Molecular and Cellular Mechanisms of Increased Angiogenesis in Multiple Myeloma: A Role for CXCL12

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DECLARATION

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Signed

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Sally K. Martin

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ABSTRACT

Multiple myeloma (MM) is an incurable haematological malignancy characterised by the clonal proliferation of plasma cells (PCs) within the bone marrow (BM). MM PC survival and expansion is dependent upon an adequate supply of oxygen and nutrients, and increased BM angiogenesis is a critical feature of MM progression. While MM PCs express and secrete a number of angiogenic factors, our current understanding of the precise mechanisms by which MM-induced angiogenesis occurs is incomplete. In this study, we collected specimens from patients with MM and the benign precursor condition MGUS, and demonstrated for the first time that circulating levels of the CXCL12 chemokine positively correlate with the degree of BM angiogenesis. Using conditioned media from a MM PC line, the contribution of MM PC-derived CXCL12 to angiogenesis was also examined and found to strongly induce vascular tube formation *in vitro*.

In several other cell types, hypoxia has been shown to up-regulate CXCL12 expression. Studies investigating the hypoxic regulation of CXCL12 in MM PCs revealed that, while acute hypoxia is unable to stimulate CXCL12 expression, prolonged hypoxia significantly up-regulates CXCL12 mRNA and protein expression. To determine the mechanism(s) responsible for this, over-expression and RNA interference technology was employed to create genetically modified MM cells in which either HIF-1α or HIF-2α were over-expressed or knocked down. These studies showed that HIF-2α is the predominant mediator of the hypoxic induction of CXCL12 in MM PCs. The ability of HIF-2α to bind to the CXCL12 promoter was confirmed using EMSA and ChIP analyses.

The role of CXCL12 in *in vivo* angiogenesis and the contribution of HIF-1α and HIF-2α were also examined. In these studies, transduced MM cells, in which HIF-1α, HIF-2α and CXCL12 were over-expressed or knocked down, were implanted into a vessel-poor, subcutaneous environment in immunocompromised mice. Tumour-induced angiogenesis was assessed after two weeks by measuring the haemoglobin content of excised implants. These studies confirmed that over-expression of CXCL12, HIF-1α and HIF-2α each stimulates a strong angiogenic response. Using the well-characterised CXCR4 antagonist, T140, CXCL12 was found to play a key role in the increased angiogenesis observed in response to HIF-1α and HIF-2α over-expression.

These novel studies have shown that CXCL12 is an important mediator of angiogenesis in MM patients, and that aberrant CXCL12 expression by MM PCs is due, in part, to its hypoxic up-regulation mediated predominantly by HIF-2.

ABBREVIATIONS

ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
Ang	angiopoietin
ARNT	aryl hydrocarbon nuclear translocator
β2M	beta-2-microglobulin
bFGF	basic fibroblast growth factor
bp	base pairs
bHLH	basic helix-loop-helix
BM	bone marrow
BMSC	bone marrow stromal cell
BSA	bovine serum albumin
CAM	chorioallantoic membrane
cDNA	complementary deoxyribonucleic acid
CFU-En	colony forming units - endothelial
ChIP	chromatin immunoprecipitation
CLL	chronic lymphoblastic leukaemia
CM	conditioned media
CML	chronic myeloid leukaemia
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EC	endothelial cell
ECM	extracellular matrix
EDTA	ethylenediaminetetra-acetic acid
eg.	for example
ELISA	enzyme-linked immunosorbent assay
EMSA	electromobility shift assay
ERK	extracellular signal-regulated kinase

FCS	foetal calf serum
Flk-1	foetal liver kinase-1 receptor
Flt-1	fms-like tyrosine kinase-1 receptor
GAPDH	glutaraldehyde 3-phosphate dehydrogenase
HBS	hypoxia inducible factor (HIF) binding site
HBSS	hank's balanced salt solution
HGF	hepatocyte growth factor
HIF	hypoxia inducible factor
hpf	high powered field of view
HRE	hypoxic response element
HUVEC	human umbilical vein endothelial cell
ID	inhibitory domain
ie.	that is
Ig	immunoglobulin
IGF	insulin-like growth factor
IL	interleukin
IRES	internal ribosome entry site
ISS	international staging system
i.u.	international units
kDa	kilodalton
LP-1-CXCL12	LP-1-pRUF-IRES-GFP-CXCL12
LP-1-CXCL12-KD	LP-1-pFIV-H1-copGFP-CXCL12 RNAi "Clone #8"
LP-1-HIF-1α	LP-1-pRUF-IRES-GFP-HIF-1α
LP-1-HIF-1α-KD	LP-1-pFIV-H1-copGFP-HIF-1α RNAi "Clone #4"
LP-1-HIF-2α	LP-1-pRUF-IRES-GFP-HIF-2α
LP-1-HIF-2α-KD	LP-1-pFIV-H1-copGFP-HIF-2α RNAi "Clone #2-6"
LP-1-pFIV	LP-1-pFIV-H1-copGFP
LP-1-pRUF	LP-1-pRUF-IRES-GFP
LP-1-scramRNAi	LP-1-pFIV-H1-copGFP-scrambled RNAi
LP-1-SFG	LP-1-SFG-$_{NES}$-TGL
M	molar
MDS	myelodysplastic syndrome
MFI	mean fluorescence intensity
MGUS	monoclonal gammopathy of undetermined significance

