Parathyroid Hormone-Related Protein (PTHrP)-Dependent Regulation of bcl-2 and Tissue Inhibitor of Metalloproteinase (TIMP)-1 in Coronary Endothelial Cells

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Key Words

Atherosclerosis • Apoptosis • Hypertension

Abstract

Background: An increased susceptibility of microvascular endothelial cells to apoptosis is considered to be an initial event leading to atherosclerosis. Parathyroid hormone-related peptide (PTHrP) is known to protect endothelial cells against apoptosis by the regulation of the anti-apoptotic gene bcl-2. As tissue inhibitor of metalloproteinase (TIMP-1) expression is regulated by bcl-2, we hypothesized that endothelial expression of PTHrP also regulates the expression of TIMP-1. **Methods:** The steady state mRNA expressions of bcl-2, bax, TIMP-1, and TIMP-2 were analyzed by real-time RT-PCR and their protein expression by immunoblotting. The tissue distribution of PTHrP was investigated in cryosections of hearts from normotensive and hypertensive rats. **Results:** Phenylephrine, an α_1 -adrenoceptor agonist, increased the expression of PTHrP, bcl-2, and TIMP-1. Transfection of endothelial cells with oligonucleotides directed against PTHrP attenuated this effect. Antisense transfection and TGF- β_1

(10 ng/ml) decreased the expression of PTHrP, bcl-2, TIMP-1, and TIMP-2, but not that of bax. Endothelial cells were identified as the main source of PTHrP in the heart. Endothelial cells in hearts from spontaneously hypertensive rats showed reduced staining with a PTHrP antibody compared to control normotensive hearts. **Conclusions:** These data suggests that the down-regulation of PTHrP favours atherosclerosis in chronic pressure overload.

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Introduction

Apoptosis has been recognized to play a fundamental role in coronary atherosclerosis [reviewed in 1]. Endothelial cells are among the cell types undergoing apoptosis in blood vessels. The concept of a relationship between endothelial cell apoptosis and development of atherosclerosis is supported by the observation that atherosclerotic plaque formation primarily localizes to regions where laminar flow of the streaming blood is disturbed by flow turbulences [2]. Furthermore, the incidence of endothelial cell apoptosis was associated with impaired endothelial function [3]. These findings support the hypothesis that a loss of endothelial protection against

apoptosis increases the risk of atherosclerosis. The realization of the mechanism by which endothelial cells control their apoptotic susceptibility is essential to understand the initial steps in the progression of atherosclerosis.

We have already shown that the susceptibility of coronary endothelial cells to apoptosis depends on the endogenous expression of parathyroid hormone-related peptide (PTHrP) [4]. Stimulation of endothelial α_{1A} -adrenergic receptors leads to an up-regulation of the anti-apoptotic protein bcl-2. This process depends on the cellular expression of PTHrP in endothelial cells [4]. In a similar way PTHrP increases the expression of bcl-2 in chondrocytes, in breast cancer cells and in the placenta [5-7]. Bcl-2 plays a major role in the regulation of cytochrome C release from mitochondria [8]. On top of this, tissue inhibitor of metalloproteinase (TIMP)-1 expression is consistently upregulated by bcl-2 overexpression in human breast epithelial cells [9]. TIMP-1 is known to exert anti-apoptotic effects in endothelial cells, possibly by interaction with matrix metalloproteinases (MMP) in capillary endothelial cells [10, 11]. MMP-independent mechanisms may also exist [12, 13]. PTHrP was initially identified as the hypercalcemic factor of malignancy [14]. The expression of PTHrP and TIMP-1 is high in metastatic breast cancer cells suggesting a coupling between PTHrP and TIMP-1 [15, 16]. PTHrP is constitutively expressed in endothelial cells and regulates the expression of bcl-2 [4, 17, 18]. As TIMP-1 is a downstream target of bcl-2, we speculated that PTHrP upregulates TIMP-1 in microvascular endothelial cells, too.

Downregulation of PTHrP in microvascular endothelial cells has been observed in hypertensive heart disease [19]. Hypertensive rats develop endothelial apoptosis under stress [20]. PTHrP is down-regulated in a TGF- β_1 -dependent way [19]. According to the aforementioned observations a down-regulation of PTHrP by either antisense approaches, or application of TGF- β_1 should result in a corresponding downregulation of bcl-2 and TIMP-1 in endothelial cells. This study was aimed to investigate such a coupling between PTHrP and TIMP-1 expression in microvascular endothelial cells and to confirm an endothelial specific downregulation of PTHrP in these cells in the presence of chronic hypertension.

