

P. MABETA

DECREASED SECRETION OF VASCULAR ENDOTHELIAL GROWTH FACTOR IS ASSOCIATED WITH INCREASED APOPTOSIS IN VASCULAR TUMOR DERIVED ENDOTHELIAL CELLS

Department of Anatomy and Physiology, University of Pretoria, Pretoria, South Africa

Bleomycin is an antineoplastic drug that has recently been employed to treat haemangiomas, the most common vascular tumors of infancy, with very good results. To better understand the mechanism of bleomycin in accelerating haemangioma regression, we have investigated the effects of the drug on vascular tumor inducing endothelial cells (sEnd.2 cells). Cell growth studies were undertaken using crystal violet staining, while morphological studies were undertaken employing transmission electron microscopy. The cells were analyzed for possible apoptosis employing flow cytometry. The expression of Bcl-2 and p53 were investigated using Western blot analysis. In addition, the production of vascular endothelial growth factor was measured using ELISA. Results showed that bleomycin inhibited the growth of these endothelial cells, even in the presence of vascular endothelial growth factor, a proangiogenic growth factor. Further, there was increased endothelial cell apoptosis, as evidenced by morphological analysis, increased acridine orange staining of cell nuclei and annexin V staining. Apoptosis was associated with an increase in the expression of p53 and a decrease in the expression of the antiapoptotic protein Bcl-2.

Key words: tumor, bleomycin, endothelial cell, apoptosis, Bcl-2, p53, annexin V, vascular endothelial growth factor

INTRODUCTION

The maintenance of the homeostasis of the vascular endothelial cell population is maintained by a balance between endothelial cell growth and apoptosis (1-4). Apoptosis is an energy-dependent process in which living cells participate in their own death in an organised manner (3, 5). It is characterized by a number of morphological and biochemical alterations (5-8). The morphological changes seen during apoptosis include shrinkage of the cell and budding of the plasma membrane. In the nucleus, initially, chromatin condensation occurs around the periphery, and the nuclear lamina begins to disappear (7). Later in the process highly condensed chromatin with cleaved DNA segregates into defined structures with an intact nuclear envelope (6, 7). Eventually, the cell breaks up into many spherical bodies called apoptotic bodies, which contain nuclear material with or without compacted organelles. These bodies are mostly engulfed by neighbouring cells, in particular macrophages (6-8).

The link between endothelial cell apoptosis and angiogenesis is evident in growing vascular tumors of infancy, also known as infantile haemangiomas (IH), indeed a decrease in endothelial cell apoptosis is a characteristic associated with the proliferating IH (9). In recent years bleomycin, an antineoplastic agent used in the treatment of various types of cancers, has been used successfully to treat these vascular tumors, although its mechanism of antitumor action remains unclear (10, 11). Initial studies aimed at elucidating bleomycin's mode of action in inducing IH regression have shown that the drug induces the

activation of caspase-3 and caspase-9 (11). Caspases are a family of cysteine proteases which play a role in the execution of apoptosis (3, 6, 11). The purpose of this study was to determine whether bleomycin induces apoptosis of vascular tumor derived endothelial cells.

MATERIALS AND METHODS

Cell culture

The endothelioma cell line, sEnd.2, derived from Pym T induced vascular tumors in the skin (thorax) of C57BL6 mice, was grown in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St Louis, MO, USA). The endothelioma (sEnd.2) cells were obtained from Professor M.S. Pepper (University of Pretoria). The medium was supplemented with 10% heat inactivated fetal calf serum (Invitrogen, USA), 2 mM L-glutamine (Invitrogen, CA, USA), and 1% penicillin-streptomycin (Highveld Biologicals, Sandringham, SA). The cell line was maintained in a 37°C incubator in a humidified atmosphere containing 5% CO₂.

