papers and washed three times. The incorporated radioactivity was measured with a β-scintillation counter (Beckman). For inhibition tests, the experimental SFM were admixed with 5 μg/mL of the anti-VEGF-C, anti-VEGF-D or anti-VEGFR-3 polyclonal antibodies (R&D Systems Inc.). Since the anti-VEGF-C and anti-VEGFR-3 antisera are not described by the manufacturer as having blocking activity, we tested this activity in preliminary experiments in which inhibition of phosphorylation of the VEGFR-3^{+} IM9 MM cell line was obtained with exposure to VEGF-C + anti-VEGF-C or to VEGF-C + anti-VEGFR-3 (*data not shown*).

Proliferation of endothelial cells and residual-stromal cells was evaluated by seeding 5×10^3 cells/well in triplicate on 96-well polystyrene plates in the same control media or in SFM admixed 1:1 with the plasma cell CM or with 1 μg/mL of VEGF-A (Sigma Chemical Co.). In the inhibition tests, these SFM were admixed with 5 μg/mL of the anti-VEGF-A, or anti-VEGFR-2 (endothelial cells) or anti-VEGFR-1 (residual-stromal cells) antisera (R&D Systems Inc.). The inhibitory activity of anti-VEGF-A and anti-VEGFR-1 antisera was assessed in preliminary experiments in which the VEGFR-1^{+} EA.hy926 endothelial cell line gave inhibition of phosphorylation when exposed to VEGF-A + anti-VEGF-A and to VEGF-A + anti- VEGFR-1 (*data not shown*). Cells were counted after 48 h by the crystal violet colorimetric method reported by Kueng *et al.*,[25] with slight modifications.[16] The media from residual-stromal cells exposed to the plasma cell CM were harvested for VEGF-C and VEGF-D measurement. Chemotaxis of endothelial cells and residual-stromal cells and inhibition experiments of this cell function were determined with the Boyden chamber technique described previously.[16] Accordingly, 1×10^5 cells suspended in 400 μL of DMEM 0.1% BSA were seeded in triplicate in the upper compartment of the chamber, and 200 mL of the media used in the proliferation assay were placed in the lower compartment, which was separated from the former by a 12-μm polycarbonate (polyvinylpyrrolidone free) filter (Costar) precoated with 0.1% gelatin. After 6 h of incubation at 37°C, cells on the upper side of the filter were removed by scraping, whereas those that had migrated to the lower side were fixed (absolute ethanol), stained (toluidine blue, Merk), and counted in ten oil × 400 immersion fields.

VEGFR and ERK-2 phosphorylation and inhibition

Plasma cells, endothelial cells or residual-stromal cells were cultured in SFM alone or admixed with their CM or VEGF as described in the proliferation assay. After 5 min at 37°C, cells were lysed for 20 min in 1 mL of ice-cold lysis buffer,[23] and centrifuged at 12,000 rpm for 15 min. Lysates were partly (50 μg proteins) tested in Western blots with the rabbit antiserum to phosphorylated ERK-2, and remaining aliquots incubated for 2 h at 4°C with rabbit preimmune serum (Santa Cruz Biotechnology Inc., CA, USA) and 50 μL of a 50% Protein A-Sepharose slurry (Sigma Chemicals Co.). Supernatants were removed and incubated overnight at 4°C with anti-VEGFR antisera and Protein A-Sepharose beads. Immunoprecipitates were washed in 20 mM HEPES,

©Ferrata Storti Foundation

pH 7.4, 137 mM NaCl, 1% Triton X-100, 10% glycerol (HNTG) buffer and tested in Western blots with an anti-phosphotyrosine MoAb (Chemicon International Inc.). In inhibition experiments, cells were treated as for inhibition of proliferation.

Results

Expression and secretion of VEGF-A by plasma cells

Immunohistochemistry on bone marrow biopsies with the anti-VEGF-A antibody showed intense expression by plasma cells from all 14 MM patients (Figure 1A, B). Secretion of VEGF-A by plasma cells was found by Western blot of their CM (Figure 1C) where levels were 1567±313 pg/mL by ELISA. This expression was very low in 8 out of 10 (80%) MGUS and in 8 out of 12 (66%) control subjects. Accordingly, the VEGF-A levels in their plasma cell CM were 170±39 and 68±13, respectively (p < 0.01 or better; Fisher and Kruskal-Wallis test followed by paired Duncan (t), Bonferroni (t) and Wilcoxon tests). Only marginal levels of VEGF-B, VEGF-C and VEGF-D were found (Figure 1C).

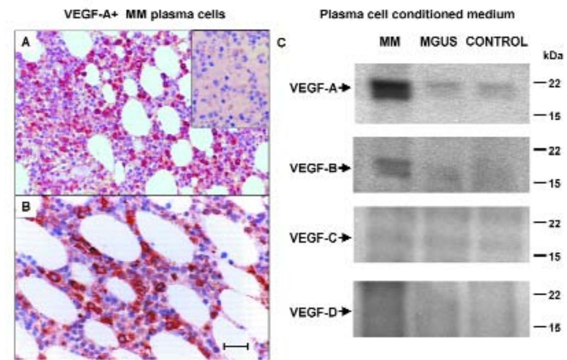

Figure 1. (A), (B) Staining with VEGF-A of bone marrow from a patient with MM. Insert: the negative control obtained with rabbit preimmune serum replacing the VEGF-A antibody. Bar = (A) 55 mm; (B) 40 mm. (C) Western blot analysis for VEGF homologs of plasma cell CM from a representative MM patient (out of 14 patients), MGUS patient (out of 12 patients) and control subject (out of 12 subjects).

