RESEARCH COMMUNICATION

Endothelin attenuates apoptosis in human smooth muscle cells

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An imbalance between proliferation and apoptosis is an important causal factor for disorders involving abnormal cell accumulation. Endothelin (ET)-1, a 21-amino-acid peptide with mitogenic and vasoconstricting activities, not only acts as a mitogen, but also attenuates paclitaxel-induced apoptosis in smooth muscle cells. In both human pericardial and prostatic smooth muscle cells, addition of ET-1 reduced paclitaxel-induced DNA fragmentation and phosphatidylserine on the cell surface,

two characteristics of apoptosis. By comparison, angiotensin II, another vasoactive peptide, did not have a significant effect on apoptosis. The effect of ET-1 was dose-dependent with an EC_{50} of 1 nM. These results suggest that ET is a potential survival factor for smooth muscle cells, and that altered activity of the ET system in disease states has potential to contribute to aberrant cell growth.

INTRODUCTION

Mammalian endothelins (ETs) 1, 2 and 3 are a family of 21-amino-acid peptides with mitogenic and vasoconstricting activities [1,2]. The effects of ETs on mammalian organs and cells are initiated by their binding to G-protein-coupled receptors found in various tissues and cells [3]. Two types of mammalian ET receptors, ET_A and ET_B , have been characterized and purified, and their cDNAs have been cloned [4–6]. ET_A receptors are selective for ET-1 and ET-2, whereas ET_B receptors bind ET-1, ET-2 and ET-3 with equal affinity.

ET-1 is thought to play important roles in various pathophysiological conditions including cell growth disorders. For example, ET-1 may be involved in the pathogenesis of restenosis in the arteries, a disorder due to the abnormal accumulation of smooth muscle cells [7,8]. Studies have also suggested that ET-1 may contribute to smooth muscle hyperplasia in the lung [9]. Recently, Nelson et al. [10,11] have shown that plasma ET-1 levels are elevated in advanced prostate cancer patients and that ET-1 may be associated with the progression of metastatic prostate cancer. Although ET is a mitogen for various cells, including smooth muscle cells [12–15] and prostate cancer cells [10,11], the growth-promoting effect of ET-1 is modest in comparison with other growth factors such as epidermal growth factor or basic fibroblast growth factor [16,17]. Based on the mitogenic effects alone, it is difficult to comprehend the significant role of ET-1 in cell growth disorders. It is now well accepted that cell growth disorders are often the result of an imbalance between the rate of proliferation and the rate of apoptosis. Therefore, we hypothesize that ET-1, in addition to its mitogenic effects, may regulate the process of cell apoptosis. We have previously characterized ET receptors in human pericardial smooth muscle cells (HPSMC) [18,19], and have shown that ET-1 stimulates the proliferation of HPSMC. In the present paper we test this hypothesis by first studying the effect of ET-1 on modulating apoptosis in these cells, and then extending the studies to human prostatic smooth muscle cells (PrSMC).

MATERIALS AND METHODS

Materials

ET-1 and angiotensin II (ANG II) were purchased from American Peptide Company (Sunnyvale, CA, U.S.A.). ^{125}I -ET-1 was from Dupont-NEN (Boston, MA, U.S.A.). A-127722 [*trans-trans*-2-(4-methoxyphenyl)-4-(1,3-benzodioxo-5-yl)-1-[(*N,N*-dibutylamino)carbonylmethyl]pyrrolidine-3-carboxylic acid] was synthesized in house. Other reagents were of analytical grade.

Cell culture

HPSMC were provided by Dr. Maria J. Vidal (Department of Cardiology, Istituto Scientifico San Raffaele, Milano, Italy) and were cultured as described previously [18,19]. PrSMC were purchased from Clonetics (San Diego, CA, U.S.A.) and were grown in SmGM media (Clonetics; Catalogue number CC-3180, media formulated for smooth muscle cells) containing 5% (v/v) fetal bovine serum (FBS). Cell viability was examined by the Trypan Blue exclusion method.

Apoptosis detection by ELISA

Cells in 96-well plates were treated with test reagents for 20 h at 37 °C. The samples were assayed for apoptosis using an ELISA kit according to the manufacturer's instructions (Boehringer-Mannheim). Briefly, cells were lysed in 200 μ l of lysis buffer (supplied in the ELISA kit). The cell lysates were collected and centrifuged at 200 *g* for 10 min. Monoclonal antibodies directed against DNA and histones in a quantitative sandwich-enzyme-based format are used in the ELISA. The amount of histone-associated DNA fragments (mono- and oligo-nucleosomes) in the cell lysates was determined at A_{405} in a spectrophotometer.