Materials and Methods

Endothelial cell cultures

Male Wistar rats (250-300 g) or aged matched spontaneously hypertensive rats were used for the isolation of coro-

nary endothelial cells. They were isolated as described before and grown for 1 or 2 days before use [19]. As reported previously, the purity of these cultures was >95% endothelial cells, as determined by the uptake of acetylated low-density lipoprotein labelled with 1,1'-diiododecyl-3,3',3'-tetramethylindocarbocyanine perchlorate, contrasted with <5% cells that were positive for α -smooth muscle actin [21].

Transfection of endothelial cells with phosphorothioated oligonucleotides

Oligonucleotides targeted to a continuous region of PTHrP mRNA in antisense (5'-TGA ACC AGC CTC CGC AGC AT-3') or sense directions (5'-ATG CTG CGG AGG CTG GTT CA-3') were synthesized (Invitrogene, Carlsbad, CA) according to the method described before [4]. To increase the stability of these oligonucleotides in cells (exonuclease resistance), four phosphorothioate-modified nucleotides were placed at each end. Coronary endothelial cells were harvested by trypsinization, incubated with 10 μ g/ml of the oligonucleotides, and exposed in an electroporation apparatus (Gene Pulser II, Bio-Rad, Munich, Germany). Transfection was performed at 400 μ F for 5 ms.

Antagonism of bcl-2 in endothelial cells

In order to address the contribution of bcl-2 in PTHrP-dependent TIMP-1 expression and apoptosis we used a synthetic peptide derived from the BH3 domain of Bad as described previously [22]. This peptide (bcl-2 binding peptide) is bound to a fatty acid to improve peptide uptake. The peptide sequence binds to bcl-2 in the cells and thereby inactivates bcl-2. As a control peptide we used a mutated peptide sequence without bcl-2 binding properties (non-bcl-2 binding peptide, negative control). Cells were incubated with these peptides for 4 h prior to incubation with phenylephrine or PTHrP. The peptides were obtained from Calbiochem (Bad Soden, Germany).

Immunoblots

Samples of endothelial cells were prepared for immunoblotting as described previously [23]. Samples containing 60 μ g of protein were loaded onto a 12.5% SDS-polyacrylamide gel electrophoresis and blotted onto PVDF (Polyvinylidene-difluoride) membranes (Millipore, Eschborn, Germany). Blots were incubated first with an antibody directed against PTHrP (antibody GF08; Calbiochem, Bad Soden, Germany) and then with an anti-mouse Ig antibody coupled to alkaline phosphatase. Where indicated, immunoblots were performed with a rabbit polyclonal antibody directed against tissue inhibitor of metalloproteinases (TIMP)-1 (H-150, Santa Cruz Biotechnology), and mouse monoclonal antibodies against bcl-2 (antibody clone 7, BD Bioscience, Heidelberg, Germany), or bax (antibody clone 6A7, BD Bioscience, Heidelberg, Germany). Samples were re-blotted on separate sheets and protein bands were visualized by amido black staining. The band corresponding to actin was used as a loading control. It was previously identified by immunoblotting with a mouse monoclonal actin antibody (antibody PC612; Calbiochem, Bad Soden, Germany).

Immunoblots were scanned and densitometrically analyzed via ImageQuant (Amersham Bioscience Inc., Piscataway, NJ).

Real-time RT-PCR

The steady state mRNA levels of PTHrP, TIMP-1, TIMP-2, bcl-2 and bax were quantified via real-time RT-PCR using iQ SYBR Green supermix (Bio-Rad, Germany) as described previously in greater detail [24]. Hypoxanthine phosphoribosyl transferase (HPRT) was used as a housekeeping gene to normalize sample contents. Primers used for determination had the following sequences: PTHrP forward: CGG TGT TCC TGC TGA GCT A, reverse: TGC GAT CAG ATG GTG AAG GA; TIMP-1 forward: CTG CAA CTC GGA CCT GGT TAT AAG G, reverse: AAC GGC CCG CGA TGA GAA ACT CC; TIMP-2 forward: ATT CCG GGA ATG ACA TCT ATG GCA AC, reverse: CTG GTA CCT GTG GTT TAG GCT CTT C; HPRT forward: CCA GCG TCG TGA TTA GTG AT, reverse: CAA GTC TTT CAG TCC TGT CC; bcl-2 forward: TTC TTT CCC CGGAAG GAT GG, reverse: GTC TGC TGA CCT CAC TTG TG; bax forward: CCT GAG CTG ACC TTG GAG CA, reverse: CCT GGT CTT GGA TCC AGA CA.