©Ferrata Storti Foundation

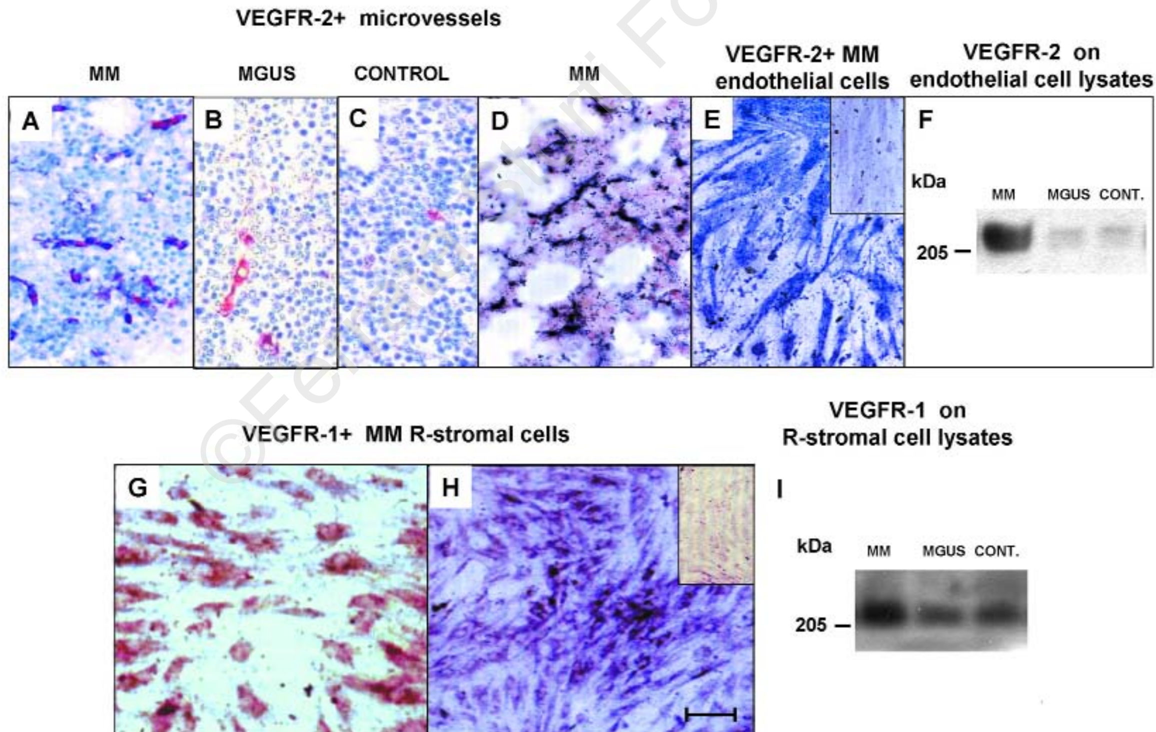

Figure 2. Staining with VEGFR-2 of bone marrow from a patient with MM (A) showing numerous, thin microvessels, (B) from a MGUS patient and (C) from a control subject showing very few microvessels. *In situ* hybridization for VEGFR-2 mRNA highlighting (D) neovessels and (E) enriched endothelial cells of the MM patient. *Insert*: RNAse-treated cells as the negative control. (F) Western blot analysis for the VEGFR-2 of endothelial cell lysates from the patients with MM, MGUS and the control subject showing enhanced expression in MM (bands' OD = MM 9.5 $\times 10^3$; MGUS 1.1 $\times 10^3$; control 1.3 $\times 10^3$). (G) Immunocytochemical staining with VEGFR-1 and (H) *in situ* hybridization for VEGFR-1 mRNA of residual-stromal cells from the patient with MM. *Insert*: RNAse-treated cells as the negative control. (I) Western blot analysis for the VEGF[R]-1 of residual-stromal cell lysates from the patient with MM (G, H) and the patient with MGUS and the control subject. The expression of VEGF[R]-1 is higher in MM (bands' OD = MM 6.8 $\times 10^3$; MGUS 2.1 $\times 10^3$; control 3.4 $\times 10^3$). The Western blot pictures refer to representative cases out of 14 patients with MM, 12 patients with MGUS and 12 control subjects. Bar: (A), (B), (C) 40 μm; (E), (H) 22 μm; (G) 12 μm.