Apoptosis detection by annexin V staining

Cells in 10-cm² Petri dishes were treated with test reagents for 48 h at 37 °C. The cells were then collected and double-stained

Abbreviations used: ET, endothelin; HPSMC, human pericardial smooth muscle cells; ANG II, angiotensin II; PrSMC, human prostatic smooth muscle cells; FBS, fetal bovine serum; PI, propidium iodide; A-127722, *trans-trans*-2-(4-methoxyphenyl)-4-(1,3-benzodioxo-5-yl)-1-[(*N,N*-dibutylamino)carbonylmethyl]pyrrolidine-3-carboxylic acid.

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with FITC-conjugated annexin V and propidium iodide (PI) using an apoptosis detection kit according to the manufacturer's instruction (R&D Systems; Catalogue number KNX50). The percentage of cells undergoing apoptosis in a quantitative manner is determined based on the principle that apoptotic cells, but not necrotic cells, bind to annexin V. Samples (10 000 cells/sample) were then analysed using an Epics Elite flow cytometer (Coulter Corporation). The samples were excited with a 488 nm argon laser set at 25 mW. The emission filters were a 515–535 nm band pass filter for FITC and a 565–585 nm band pass filter for PI. Data were analysed using the Epics Elite software.

Binding of ^{125}I -labelled ET to cells

Cells (approx. 90 % confluency) in 48-well culture plates were incubated with ^{125}I -labelled ET-1 in 0.2 ml/well of Earle's solution (140 mM NaCl/5 mM KCl/1.8 mM CaCl_2 /0.8 mM MgSO_4 /5 mM glucose/25 mM Hepes, pH 7.4) containing protease inhibitors (5 $\mu\text{g}/\text{ml}$ pepstatin A, 0.01 mM phosphoramidon and 0.1 mM PMSF) for 4 h at 4 °C. After incubation, the cells were washed twice with PBS (0.5 ml/well), followed by solubilization with 0.1 M NaOH (0.2 ml/well) before estimating radioactivity. Non-specific binding was determined in the presence of 1 μM ET-1. Binding results were analysed by the radioligand-binding analysis program EBDA & LIGAND (Bio-soft, MO, U.S.A.) to calculate B_{max} , K_d and Hill coefficient (h).

RESULTS AND DISCUSSION

The presence of mono- and oligo-nucleosomes, an indication of DNA fragmentation, is one of the characteristics of cells undergoing apoptosis [20]. Employing an ELISA which measures mono- and oligo-nucleosomes in cell lysates, we first compared the effects of serum withdrawal and paclitaxel, an anticancer agent known to induce apoptosis [21], on inducing apoptosis in HPSMC. The A_{405} value, a quantitative measurement of histone-associated DNA fragments, was in the range 0.007–0.078 with a mean $\pm$ S.D. of 0.025 ± 0.022 ($n = 9$) for cells cultured in 10 % (v/v) FBS. Upon serum withdrawal for 48 h the value remained in the same range, suggesting that serum withdrawal for 48 h did not have a significant effect on inducing apoptosis in HPSMC. In contrast, paclitaxel effectively induced apoptosis in HPSMC in a dose-dependent manner (Figure 1). The A_{405} value was 0.047 ± 0.012 for cells in 10 % (v/v) FBS in the absence of paclitaxel; in the presence of paclitaxel at 1 and 10 μM the A_{405} values increased to 0.17 ± 0.03 and 0.15 ± 0.02 respectively. The results suggest that paclitaxel is effective in inducing apoptosis in HPSMC. Although the mechanism of paclitaxel-induced apoptosis is poorly understood, recent reports have suggested that paclitaxel may cause the phosphorylation and inactivation of bcl-2, a mammalian homologue of the anti-apoptotic *ced9* gene product of *Caenorhabditis elegans* [22], thus inducing cells to go into apoptosis.