Immunohistochemistry

Cryosections (10 μ m) were cut from shock-frozen hearts ($n=4$, Wistar or spontaneously hypertensive rats each), fixed with 4% paraformaldehyde (PFA) for 20 min, washed, air-dried and incubated for 1 h in 5% normal goat serum containing 5% bovine serum albumin in 0.005 M phosphate-buffered saline (PBS). For single antibody incubation with anti-PTHrP and for anti-cav-1 $\alpha\beta$ /anti-PTHrP double-labelling, primary antibodies were diluted in 0.005 M phosphate buffer containing 0.01 % NaN_3 and 4.48 g/l NaCl, and applied overnight at room temperature. Secondary antibodies were applied for 1 h, and slides destined for confocal laser scanning microscopy (CLSM) were incubated for 10 min with TO-PRO-3 (diluted 1:1,000; Invitrogen, Karlsruhe, Germany), a monomeric cyanine nucleic acid stain used for nuclear counterstaining. Antibodies and their sources were as follows: anti-caveolin (cav)-1 $\alpha\beta$ (1:200), monoclonal from mouse (clone 2297; Transduction Laboratories, Heidelberg, Germany); anti-PTHrP (1:800), polyclonal from rabbit (antibody GF08; Calbiochem, Bad Soden, Germany). Secondary antibodies used in this study for immunohistochemistry were FITC-conjugated donkey-anti-mouse-Ig, F(ab')₂ fragments (1:200; Dianova, Hamburg, Germany), and Cy3-conjugated donkey-anti-rabbit-Ig (1:2,000; Chemicon, Temecula, CA, USA).

Sections were rinsed, postfixed for 10 min in 4% PFA, rinsed again and coverslipped with carbonate-buffered glycerol (pH 8.6). Slides were evaluated with an epifluorescence microscope (Zeiss, Jena, Germany) using appropriate filter sets and with CLSM (Leica-TCS SP2 AOBS; Leica, Mannheim, Germany). The CLSM settings were as follows: Detection of FITC: 20% laser power at 488 nm, detection at 490-540 nm; Cy3: 100% laser power at 543 nm, detection at 555-620 nm; Cy5: 50% laser power at 633 nm, detection at 639-738 nm. Z-Series through endothelial cells were taken for more detailed analysis of the subcellular localisation of PTHrP.

Controls included (1) omission of the primary antibody and (2) pre-absorption of the anti-PTHrP-antibody with 200 μ g/ml of its antigenic peptide. The specificity of the anti-cav-1 $\alpha\beta$ was previously shown in experiments with

cav-1 deficient mice [25].

Apoptosis assay

For induction of apoptosis, cultures were irradiated with 254 nm UV light at 80 J/m² as described previously [26]. Then cultures were incubated for an additional 20 h in the standard incubator. To quantify apoptosis, the medium was removed and replaced by 1 ml of PBS with the addition of HOE33258 (5 μ g/ml) at 37°C for additional 30 min. Cultures were analyzed using a fluorescence microscope, and apoptotic cells were identified by clear nuclear-chromatin condensation as previously described [4]. At least 5 images were counted per culture dish and a total of 5 culture dishes were counted. The average number of total cell counted was 2551 $\pm$ 145 cells for each condition.

Statistics

Data are given as means $\pm$ s.e.m. from n different culture preparations. Statistical comparison between groups was performed by one-way analysis of variance and use of Student-Newman-Keuls test for post hoc analysis. A P value of 0.05 was considered as statistically significant. Comparisons between two groups were performed by means of a t -test for independent samples with a critical value equal to 0.05.

Results

Effect of PTHrP up-regulation on the endothelial expression of pro- and anti-apoptotic genes

In the first set of experiments we investigated the effect of elevated endothelial expression of PTHrP on bcl-2 and TIMP-1 expression. According to our previously established protocol, we used the stimulation of α_{1A} -adrenoceptors to increase PTHrP protein expression [4]. This procedure does not elevate PTHrP mRNA expression [4]. Therefore, the PTHrP mRNA expression was not analyzed in this set of experiments. Cells were exposed to phenylephrine, an α -adrenoceptor agonist, for either 24 or 48 hours. Thereafter, expressions of bcl-2, bax, TIMP-1, and TIMP-2 were investigated. The mRNA expression of bcl-2 and TIMP-2 remained unchanged during cultivation without application of phenylephrine throughout the cultivation period. The expression of bax increased with cultivation leading to 1.7 $\pm$ 0.2-fold higher level after 48 h in culture ($n=3$, $p<0.05$). The expression of TIMP-1 declined during cultivation leading to 0.6 $\pm$ 0.2-fold level after 48 h in cultivation. Fig. 1 shows the effect of phenylephrine on these values at 24 h (Fig. 1A) and 48 h (Fig. 1B). Phenylephrine increased the mRNA expression of bcl-2 and TIMP-1 in cells exposed to the agonist for 48 hours. It did not increase the mRNA expression of either bax or TIMP-2 at 48 hours (Fig. 1B). To test a