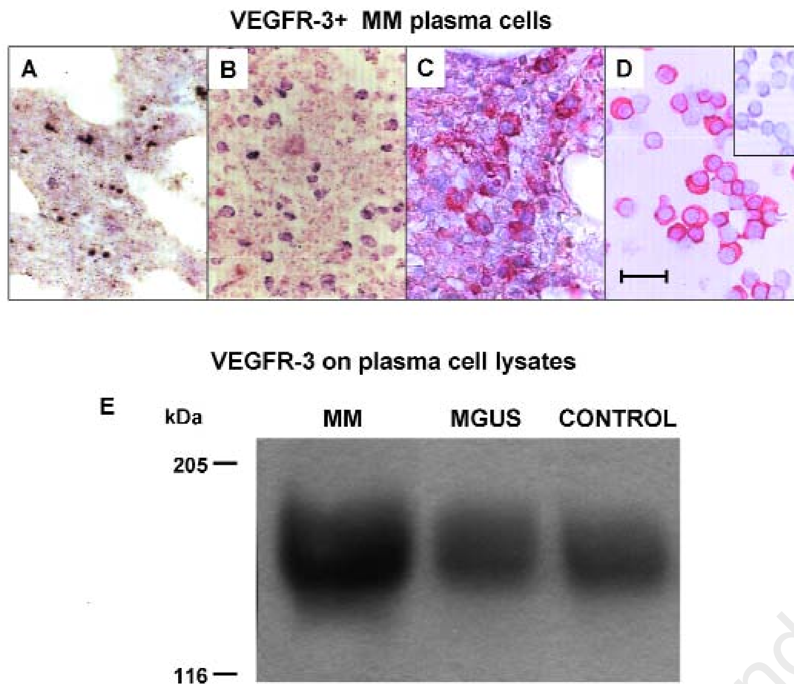

Figure 3. (A), (B) *In situ* hybridization for VEGFR-3 mRNA of bone marrow from two patients with MM and staining with VEGFR-3 of (C) bone marrow and (D) enriched plasma cells of the patient (B). *Insert*: the negative control obtained with rabbit preimmune serum replacing the VEGFR-3 antibody. Bar: (A) 40 μm; (B), 22 μm (C), (D) 16 μm. (E) Western blot analysis for the VEGFR-3 of plasma cell lysates from the patient (B), a patient with MGUS and a control subject. The VEGFR-3 expression is higher in MM (bands' OD = MM 10.6×10³; MGUS 2.2×10³; control 2.6×10³). Western blot pictures refer to representative cases out of 14 patients with MM, 12 patients with MGUS and 12 control subjects.

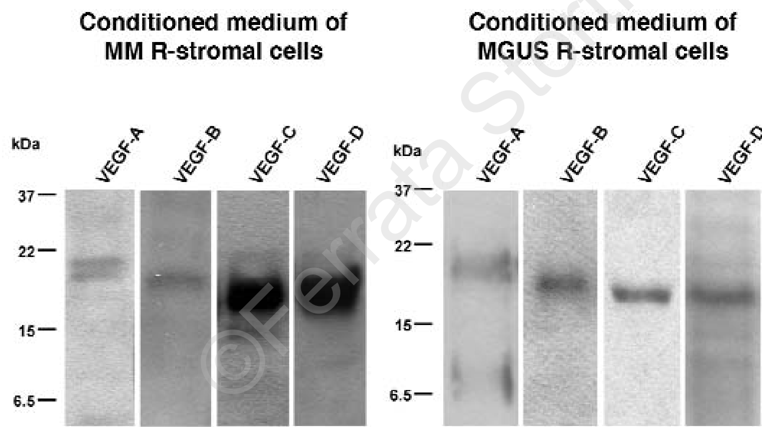

Figure 4. Western blot for VEGF homologs of CM of residual[R]-stromal cells from a representative patient with MM (out of 14) and another with MGUS (out of 12). Note the pronounced presence of VEGF-C and VEGF-D in MM.

Expression of VEGFR-2 by microvascular endothelial cells

In the MM patients, VEGFR-2 immunohistochemical staining of biopsy sections adjacent to those just described highlighted microvessels (Figure 2A) whose area was 0.38±0.22 mm² × 10⁻². Overlapping areas (0.45±0.27) were obtained by FVIII-RA staining. VEGFR-2 expression by microvascular endothelial cells was confirmed both on sections (Figure 2D) and enriched cells (Figure 2E) by mRNA *in situ* hybridization and by Western blot of the translated protein (Figure 2F; 9.8±2.5 ×10³ OD). In the MGUS patients, the two microvessel areas shrank significantly (0.07±0.03 and 0.09±0.02, $p < 0.01$; same paired tests), and were virtually equal to those of the control subjects (0.07±0.02 and 0.08±0.01) (Figure 2B, C). VEGFR-2 expression by endothelial cells was thus marginal (Figure 2F; MGUS: 1.3±0.7 × 10³ OD; control subjects: 1.2±1 × 10³ OD; $p < 0.01$ or better, compared with MM; same paired tests). VEGFR-1 and VEGFR-3 expression was extremely weak (*data not shown*).

©Ferrata Storti Foundation

Table 1. Effect of plasma cell conditioned media (CM) on proliferation and chemotaxis of endothelial cells and residual stromal cells in the 14 patients with multiple myeloma.

Sample	*Endothelial cells*		*Residual-stromal cells*	
	×10³	*Migrated cells*	*×10³*	*Migrated cells*
Starvation serum-free medium (SFM) (negative control)	5.2±1.1	1±1	5.8±1.6	1±1
Complete medium (positive control)	37.9±5.7#	121±25#	40.6±3.5#	141±34#
SFM + plasma cell CM	22.1±4.0#	68±18#	32.1±4.8#	104±22#
SFM+ VEGF-A	13.3±6.4†	33±13†	21.5±3.0‡	63±16‡
SFM + plasma cell CM + anti-VEGF-A	9.3±1.6	20±11	13.2±3.9	51±18
SFM + VEGF-A + anti-VEGF-A	6.1±1.2	12±4	7.6±2.2	8.7±5.1
SFM + plasma cell CM + anti-VEGFR-2	11.3±2.2	27±9	nt	nt
SFM + VEGF-A + anti-VEGFR-2	12.4±2.7	21±8	nt	nt
SFM + plasma cell CM + anti-VEGFR-1	nt	nt	15.2±2.6	41±12
SFM + VEGF-A + anti-VEGFR-1	nt	nt	16.8±2.4	46±16

For proliferation assay, low-density cultures (5×10³ cells/well) of endothelial cells and residual stromal cells were exposed to the indicated control or experimental media and counted after 48 h. For chemotaxis assay, 1×10⁵ cells were seeded to the upper compartment of Boyden chambers, whereas the media were added to the lower compartment. Cells which had migrated to the lower surface of a gelatin-coated filter were counted after 6 h. Results are expressed as mean ± 1 SD of three determinations per patient in the whole patient group. †p < 0.05, ‡p < 0.03 and #p < 0.01 versus the negative control (Wilcoxon's rank test). nt = not tested.