MIP	macrophage inflammatory protein
mg/ mL/ mm/ mM	milligram/ millilitre/ millimetre/ millimolar
MM	multiple myeloma
MMP	matrix metalloproteinase
MM PC	multiple myeloma plasma cell
M-protein	monoclonal paraprotein
mRNA	messenger ribonucleic acid
MVD	microvessel density
NHL	non-Hodgkin's lymphoma
nm	nanometres
OC	osteoclast
OD	optical density
ODD	oxygen-dependent degradation
PAS	Per-ARNT-Sim
PB	peripheral blood
PBS	phosphate buffered saline
PC	plasma cell
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PE	phycoerythrin
pFIV	pFIV-H1-copGFP
PHD	prolyl hydroxylase domain
PI3K	phosphoinositide-3-kinase
pRUF	pRUF-IRES-GFP
RCC	renal cell carcinoma
rh	recombinant human
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
RPMI	Roswell Park Memorial Institute
RT	room temperature
SDF-1	stromal-derived factor-1
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SMC	smooth muscle cell

TAD	transactivation domain
T140	4F-Benzoyl-TE14011 (*in vitro*)
	4F-Benzoyl-TN14003 (*in vivo*)
TGF	transforming growth factor
TNF	tumour necrosis factor
TrHBMEC	transformed human bone marrow endothelial cell
µg/ µl/ µm/ µM	microgram/ microlitre/ micrometre/ micromolar
VEGF	vascular endothelial growth factor
VHL	von Hippel Lindau
VLA	very late antigen
v/v	volume per volume
WST-1	4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate
w/v	weight per volume
x g	times gravity

LIST OF PUBLICATIONS

Scientific Manuscripts

1. **SK Martin**, P Diamond, LB To, D Peet, S Gronthos and ACW Zannettino (2008). CXCL12 expression is regulated by HIF-2α in multiple myeloma plasma cells. *Manuscript in Preparation.*

2. P Diamond, A Labrinidis, **SK Martin**, AN Farrugia, S Gronthos, LB To, N Fujii, P O'Loughlin, A Evdokiou and ACW Zannettino (2008). Targeted disruption of the CXCL12/CXCR4 axis inhibits osteolysis in a xenogenic transplantation model of myeloma-associated bone loss. *Submitted to Journal of Bone and Mineral Research.*

3. A Labrinidis, P Diamond, **SK Martin**, S Hay, V Liapis, N Sims, GJ Atkins, T Vincent, DM Findlay, ACW Zannettino and A Evdokiou (2008). Apo2L/TRAIL inhibits tumour progression and bone destruction in a murine model of multiple myeloma. *Submitted to Clinical Cancer Research.*

4. A Labrinidis, P Diamond, **SK Martin**, S Hay, V Liapis, DM Findlay, ACW Zannettino and A Evdokiou (2008). Multimodal imaging analysis of tumour progression and bone destruction in a murine model of multiple myeloma. *Submitted to International Journal of Oncology.*

5. ACW Zannettino, **SK Martin**, S Gronthos, LB To and DJ Peet (2008). The role of CXCL12 in multiple myeloma. *Cancer Research Progress*. Nova Publishers. Edited by Henrik N. Kristiansen.

6. **SK Martin**, AL Dewar, AN Farrugia, N Horvath, S Gronthos, LB To and ACW Zannettino (2006). Tumour angiogenesis is associated with plasma levels of stromal-derived factor-1α in patients with multiple myeloma. *Clinical Cancer Research,* 12(23): 6973-6977.

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1. 30th Annual Meeting of the American Society for Bone and Mineral Research, Montreal, Canada, September 2008. *CXCL12 Expression is Regulated by HIF-2α in Multiple Myeloma Plasma Cells*. **SK Martin**, P Diamond, LB To, D Peet, S Gronthos and ACW Zannettino.

2. 30th Annual Meeting of the American Society for Bone and Mineral Research, Montreal, Canada, September 2008. *CXCL12 Stimulates Osteoclastic Bone Resorption in a Novel Mouse Model of Human Multiple Myeloma*. P Diamond, A Labrinidis, **SK Martin**, AN Farrugia, S Gronthos, LB To, A Evdokiou and ACW Zannettino

3. 2nd International Conference on Osteoimmunology: Interactions of the Immune and Skeletal Systems, Rhodes, Greece, June 2008. *The Role of the Chemokine CXCL12 in Induction of Osteoclast Activity and Osteolytic Bone Disease in Multiple Myeloma*. P Diamond, S Hampton-Smith, A Labrinidis, **SK Martin**, S Gronthos, LB To, N Fujii, P O'Loughlin, A Evdokiou and ACW Zannettino.

4. 11th International Myeloma Workshop, Kos, Greece, June 2007. *CXCL12 Stimulates Osteoclastic Bone Resorption in a Novel Mouse Model of Human Multiple Myeloma*. P Diamond, A Labrinidis, **SK Martin**, AN Farrugia, S Gronthos, LB To, A Evdokiou and ACW Zannettino.

5. 11th International Myeloma Workshop, Kos, Greece, June 2007. *Tumour Angiogenesis is Associated with Plasma Levels of Stromal-Derived Factor-1α in Patients with Multiple Myeloma*. **SK Martin**, AL Dewar, AN Farrugia, N Horvath, S Gronthos, LB To and ACW Zannettino.

6. 10th International Myeloma Workshop, Sydney, Australia, April 2005. *Myeloma Plasma Cell-Derived SDF-1α and RANKL act Synergistically to Stimulate Neo-Angiogenesis*. **SK Martin**, AN Farrugia, P Diamond, LB To and ACW Zannettino.

7. 10th International Myeloma Workshop, Sydney, Australia, April 2005. *Elevated Serum Levels of CXCL12 is Associated with Increased Osteoclast Activity and Osteolytic Bone Disease in Multiple Myeloma Patients*. AN Farrugia, S Gronthos, A Kortesidis, J Manavis, LB To, **SK Martin**, P Diamond, H Tamamura, T Lapidot, N Fujii and ACW Zannettino.