mechanistic link between the upregulation of PTHrP protein and the corresponding changes in bcl-2 and TIMP-1 expression, endothelial cells were also transfected with antisense oligonucleotides directed against PTHrP. Expression values were compared to values in cells being transfected with sense oligonucleotides. The observed increased expression of either bcl-2 mRNA or TIMP-1 mRNA at 48 h was attenuated in cells transfected with oligonucleotides directed against PTHrP (Fig. 1B). Expression of either bax mRNA or TIMP-2 mRNA were not altered at 48 h in presence or absence of PTHrP antisense (Fig. 1B). The results were slightly different at the earlier time point. Phenylephrine increased the expression of either TIMP-1 or TIMP-2 within 24 hours. This early response was not attenuated by transfection with oligonucleotides directed against PTHrP (Fig. 1A).

Effect of PTHrP down-regulation on bcl-2 and TIMP-1 expression

In the next step we investigated the effect of downregulation of PTHrP on the corresponding mRNA expression of bcl-2, bax, TIMP-1, and TIMP-2. The following strategies were applied to down-regulate PTHrP: Antisense transfection experiments were used in which the expression of the genes under investigation was compared to those in sense transfected cells. These experiments also repeated exactly the previously described effect of PTHrP expression on bcl-2 protein expression in these cells and on apoptotic susceptibility [4]. In a second approach TGF- β_1 was used because it is known to moderately down-regulate PTHrP in pathophysiological conditions [16]. When antisense transfection was performed a downregulation was observed for the mRNA expression of bcl-2, TIMP-1, and TIMP-2 at 48 hours, while the expression of bax mRNA did not change (Fig. 2). TGF- β_1 caused a strong and concentration-dependent reduction in PTHrP mRNA expression within 24 h. This was accompanied by a reduction in the mRNA expression of bcl-2, TIMP-1, and TIMP-2. These changes in mRNA expression were transient in nature, except for TIMP-2 that was still reduced at 48 hours (Fig. 2). In summary, the real-time RT-PCR analysis of cultured microvascular endothelial cells indicated a strong correlation between basal PTHrP expression and TIMP-1 and bcl-2 mRNA levels.

Effects of PTHrP expression on bcl-2 and TIMP-1 protein expression

The aforementioned results described the effects of either down- or up-regulation of PTHrP in endothelial

Fig. 1. Effect of phenylephrine (PE, 1 μ M) on the mRNA expression of bcl-2, bax, TIMP-1 and TIMP-2. A) Basal expression of untreated control cells after 24 h of cultivation was set as 100%. In case of antisense (AS) experiments the controls were exposed to sense oligonucleotides (S). B) Basal expression of untreated control cells after 48 h of cultivation was set as 100%. mRNA expression was determined by real time RT-PCR and the values were normalized to hypoxanthine phosphoribosyl transferase (HPRT) as a house-keeping gene. Data are means $\pm$ s.e. from n=6 experiments. *, $p < 0.05$ vs. control; #, $p < 0.05$ vs. PE.

cells on the mRNA expression of bcl-2, bax, TIMP-1, and TIMP-2. In the following these data were validated on the protein level (Fig. 3a). Antisense transfection compared to sense transfected cells or TGF- β_1 reduced the protein expression of PTHrP to a similar extent (Fig. 3b). In agreement with the results on mRNA expression, this was accompanied by a significant reduction of bcl-2 and TIMP-1 expression whereas the expression of bax remained unchanged (Fig. 3). In contrast, phenylephrine increased the expression of PTHrP and that of TIMP-1 and bcl-2 (Fig. 3). Notably, bax protein expression was

Fig. 2. Effect of PTHrP down-regulation on the mRNA expression of bcl-2, bax, TIMP-1 and TIMP-2. A) Basal expression of untreated control cells after 24 h of cultivation was set as 100%. In case of antisense experiments the controls were exposed to sense oligonucleotides. Cells were either antisense transfected (AS), or exposed to TGF- β 1 (1 or 10 ng/ml). B) Basal expression of untreated control cells after 48 h of cultivation was set as 100%. mRNA expression was determined by real time RT-PCR and the values were normalized to hypoxanthine phosphoribosyl transferase (HPRT) as a house-keeping gene. Data are means $\pm$ s.e. from n=6 experiments. *, $p < 0.05$ vs. control.

reduced in the presence of phenylephrine although the bax mRNA levels were not changed.