Expression of VEGFR-1 by residual-stromal cells

In all MM patients, both immunocytochemical staining and the *in situ* hybridization for VEGFR-1 mRNA of residual stromal cells showed intense expression (Figure 2G, H), which was confirmed at the protein level by Western blot (Figure 2I; 7±3.1 ×10³ OD), whereas it was faint in MGUS and control subjects (Figure 2I; MGUS: 2.3±1.1 ×10³ OD; control subjects: 3.2±1.8×10³ OD; $p < 0.01$). The expression of VEGFR-2 and VEGFR-3 was always weak or absent when assessed by Western blotting (*data not shown*).

Expression of VEGFR-3 by plasma cells

Intense expression of VEGFR-3 mRNA by plasma cells of all MM patients was shown by *in situ* hybridization (Figure 3A, B). VEGFR-3 protein was also detected by immunohistochemistry of biopsies (Figure 3C) and cytospun cells (Figure 3D), and by Western blot of cell lysates (Figure 3E; 9.6±3.3 × 10³ OD). It was significantly lower in the MGUS and control groups, as evaluated by Western blot (Figure 3E; MGUS: 2.9±1.5×10³ OD; control subjects: 2.3±1.4×10³ OD; $p < 0.01$).

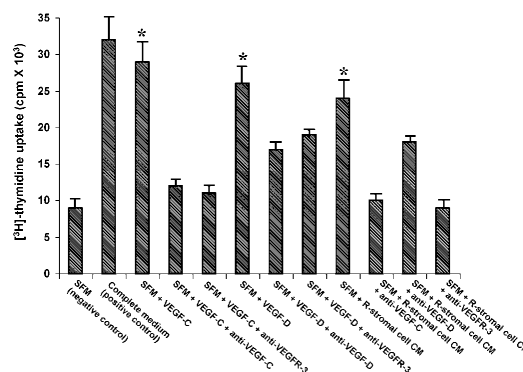

Figure 5. Effect of residual [R]-stromal cell CM on plasma cell [3H]thymidine incorporation in the 14 MM patients. Cells (2×10⁵ cells/mL) in suspension were incubated in starvation serum-free medium (SFM, negative control), complete medium (positive control), or in SFM admixed with the CM of residual-stromal cells or with the indicated VEGF homologs and the respective antibodies and pulse-labeled with 7.4 kBq /well [³H]thymidine. After 72 h the incorporated radioactivity was measured. Results are given as mean ± 1 SD in a triplicate experiment per patient in the whole patient group. *$p < 0.01$ by Wilcoxon rank test compared to the negative control.

Secretion of VEGF-C and VEGF-D by residual-stromal cells

VEGF secretion was assessed in the CM of MM residual stromal cells. By using Western blot, only VEGF-C and VEGF-D were intense (Figure 4). Their ELISA levels were 841±123 and 492±51 pg/mL protein and rose to 1122 + 215 and 570 + 84 after cell exposure to plasma cell CM. In contrast, levels of VEGF-A and VEGF-B were 136±42 and 122±25. The CM of MGUS and control subjects gave comparable low amounts (Figure 4): 50-140 pg/mL for VEGF-A, 54-205 for VEGF-B, 120-305 for VEGF-C and 85-160 for VEGF-D.

Functional studies

To mimic functional events within the bone marrow microenvironment, endothelial and residual stromal cells were exposed to the plasma cell CM, and plasma cells to the residual stromal cell CM. Both endothelial and residual stromal cells displayed a significant ($p < 0.01$; Wilcoxon rank test) increase

©Ferrata Storti Foundation

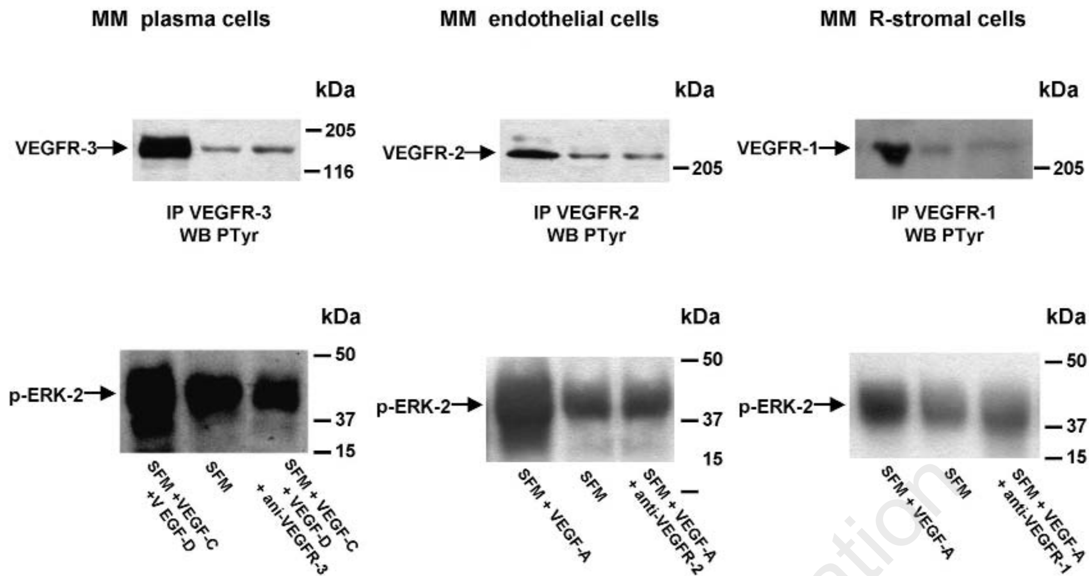

Figure 6. Induction of activated VEGFRs in MM plasma cells, endothelial cells and residual [R] stromal cells by interaction of VEGFRs with the indicated VEGF homologs. Cells were cultured in SFM alone or admixed with the VEGF homologs. After 5 min at 37°C, cells were lysed, the extracts immunoprecipitated (IP) with the indicated anti-VEGFR antibody, and the immunoprecipitates were analyzed by Western blot with anti-phosphotyrosine antibody (WB PTyr). ERK-2 phosphorylation (p-ERK-2) in cells incubated with the VEGF homologs and its lowering with SFM or addition of anti-VEGFRs antibodies. Similar phosphorylation events were obtained when the VEGF homologs were replaced by the residual stromal cell CM (in plasma cells) and by the plasma cell CM (in endothelial and residual-stromal cells), or when the anti-VEGFRs antibodies were replaced by the anti-VEGF homolog antibodies (*lanes not shown*).