We then tested the effect of ET-1 in the presence of paclitaxel in HPSMC. In a typical experiment, the A_{405} value was 0.05 ± 0.01 (mean $\pm$ S.D., $n = 4$) for cells in 10 % (v/v) FBS without paclitaxel. ET-1 (10 or 100 nM) alone had no effect on the basal A_{405} value. When cells were treated with 1 μM paclitaxel for 20 h at 37 °C, the A_{405} value increased to 0.52 ± 0.12 . When ET-1 (10 nM) was added together with paclitaxel, the A_{405} value decreased to 0.31 ± 0.08 , a 45 % reduction in DNA fragmentation. When ET-1 was increased to 100 nM, the A_{405} value further decreased to 0.14 ± 0.03 , a 81 % decrease in DNA fragmentation. When serum-deprived cells (cultured in serum-free media for 48 h) were treated with 1 μM paclitaxel for 20 h at 37 °C in the presence of ET-1 (10 or 100 nM), a similar observation, that ET-1 attenuated

Figure 1 Paclitaxel induced DNA fragmentation in HPSMC

Cells cultured in 10 % (v/v) FBS were treated with increasing concentrations of paclitaxel for 20 h at 37 °C. Apoptosis was measured by an ELISA which detects histone-associated DNA fragments. Each value represents the mean $\pm$ S.D. of three determinations. Unpaired Student's t test (two-tailed) using StatView II (Abacus Concepts): * $P = 0.003$, ** $P = 0.002$ when compared with the sample in the absence of paclitaxel. The results shown are representative of four experiments.

paclitaxel-induced apoptosis, was made. By comparison, ANG II (1–100 nM) did not have a significant effect: the A_{405} values with and without 100 nM ANG II in the presence of 1 μM paclitaxel were 0.23 ± 0.01 and 0.17 ± 0.05 respectively.

Figure 2 shows that ET-1 reduced apoptosis in a dose-dependent manner with an EC_{50} at approx. 1 nM. This effect is consistent with the EC_{50} values in a number of other ET-1-mediated biological responses [18,19]. These results show that ET-1 exhibits a profound effect to attenuate paclitaxel-induced apoptosis.

It may be worth mentioning that the absorbance values from

Figure 2 Effect of ET-1 on modulating HPSMC apoptosis was dose-dependent

Cells in media containing 10 % (v/v) FBS were treated with (●) or without (▲) paclitaxel (1 μM) in the presence of increasing concentrations of ET-1 for 20 h at 37 °C. Apoptosis was measured as in Figure 1. Each value represents the mean $\pm$ S.D. of three determinations. Unpaired Student's t test (two-tailed): * $P = 0.003$, ** $P < 0.001$ when compared with the sample treated with paclitaxel in the absence of ET-1. Results shown are representative of three different experiments.

Figure 3 ET-1-attenuated paclitaxel-induced phosphatidylserine processing in HPSMC

HPSMC were treated with or without paclitaxel in the presence or absence of ET-1 for 48 h at 37 °C. Cells were stained with FITC-conjugated annexin-V and propidium iodide (PI), and immediately analysed by flow cytometry. (A) Control, untreated; (B) paclitaxel (10 μ M) alone; (C) paclitaxel (10 μ M) plus ET-1 (10 nM); (D) paclitaxel (10 μ M) plus ET-1 (100 nM). Normal cells (quadrant 3) exhibited minimal (background) staining for annexin V and PI. Cells in quadrant 1 were annexin V negative and PI positive, and were considered as necrotic cells. Cells in quadrant 2 stained positive for both annexin V and PI and represented late apoptotic cells, since late apoptotic cells tend to take up PI after losing membrane integrity. Annexin V positive- and PI negative-staining cells in quadrant 4 were early apoptotic cells. The results shown are representative of five different experiments. The number of cells (in %) in each quadrant is indicated.

the ELISA assay vary from experiment to experiment. For example, the *A* value at 1 μ M paclitaxel in Figure 1 is 0.17 but the *A* value in Figure 2 under the same condition is 0.32. One reason for this variation might be due to the fact that different assay kits were used for different experiments. In addition, although the cell number/well in each experiment and the conditions for each experiment were kept as constant as possible, smooth muscle cells in culture tend to form multi-layered growth patterns (hill-and-valley patterns), and variation in cell density from experiment to experiment is unavoidable. Nevertheless, a consistent observation was made among different independent experiments with regard to the effects of ET-1 and other test reagents. The average decrease in DNA fragmentation for cells in 10 % FBS with 1 μ M paclitaxel and 10 nM ET-1 from nine independent experiments was 47 ± 19 % (mean $\pm$ S.D.) and with 100 nM ET-1 from five independent experiments was 67 ± 22 % (mean $\pm$ S.D.).