As TIMP-1 has been identified as a bcl-2 -dependent regulated gene we also investigated whether TIMP-1 protein induction by phenylephrine and PTHrP depends on bcl-2. Therefore, we repeated the above mentioned experiments in the presence of bcl-2 blocking

Fig. 3. Effect of antisense transfection (AS), TGF- β 1 (10 ng/ml), or phenylephrine (PE) on the protein expression of PTHrP, bcl-2, bax, and TIMP-1 after 48 h. A) Representative immunoblots. B) Quantification of the band intensities. Basal expression of untreated control cells after 48 h of cultivation was set as 100%. In case of antisense experiments the controls were exposed to sense oligonucleotides. Data are means $\pm$ s.e. from n=6 experiments. *, $p < 0.05$ vs. control.

peptides or control peptides. Phenylephrine increased TIMP-1 protein expression in cells incubated with a mutated bcl-2 binding peptide (negative control) by $46.9 \pm 9.5\%$ ($n=6$, $p < 0.05$) but not in cells incubated with a non-mutated bcl-2 binding peptide ($1.9 \pm 3.3\%$ ($n=6$, n.s.)). Similarly, PTHrP increased TIMP-1 protein by $29.0 \pm 2.2\%$ ($n=4$, $p < 0.05$) in cells treated with a mutated bcl-2 binding peptide (negative control) but not in those cells treated with a non-mutated bcl-2 binding peptide ($-5.3 \pm 9.0\%$ ($n=4$, n.s.)).

Effect of bcl-2 antagonism, TIMP-1, and TIMP-2 protein on apoptosis

Having shown that phenylephrine increases bcl-2 expression via PTHrP, we further investigated whether

Fig. 4. Effect of phenylephrine, PTHrP, TIMP-1, and TIMP-2 on endothelial cell apoptosis. Cells were incubated with a bcl-2 binding peptide (bcl-2 pos. peptide, 10 μ M) or a mutated bcl-2 binding peptide (bcl-2 neg. peptide, 10 μ M) for 4 h and then exposed to phenylephrine (1 μ M) or PTHrP (100 nM) for another 24 h before they were exposed to UV (254 nm, 80 J/m²). The number of apoptotic cells was calculated by counting cells with clear chromatin condensation compared with the total number of cells. The 100% value corresponds to 4.34 $\pm$ 1.18 % apoptotic cells. Data are means $\pm$ s.e. from n=20 culture dishes. *, p<0.05 vs. control; #, p<0.05 between pairs as indicated.

this is sufficient to evoke an anti-apoptotic effect. In untreated control cultures a total of 4.34 $\pm$ 1.18 % apoptotic cells were counted. If endothelial cells were treated with PTHrP and the bcl-2 binding and neutralizing peptide, UV-induced apoptosis increased this number by 39% (6.03 $\pm$ 1.65 %, p<0.05 vs. control). However, in endothelial cells exposed to PTHrP and the non-binding bcl-2 control peptide 4.77 $\pm$ 0.67 % apoptotic cells were counted (n.s. from controls). These data argue for a bcl-2 -dependent anti-apoptotic effect of PTHrP. In a similar way bcl-2 antagonism attenuated the protective effect of phenylephrine (Fig. 4).

As PTHrP seems to be involved in the up-regulation of TIMP-1 and at least in part also to that of TIMP-2 we investigated also the extent of UV-induced apoptosis in cells treated with these two agonists. In this set of experiments the basal number of apoptotic cells was 3.17 $\pm$ 0.77 % and that was increased in the presence of TIMP-1 (500 ng/ml) by 24% to 3.93 $\pm$ 0.81 % (p<0.05 vs.

Fig. 5. Comparison of PTHrP, bcl-2, bax, and TIMP-1 protein expression in endothelial cells isolated from normotensive Wistar rats or spontaneously hypertensive rats (SHR). A) Representative immunoblots. B) Quantification of the band intensities. Mean expression in endothelial cells from normotensive rats are set as 100%. Data are means $\pm$ s.e. from n=4 isolations. *, p<0.05 vs. Wistar.

control) and by 53% to 4.89 $\pm$ 0.85 % (p<0.05 vs. control; p<0.05 vs. TIMP-1) in the presence of TIMP-2. Thus, in the presence of TIMP-1 the cells were less susceptible against apoptosis than those treated with TIMP-2, showing that TIMP-2 doesn't protect cells (Fig. 4).

Myocardial expression and distribution of PTHrP and modification in chronic hypertensive rat hearts

Finally, we investigated the expression of PTHrP in the endothelial cells from spontaneously hypertensive rats and the distribution of PTHrP in the ventricle. Endothelial cells from either normotensive or spontaneously hypertensive rats were isolated and immediately frozen into fluid nitrogen without further cultivation. In endothelial cells from spontaneously hypertensive rats the expression of PTHrP and bcl-2 was reduced by 40% compared to normotensive rats. However, neither the expression of bax nor that of TIMP-1 was differentially regulated (Fig.

