CHAPTER 5

Bleomycin, mitomycin C, and cytoskeletal-disrupting agents induce apoptosis in bovine microvascular endothelial cells

5.1. Introduction

A balance between endothelial cell growth and apoptosis is crucial for the maintenance of homeostasis of the vascular endothelial cell population.[1,2] Angiogenic growth factors, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) play a role in the maintenance of endothelial cell survival and inhibit EC apoptosis by modulating gene expression, and through post-transcriptional regulation of anti-apoptotic protein kinases (Fig 5.1). [2,3,4] Studies have shown that deprivation of these growth factors leads to apoptosis of endothelial cells. [1,2] Apoptosis is an energy-dependent process in which living cells participate in their own death in an organised manner, and which involves the activation of a group of cysteine proteases called caspases. [5-7]

Apoptosis is characterised by a number of morphological and biochemical alterations in cells. [6,8] The morphological changes associated with apoptosis include cell shrinkage and budding of the plasma membrane. In the nucleus, initially, chromatin condensation occurs around the periphery, and the nuclear laminar begins to disappear. [8] Later in the process, highly condensed chromatin with cleaved DNA segregates into defined structures which have an intact nuclear envelope. [7,8] Eventually, the cell breaks up into many spherical bodies called apoptotic bodies, which contain compacted organelles and/or nuclear material. These bodies are mostly engulfed by neighbouring cells, in particular macrophages.[7,8] Biochemical determinants of apoptosis include the formation of internucleosomal DNA fragments of 180 to 200 base pairs.[8,9]

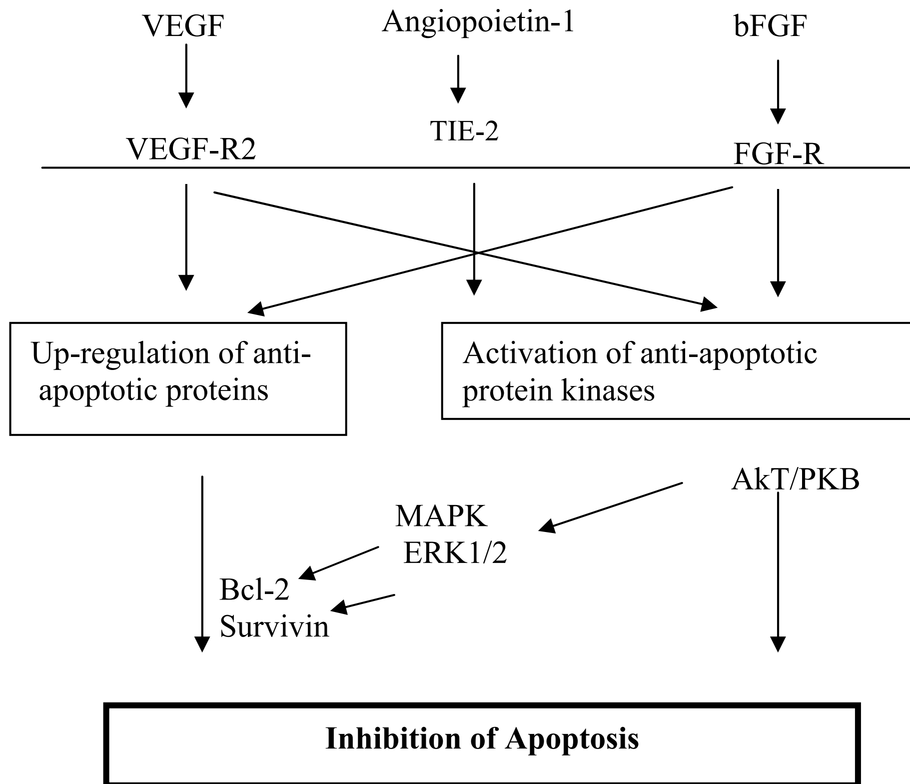


Figure 5.1. Regulation of endothelial cell apoptosis by angiogenic factors.