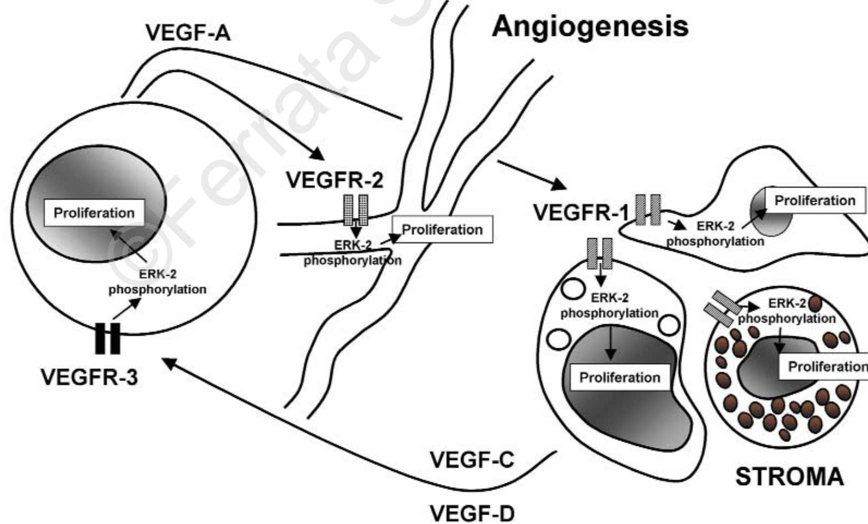

Figure 7. The hypothetical paracrine pathway of VEGF for blood vessel formation and plasma cell growth within the bone marrow of MM. VEGF-A is prevalently produced by plasma cells and stimulates proliferation and chemotaxis of endothelial cells (both necessary for angiogenesis) via VEGFR-2, and of residual stromal cells via VEGFR-1. Activation of these cells results in VEGF-C and VEGF-D production that stimulates plasma cell growth via VEGFR-3. Proliferation of all cell types parallels phosphorylation of VEGFRs and ERK-2.

©Ferrata Storti Foundation

in proliferation and chemotaxis when exposed to SFM plus the plasma cell CM versus the SFM alone (Table 1). This increase was less marked when the SFM was supplemented with VEGF-A, probably because the CM contains additional growth and chemotactic factors, such as fibroblast growth factor-2 (FGF-2)[16] and hepatocyte growth factor.[26] Neither proliferation nor chemotaxis, in fact, was fully abolished when the anti-VEGF-A antibody was added to the CM, whereas abolition occurred when the antibody was added to the SFM admixed with the VEGF-A only. Similar results were obtained in endothelial cells when the anti-VEGFR-2 antibody was added to the plasma cell CM or to VEGF-A, and in residual stromal cells with the anti-VEGFR-1 antibody (Table 1).

We evaluated, in short-term cultures,[24] the $[^3H]$-thymidine incorporation by plasma cells stimulated with residual stromal cell CM, VEGF-C or VEGF-D. As shown in Figure 5, the SFM containing the residual stromal cell CM led to a significant increase in DNA synthesis rate when compared with negative control. This increase was reduced by the anti-VEGF-C and, to a lesser extent, by anti-VEGF-D antibodies. A slightly weaker stimulus was given by the SFM containing the VEGF-C or VEGF-D, once again because the CM may contain additional plasma cell growth factors, such as IL-6,[27] granulocyte macrophage-colony stimulating factor,[28] and FGF-2.[29] The DNA synthesis rate also reduced when either the CM or VEGF-C and VEGF-D were admixed with the anti-VEGFR-3 antibody.

VEGFR and ERK-2 phosphorylation and inhibition

Engagement of a VEGFR promotes its activation and phosphorylation, followed by the recruitment and phosphorylation of several proteins,[30,31] and activation of intracellular signaling pathways.[32] To determine whether this was the case in our study, the endothelial and residual stromal cells of each MM patient were exposed for 5 min to the plasma cell CM or VEGF-A, and their plasma cells to the residual stromal cell CM or VEGF-C and VEGF-D, either without or with the relative anti-VEGFs or anti-VEGFRs neutralizing antibodies. As shown in Figure 6, VEGFR-1 of residual stromal cells, VEGFR-2 of endothelial cells and VEGFR-3 of plasma cells were rapidly phosphorylated after interaction with their VEGF homologs. The transduction pathways of the VEGFR signal led to ERK-2 phosphorylation. That phosphorylation of VEGFR and ERK-2 follows the VEGF/VEGFR interaction was proved by its inhibition when anti-VEGF or anti-VEGFR neutralizing antibodies were added.

