FEBS Letters 419 (1997) 166–170 FEBS 19590

A cyclic peptide analogue of loop III of PDGF-BB causes apoptosis in human fibroblasts

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Received 29 October 1997

Abstract A cyclic peptide analogue of platelet-derived growth factor-BB (PDGF-BB), P1 [^{77}IVRKK^{81}-C-^{73}RKIE^{76}], has recently been shown to inhibit specifically [^{125}I]PDGF-BB/receptor binding, and PDGF-BB-induced DNA synthesis in cells expressing PDGF receptors. Here we demonstrate that P1 induces apoptosis in exponentially growing human fibroblasts as confirmed by characteristic changes in cell and nuclear morphology, by TUNEL staining and by flow cytometry. Following incubation with P1 (100 μM), the percentage of cells exhibiting DNA fragmentation increased from 24% after 8 h to 76% after 28 h as exponentially growing cells progressed through the cell cycle. We conclude from these findings taken together that apoptosis accounts for the major proportion of P1-induced cell death. Omission of the Cys residue from P1 or replacement by Ser did not alter the potency of the peptide confirming that peptide dimerisation is not important for its activity. PDGF-BB, EGF, FGF, thrombin and foetal bovine serum were not able to rescue cells from the effects of P1. P1 is a useful tool for investigation of the balance of cellular proliferation/apoptosis in wound healing, atherosclerosis and restenosis, and constitutes a basis from which to design compounds with greater potency.

Key words: Apoptosis; Cyclic peptide; Platelet-derived growth factor; Human dermal fibroblast

1. Introduction

In tissues capable of self-renewal cellular proliferation exists in a regulated equilibrium with cell death, the latter occurring frequently via apoptosis [1,2]. A demonstration of this equilibrium has been made in arteries following balloon injury in which smooth muscle cell (SMC) content has been observed to increase by only 10% of the cell number predicted from the rate of cell proliferation [3]. This discrepancy may be explained by the concurrence of cell death alongside proliferation [3–6]. Apoptosis as a mode of cell death in the arterial vessel wall has been demonstrated in studies of SMC death in human atherosclerotic lesions [6–8], and following balloon injury [8,9]. In vivo, immune cytokines including interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and interleukin-1β may initiate SMC apoptosis [10], with protection from apoptosis being supplied by survival factors [11], or through interactions of SMC with the extracellular matrix [12,13] or endothelial cells at the luminal surface [8,9]. Although the molecular basis of apoptosis is largely unknown, events originally considered to be solely associated with the induction of cellular proliferation, for example receptor phosphorylation [14] and activation of transcription factors including c-*myc* [15,16], c-*fos* [17] and c-*jun* [18], have recently been shown to occur during apoptosis. Development of agents which reduced the cellular proliferation associated with atherosclerosis and restenosis by triggering apoptosis would have therapeutic potential.

Platelet-derived growth factor (PDGF) is a major connective tissue cell mitogen and chemotactic factor [19,20] which has roles in normal development [21,22], wound healing [23] as well as in several aberrant conditions including atherosclerosis and fibrosis [19,24]. The mitogenic biological activity of PDGF isoforms, homo- and heterodimers of A- and B-chains is mediated through binding to α and β cell surface receptor subunits initiating dimerisation of transcellular domains to homo- and heterodimers (reviewed in [25]). High affinity receptors for growth factors including PDGF activate cellular tyrosine kinases leading to subsequent receptor autophosphorylation and ultimately induction of transcriptional activators and subsequent cellular proliferation [25,26].

Recently we reasoned that cyclisation of peptides corresponding to PDGF-BB$^{73-81}$ (loop III) would generate compounds capable of mimicking more closely the natural conformations of this region within PDGF-BB [27] whilst also reducing the conformational entropic penalty barrier observed in receptor/peptide interactions [28,29]. Cyclic PDGF$^{73-81}$ (**P1**) acts as a potent inhibitor of PDGF-BB-induced DNA synthesis in human fibroblasts and competitively inhibits the binding of PDGF-BB but not epidermal growth factor (EGF) to each of the two respective cellular receptors. The inhibitory action of **P1** upon DNA synthesis is restricted to cells expressing PDGF receptors as the peptide had no effect upon the PDGF receptor-negative cell lines A431 and COS-1 [27].