Adapted from Chavakis and Dimmeler Arterioscler. Thromb Vasc Biol 2002; 22: 887-893

According to Elmore (2007), microscopic observation of morphological changes combined with DNA fragmentation assays are two reliable independent techniques that define cell death occurring by apoptosis. [9]

In this chapter the effects of test drugs on endothelial cell morphology were determined using light microscopy (because of the simplicity and cost-effectiveness of the technique). The potential apoptotic effects of test drugs were further assessed using an assay of DNA fragmentation and acridine orange staining.

5.2. Materials and Method

5.2.1. Cell Morphology

Viable BME cells were seeded aseptically onto heat-sterilised coverslips in six-well culture plates at a density of 2 x 10^5 cells per well. Cells were exposed to bleomycin, mitomycin C, 2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole, or to drug vehicles for 48 hours. The vehicle for bleomycin, mitomycin C, and the vinca alkaloids was saline, while the vehicle for 2-ME, taxol, colchicine, nocodazole and cytochalasin D was 0.05% DMSO. At termination, coverslips were inserted into staining dishes and cells were fixed in Bouin's fixative for 30 minutes. Cells were then left in 70% ethanol for 20 minutes and then rinsed with tap water. Thereafter, cells were stained with Mayer's haemalum for 15 minutes (twice), rinsed with tap water, and subjected to 1% eosin for two minutes. Cells were then rinsed for five minutes in each of the following solvents: 70%, 90%, 100% ethanol and xylol. Finally, coverslips were mounted onto microscope slides with mounting resin. A Nikon Optiphot light microscope was used to study cell morphology and photos were taken using a Nikon digital camera.

5.2.2. DNA fragmentation

Bovine microvascular endothelial cells were seeded into 60 mm tissue culture dishes at a density of 1x10^6 cells/plate. After 24 hours cells were exposed to bleomycin, mitomycin C, 2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole, and cytochalasin D or vehicles for 48 hours. At the end of the incubation, floating cells were recovered from culture media by centrifugation, and adherent cells were washed twice with PBS. Floating and adherent cells were lysed together in 250 µl/tissue culture dish containing 20 mM Tris-HCl (pH 7.4), 5 mM EDTA (pH 7.4), 0.4% Triton X-100. Cell lysates were centrifuged at 13,000 rpm for 30 minutes at 4°C to pellet nuclei.

Supernatants were extracted with equal volumes of phenol-chloroform, precipitated with 0.5 vol. of 7.5 M ammonium acetate (pH 7.5), and three volumes of ethanol, and then centrifuged at 13,000 rpm for 30 minutes at 4°C. Samples were left overnight at -20°C. Pellets were resuspended in 10 mM Tris-HCl (pH 8.0),1 mM EDTA (pH 8.0), containing DNase-free pancreatic ribonuclease (20 µg/ml), incubated at 37°C for 30 minutes and loaded on to 1.8% agarose gels containing ethidium bromide (1 µg/ml). [1,10]

Samples were then electrophoresed at 10 V/cm for two hours. DNA was visualised by UV fluorescence. The appearance of ladder bands signifies DNA cleavage, one of the hallmarks of apoptosis.

5.2.3. Acridine-Orange staining

The mode by which the test drugs induced cell death was further assessed using acridine orange (AO) staining. Cells were seeded in 34.6 mm diameter wells at a density of 200 000 cells per well. After 24 hours, BME cells were grown in the presence of the various drug preparations/vehicles for 48 hours.

Cells were harvested as follows: medium and floating cells were transferred to 15 ml tubes. The rest of the adherent cells were detached with 0.25% trypsin-1mM EDTA. The floating and detached cells from the same sample were pooled together in 15 ml tubes, pelleted by centrifugation at 1000 rpm for 5 minutes using a Beckman Model centrifuge, and cell pellets were washed with 1 ml PBS, and subsequently re-suspended in 25 µl PBS. Cells were then incubated with 10 µg/ml acridine orange for 5 minutes. Stained cell suspensions (10 µl) were placed on microscope slides and covered with coverslips. [11,12]

Cells were viewed and counted using a Zeiss Axiovert inverted microscope at 400X magnification. Pictures were taken with a Nikon digital camera. Apoptosis was identified as nuclear chromatin condensation. Randomly selected microscopic fields were examined for this experimental condition, and the percentage of cells undergoing apoptosis in each field was determined. Results are expressed as the mean percentage (± SD) of cells with evidence of apoptosis. Tests were done in triplicate.