Discussion

Here we show (outlined in Figure 7) a previously undescribed paracrine loop for angiogenesis and tumor growth in the bone marrow of MM patients. Plasma cells secrete VEGF-A and this induces proliferation and chemotaxis in endothelial cells (both being mandatory for the development of angiogenesis)[33] through VEGFR-2, a VEGF-A receptor prevalently expressed on these cells. VEGF-A has so far been regarded as an angiogenesis inducer in MM.[10-13] Conversely, secretion of VEGF-A and expression of VEGFR-2 were very low (or inconstant) in MGUS and the controls. Accordingly, the VEGFR-2$^+$ and FVIII-RA+ microvessel areas are larger in MM patients. This offers a functional confirmation of the vascular phase in the MM bone marrow, as previously demonstrated by others[34-37] and ourselves.[16,38] Together with VEGF-A, the synergistic[39] FGF-2 (also secreted by plasma cells)[16] is directly involved in induction of this phase. Because VEGFR-2 mediates major angiogenic and permeability actions of VEGF-A,[40] its expression together with that of aquaporin 1 by endothelial cells in MM[41] suggests that neovessels are hyperpermeable, which implies increased interstitial pressure, hypoxia and further stimulation of VEGF-A secretion.

Plasma cell VEGF-A also stimulates the proliferation and chemotaxis of residual stromal cells via VEGFR-1, which is prevalently expressed on these cells. Bellamy *et al.*[10] have shown that bone marrow stromal cells express VEGFR-2. This has been confirmed by Dankbar *et al.*,[14] who have also shown IL-6 secretion following the VEGF-VEGFR-2 interaction, which suggests another paracrine loop for plasma cell growth. Here we split stromal cells into endothelial cells and residual stromal cells and found that expression of VEGFR-2 and VEGFR-1 was confined to these populations, respectively. In MM the residual stromal cells secreted VEGF-C and VEGF-D abundantly into their CM. This occurred constitutively and after cell exposure to plasma cell CM. Both homologs are ligands of VEGFR-3, a receptor intensely expressed on plasma cells. Since antibodies to VEGF-C and less so to VEGF-D reduce proliferation of plasma cells exposed to the CM, both homologs are suggested to stimulate plasma cell proliferation via VEGFR-3. At variance with MM, secretion of VEGF-C and VEGF-D and expression of VEGFR-3 are very weak in MGUS and control subjects. To date, plasma cells have been shown to express VEGFR-1,[12] and VEGFR-2 only slightly,[14] and proliferate in response to VEGF. Overall data suggest a wide expression of VEGFRs by plasma cells and full opportunities to proliferate in response to the array of VEGF homologs. Much in the same way as MM, autocrine and paracrine proliferative responses to the VEGF-VEGFR interaction have been demonstrated in leukemic cells.[42,43]

We show that proliferation of endothelial cells, residual stromal cells and plasma cells linked to VEGFR-2, VEGFR-1 and VEGFR-3 triggering paral-

© Ferrata Storti Foundation

lels ERK-2 phosphorylation which, in fact, mediates DNA synthesis.[44] Podar *et al.*[12] have described an autocrine VEGF-VEGFR-1 loop for plasma cell growth and migration involving ERK-1/ERK-2 and protein kinase C (PKC) phosphorylation, respectively. VEGF-induced migration following β_1-mediated adhesion to fibronectin also involves PKC.[45]

To sum up, our data suggest that a paracrine loop for tumor angiogenesis and growth is operative in MM. Its afferent arm is plasma cell VEGF-A and endothelial cell VEGFR-2/residual stromal cell VEGFR-1. The efferent arm is residual stromal cell VEGF-C/VEGF-D and plasma cell VEGFR-3. In MGUS (and control subjects) this loop is down-regulated and perhaps functional at a very low *tone*. As in solid tumors,[46-48] progression to MM[49] parallels overexpression of VEGF-A, which induces angiogenesis (and further plasma cell growth as evaluated by the labeling index)[38] and residual stromal cell activation, the latter resulting in VEGF-C/VEGF-D overexpression, and ultimately in growth. Thus, progression enhances the functional *tone* of the loop which supports progression itself. This hypothesis is in line with the higher serum VEGF levels in patients with MM than in those with MGUS, and the correlation of VEGF with worse prognosis.[50,51] Our data also suggest that VEGF and its signaling proteins (particularly ERK) may be appropriate targets[52] in the management of MM.