In the present work we establish that **P1** is able to induce apoptosis in human fibroblasts, an event which is not dependent upon peptide dimerisation. PDGF-BB and other growth factors likely to be present in the microenvironment of the damaged vessel wall in atherosclerosis and restenosis are not able to rescue cells from **P1**-induced apoptosis. We also provide evidence that high concentrations of PDGF-BB may also induce cell death and DNA fragmentation in human fibroblasts.

2. Materials and methods

2.1. Materials

All chemicals were of the highest grade and were from Sigma Chemical Company Ltd., Poole, UK, unless stated. Other materials

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Abbreviations: PDGF, platelet-derived growth factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum

PII S0014-5793(97)01446-4

suppliers were: peptide synthesis reagents (PerSeptive Biosystems, Hertford, UK), tissue culture reagents (Life Technologies Ltd., Paisley, UK), plastics [Falcon] (Marathon Laboratory Supplies, London, UK), methyl-[^{3}H]thymidine (1.85 TBq/mmol) (ICN Biomedicals Ltd., Thame, UK), recombinant human PDGF-BB, EGF and fibroblast growth factor (FGF, 146-amino acid isoform) (R&D Systems Europe Ltd., Abingdon, UK), thrombin (gift from Dr. J-M. Freysinnet, University Louis Pasteur de Strasbourg), ethidium homodimer and calcein AM (Molecular Probes Inc., Eugene, OR, USA), fluorescent mounting medium (Dako Corporation, California, USA), fluorescein apoptosis detection kit (Promega, Southampton, UK), propidium iodide solution (Coulter Electronics Ltd., Luton, UK) and Vectashield mounting medium with 4,6-diamidino-2-phenylindole (DAPI) from Vector Laboratories Inc., California, USA. The HFFF2 cell line (human dermal fibroblast) was from the European Tissue Culture Collection, Porton Down, UK, and was maintained in complete DMEM (DMEM supplemented with 4 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin) as previously reported [27].

2.2. Peptide synthesis

Peptides were synthesised as previously reported [27]. The purity and peptide content were determined by analytical RP-HPLC and ESI mass spectrometry. The cyclic peptides and corresponding PDGF-BB residue numbers used in this study were: ^{77}IVRKK^{81}-C-^{73}RKIE^{76} (**P1**), ^{77}IVRKK^{81}-^{73}RKIE^{76} (**P2**) and ^{77}IVRKK^{81}-S-^{73}RKIE^{76} (**P3**). A linear peptide ^{73}RKIEIVRKK^{81}-C (**P4**) was also synthesised.

2.3. The effects of peptides on DNA synthesis

Previously reported methodology was used [27] except that 0.2% FBS was used in growth arrest medium. Coaddition to cells of peptides was with PDGF-BB (15 ng/ml, 0.5 nM), EGF (1 ng/ml, 0.167 nM), FGF (1 ng/ml, 0.063 nM) or thrombin (4 U/ml), added at time zero. Inhibition of DNA synthesis was expressed as reduction in [^{3}H]thymidine calculated as a percentage of stimulation observed in the absence of peptide. Three different synthetic batches of **P1** were shown to inhibit PDGF-BB-induced DNA synthesis with a mean EC_{50} of 5.27 μM (±S.D. = 2.3 μM), in general agreement with the previously reported value of 9.5 μM [27].

2.4. Discrimination of live and dead cells by microscopy

HFFF2 cells were grown to 40% confluence (2 days) on coverslips and the medium was replaced by complete DMEM containing 8% FBS together with **P1** (100 μM), and cultured continued for 8 h. Coverslips were then washed with PBS (3×2 min) and incubated under PBS containing 4 μM ethidium homodimer and 3 μM calcein (30 min). Cell-permeant calcein AM is converted to fluorescent calcein by intracellular esterases within viable cells, whilst ethidium homodimer only enters dead cells giving a red fluorescent staining of nucleic acids [30]. After washing with PBS (2×3 min) and distilled water (1×2 min), coverslips were mounted and examined immediately under a triple band (fluorescein, rhodamine, DAPI) filter. Exponentially growing cells treated with saponin (0.1%, 10 min) were used as control dead cells.