1. In vitro cell growth inhibition assay

Cell growth was assessed using the crystal violet nuclear staining assay (12). The cells were seeded into 24-well culture plates at a density of 10,000 cells per well for 24 hours, and then treated with bleomycin (0.1–100 µg/ml), 0.9% saline or to a combination of varying doses of bleomycin (0.1–100 µg/ml) and

30 ng/ml of VEGF (Sigma-Aldrich, St Louis, MO, USA). Growth was terminated after 24 hours by fixation with 1% glutaraldehyde in PBS for 15 minutes, followed by staining with a 0.1% crystal violet solution (Sigma-Aldrich, St Louis, MO, USA) solution for 30 minutes. The chromophore was extracted with 0.1% Triton X-100 (Sigma-Aldrich, St Louis, MO, USA) per well. Absorbance was read at 570 nm on an ELx 800 Universal Microplate Reader (Bio-Tek instruments Inc, VT, USA). Three wells were analysed for each concentration. Studies were repeated three times.

2. Annexin V assay

For flow cytometry analysis, an annexin-V biotin apoptosis detection kit was used. During early apoptosis, phosphatidyl serine translocates from the inner side of the cell membrane to the outer surface (12). The assay takes advantage of the fact that FITC-labeled annexin V can bind to the exposed phosphatidylserine. The assay was performed in accordance with the manufacturer's protocol (BioVision, Mountain View, USA).

Briefly, 24 hours after drug treatment or exposure to saline, both floating and trypsinized adherent sEnd.2 cells (5×10^5) were collected, suspended in PBS, resuspended in 200 μ l of binding buffer containing 5 μ l of annexin-V and 5 μ l of PI, and then incubated for 5 minutes in the dark at room temperature. Cells were washed in binding buffer, fixed in 2% formaldehyde, and incubated in 5 μ g/ml avidin-fluorescein. Following resuspension in PBS, analysis was performed using flow cytometry (Beckman Coulter, Johannesburg, SA).

3. Transmission electron microscopy

For morphology studies, sEnd.2 cells were seeded in a 25 cm² flasks at a density of 1.25×10^6 cells per flask and allowed to attach overnight. Cells were treated with bleomycin or saline (control). After 24 hours cells were fixed in 2.5% glutaraldehyde (Sigma-Aldrich, St Louis, MO, USA) in 0.075 M phosphate buffer (pH 7.4) for 5 minutes.

Cells were rinsed three times with 0.075 M phosphate buffer and post fixed in 1% OsO₄ in Millonig's buffer for 1 hour, then in Millonig's buffer for 10 minutes and rinsed in distilled water for 20 minutes. This was followed by a dehydration series of varying ethanol concentration (50%, 70%, 80%, and 96%, 100%), and subsequently propylene oxide.

The cells were then embedded in PO: Epoxy resin/ 2:1, followed by polymerization in 100% Epoxy resin. Samples were viewed using a Multi-Purpose Philips 301 Transmission Electron Microscope (Apollo Scientific SA, Midrand, South Africa).

4. Protein extraction and Western blotting analysis

Cells were plated and culture in complete medium and allowed to attach overnight. This was followed by the addition of bleomycin (0–20 μ g/ml), and incubation for 24 hours. Cells were lysed with cell lysis buffer containing 10 mM Tris (pH 7.4), 1 mM EDTA, 25 mM NaF, 100 μ M Sodium orthovanadate and 1 mM DTT. The lysates were then sonicated for 10 s twice and the supernatants were boiled in SDS sample buffer for 5 minutes. Protein concentration was determined by using a Bradford method.

Protein aliquots of control and bleomycin-treated cells were separated on 15% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. The membranes were dried, preblocked with 5% non-fat milk in phosphate-buffered saline and 0.1% Tween-20, then incubated

with a primary antibody for p53, Bcl-2, both diluted 1:500 (Sigma-Aldrich, St Louis, MO, USA) and detected with horseradish peroxidase-conjugated anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Alpha-actin (Sigma-Aldrich, St Louis, MO, USA) was used as an internal control to confirm equal loading of protein. The protein bands were detected with a FluorChem Imaging System (Proteinsimple, CA, USA).

5. Measurement of vascular endothelial growth factor

The cell line, sEnd.2, was cultured in 24-well plates at a density of 10,000 cells for 24 hours and subsequently exposed to saline or varying doses of bleomycin (0.001–100 μ g/ml). After 24 hours of exposure to the drug, the levels of vascular endothelial growth factor (VEGF) were measured in sEnd.2 cell supernatants using a Quantikine VEGF ELISA kit (R & D, Minneapolis, USA), according to the manufacturer's instructions. Briefly, 50 μ l of assay diluent followed by 50 μ l of standard, control or sample were added to each well in a 96-well plate and incubated for 2 hours.