References

1. Ferrara N. Role of vascular endothelial growth factor in regulation of physiological angiogenesis. Am J Physiol Cell Physiol 2001;280:C1358-C66.
2. Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M, et al. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat Med 2001;7:575-83.
3. Olofsson B, Jeltsch M, Eriksson U, Alitalo K. Current biology of VEGF-B and VEGF-C. Curr Opin Biotechnol 1999;10:528-35.
4. Witmer AN, Van Blijswijk BC, Dai J, Hofman P, Partanen TA, Vrensen GF, et al. VEGFR-3 in adult angiogenesis. J Pathol 2001;195:490-7.
5. Larrivee B, Karsan A. Signaling pathways induced by vascular endothelial growth factor. Int J Mol Med 2000;5:447-56.
6. Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J. Vascular-specific growth factors and blood vessel formation. Nature 2000; 407:242-8.
7. Skobe M, Hamberg LM, Hawighorst T, Schirner M, Wolf GL, Alitalo K, et al. Concurrent induction of lymphangiogenesis, angiogenesis, and macrophage recruitment by vascular endothelial growth factor-C in melanoma. Am J Pathol 2001;159:893-903.
8. Skobe M, Hawighorst T, Jackson DG, Prevo R, Janes L, Velasco P, et al. Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. Nat Med 2001;7:192-8.
9. Makinen T, Jussila L, Veikkola T, Karpanen T, Kettunen MI, Pulkkanen KJ, et al. Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. Nat Med 2001;7:199-205.
10. Bellamy WT, Richter L, Frutiger Y, Grogan TM. Expression of vascular endothelial growth factor and its receptors in hematopoietic malignancies. Cancer Res 1999;59:728-33.
11. Bellamy WT. Expression of vascular endothelial growth factor and its receptors in multiple myeloma and other hematopoietic malignancies. Semin Oncol 2001;28:551-9.
12. Podar K, Tai YT, Davies FE, Lentzsch S, Sattler M, Hideshima T, et al. Vascular endothelial growth factor triggers signaling cascades mediating multiple myeloma cell growth and migration. Blood 2001;98:428-35.
13. Di Raimondo F, Azzaro MP, Palumbo G, Bagnato S, Giustolisi G, Floridia P, et al. Angiogenic factors in multiple myeloma: higher levels in bone marrow than in peripheral blood. Haematologica 2000;85:800-5.
14. Dankbar B, Padro T, Leo R, Feldmann B, Kropff M, Mesters RM, et al. Vascular endothelial growth factor and interleukin-6 in paracrine tumor-stromal cell interactions in multiple myeloma. Blood 2000;95:2630-6.
15. Durie BGM, Salmon SE. Multiple myeloma, macroglobulinemia and monoclonal gammapathies. In: Hoffbrand AV, Brain MC, Hirsh J, editors. Recent Advances in Hematology. New York, NY: Churchill Livingstone; 1977. p. 243-61.
16. Vacca A, Ribatti D, Presta M, Minischetti M, Iurlaro M, Ria R, et al. Bone marrow neovascularization, plasma cell angiogenic potential and matrix metalloproteinase-2 secretion parallel progression of human multiple myeloma. Blood 1999;93:3064-73.
17. Billadeau D, Ahmann G, Greipp P, Van Ness B. The bone marrow of multiple myeloma patients contains B-cell populations at different stages of differentiation that are clonally related to malignant plasma cells. J Exp Med 1993;178:1023-31.
18. Dominici M, Campioni D, Lanza F, Luppi M, Barozzi P, Pauli S, et al. Angiogenesis in multiple myeloma: correlation between in vitro endothelial colonies growth (CFU-Em) and clinical-biological features. Leukemia 2001;15:171-6.
19. Valdembri D, Serini G, Vacca A, Ribatti D, Bussolino F. In vivo activation of JAK2/STAT-3 pathway during angiogenesis induced by GM-CSF. FASEB J 2002;16:225-7.
20. Herley MT, Yu Y, Whitney RG, Sato JD. Characterization of the VEGF binding site on the Flt-1 receptor. Biochem Biophys Res Commun 1999;262:731-8.
21. Terman BI, Dougher-Vermazen M, Carrion ME, Dimitrov D, Armellino DC, Gospodarowicz D, et al. Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. Biochem Biophys Res Commun 1992;187:1579-86.
22. Aprelikova O, Pajusola K, Partanen J, Armstrong E, Alitalo R, Bailey SK, et al. FLT4, a novel class III receptor tyrosine kinase in chromosome 5q33-qter. Cancer Res 1992;52:746-8.
23. Vacca A, Ria R, Presta M, Ribatti D, Iurlaro M, Merchionne F, et al. $\alpha V\beta 3$ integrin engagement modulates cell adhesion, proliferation, and protease secretion in human lymphoid tumor cells. Exp Hematol 2001;29:993-1003.
24. Klein B, Zhang XG, Jourdan M, Content J, Houssiau F, Aarden L, et al. Paracrine rather than autocrine regulation of myeloma-cell growth and differentiation by interleukin-6. Blood 1989;73:517-26.
25. Kueng W, Silber E, Eppenberger U. Quantification of cells cultured on 96-well plates. Anal Biochem 1989;182:16-9.
26. Borset M, Seidel C, Hjorth-Hansen H, Waage A, Sundan A. The role of hepatocyte growth factor and its receptor c-Met in multiple myeloma and other blood malignancies. Leuk Lymphoma 1999;32:249-56.
27. Barillé S, Bataille R, Amiot M. The role of interleukin-6 and interleukin-6/interleukin-6 receptor-α complex in the pathogenesis of multiple myeloma. Eur Cytokine Netw 2000; 11:546-51.
28. Klein B. Cytokine, cytokine receptors, transduction signals, and oncogenes in human multiple myeloma. Semin Hematol 1995;32:4-19.
29. Ribatti D, Vacca A, Nico B, Crivellato E, Roncali L, Dammacco F. The role of mast cells in tumour angiogenesis. Br J Haematol 2001;115:514-21.
30. Seetharam L, Gotoh N, Maru Y, Neufeld G, Yamaguchi S, Shibuya M. A unique signal transduction from FLT tyrosine kinase, a receptor for vascular endothelial growth factor (VEGF). Oncogene 1995;10:135-47.
31. Kroll J, Waltenberger J. The vascular endothelial growth factor receptor KDR activates multiple signal transduction pathways in porcine aortic endothelial cells. J Biol Chem 1997;272:32521-7.
32. Ortega N, L'Faqihi FE, Plouet J. Control of vascular endothelial growth factor angiogenic activity by the extracellular matrix. Biol Cell 1998;90:381-90.
33. Folkman J, Browder T, Palmblad J. Angiogenesis research: guidelines for translation to clinical application. Thromb Haemost 2001;86:23-33.
34. Rajkumar SV, Mesa RA, Fonseca R, Schroeder G, Plevak MF, Dispenzieri A, et al. Bone marrow angiogenesis in 400 patients with monoclonal gammopathy of undetermined significance, multiple myeloma, and primary amyloidosis. Clin Cancer Res 2002;8:2210-6.