In addition to the presence of mono- and oligo-nucleosomes,

the process of phosphatidylserine export to the outer plasma membrane is another characteristic of cells undergoing apoptosis [23–25]. Using a double-staining method which determines the binding of FITC-conjugated annexin V to phosphatidylserine on the cell surface, and the staining of DNA by PI, we set out to confirm the above observation. Figure 3(A) shows that, in untreated HPSMC a majority of cells (93 %) were normal and remained in quadrant 3 with minimal (background) staining by both annexin V and PI. ET-1 alone (10 or 100 nM) had no effect on untreated cells. When cells were treated with 10 μ M paclitaxel (Figure 3B), the number of normal cells decreased to 21 % of the total cell population, whereas the number of late apoptotic cells (quadrant 2) increased from 2.9 % to 35.1 %, and that of early apoptotic cells (quadrant 4) increased from 2.7 % to 36.9 %. Paclitaxel did not induce necrosis in these cells significantly; the number of cells in quadrant 1 increased from 1.7 % to 7.3 % after paclitaxel treatment. When cells were treated with paclitaxel in the presence of 10 nM ET-1 (Figure 3C), normal cells increased

Table 1 A-127722 abolished the effect of ET-1 on attenuating paclitaxel-induced apoptosis in HPSMC

HPSMC were treated with or without paclitaxel (1 μ M) in the presence or absence of ET-1 or ET-3 (10 nM) and/or A-127722 (1 or 10 μ M) for 20 h at 37 °C. Apoptosis was measured as described in the legend to Figure 1. Each value represents mean $\pm$ S.D. of four determinations. The results shown are representative of two experiments. Unpaired Student's *t* test (two tailed): no paclitaxel, $P = 0.10$ – 0.28 , indicating that the difference among the four values was not statistically significant; with paclitaxel, * $P < 0.001$ when compared with control, ** $P = 0.13$ when compared with control, † $P = 0.003$ when compared with +ET-1, ‡ $P = 0.003$ when compared with +ET-1. ND, not determined.

Treatment	Apoptosis (A_{405})	
	No paclitaxel	With paclitaxel
Control	0.007 $\pm$ 0.003	1.181 $\pm$ 0.091
+ ET-1	0.029 $\pm$ 0.019	0.498 $\pm$ 0.082*
+ ET-3	0.010 $\pm$ 0.003	1.060 $\pm$ 0.102**
+ ET-1 + A-127722 (1 μ M)	ND	0.953 $\pm$ 0.033†
+ ET-1 + A-127722 (10 μ M)	0.017 $\pm$ 0.010	1.424 $\pm$ 0.036‡

Table 2 ET-1 attenuated paclitaxel-induced apoptosis in PrSMC

PrSMC cells in media containing 5% (v/v) FBS were treated with or without paclitaxel (1 μ M) in the presence or absence of ET-1 (10 nM) or ANG II (10 nM) for 20 h at 37 °C. Apoptosis was measured as described in the legend to Figure 1. Each value represents the mean $\pm$ S.D. of three determinations. Unpaired Student's *t* test (two tailed); * $P = 0.01$, † $P = 0.4$ when compared with control with paclitaxel. The results shown are representative of two different experiments.

Treatment	Apoptosis (A_{405})	
	No paclitaxel	With paclitaxel
Control	0.029 $\pm$ 0.008	0.565 $\pm$ 0.063
+ ANG II	0.030 $\pm$ 0.003	0.513 $\pm$ 0.094†
+ ET-1	0.047 $\pm$ 0.012	0.299 $\pm$ 0.015*

to 43% and the numbers of early and late apoptotic cells decreased to 32.2 and 20.8% respectively. When the concentration of ET-1 was increased to 100 nM (Figure 3D), normal cells in quadrant 3 increased to 71% and the numbers of early and late apoptotic cells further decreased to 16.2 and 7.5% respectively. Again, 100 nM ANG II had no significant effect on paclitaxel-induced apoptosis.

To study the involvement of ET receptor subtypes, we tested A-127722, an antagonist selective for the ET_A receptor [26]. Table 1 summarizes the results from a study using the ELISA method. ET-1 (10 nM) reduced paclitaxel-induced apoptosis by 58%, whereas ET-3 (10 nM) had no significant effect. In the presence of 1 μ M or 10 μ M A-127722, attenuation of apoptosis by ET-1 was abolished. A similar observation was made when the double-staining method was used; treatment of HPSMC with 1 μ M paclitaxel for 48 h at 37 °C resulted in an increase in the number of apoptotic cells from 4.7% to 33.4%, whereas ET-1 (100 nM) attenuated paclitaxel-induced apoptosis by 42%, which was completely abolished by A-127722 (10 μ M). These results suggest that the ET_A subtype is involved in modulating apoptosis in HPSMC. The observation is consistent with our previous reports that ET-1-mediated biological responses, such as phosphatidylinositol hydrolysis and arachidonic acid release, are mediated by the ET_A receptor in HPSMC [18,19]. In-depth

studies will be required to investigate whether the ET_B receptor also plays a role in modulating apoptosis.

