ROLE OF OSTEO-PROGENITORS IN THE PATHOGENESIS OF VASCULAR CALCIFICATION

Thesis submitted by

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MSc. (INDIA)

AUGUST 2009

for the degree of Doctor of Philosophy (Vascular Biology)
in the School of Medicine and Dentistry
James Cook University, QLD
AUSTRALIA
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DECLARATION

I, the undersigned declare that this research investigation is carried out on my own and it has not been previously submitted anywhere for another degree or diploma at any university or institution of tertiary education in or out of Australia. Information derived from the published or unpublished works of others has been acknowledged in the text and a list of references is given.

Shripad Nagesh PAL,

August 2009

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DECLARATION ON ETHICS

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *National Statement on Ethics Conduct in Research Involving Human* (1999), the *joint NHMRC/AVCC Statement and Guidelines on Research Practice* (1997), the *James Cook University Policy on Experimental Ethics. Standard Practices and Guidelines* (2001), and the *James Cook University Statement and Guidelines on Research Practice* (2001). The proposed research methodology received clearance from the James Cook University Experimental Ethics Review Committee (approval number H-2196).

Shripad N. Pal

August 2009
ACKNOWLEDGEMENTS

The completion of this research thesis would have remained an unfulfilled dream without the untiring and selfless support from a number of people throughout my research student tenure. It is to all of them that I wish to extend my heartfelt gratitude without which this task would not have been possible.

First and foremost, I thank the Almighty Lord for providing me the opportunity, determination and perseverance which I could invest bit by bit, day by day to reach this stage. Secondly, I owe a lot to my parents (Nagesh K Pal and Mangala N Pal), my younger brother Pratik, my dearest wife Rucha and her family who loved, cared, supported, and believed in me in the toughest of times. This journey would not have been possible without their love, prayers and blessings.

In Australia, I am sincerely thankful to my supervisor Professor Jonathan Golledge, co-supervisors Dr Lynn Woodward and Dr Mirko Karan. I am grateful for their able supervision, professional guidance, and many words of thought during my research. I really appreciate them for their continuing belief in me which kept on reigniting the lamp of hope throughout the PhD candidature, especially during the darkest of research moments. Professor Golledge also provided me with all the necessary financial support required for my research candidature and survival in Townsville for which I am deeply indebted.

Sincere thanks and thoughts go towards Dr Catherine Rush and Dr Ann Van Campenhout for their willingness to pass on their research experience. It’s their patience and selfless guidance that helped me to efficiently focus on the candidature. I extend my appreciation to Dr Monsur Kazi, Dr Paula Clancy, Mrs Frances Wood and Ms. Simone Mangan for their friendship, words of support, guidance and technical assistance throughout my tenure. The time and efforts of Dr Bradford Cullen and Dr Julie Mudd in their work with the mouse model which provided me with tissue samples is gratefully acknowledged.

A warm and a heartfelt thanks goes towards my fellow PhD colleague, office buddy and partner in crime, Venkat Vangaveti, for his company, tolerance, and support. Very special thanks go to Dr. Adam Parr, Ms Barbara Bradshaw from the Townsville General Hospital and Sullivan and Nicolaides Pathology, Townsville for assisting me in arranging the patient samples without which the research investigation would not have been complete. I also
extend my gratitude towards the Biomedical and Tropical veterinary sciences faculty for providing all the required facility for undertaking mouse work.

I am also obliged to thank my faculty, The School of Medicine and Dentistry and Graduate research school, James Cook University for all financial and administrative support that assisted me in completing my research candidature.