5.3. Results

5.3.1. Cell Morphology

Hematoxylin and eosin (H&E) staining of bleomycin treated-BME cells was undertaken to determine the effects of the drug on cell morphology. In control cultures, cells in interphase and telophase and were observed (fig 5.2 A;B).

Condensed chromatin and structures that resembled apoptotic bodies were evident in bleomycin, mitomycin C, colchicine, nocodazole, and taxol treated cultures. In 2-ME treated cultures, cells in metaphase were observed, however, only some of the chromosomes in these cells were aligned along the equator, while other chromosomes were aligned closer to the poles (fig 5.2 F). Cells which appeared round with dense nuclear chromatin fragments were also observed in 2-ME treated cultures, and according to Elmore (2007), such morphologic appearance is a characteristic of apoptosis. [9]

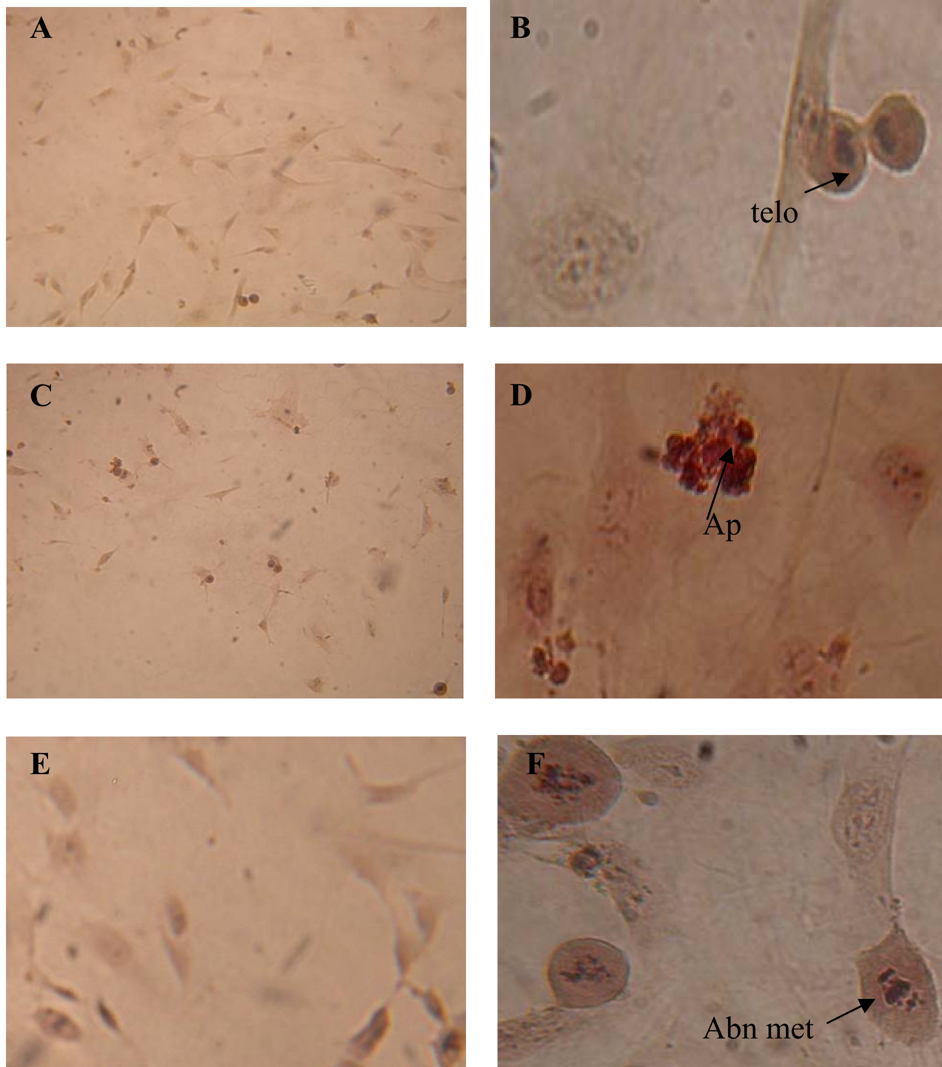