Each well was aspirated and washed four times with wash buffer. After aspirating the wells, 200 μ l of mouse VEGF conjugate was added to each well and the plates were covered with an adhesive strip and incubated for another 2 hours. The wells were aspirated, and 100 μ l of substrate solution was added to each well and the plate incubated for 30 minutes at room temperature. Subsequently, 100 μ l of stop solution was added to each well. The optical density of each well was read at 450 nm on an ELx 800 Universal Microplate Reader (Bio-Tek instruments Inc, VT, USA).

6. Statistical analysis

Data were analysed using One-way analysis of variance (ANOVA), followed by a post hoc test, Bonferoni's method. Results are presented as means \pm standard deviation (S.D.). Significance was designated at $P < 0.05$.

RESULTS

In this study the effects of bleomycin on sEnd.2 cell growth were evaluated. Also, the combined effects of bleomycin and VEGF on the growth of these cells were investigated. Bleomycin induced a decrease in sEnd.2 cell numbers in a dose-dependent manner, with an IC₅₀ of approximately 8.80 μ g/ml (*Fig. 1*). The reduction in cell growth was partially prevented in cells treated with a combination of bleomycin and VEGF, and the IC₅₀ value was approximately 12.4 μ g/ml (*Fig. 1*). Based on the data from growth studies, a dose range of 5–20 μ g/ml bleomycin was chosen for further studies.

After observing that bleomycin decreased sEnd.2 cell viability in a concentration-dependant manner, possible apoptotic induction by this drug was evaluated. Transmission electron microscopy studies revealed morphological features that are consistent with apoptosis in bleomycin treated cultures, namely membrane blebbing and nuclear fragmentation (*Fig. 2*). Flow cytometry analysis was undertaken and showed that bleomycin induced endothelial cell apoptosis in a dose-dependent manner (*Fig. 3*). Control cells did not stain with either annexin or PI (*Fig. 3*), while 53.9% of the cells treated with bleomycin stained positive for annexin (*Fig. 3*), indicating that the cells underwent apoptosis.

The effects of bleomycin on the expression of the anti-apoptotic Bcl-2 protein were examined by Western blot analysis. As shown in *Fig. 4*, bleomycin decreased the expression of Bcl-

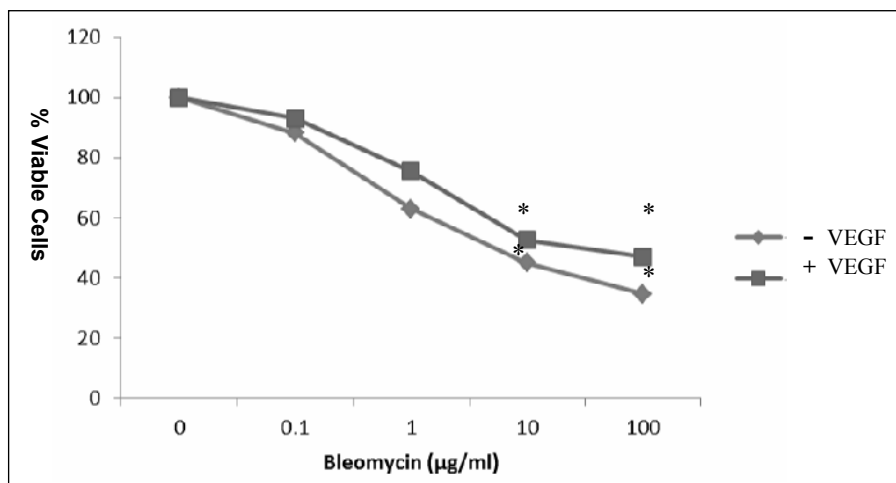


Fig. 1. Effect of bleomycin on endothelial cell growth. Cell growth was determined in control and bleomycin or bleomycin + VEGF (30 ng/ml)-treated sEnd.2 cells using crystal violet staining. Cell numbers are expressed as a percentage of cells relative to 100% control. The assay was repeated three times; three wells were analysed per concentration. Data shown are representative of two separate experiments. Three wells were analyzed per treatment dose. Values are mean \pm S.D. * $P < 0.05$.