Last but not the least; I would convey a heartfelt thankyou to all the friends in Townsville, both compatriots and local fellows who supported me socially throughout my candidature. Without their first-hand social and emotional support it would have been tough to survive and thrive in Townsville.
LIST OF ABBREVIATIONS

µg/kg: Microgram/kilogram
µl: Microliter
A.A: Aortic arch
AAA: Abdominal aortic aneurysm
ACI: Aortic calcification index
ACK: Ammonium chloride and potassium chloride
ALP: Alkaline phosphatase
ApoE: Apolipoprotein E
BL/6: Black 6
BM: Bone marrow
BMP-2: Bone matrix protein-2
BMT: Bone marrow transplantation
COV: Coefficient of variation
CT: Computed tomography
CTA: Computed tomography angiogram
CVC: Calcifying vascular cells
D/W: Distilled water
DAPI: 4', 6-diamidino-2-phenylindole
dL: Deciliter
EDTA: Ethylene diamine tetra-acetic acid
ELISA: Enzyme linked Immunosorbent assay
EPC: Endothelial progenitor cells
ESRD: End stage renal disease
FC: Flow cytometry
FCS: Fetal calf serum
FSC: Forward scatter
G-CSF: Granulocyte colony-stimulating factor
GFP: Green fluorescent protein
GM-CSF: Granulocyte-macrophage colony-stimulating factor
HDL: High density lipoprotein
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hMSC: Human mesenchymal stem cells
HSC: Hematopoietic stem cells
hVSMC: Human vascular smooth muscle cells
I. R: Infra renal
I-CAM-1: Intercellular adhesion molecule-1
IL- 6: Interleukin 6
IL-1β: Interleukin 1- beta
IL-8: Interleukin 8
IQR: Inter quartile range
LDLR: Low-density lipoprotein receptor
MACS: Magnetic assorting cell separator
mg: Milligrams
MGP: Matrix gamma-carboxyglutamic acid protein
Min: Minutes
ml: Milliliter
mM: Milimolar
mm³: Cubic milimeter
Mmol/L: Milimolar/liter
MNC: Mononuclear cells
MS: Magnetic separation
MSC: Mesenchymal stem cells
ng/ml: Nanogram/milliliter
nm: Nanometer
O.D: Optical density
°C: Degrees centigrade
OCN⁺: Osteocalcin positive
OPG⁻/: Osteoprotegerin deficient
OPG⁺/+: Osteoprotegerin present
OPN: Osteopontin
PBS: Phosphate buffered saline
PDGF: Platelet derived growth factor
PE: Phycoerythrin
pg/ml: picogram/milliliter
PPI: Pyrophosphate
R.T: Room temperature
RANK: Receptor activator of nuclear kappa
RANK-L: Receptor activator of nuclear kappa ligand
RBC: Red blood cells
rpm: Revolutions per minute
S.D: Standard deviation
S.R: Supra renal
SCF: Stem cell factor
SDF-1α: Stromal cell derived factor 1 alpha
SMC: Smooth muscle cells
SNP: Sullivan Nicolaides pathology
SSC: Side scatter
T.A: Thoracic arch
TGF-β: Tumor growth factor-beta
TNF α: Tumor necrosis factor- alpha
V-CAM-1: Vascular cell adhesion molecule-1
VEGF: Vascular endothelial growth factor
VSMC: Vascular smooth muscle cells
ABSTRACT

Vascular calcification, until recently, was considered to be a passive process which occurred as a nonspecific response to tissue injury or necrosis. Since the severity of vascular calcification has been correlated with that of atherosclerosis and its risk factors, it was postulated that the process is linked to these events. However recent findings from a number of mouse model studies suggest that the mechanisms involved in vascular calcification may be distinct from those underlying atherosclerosis.

Current theories regarding the pathogenesis of vascular calcification suggest a number of possible mechanisms. These include passive models in which vascular calcification is observed as a result of loss of molecular inhibitors and those where active cell mediated process is involved. Calcification has also been reported as result of apoptosis or death of vascular smooth muscle cells (VSMC). Current evidence favours a cell mediated mechanism of vascular calcification.