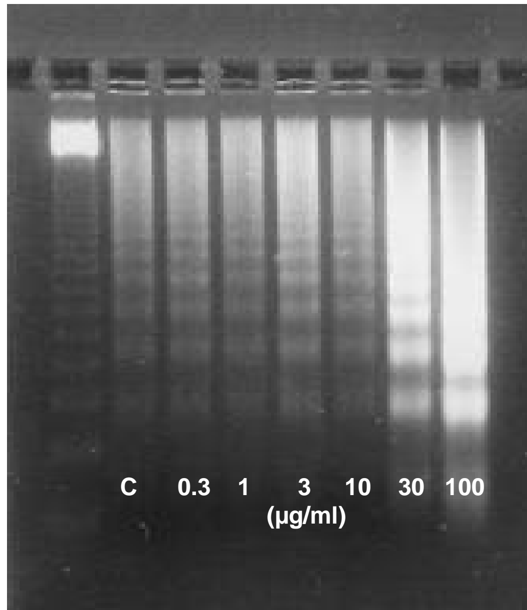
Figure 5.2. Photomicrographs of haematoxylin and eosin (H&E) stained BME cells after 48 hours of exposure to bleomycin or 2-ME.

A and B: Control cells were exposed to vehicle (DMSO) only. Most cells were in interphase, cells in telophase were also observed (arrow). C and D: Cells exposed to bleomycin. The arrow indicates an apoptotic cell. E and F: Cells exposed to 2-ME, the arrow indicates a cell undergoing abnormal metaphase. (A, C, E: original magnification 100X; B, D, F: original magnification 400X)

Ap = apoptosis; Abn met = abnormal metaphase; telo = telophase

5.3.2. DNA Fragmentation

Bleomycin



Mitomycin C

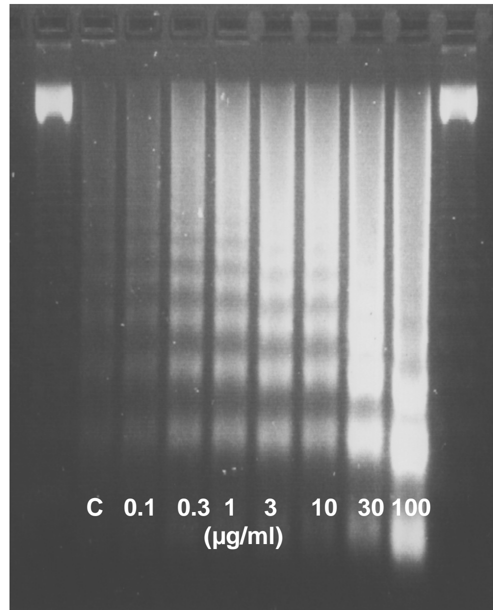
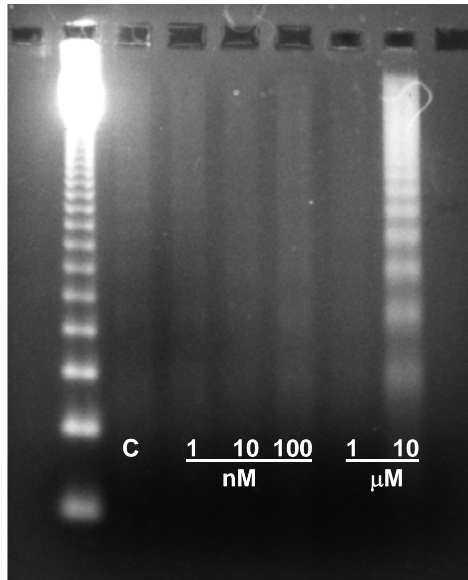


Figure 5.3. Analysis of internucleosomal fragmentation in DNA preparations following exposure of BME cells to bleomycin and mitomycin C.