Fig. 6. PTHrP-immunolabelling, atrium (A) and ventricle (B). PTHrP-immunolabelling is located predominantly in microvascular (C, arrows) and arterial endothelial cells (A, arrowheads) and with less intensity in vascular smooth muscle cells (SMC). Labelling of cardiomyocytes (CM, doubled arrowheads) is very weak to undetectable. C and D. Preabsorption of the PTHrP antibody with the corresponding peptide abolishes immunoreactivity. Bar represents 20 μ m throughout.

5). PTHrP-immunolabelling was observed in endothelial cells. Endothelial labelling intensity was highest in the aorta and decreased from coronary arteries and veins to the microvasculature (Fig. 6). More importantly the PTHrP staining was lower in hearts from spontaneously hypertensive rats compared to those from normotensive rats (Fig. 7). These data confirmed that endothelial cells are the main source of PTHrP in the myocardium. The previously described reduction in myocardial PTHrP expression in hypertensive hearts is indeed predominantly caused by a reduction in endothelial PTHrP expression.

Discussion

This study was intended to investigate the role of PTHrP in endothelial expression of bcl-2 and TIMP-1. The main finding of our study is that an up-regulation of PTHrP by stimulation of α -adrenoceptors up-regulates bcl-2 and TIMP-1 expression. The conclusion is based on experiments in which cell transfection with antisense oligonucleotides directed against PTHrP attenuated the effect of phenylephrine on PTHrP, bcl-2 and TIMP-1 expression. Noteworthy, the finding that phenylephrine increases bcl-2 and TIMP-1 mRNA expression was con-

Fig. 7. Normotensive vs. hypertensive rat heart, immunohistochemistry. (A-B) PTHrP-immunolabelling in hearts from normotensive rats (A) compared to spontaneously hypertensive rats (B). Intensity of PTHrP-labelling is lower in the capillary endothelial cells in the ventricles (arrowheads) from hypertensive rats compared to those from normotensive rats. Capillaries of the atrium (double-arrowheads) of hypertensive rats appear enlarged. (C-D) Endothelial cells in hearts of hypertensive rats labelled for caveolin-1 (cav-1; C) are also PTHrP-immunoreactive. Additionally, atrial cardiomyocytes show weak perinuclear PTHrP-labelling (asterix). arrow: atrium-ventricle-border, bar represents 20 μ m throughout.

firmed by immunoblotting indicating the physiological relevance of our finding. In pathophysiological conditions such as chronic hypertension, ventricular expression of PTHrP is downregulated in a TGF- β_1 -dependent way. We found that TGF- β_1 downregulates PTHrP, bcl-2 and TIMP-1 in a concentration dependent way. Furthermore, neither phenylephrine nor PTHrP were able to increase the expression of TIMP-1 in the presence of bcl-2 binding peptides. Moreover, histochemical studies identified endothelial cells as the main source of PTHrP in the ventricle and as the main target of PTHrP down-regulation in the ventricle.

Our finding that an increased PTHrP expression in endothelial cells increases the expression of bcl-2 but not that of bax suggests that an increased PTHrP expression lowers the susceptibility of endothelial cells against apoptosis. Following this argumentation a loss of endothelial PTHrP expression will increase the susceptibility of endothelial cells against apoptosis. Indeed, we reconfirmed in this study our former finding that PTHrP protects coronary endothelial cells against UV-induced apoptosis. Moreover, here we show that the anti-apoptotic effect of PTHrP and phenylephrine is attenuated if bcl-2 activity is blocked. This supports the hypothesis that reduced expression of PTHrP in microvascular endothelial cells shifts these cells to apoptosis, an initiating event in the progression of atherosclerosis. Endothelial cells are not unique in this relationship. Intracranial PTHrP protects against serum starvation-induced apoptosis in MCF7 breast cancer cells and chondrocytes [27-29]. In MCF7 and HEK293 cells this anti-apoptotic effect of PTHrP was mediated at least in part by shifting the ratio of bcl-2 to bax to higher values [27, 28]. As mentioned in the introduction, breast cancers are characterized by an increased expression of PTHrP, bcl-2, and TIMP-1. Moreover, TIMP-1 inhibited apoptosis of human breast epithelial cells [9]. However although TIMP-1 protects human breast cancer cells against apoptosis we did not find a similar protective effect on coronary endothelial cells.