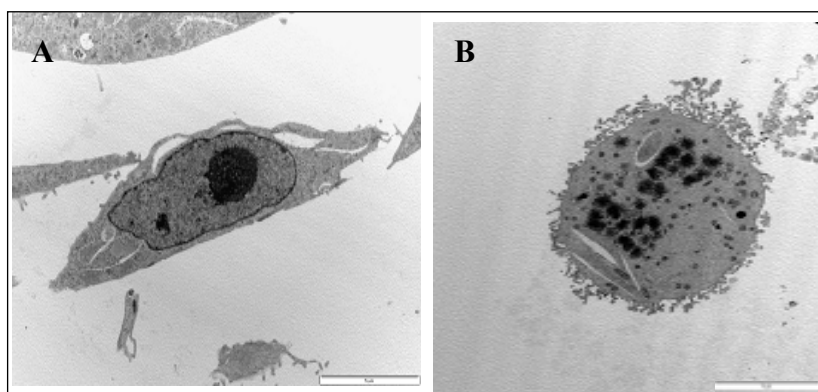


Fig. 2. Bleomycin induces apoptosis of sEnd.2 cells. Bleomycin induces apoptosis of mouse endothelioma cells. Transmission electron micrographs of control (A) and bleomycin-treated sEnd.2 cells (B). Bar 5 μm .

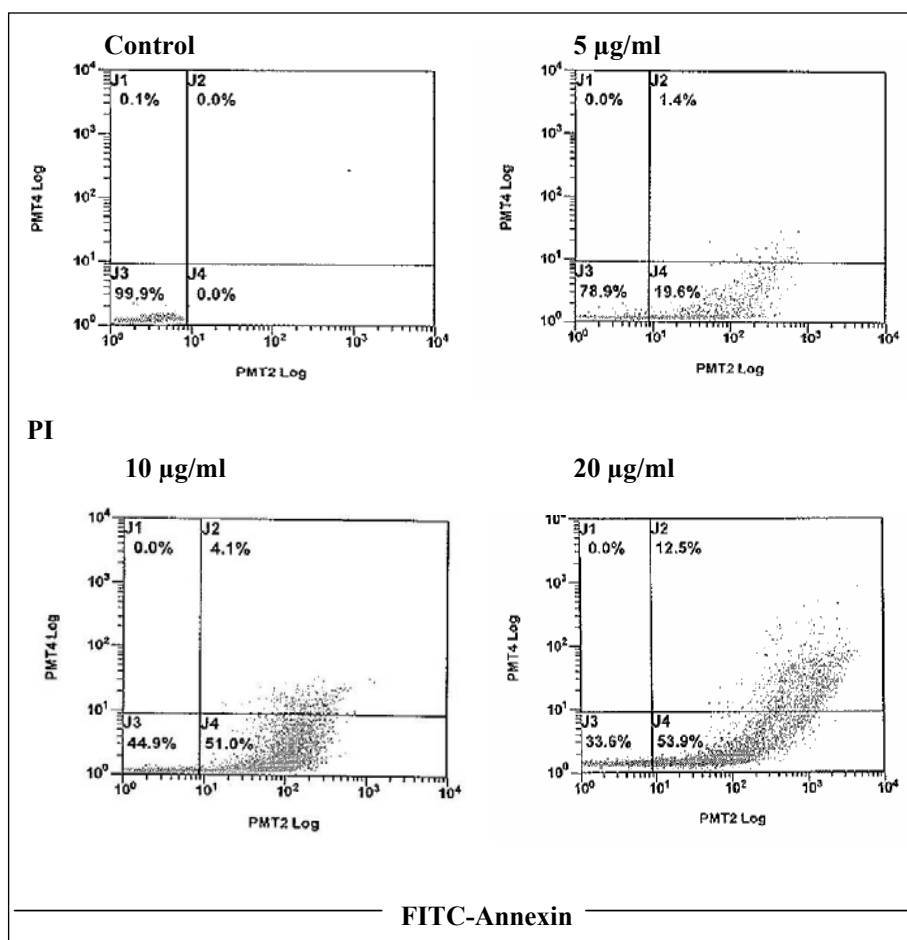


Fig. 3. Flow cytometry of control and bleomycin-treated sEnd.2 cells using a double-staining method with FITC-conjugated annexin-V and propidium iodide (PI). The J4 quadrant represents the percentage of early apoptotic cells (annexin V-stained cells) and the J2 quadrant the percentage of late apoptotic cells (annexin V+PI-stained cells). The J1 quadrant represents necrotic cells (PI positive cells) while the J3 quadrant represents live cells.