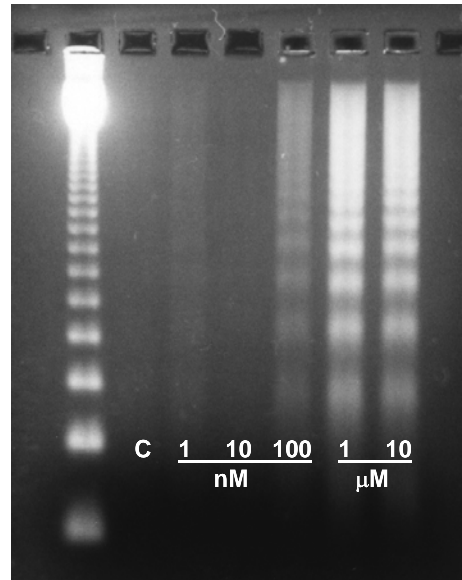
Bleomycin induced DNA strand breaks in a dose-dependent manner (fig 5.3). The most intense ladders were observed following exposure to 30 and 100 µg/ml of bleomycin. DNA strand breaks were also observed in samples exposed to mitomycin C at doses of 0.3 µg/ml and above (fig 5.3). The intensity of the ladders increased with increasing drug concentration.

With respect to cytoskeletal-disrupting drugs, internucleosomal DNA fragmentation was detected only after exposure to the highest dose of 2-ME (10 µM), while taxol and colchicine induced DNA strand breaks at doses of 100 nM and above (fig 5.4). However, no difference in intensity was observed between samples exposed to 1 and 10 µM of taxol. In the case of cytochalasin D treated cells, internucleosomal strand breaks were observed following exposure to doses of 1 and 10 µM, while fragmentation of DNA was detected at 1 and 10 µM in nocodazole-treated cells, and again, increasing drug concentration did not result in increased intensity of DNA strands. Results of vincristine and vinblastine treated cultures were inconclusive.