2.5. Measurement of cell viability

Exponentially growing HFFF2 cells at 50% confluence, in 24-well plates, were cultured with complete DMEM containing 8% FBS and varying concentrations of **P1** for 8 h or 28 h. Cells were scraped to harvest both adherent cells and floating cells, centrifuged (600×*g*, 6 min), washed with PBS, incubated with 400 μl PBS/6 μM calcein AM (30 min, dark) and fluorescent intensity (FI) measured in a luminescence spectrophotometer (excitation at 485 nm, emission read at 530 nm). Positive controls were prepared untreated cells, and negative controls (of dead cells) were prepared by treating the cells with 0.1% saponin in PBS for 30 min. Triplicates were performed for each experimental condition, and the total percentage of live cells calculated using the formula:

$$\%\ \text{Live cells} = \frac{100 \times \text{sample FI} - \text{FI of saponin} - \text{treated cells}}{\text{FI of untreated cells} - \text{FI of saponin} - \text{treated cells}}$$

2.6. Terminal deoxynucleotidyl transferase mediated dUTP nick-end labelling (TUNEL) assay

HFFF2 cells seeded onto 24-well plates (10 000 cells/well) were grown to 40% confluence (2 days) in complete DMEM containing 8% FBS, at which time fresh medium containing varying concentrations of **P1** was added. Duplicates of each experimental condition were performed. Following culture for 8 h or 28 h, adherent cells were removed by scraping and the total cell population transferred onto glass microscope slides (cytospin, 600 rpm, 5 min) and fixed (100% methanol). Cells were permeabilised by immersion in PBS containing 0.2% Triton X-100 for 5 min and the TUNEL reaction was performed as specified by the manufacturer. Slides were mounted using Vectashield mounting medium containing DAPI (4.3 μM). TUNEL-positive cell nuclei were counted when slides were viewed under a fluorescein filter and are expressed as a percentage of the total number of nuclei counted when slides were viewed under a fluorescein/rhodamine/DAPI filter.

*2.7. FACS analysis of cells treated with **P1***

HFFF2 cells were seeded into 25-cm^2 flasks (400 000 cells/flask) and cultured for 48 h in complete DMEM containing 8% FBS. The medium was replaced with 4 ml of complete DMEM/8% FBS containing either **P1** (100 μM) or PDGF-BB (16.7 nM) and culture continued for 28 h. Cells were dislodged from the plastic by scraping, harvested by centrifugation (600×*g*, 5 min, 4°C), washed once with PBS and fixed by incubation in ice-cold 70% ethanol for 30 min. After a single wash with PBS, cells were incubated with 700 μl propidium iodide at 4°C for 30 min in the dark. Samples were then analysed by flow cytometry using a Coulter Elite Flow cytometer.

3. Results and discussion

P1, **P2** (minus Cys) and **P3** (Cys replaced by Ser) gave similar dose-dependent profiles for the inhibition of PDGF-BB-induced DNA synthesis in human fibroblasts, having EC_{50} values in the range of 5.1–7.05 μM (Fig. 1). A linear counterpart of **P1**, **P4**, had no effect upon PDGF-BB-induced [^{3}H]thymidine incorporation when tested up to 100 μM. We conclude that the presence of Cys is not important for the inhibitory activity of **P1**. This suggests that ligand dimerisation and consequent receptor dimerisation are not required, and that the effects of **P1**, **P2** and **P3** are mediated through monomeric receptor binding. This is in contrast to the mitogenic response elicited by dimeric PDGF-BB, in which receptor dimerisation is crucial in obtaining a mitogenic response [25].

During these experiments, we observed that **P1** added alone caused some cell death when used at higher concentrations. This may explain why the [^{3}H]thymidine incorporation level observed when HFFF2 was incubated with **P1** at 100 μM is

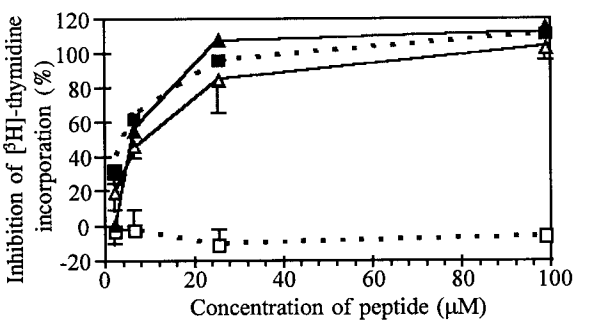

Fig. 1. The inhibition of PDGF-induced DNA synthesis in human fibroblasts by peptides derived from loop III of PDGF-BB. **P1** (filled squares), **P2** (open triangles), **P3** (filled triangles) or **P4** (open squares) were added to growth-arrested HFFF2 cells in combination with PDGF-BB (0.5 nM). Percentage inhibition of [^{3}H]thymidine incorporation was calculated relative to cells not treated with peptide. The results shown are the mean of triplicate determinations (±S.E.M.).