To get a deeper insight into the specificity of PTHrP on the expression of TIMPs we also analyzed the expression of either TIMP-2 or TIMP-3. TIMP-3 has been correlated with pro-apoptotic pathways in several cultured cells, i.e. in retinal pigment epithelial, in MCF-7 cells, in human DLD colon carcinoma cells, in rat vascular smooth muscle cells, and melanoma cell lines [30-33]. Since in none of the experiments any changes in TIMP-3 expression were observed the data on the regulation of TIMP-3 expression are not shown. The lack of influence of PTHrP on the endothelial expression of TIMP-3 as a

pro-apoptotic agonist may not be a surprise because PTHrP acts as an anti-apoptotic peptide.

TIMP-2 is indifferent in respect to apoptosis. It has been shown to exert anti-apoptotic properties in metaphoric mesenchymes and a melanoma cell line but also to exert pro-apoptotic properties in human T-lymphocytes [34-36]. The data of TIMP-2 mRNA expression are shown in this study but they are not conclusive. Phenylephrine increased TIMP-2 expression within 24 h and this effect of phenylephrine on TIMP-2 expression was PTHrP-dependent. However, phenylephrine-dependent up-regulation of TIMP-2 was transient and after 48 h no effect was observable. On the other hand, TGF- β_1 decreased the expression of TIMP-2 along with a down-regulation of PTHrP. None of these two experiments is providing direct and strong evidence to assume a PTHrP-dependent regulation of TIMP-2 because phenylephrine increases TIMP-2 expression prior to a significant up-regulation of PTHrP on the protein level [4].

In contrast to TIMP-3 and TIMP-2 phenylephrine increased TIMP-1 in a PTHrP-dependent way. The effect was found after 48 h. At that time PTHrP protein levels in cultured endothelial cells are higher than in control cultures [4]. Transfection with antisense oligonucleotides against PTHrP attenuated this effect. Antisense oligonucleotides directed against PTHrP reduced the basal TIMP-1 expression as well. TGF- β_1 reduced the protein expression of TIMP-1, bcl-2, and PTHrP but not that of bax. On a mechanistic basis it is interesting to know whether TIMP-1 expression in endothelial cells is regulated directly by PTHrP or indirectly via bcl-2. In our study we used a cell-permeable bcl-2 binding protein to address this question. Blocking cellular bcl-2 binding attenuated the ability of phenylephrine to increase TIMP-1 expression. Therefore, the results are in agreement with previous findings, that bcl-2 regulates the expression of TIMP-1. Nevertheless, we did not find a difference in TIMP-1 expression between endothelial cells from spontaneously hypertensive rats compared to those from normotensive rats. It seems that the observed phenylephrine effect on cultured endothelial cells counterbalanced the culture-dependent down-regulation of TIMP-1 that does not occur in vivo under the conditions investigated here.

If PTHrP modifies TIMP-1 expression via bcl-2 the other big issue is to identify the mechanisms by which PTHrP modifies bcl-2 expression. At present, we cannot completely solve this question. On the one hand, PTHrP can translocate into the nucleus and act via binding to transcription factors [4]. Of note, proliferating endothe-

lial cells have low levels of nuclear PTHrP but relative high numbers of apoptosis [4]. In contrast, growth arrested endothelial cells have higher values of nuclear PTHrP but lower levels of basal apoptosis. On the other hand, PTHrP can be released from cells and act in an autocrine manner [37]. This may activate pro-survival kinases acting on transcription factors which increase the expression of bcl-2. As the entire specificity of the endothelial PTHrP receptor is not clear at present, it is difficult to deal with this event in more detail.

At least, this study gives some hints about the pathophysiological relevance of our findings. As two molecules, bcl-2 and its down-stream target TIMP-1, are regulated in a PTHrP-dependent way, a crucial role for PTHrP expression with respect to the protection of endothelial cells against apoptosis and vascular stability can be suggested. Hypertension is considered as one of the main risk factors contributing to the initiation of atherosclerosis [1]. The ventricular expression of PTHrP is differentially regulated in hypertension. By acute changes in pressure the PTHrP expression increases and endothelial cells release PTHrP [38, 39]. In a chronic situation, however, PTHrP is downregulated by TGF- β_1 [19]. In spontaneously hypertensive rats, ventricular TGF- β_1 expression is regulated by angiotensin II [40]. This cytokine is involved in the transition of compensated hypertrophy to heart failure and is known to induce apoptotic cell death with

downregulation of bcl-2 in endothelial cells, thereby favouring the formation of atherosclerotic plaques [41, 42]. In our study, we documented an intensive PTHrP staining *in situ* but observed a reduction in the vessels of chronic pressure overloaded hearts. In isolated endothelial cells from spontaneously hypertensive rats PTHrP and bcl-2 expression were lower than those in cells from normotensive rats. This scenario seems likely to contribute to the increased risk of atherosclerosis in chronic hypertension. Spontaneously hypertensive rats with a regular chow normally do not develop atherosclerosis. However, hypertension in combination with stress induces vascular apoptosis in these cells [20]. Of note, the total number of apoptotic cells is low. Kobiyashi et al. [20], counted 0.5-0.7 apoptotic cells per mm² in intact vessels. Nevertheless, the increased apoptotic susceptibility increases the risk of the development of atherosclerosis. In this line, we suggest that the above mentioned mechanism is part of a rather complex scenario that favours the onset of atherosclerosis in prolonged hypertensive heart disease.