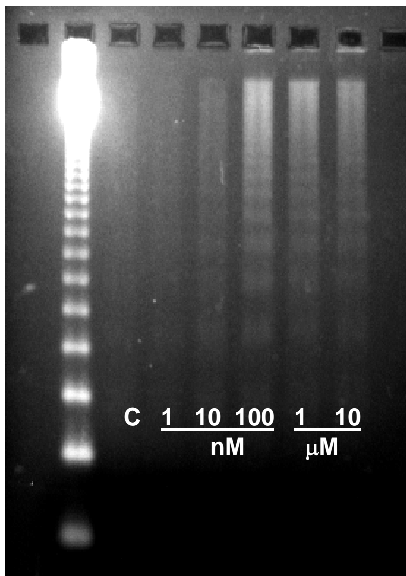
2-Methoxyestradiol



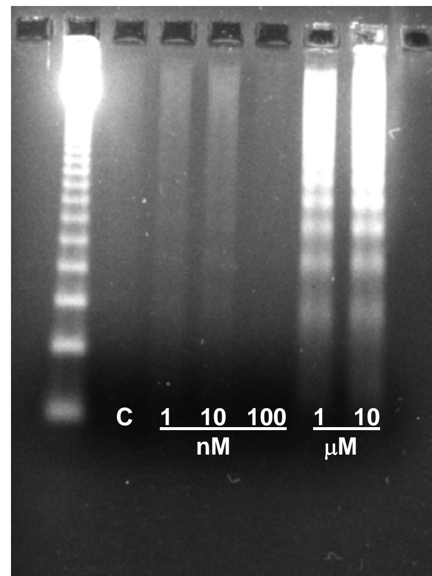
Taxol



Colchicine



Nocodazole



Cytochalasin D

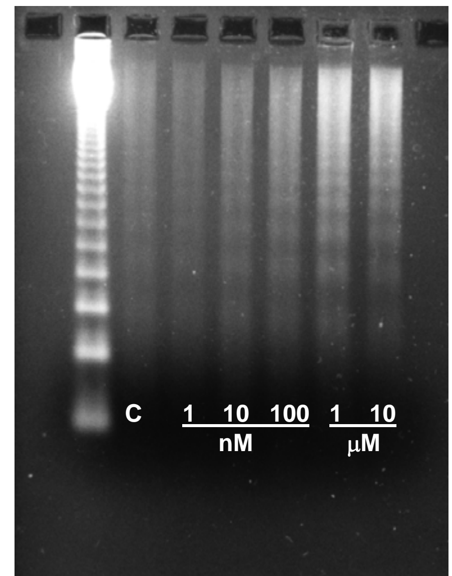


Figure 5.4. Analysis of internucleosomal fragmentation in DNA preparations following exposure to cytoskeletal-disrupting drugs. DNA isolated from untreated preparations was compared to DNA from cells exposed to varying concentrations of the different drugs.

5.3.3. Acridine Orange Staining

Drug-induced apoptosis was confirmed by endothelial cell nuclear acridine orange incorporation. Bovine microvascular endothelial cells were incubated with bleomycin, mitomycin C, 2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole and cytochalasin D for 48 hours, and AO staining was subsequently performed. Table 5.1 shows levels of apoptosis as determined by AO labelling of BME cells treated with the various test drugs. Bleomycin increased AO staining of BME cell nuclei (fig 5.5 B) when compared to the control (fig 5.5 A). Increased AO staining of BME cell nuclei was also observed in cultures treated with mitomycin C, -methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole and cytochalasin D (table 5.1).

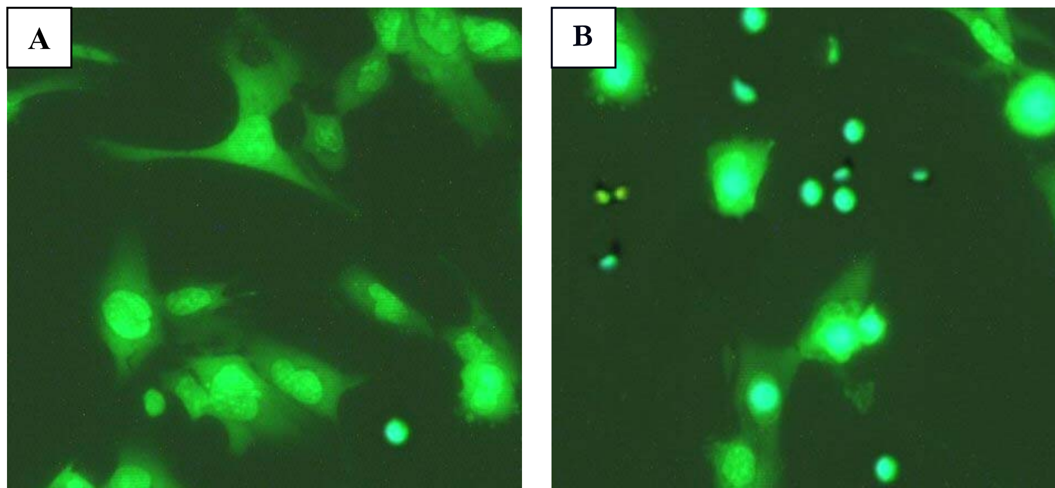


Figure 5.5. Micrographs of acridine orange (AO) stained BME cells after 48 hours of exposure to (A)- vehicle (saline) or (B)-bleomycin. Magnification x400