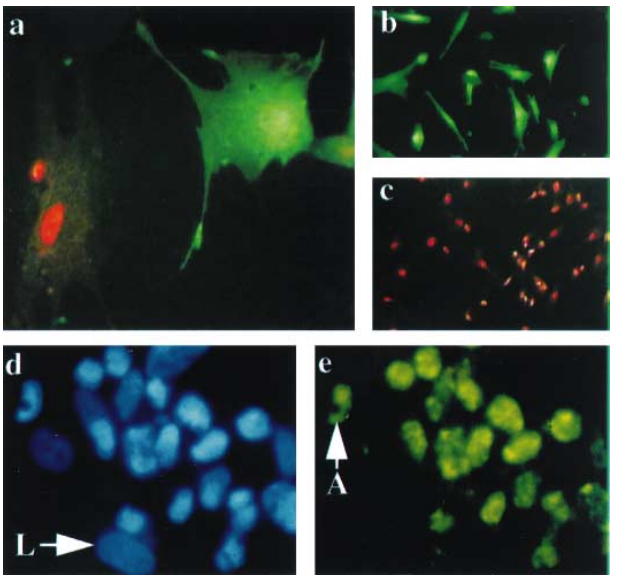

Fig. 2. The effect of **P1** on exponentially growing human fibroblasts. Immunofluorescent micrographs of HFFF2 cells; (a) treated with 100 μM **P1** for 8 h (magnification ×200), (b) untreated, (c) permeabilised by treatment with 0.1% saponin for 10 min (magnification ×40). Cells were double-stained with ethidium homodimer (nuclei of dead cells stain red) and calcein (live cells stain green). Immunofluorescent micrographs (magnification ×166) of the same HFFF2 cells treated for 28 h with **P1** (100 μM) and dual-labelled (d) by DAPI (shows the total number of nuclei in blue) and (e) with the TUNEL assay (stains only those nuclei with fragmented DNA yellow/green). Less than 2% of untreated cells were TUNEL-positive (not shown). Arrows indicate: A, dead cell showing apoptotic morphology and L, live cell.

below that of the 0.2% FBS control, giving an apparent inhibition of [^{3}H]thymidine incorporation greater than 100% (Fig. 1). Histological examination of cells treated with **P1** (100 μM, 8 h) showed the presence of some dead cells, the majority of which exhibited a decrease in volume, membrane blebbing, nuclear condensation and often the presence of apoptotic bodies (Fig. 2a), all of which are characteristic of cells undergoing apoptosis [31,32].

Following preliminary studies, quantification of TUNEL staining [33] was examined following 8 h and 28 h incubation of cells with **P1**. We considered 8 h to be the shortest time period at which significant cell death and DNA fragmentation was observed and could be reliably assessed, and 28 h represented the doubling time of settled, exponentially growing HFFF2 cells. The total numbers of cell nuclei stained with DAPI (Fig. 2d) were compared with the numbers of nuclei containing fragmented DNA labelled at the 3′-OH end by fluorescein-12-dUTP (Fig. 2e). TUNEL-labelled nuclei (Fig. 2e) appear brighter in the DAPI image (Fig. 2d) due to the cooperative effects of both TUNEL and DAPI fluorescence. The images clearly show that TUNEL-positive nuclei are smaller with condensed chromatin, two of the classical morphological features of apoptosis. The percentage of TUNEL-positive cells increased after incubation for 8 h and 28 h with **P1** in a dose-dependent manner (Fig. 3), reaching a maximum of 76% at 100 μM **P1** (28 h). The viability of exponentially growing HFFF2 cells decreased with increasing concentrations of **P1** in a dose-dependent manner (Fig. 3), reaching a minimum of 8% at 100 μM **P1** (28 h). Analysis of the sub-G_0/G_1 peak, representing cells with less than diploid DNA content, by flow cytometry confirmed DNA fragmentation which reached 48% of events for exponentially growing cells treated with **P1** (100 μM, 28 h), as compared to 6.8% for untreated cells (Fig. 4). As observed with the TUNEL assay, the sub-G_0/G_1 peak was lower after 8 h incubation with **P1**, being 12%.