2 protein compared to the control cells. The expression of p53 protein was also examined. The results revealed an induction of p53 protein (Fig. 4) in bleomycin-treated cells, and the effect appeared to be dose-dependent. To determine the effect of bleomycin on VEGF secretion, the levels of this pro-angiogenic growth factor were measured. At concentrations of 0.1 µg/ml of bleomycin, the secretion of VEGF was marginally inhibited (Fig. 4). Higher doses of the drug showed greater potency in inhibiting the secretion of VEGF (Fig. 4).

DISCUSSION

The present study showed that bleomycin inhibited the growth of vascular tumor-derived endothelial cells and induced the apoptosis of these cells. Previous reports have linked increased endothelial cell apoptosis with the inhibition of angiogenesis (1, 5, 13). Therefore bleomycin-induced endothelial cell apoptosis may play a role in promoting the regression of these vascular tumors which are characterized by excessive angiogenesis.

It is also interesting to note that in the present study, VEGF levels were high in cell culture supernatants of endothelioma cells, and that the levels of this growth factor decreased significantly in cultures treated with bleomycin. Previously, an increased expression of VEGF was observed in the tissue samples of haemangioma patients (14). In another study, high levels of VEGF were measured in the serum samples of haemangioma patients (15). Although the precise role of VEGF in haemangioma development is not known, VEGF plays a role in protecting endothelial cells against apoptotic death and in promoting angiogenesis (5). Indeed, in a study conducted by Ahluwalia and Tarnawski (16) on myocardial microvascular endothelial cells, an increased expression of the VEGF gene in these cells contributed to an increase in angiogenesis. In another study, myoblasts that overexpressed VEGF (and FGF 4), exhibited an increased 'proangiogenic potential' (17). Studies have further revealed that Lumican, an extracellular matrix proteoglycan, inhibits endothelial cell migration partly by inhibiting VEGF stimulated nitric oxide secretion (18). In addition, a decrease in VEGF expression was shown to be associated with decreased angiogenesis (18). As such, the inhibition of VEGF may have therapeutic significance.

This study has further shown that p53 is down regulated in these endothelial cells and that the protein becomes up-regulated following treatment with bleomycin. A previous study has reported that the loss of p53 is associated with an increase in the expression of VEGF and that such a loss of p53 function contributes to the increased neovascularization seen during tumorigenesis (19).

The findings from this study further revealed that bleomycin decreased the expression of the antiapoptotic protein Bcl-2. Previously we showed that the inhibition of haemangioma growth in a murine model correlated with decreased localization of the anti-apoptotic protein Bcl-2 and of the vascular endothelial growth factor receptor 2 (VEGFR-2) in tissue samples (11). Another study undertaken by Fontanini *et al.* (20) has revealed that Bcl-2 controls the development of tumor angiogenesis in non-small cell lung carcinoma, with putative mediation by VEGF. Other studies have shown that VEGF upregulates the expression of Bcl-2 (21, 22). Thus there appears to be a link between the expression levels of p53, Bcl-2, VEGF and the process of angiogenesis.