Table 5.1. Test drugs induce apoptosis in BME cells

Control (Saline)	5.92 ± 4.28
(DMSO)	7.35± 11.25
Bleomycin	
0.1 µg/ml	11.26 ± 9.62
1 µg/ml	18.53 ± 11.65*
10 µg/ml	21.03 ± 11.61*
100 µg/ml	41.72 ± 16.35*
Mitomycin	
0.1 µg/ml	22.55 ± 18.6*
1 µg/ml	33.76 ± 18.21*
10 µg/ml	58.10 ± 20.42*
Colchicine	
0.01 µM	6.21 ± 4.02
0.1 µM	10.25 ± 12.05
1 µM	33.52 ± 11.26*
10 µM	31.05 ± 9.65*
Nocodazole	
0.01 µM	12.53 ± 9.36
0.1 µM	21.4 ± 12.22*
1 µM	43.87 ± 9.75*
10 µM	40.25 ± 8.15*
2-ME	
0.01 µM	18.57 ± 8.05
0.1 µM	14.23 ± 12.22
1 µM	32.81 ± 12.50*
10 µM	52.21 ± 10.02*
Taxol	
0.01 µM	17.82 ± 6.22
0.1 µM	28.74 ± 13.64*
1 µM	45.37 ± 10.80*
10 µM	44.00 ± 8.01*
Vincristine	
0.001 ng/ml	5.92 ± 6.10
0.1 ng/ml	5.87 ± 4.25
1 ng/ml	8.22 ± 9.65
10 ng/ml	27.26 ± 11.20*
Vinblastine	
0.001 ng/ml	6.04 ± 8.22
0.1 ng/ml	12.01 ± 8.75
1 ng/ml	10.72 ± 12.20
10 ng/ml	37.20 ± 11.26*

Confluent monolayers of BME cells were exposed to the agents indicated above, and apoptosis (acridine orange labelling) was determined after 48 hours. Values are the mean ± SD. Results are pooled from at least three separate experiments. An average of 12 fields was analyzed per treatment dose in each experiment; *Significant difference between control and drug-treated cells: $P < 0.05$. The control for bleomycin, mitomycin C, and vinca alkaloids was saline; the vehicle for other drugs was DMSO.

5.4. Discussion

The inhibition of apoptosis has been cited as an essential mechanism during angiogenesis. Apoptosis is a physiological process through which cells are eliminated from the body as a normal part of development and homeostasis. [2] The characteristic morphological and biochemical changes that define apoptosis have served as tools for the detection and analysis of this process. [9] To determine possible apoptosis induction by test drugs, effects of these drugs on cell morphology were investigated using light microscopy.

In control (DMSO-treated) cultures, normal cells in various stages of mitosis were observed (fig 5.2 B). Similar observations were made for cells treated with saline. In drug-treated cultures, including bleomycin-treated cultures, cells showing features of apoptosis were observed (fig 5.2 D). Morphological studies also revealed that 2-ME induced metaphase characterized by abnormal alignment of chromosomes (fig 5.2 F), possibly due to disruption of microtubules. Apoptotic cells were also observed in 2-ME treated cultures (fig 5.2 F). In a previous study conducted on the breast cancer cell line, MCF-7, 2-ME was shown to induce a metaphase block which ultimately lead to the induction of apoptosis. It is possible that in this study, the 2-ME induced abnormal alignment of chromosomes during metaphase prevented cells from proceeding beyond this phase of the cell division cycle, and resulted in BME cell morphology characteristic of apoptosis. Microscopic observations of cells treated with vinca alkaloids were inconclusive. While light microscopy provides useful information on morphological changes in cells undergoing apoptosis, chromatin condensation and DNA fragmentation have been cited as early and relatively unequivocal hallmarks of apoptosis. [6.,9,10]

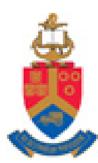
It has also been suggested that as a rule, classification of cell death in a given model should always include morphological examination coupled to at least one other assay. [6] Therefore, in this study, DNA fragmentation and chromatin condensation were assessed to further determine possible apoptosis.