No one criterion alone can be said to be indicative of apoptosis. We have shown that exponentially growing HFFF2 cells treated with **P1** undergo cell shrinkage, membrane blebbing, nuclear condensation and generation of apoptotic bodies, using different staining methods. We have also shown that **P1** induces DNA fragmentation as assessed by both TUNEL staining and flow cytometry and that this is time-dependent in exponentially growing cells. From a combination of these criteria, we conclude that apoptosis accounts for the major

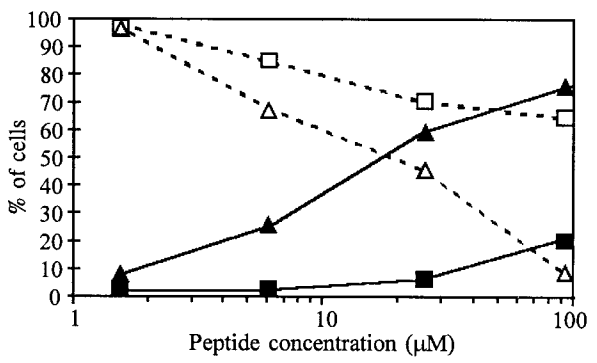

Fig. 3. The influence of **P1** upon cell viability and DNA fragmentation in exponentially growing human fibroblasts. HFFF2 cells were cultured for 8 h and 28 h in the presence of varying concentrations of **P1**. Percent viability was determined by calcein staining, as described in Section 2, after 8 h (open squares) or 28 h (open triangles) treatment with **P1**. Results are the mean of triplicate determinations (S.E.M. ≤ 2.75% in all cases). TUNEL-positive cells with shrunken nuclei are expressed as a percentage of the total number of nuclei (DAPI-stained) after treatment of cells with **P1** for 8 h (filled squares) or 28 h (filled triangles). At least 350 cells were counted for each condition.

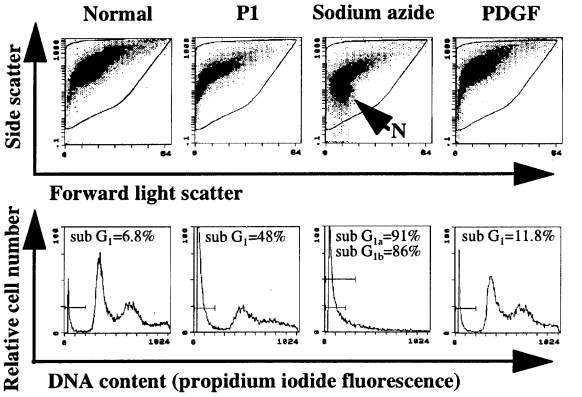

Fig. 4. Flow cytometric analysis of exponentially growing human fibroblasts following treatment with **P1**. HFFF2 cells were cultured for 28 h with **P1** (100 μM) or PDGF-BB (0.5 nM), fixed, stained with propidium iodide and analysed by flow cytometry (15000 events) as described in Section 2. HFFF2 cells treated for 4 h with sodium azide (1 M) were included as an example of the profiles produced by necrotic cells [34]. Arrow N indicates an extra population of cells seen in the forward/side scatter plot in necrotic cells. The percentage of sub-G_0/G_1 events is indicated for each of the DNA content histograms.

Table 1
Inhibition of DNA synthesis in HFFF2 cells treated with **P1** in the presence of various growth factors

Growth factor	Assay duration (h)	EC_{50} inhibition (μM)	S.D.	EC_{100} inhibition (μM)
PDGF (15 ng/ml)	28	5.27	2.30	25
EGF (1 ng/ml)	28	7.37	0.84	20
FGF (0.5 ng/ml)	28	2.50	0.50	6
Thrombin (4 U/ml)	28	3.35	0.75	19
Thrombin (4 U/ml)	44	8.00	1.00	25
FBS 8%	28	15.00	5.00	45

This table shows the concentrations of **P1** which cause 50% (EC_{50}) and 100% (EC_{100}) inhibition of growth factor-induced [^{3}H]thymidine incorporation in quiescent human dermal fibroblasts (HFFF2) stimulated with various mitogens. Linear peptide, ^{73}RKIEIVRKK^{81}-C (**P4**), which corresponds in amino acid sequence to **P1**, showed no ability to inhibit DNA synthesis in the presence of any of the mitogens used.

proportion of cell death induced by **P1**, and that this phenomenon is cell cycle-dependent possibly reflecting the need for exponentially growing cells to reach the G_1/S checkpoint prior to apoptosis. Also, **P1**-induced apoptosis occurs in exponentially growing human fibroblasts in the presence of 8% FBS, optimal conditions for growth in which no shortage of progression factors should exist.