In summary, according to the present data, bleomycin treatment resulted in an increase in the expression of p53 and a reduction in the expression of Bcl-2, which may have then activated downstream molecular events leading to apoptotic cell death. Bleomycin also inhibited the production of VEGF, a

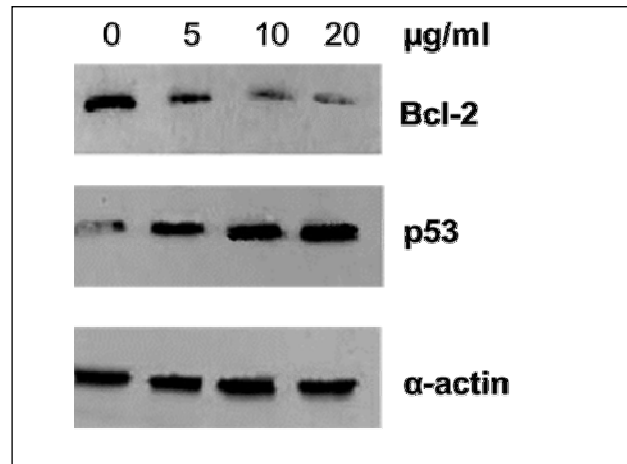


Fig. 4. Representative western blots of p53, Bcl-2 and α -actin in mouse endothelial cells. The increased expression of pro-apoptotic p53 and decreased expression of the anti-apoptotic bcl2 could reflect increased apoptosis in cell cultures treated with bleomycin.

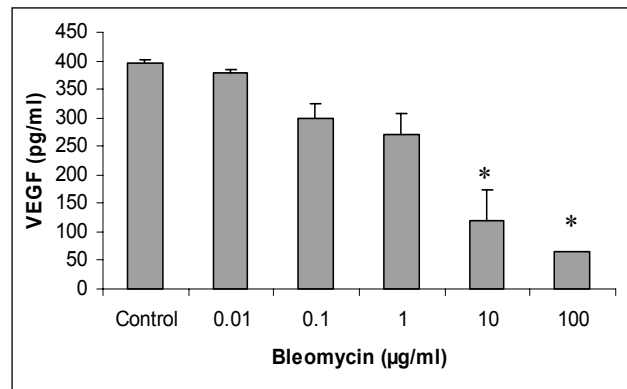


Fig. 5. Levels of VEGF in cell supernatants 24 hours after exposure of cells to various concentrations of bleomycin were measured using ELISA. Results shown represent the mean VEGF concentration in pg/ml of cell culture supernatant \pm S.D. from three independent experiments conducted in triplicate for each treatment condition. * $P < 0.05$ vs. control.

growth factor which inhibits endothelial cell apoptosis. Although there is no animal model which faithfully represents IH as they occur in human patients, a number of models have been used to study haemangioma development, including the PymT model (23). It is possible that bleomycin may induce haemangioma regression in patients in part through the inhibition of VEGF secretion and the induction of endothelial cell apoptosis. Further studies are required to elucidate the link between p53, Bcl-2, and VEGF, and the induction of angiogenesis, and to establish if this association is mimicked in human patients, and how it can be exploited to enhance antiangiogenic therapy strategies.

Acknowledgements: The author wishes to thank Dr R. Cockeran (Department of Immunology, University of Pretoria) for assistance with the Flow Cytometer, and Ms S. Nkadimeng, Ms L. du Plessis and Ms E. van Wilpe (Department of Anatomy and Physiology, University of Pretoria) for assistance with the Electron Microscope. This study was supported by the National Research Foundation and the University of Pretoria.

Conflict of interests: None declared.