When DNA extracted from cells treated with bleomycin, mitomycin C, 2-ME, taxol, nocodazole and cytochalasin D was analyzed using gel electrophoresis, characteristic internucleosomal “ladders” of DNA fragments were found (fig 5.3 and 5.4). In respect to the intensity of DNA ladders, a dose response was apparent in samples treated with bleomycin and mitomycin C, with greater evidence of DNA fragmentation in cells exposed to higher drug doses (fig 5.3).

Internucleosomal DNA fragmentation is a biochemical indicator of apoptosis, and is attributable to the activation of endogenous endonucleases which cleave DNA. [9]

Apoptosis was also determined by AO labelling of BME cells treated with the various test drugs. Bleomycin increased acridine orange staining of BME cell nuclei (fig 5.5 B). Increased AO-staining of cell nuclei was also observed in cells treated with test drugs, indicating that these drugs induced apoptosis.

In this chapter it was demonstrated that test drugs induced BME cell apoptosis. Since a balance between endothelial cell proliferation and apoptosis ensures homeostasis of the vascular endothelial cell population, [1] and proangiogenic factors promote neovessel formation by inhibiting apoptosis, increased endothelial cell apoptosis may lead to angiogenesis inhibition. Indeed, several potent angiogenesis inhibitors, such as angiostatin and endostatin, were reported to inhibit angiogenesis in part by inducing endothelial cell apoptosis. [3] Furthermore, apoptosis has been shown to increase five-fold in haemangiomas undergoing regression. [13] Therefore the apoptotic effects of these test drugs on endothelial cells suggests that they may have potential in inhibiting the excessive angiogenesis associated with haemangioma development.



References

1. Polunovsky VA, Wendt CH, Ingber DH, Peterson MS, Bitterman PB. Induction of endothelial cell apoptosis by TNFa: Modulation by inhibitors of protein synthesis. Exp Cell Res1994; 214:584-94.

2. Chavakis E, Dimmeler S. Regulation of endothelial cell survival and apoptosis during angiogenesis. Arterioscler Thromb Vasc Biol 2002; 22: 887-93.

3. Folkman, J. Angiogenesis and apoptosis. Cancer Biol 2003; 13:159-67.

4. Pollman MJ, Naumovski L, Gibbons GH. Endothelial cell apoptosis in capillary network remodelling. J Cell Physiol 1999; 178:359-70.

5. Choy JC, Granville DJ, Hunt DWC, *et al*. Endothelial cell apoptosis: Biochemical characteristics and potential implications for atherosclerosis. J Mol Cell Cardiol 2001; 33:1673-90.

6. Catchpoole DR, Stewart BW. Formation of apoptotic bodies is associated with internucleosomal DNA fragmentation during drug-induced apoptosis. Exp Cell Res 1995; 216:169-77.

7. Huerta S, Coulet EJ, Huerta-Yepez S, *et al.* Screening and detection of apoptosis. J Surg Res 2007; 139:135-56.

8. Lopez-Larraza DM, Padron J, Ronci NE, *et al*. Chromatin condensation and differential sensitivity of mammalian and insect cells to DNA strand breaks induced by bleomycin. Mutation Res 2006; 600:93-101.

9. Elmore S. Apoptosis: A review of programmed cell death. Tox Path 2007; 35:495-16.

10. Tounekti O, Belehradek J, Mir LM. Relationships between DNA fragmentation, chromatin condensation, and changes in flow cytometry profiles detected during apoptosis. Exp Cell Res 1995; 217:506-16.

11. Willingham MC. Cytochemical methods for the detection of apoptosis. J Histochem Cytochem 1999; 47:1101-9.

12. Ribble D, Goldstein N, Norris D, Shellman Y. A simple technique for quantifying apoptosis in 96-well plates. BMC Biotechnol 2005; 5:12-24.

13. Hasan Q, Roger BM, Tan ST, Gush J, Davis PF. Clusterin/ApoJ expression during the development of hemangioma. Human Pathology 2000; 31: 6 691-7.