Cells treated with high concentrations of PDGF-BB (16.7 nM) show an approximate doubling of DNA fragmentation when compared to untreated cells, with a sub-G_0/G_1 peak representing 11.8% of total events (Fig. 4). EGF (16.7 nM) had no effect upon DNA fragmentation (data not shown). Cells treated with high concentrations of PDGF-BB (16.7 nM) show similar morphological characteristics as those treated with **P1** including the apoptotic features of cell shrinkage and membrane blebbing, although cells treated with the same concentration of EGF appeared unchanged (Fig. 5). Apoptosis induced by high concentrations of PDGF has previously been reported in growth-arrested murine fibroblasts [35]. It would seem that although normal signalling of the PDGF receptor leads to a mitogenic signal and even rescue from apoptosis [36], when present at high concentration PDGF may induce apoptosis. Such high concentrations may occur in microenvironments present in areas of platelet deposition, tissue remodelling and wound healing.

P1 was able to inhibit mitogen-induced DNA synthesis in HFFF2 cells in the presence of PDGF-BB, EGF, FGF, thrombin, and FBS with effective EC_{50} values in the range of 2.5–15 μM (Table 1). 100% inhibition of proliferation was achieved for each of the single mitogens at ≤25 μM of **P1** and for FBS at a concentration of 45 μM. Cells treated with thrombin were also pulsed between 38 and 44 h to determine the effect on cells proliferating due to the secondarily induced responses of thrombin action (e.g. the PDGF autocrine pathway). Independent of the growth factor under test, at high concentrations of **P1** (25–100 μM), all cells showed cell death and had the morphology of apoptotic cells, represented in Fig. 5c.

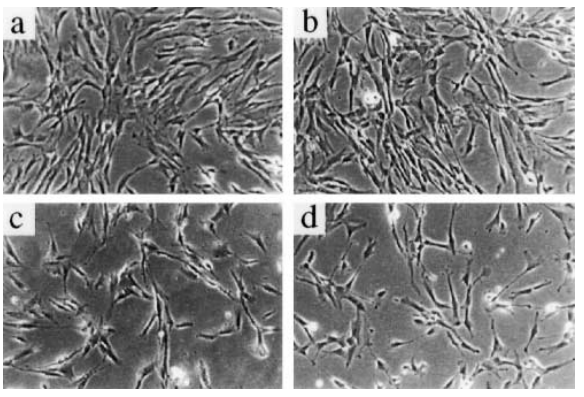

Fig. 5. Appearance of exponentially growing human fibroblasts (magnification ×47.5) cultured for 28 h in the presence of: (a) 8% FCS, (b) 8% FCS+EGF (16.7 nM), (c) 8% FCS+**P1** (100 μM), (d) 8% FCS+PDGF (16.7 nM). Cells were all at 40% confluence before commencement of the experiment.

Apoptosis is an active in vivo mechanism by which unwanted cells commit suicide and are subsequently removed by phagocytosis, thus avoiding release of intracellular contents and associated necrotising inflammatory response [37]. Disruption of the proliferative/apoptotic equilibrium is a potential therapeutic strategy from which to regulate normal and abnormal states of cellular proliferation during wound healing, atherosclerosis and restenosis. **P1** may be a useful compound with which to study the induction of apoptosis in vascular tissue, and may constitute a starting compound from which to develop further apoptosis-inducing agents.

Acknowledgements: We wish to express our thanks to Drs. Sue Smith and Bupinder Sirha for assistance in FACS analysis, to Dr. Jacqui Schmitt for advice about the TUNEL assay, to Dr. Florea Lupu for advice regarding microscopy and to Ulla Dennehy and Sally Mill for their help in tissue culture. Our gratitude is also expressed to Dr. Richard Knight and Dr. David O'Regan for encouragement, many useful discussions, and constructive criticism in the preparation of the manuscript. This investigation was made possible by a generous donation from the Estate of the late Leopold Muller.

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