©Ferrata Storti Foundation

35. Sezer O, Niemoller K, Kaufmann O, Eucker J, Jakob C, Zavrski I, et al. Decrease of bone marrow angiogenesis in myeloma patients achieving a remission after chemotherapy. Eur J Haematol 2001;66:238-44.
36. Laroche M, Brousset P, Ludot I, Mazieres B, Thiechart M, Attal M. Increased vascularization in myeloma. Eur J Haematol 2001;66:89-93.
37. Moehler M, Neben K, Ho D, Goldschmidt H. Angiogenesis in hematologic malignancies. Ann Hematol 2001;80:695-705.
38. Vacca A, Ribatti D, Roncali L, Ranieri G, Serio G, Silvestris F, et al. Bone marrow angiogenesis and progression in multiple myeloma. Br J Haematol 1994;87:503-8.
39. Ribatti D, Nico B, Morbidelli L, Donnini S, Ziche M, Vacca A, et al. Cell-mediated delivery of fibroblast growth factor-2 and vascular endothelial growth factor onto the chick chorioallantoic membrane: endothelial fenestration and angiogenesis. J Vasc Res 2001;38:389-97.
40. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 1995;376:62-6.
41. Vacca A, Frigeri A, Ribatti D, Nicchia GP, Nico B, Ria R, et al. Microvessel overexpression of aquaporin 1 parallels bone marrow angiogenesis in patients with active multiple myeloma. Br J Haematol 2001;113:415-21.
42. Fiedler W, Graeven U, Ergun S, Verago S, Kilic N, Stockschlader M, et al. Vascular endothelial growth factor, a possible paracrine growth factor in human acute myeloid leukemia. Blood 1997;89:1870-5.
43. Dias S, Choy M, Alitalo K, Rafii S. Vascular endothelial growth factor (VEGF)-C signaling through FLT-4 (VEGFR-3) mediates leukemic cell proliferation, survival, and resistance to chemotherapy. Blood 2002;99:2179-84.
44. Dudley DT, Pang L, Decker SJ, Bridges AJ, Saltiel AR. A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc Natl Acad Sci USA 1995;92:7686-9.
45. Podar K, Tai YT, Lin BK, Narsimhan RP, Sattler M, Kijima T, et al. Vascular endothelial growth factor (VEGF) -induced migration of multiple myeloma cells is associated with β1-integrin- and PI3-kinase-dependent PKCα activation. J Biol Chem 2002;277: 7875-81.
46. Guidi AJ, Abu-Jawdeh G, Berse B, Jackman RW, Tognazzi K, Dvorak HF, et al. Vascular permeability factor (vascular endothelial growth factor) expression in cervical neoplasia. J Natl Cancer Inst 1995;87:1237-45.
47. Abu-Jawdeh G, Faix JD, Niloff J, Tognazzi K, Manseau E, Dvorak HF, et al. Strong expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in ovarian borderline and malignant neoplasm. Lab Invest 1996;74:1105-15.
48. Chow NH, Hsu PI, Lin XZ, Yang HB, Chan SH, Cheng KS, et al. Expression of vascular endothelial growth factor in normal liver and hepatocellular carcinoma: an immunohistochemical study. Hum Path 1997;28:698-703.
49. Zhan F, Hardin J, Kordsmeier B, Bumm K, Zheng M, Tian E, et al. Global gene expression profiling of multiple myeloma, monoclonal gammopathy of undetermined significance, and normal bone marrow plasma cells. Blood 2002;99:1745-57.
50. Sezer O, Jacob C, Euker J, Niemoller K, Gatz F, Wernecke K, et al. Serum levels of the angiogenic cytokines basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) in multiple myeloma. Eur J Haematol 2001;66:83-8.
51. Iwasaki T, Hamano T, Ogata A, Hashimoto N, Kitano M, Kakishita E. Clinical significance of vascular endothelial growth factor and hepatocyte growth factor in multiple myeloma. Br J Haematol 2002;116:796-802.
52. Hideshima T, Chauhan D, Podar K, Schlossman RL, Richardson P, Anderson KC. Novel therapies targeting the myeloma cell and its bone marrow microenvironment. Semin Oncol 2001;28:607-12.

Pre-publication Report & Outcomes of Peer Review

Contributions

AV was primarily responsible for this paper, from conception to submitted manuscript. The other authors, listed according to decreasing individual contribution to the paper, worked equally on the laboratory experiments, interpretation of data and statistical analyses. The last author had a major role as a senior author in interpreting the data and preparing the article.

Funding

Supported by the Associazione Italiana per la Ricerca sul Cancro (AIRC, Milan) and Ministry of Education, University and Research (MIUR, "Molecular Engineering – C03" – SP4 funds), Italy. R.R. is recipient of a fellowship from the European Community #94/342/CE.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Dr. S. Vincent Rajkumar, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Dr. Rajkumar and the Editors. Manuscript received July 4, 2002; accepted December 30, 2002.

In the following paragraphs, Dr. Rajkumar summarizes the peer-review process and its outcomes.

What is already known on this topic

Increased bone marrow angiogenesis is a feature of multiple myeloma and has prognostic value in the disease. The increased angiogenesis is felt to be mediated by the expression of angiogenic cytokines such as vascular endothelial growth factor A (VEGF-A) by the neoplastic myeloma cells.

What this study adds

This study shows that VEGF-A is secreted by myeloma cells and leads to proliferation and chemotaxis of endothelial cells mediated through VEGF receptor 2 expressed on these cells. In contrast, the secretion of VEGF-A and expression of VEGF receptor 2 were low in patients with MGUS and in controls. This study, along with other previously published studies, provides further rationale for development of novel agents and trials targeting the VEGF pathway in myeloma.

Caveats

More work is still needed to define the pathogenetic role of VEGF and angiogenesis in myeloma. The results with VEGF-C, VEGF-D, and VEGFR3 need further confirmation.