The origin of the cells responsible for vascular calcification is not clearly defined. One novel source of cells controlling vascular calcification is from the bone marrow (BM). A circulating immature BM-derived population has been identified. A small subset of this BM population has been reported to possess bone forming properties in vitro and hence called osteo-progenitors. In the present investigation, it was hypothesized that these circulating osteo-progenitors contribute to vascular calcification. It was postulated that the osteo-progenitors are recruited from the BM environment under the influence of stem cell mobilising cytokines such as stromal cell derived factor-1 α (SDF-1α), granulocyte-colony stimulating factor (G-CSF) and stem cell factor (SCF). Further, it was suggested that these stem cell mobilising cytokines facilitate the homing of immature circulating osteo-progenitors to vascular lesions and contribute to calcification.

These hypotheses were tested in two mouse models and one human patient cohort. The aims of the investigation included:

a) To establish a suitable mouse model for vascular calcification studies.

b) To assess the association of the circulating osteo-progenitor population with the severity of aortic calcification in mouse models.

c) To identify if the osteo-progenitor population was deposited within the vasculature at sites of vascular calcification.
d) To assess the relationship between the circulating osteo-progenitor population and aortic calcification in a human patient group suffering from peripheral artery disease.
e) To assess the relationship between the stem cell mobilising cytokines and the severity of vascular calcification in a mouse model and a human patient cohort.

The findings of this work suggest, for the first time, an association between circulating osteocalcin positive mononuclear cells (OCN\(^+\) MNC) and aortic calcification in two mouse models and a human patient cohort diagnosed with peripheral artery disease. It was found that the severity of vascular calcification was increased in 52 week old osteoprotegerin knockout (OPG\(^{-/-}\)) mice and even more elevated in younger (14 week old) OPG\(^{-/-}\) mice receiving controlled doses of calcitriol. It was further observed that in both mouse models the percentage of circulating OCN\(^+\) MNC was correlated to the aortic calcium content. These results suggest a possible role for BM-derived osteo-progenitors in vascular calcification. It was also observed that OCN\(^+\) population deposited within the vasculature was directly associated with the severity of extractable aortic calcium in the OPG\(^{-/-}\) mouse model. These results suggest a three-way association between osteo-progenitor population in circulation, its cellular deposition within vasculature and the severity of aortic calcification.

The investigation undertaken in the human patient cohort also supported the initial hypothesis and confirmed the research findings obtained from the two mouse models. In the patient study the percentage of circulating OCN\(^+\) MNC was observed to be associated with the severity of infra-renal aortic calcification.

The present study also supported the hypothesis that the stem cell mobilizing cytokines could be involved with the release of osteo-progenitors and may facilitate their homing to the vasculature. The concentrations of SDF-1\(\alpha\), G-CSF and SCF were associated with the percentage of circulating OCN\(^+\) MNC and the severity of aortic calcification in the mouse models and patient cohort investigated. These results suggest that the BM-derived osteo-progenitors are mobilised into the peripheral circulation from the marrow environment under the influence of these cytokines. Further, the circulating osteo-progenitors may home to vascular lesions and differentiate into bone-forming cells. This process may contribute to the pathogenesis of vascular calcification.
Further work, however, is necessary to confirm the role of these BM-derived immature cells in vascular calcification as there are a number of limitations of the present investigation. Firstly, both mouse models employed were based on OPG deficiency. Thus it is possible that the increased OCN$^+$ MNC was related to this rather than the aortic calcification in these animals. Secondly, the role of OPG within the vasculature is also not entirely clear. While depletion of OPG in mouse models is reported to induce vascular calcification, in patients serum OPG levels are positively associated with peripheral artery disease. This difference in results between mouse models and human patient illustrates the current uncertainty regarding the role of OPG in cardiovascular disease. The patient group investigated in this study was small. A larger group would be ideal to confirm the association between circulating osteoprogenitors and aortic calcification. The absence of a healthy control group is a further limitation of the human investigation.

Overall, the current research suggests an important new mechanism underlying vascular calcification with implications for treatment. Results obtained from this study may also be useful in the investigation of other pathology types, and may assist in establishing collaboration with external groups. Since vascular calcification is also linked to other clinical conditions such as atherosclerosis, diabetes, obesity and bone related disorders, this investigation can build on those areas within research groups with broader clinical perspectives.
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