To investigate whether the modulating effect of ET on apoptosis is unique for HPSMC, we extended the studies to PrSMC. Because ET receptors have not been characterized in these cells, we first studied the expression of ET receptors using binding assays. In saturation binding studies in intact cells incubated at 4 °C for 4 h, ¹²⁵I-labelled ET-1 binding reached a plateau at approx. 1.0 nM ET-1. Scatchard analysis yielded a straight line with values of B_{max} and K_d at 870 fmol/10⁶ cells and 0.37 nM respectively; the Hill coefficient was 1.006 and the number of receptors was calculated to be 524000 sites per cell. As a comparison, the number of receptors in HPSMC is 120400 sites/cell [18]. To study the effect of ET on apoptosis, ELISA was used to detect DNA fragmentation and the results are summarized in Table 2. Clearly, addition of ET-1 (10 nM) decreased paclitaxel-induced DNA fragmentation by 52%, and 10 nM ANG II again had no significant effect on attenuating paclitaxel-induced apoptosis. ET-1 or ANG II alone had no effect on the basal A_{405} values. The results suggest that ET-1 also attenuates paclitaxel-induced apoptosis in PrSMC.

We have shown in the present study that ET-1 is able to attenuate chemically-induced apoptosis in smooth muscle cells. A few questions remain unanswered. First, in the present study we have not extended our investigations beyond human pericardial and prostatic smooth muscle cells. It would be of interest to know whether ET-1 exerts similar effects on modulating apoptosis in other cell types. A second question is whether the effect of ET-1 is unique to paclitaxel-induced apoptosis. Preliminary studies in our laboratory suggest that ET-1 also protects cells from apoptosis induced by other tubulin-interacting compounds in HPSMC. An important next step would be to investigate whether ET-1 protects against apoptosis induced by other stimuli such as radiation, DNA-damaging agents, etc. We are presently exploring different conditions to induce apoptosis in smooth muscle cells in order to address this issue. Finally, the mechanism by which ET-1 affects apoptosis is not clear. In parallel studies we have observed that ET-1 activates phosphatidylinositol 3-kinase and mitogen-activated protein kinase in HPSMC. Both phosphatidylinositol 3-kinase and mitogen-activated protein kinases have been shown to play a role in the ability of insulin-like growth factor-1 to modulate apoptosis [27]. It is possible that activation of phosphatidylinositol 3-kinase and/or mitogen-activated protein kinases is involved in the effect of ET on apoptosis.

The finding that ET-1 protects smooth muscle cells from apoptosis has an immediate impact on our understanding of the pathogenic role of ET in cell growth disorders, such as restenosis, benign prostatic hyperplasia and cancer. Studies have suggested that an increased rate of cellular proliferation may not be the only factor in causing cell growth disorders, and apoptosis may play an equally pivotal role [28–31]. Although both ET and ANG II have been shown to exhibit similar mitogenic activities for smooth muscle cells [12–17], evidence exists to suggest that ET may play a more important role in disorders involving excess cell accumulation than does ANG II. For example, in a porcine restenosis model, an ET_A receptor antagonist exhibits efficacy in preventing restenosis, while captopril, an angiotensin converting enzyme inhibitor, is not effective [32]. Angiotensin converting enzyme inhibitors are not effective in the prevention of restenosis in humans [33–35]. ET receptor antagonists have not yet been tested in human restenosis studies. Furthermore, Yamada et al. [36] have shown that ANG II may actually enhance apoptosis induced by serum or growth-factor starvation in pheochromocytoma cells and fibroblasts. Thus it is apparent

that vasoactive agents, such as ET and ANG II, have substantially different effects on cell growth and apoptosis, which may have different implications for vascular disorders.

From these results, we would like to propose that ET-1 is a survival factor for smooth muscle cells. The present study, together with earlier studies describing ET-1 as a mitogen, strongly suggest that ET may play an important role in the pathogenesis of cell growth disorders.

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