REFERENCES

1. Chavakis E, Dimmeler S. Regulation of endothelial cell survival and apoptosis during angiogenesis. *Arterioscler Thromb Vasc Biol* 2002; 22: 887-893.
2. Mabeta P, Pepper MS. A comparative study on the anti-angiogenic effects of DNA-damaging and cytoskeletal-disrupting agents. *Angiogenesis* 2009; 12: 81-90.
3. Choy JC, Granville DJ, Hunt DW, McManus BM. Endothelial cell apoptosis: biochemical characteristics and potential implications for atherosclerosis. *J Mol Cell Cardiol* 2001; 33: 1673-1690.
4. Folkman J. Angiogenesis and apoptosis. *Semin Cancer Biol* 2003; 13:159-167.
5. Polunovsky VA, Wendt CH, Ingber DH, Peterson MS, Bitterman PB. Induction of endothelial cell apoptosis by TNF- α : modulation by inhibitors of protein synthesis. *Exp Cell Res* 1994; 214: 584-594.
6. Catchpoole DR, Stewart BW. Formation of apoptotic bodies is associated with internucleosomal DNA fragmentation during drug-induced apoptosis. *Exp Cell Res* 1995; 216: 169-177.
7. Lopez-Larraz DM, Padron J, Ronci NE, Vidal Rioja LA. Chromatin condensation and differential sensitivity of mammalian and insect cells to DNA strand breaks induced by bleomycin. *Mutation Res* 2006; 600: 93-101.
8. Huerta S, Goulet EJ, Huerta-Yepez S, Livingston EH. Screening and detection of apoptosis. *J Surg Res* 2007; 139: 143-156.
9. Mulliken JB, Glowacki J. Hemangiomas and vascular malformations in infants and children: a classification based on endothelial characteristics. *Plast Reconstr Surg* 1983; 69: 412-422.
10. Pienaar C, Graham R, Geldenhuys S, Hudson DA. Intralesional bleomycin for the treatment of hemangiomas. *Plast Reconstr Surg* 2006; 117: 221-226.
11. Mabeta P, Pepper MS. Inhibition of hemangioma development in a syngeneic mouse model correlates with bcl-2 suppression. *Angiogenesis* 2012; 15: 131-139.
12. Gillies RJ, Didier N, Denton M. Determination of cell numbers in monolayer cultures. *Anal Biochem* 1986; 159:109-113.
13. Merchant SH, Gonchoroff NJ, Hutchison RE. Apoptotic index by annexin V flow cytometry: adjunct to morphologic and cytogenetic diagnosis of myelodysplastic syndromes. *Cytometry* 2001; 46: 28-32.
14. Takahashi K, Mulliken JB, Kozakewich HP, Rogers RA, Folkman J, Ezekowitz RA. Cellular markers that distinguish the phases of hemangioma during infancy and childhood. *J Clin Invest* 1994; 93: 2357-2364.
15. Przewratil P, Sitkiewicz A, Andrzejewska E. Serum levels of vascular endothelial growth factor and basic fibroblast growth factor in children with haemangiomas and vascular malformations-a preliminary report. *Pediatr Dermatol* 2009; 26: 399-404.
16. Ahluwalia A, Tarnawski AS. Activation of the metabolic sensor - AMP activated protein kinase reverses impairment of angiogenesis in aging myocardial microvascular endothelial cells. Implications for the aging heart. *J Physiol Pharmacol* 2011; 65: 583-587.
17. Bialas M, Krupka M, Janeczek A, *et al.* Transient and stable transfections of mouse myoblasts with genes coding for pro-angiogenic factors. *J Physiol Pharmacol* 2011; 62: 219-228.
18. Brezillon S, Zeltz C, Schneider L, *et al.* Lumican inhibits B16F1 melanoma cell lung metastasis. *J Physiol Pharmacol* 2009; 60(Suppl 4): 15-22.
19. Ravi R, Mookerjee B, Bhujwalla ZM, *et al.* Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. *Genes Dev* 2000; 14: 34-44.
20. Fontanini G, Boldrini L, Vignati S, *et al.* Bcl2 and p53 regulate vascular endothelial growth factor (VEGF)-mediated angiogenesis in non-small cell lung carcinoma. *Eur J Cancer* 1998; 34: 718-723.
21. Beierle EA, Strande LF, Chen MK. VEGF upregulates Bcl-2 expression and is associated with decreased apoptosis in neuroblastoma cells. *J Pediatr Surg* 2002; 37: 467-471.
22. Pidgeon GP, Barr M P, Harmey JH, Foley DA, Bouchier-Hayes DJ. Vascular endothelial growth factor (VEGF) upregulates BCL-2 and inhibits apoptosis in human and murine mammary adenocarcinoma cells. *Br J Cancer* 2001; 85: 273-278.
23. Sabapathy KT, Pepper MS, Kiefer F, *et al.* Polyoma middle T-induced vascular tumor formation: the role of the plasminogen activator/plasmin system. *J Cell Biol* 1997; 137: 953-963.

Received: March 13, 2013

Accepted: June 23, 2013

Author's address: Dr. Peace Mabeta, Department of Anatomy and Physiology, P/Bag X04, University of Pretoria, Pretoria 0001, South Africa.
E-mail: peace.mabeta@up.ac.za