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References

- Rössig L, Dimmeler S, Zeiher AM: Apoptosis in the vascular wall and atherosclerosis. *Basic Res Cardiol* 2001;96:11-22.
- Davies PF, Remuzzi A, Gordon EJ, Dewey CF Jr, Gimbrone MA Jr: Turbulent fluid shear stress induces vascular endothelial cell turnover in vitro. *Proc Natl Acad Sci* 1986;83:2114-2117.
- Asai K, Kudej RK, Shen YT, Yang GP, Takagi G, Kudej AB, Geng YJ, Sato N, Nazareno JB, Vatner DE, Natividad F, Bishop SP, Vatner SF: Peripheral vascular endothelial dysfunction and apoptosis in old monkeys. *Arterioscler Thromb Vasc Biol* 2000;20:1493-1496.
- Schorr K, Taimor G, Degenhardt H, Weber K, Schlüter K-D: Parathyroid hormone-related peptide is induced by stimulation of α_1A -adrenoceptors and improves resistance against apoptosis in coronary endothelial cells. *Mol Pharmacol* 2003;63:111-118.
- Amling M, Neff L, Tanaka S, Inoue D, Kuida K, Weir E, Philbrick WM, Broadus AE, Baron R: Bcl-2 lies downstream of parathyroid hormone-related peptide in signaling pathway that regulates chondrocytes maturation during skeletal development. *J Cell Biol* 1997;136:205-213.
- Sepulveda VAT, Shen X, Falzon M: Intracrine PTHrP protects against serum starvation-induced apoptosis and regulates the cell cycle in MCF-7 breast cancer cells. *Endocrinology* 2002;143:596-606.
- Thota CS, Reed LC, Yallampalli C: Effects of parathyroid hormone like hormone PTHLP antagonist, PTHLP(7-34) on fetoplacental development and growth during midgestation in rats. *Biol Reproduction* 2005;73:1191-1198.
- Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD: The release of cytochrome C from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 1997; 275:1132-1136.
- Li G, Fridman R, Kim HR: Tissue inhibitor of metalloproteinase-1 inhibits apoptosis of human breast epithelial cells. *Cancer Res* 1999;59:6267-6275.
- Vorotnikova E, Tries M, Braunhut S: Retinoids and TIMP1 prevent radiation-induced apoptosis of capillary endothelial cells. *Radiat Res* 2004;161:174-184.
- Boulday G, Fitau J, Coupel S, Souillou JP, Charreau B: Exogenous tissue inhibitor of metalloproteinase-1 promotes endothelial cell survival through activation of the phosphatidylinositol 3-kinase/akt pathway. *Ann N Y Acad Sci* 2004;1030:28-36.
- Guedez L, Courtemanch L, Stetler-Stevenson M: Tissue inhibitor of metalloproteinase (TIMP)-1 induces differentiation and an antiapoptotic phenotype in germinal center B cells. *Blood* 1998a;92:1342-1349.

- 13 Guede L, Stetler-Stevenson WG, Wolff L, Wang J, Fukushima P, Mansoor A, Stetler-Stevenson M: In vitro suppression of programmed cell death of B cells by tissue inhibitor of metalloproteinase-1. *J Clin Invest* 1998; 102:2002-2010.
- 14 Philbrick WM, Wysolmerski JJ, Galbraith S, Holt E, Orloff JJ, Yang KH, Vasavada RC, Weir EC, Broadus AE, Stewart AF: Defining the roles of parathyroid hormone-related peptide in normal physiology. *Physiol Rev* 1996;76:127-173.
- 15 Sterling JA, Oyajobi BO, Grubbs B, Padalecki SS, Munoz SA, Gupta A, Story B, Thao M, Mundy GR: The hedgehog signaling molecule Gli2 induces parathyroid hormone-related peptide expression and osteolysis in metastatic human breast cancer cells. *Cancer Res* 2006;66:7548-7553.
- 16 Yoshiji H, Gomez DE, Thorgeirsson UP: Enhanced RNA expression of tissue inhibitor of metalloproteinases-1 (TIMP-1) in human breast cancer. *Int J Cancer* 1996;69:131-134.
- 17 Ishikawa M, Ouchi Y, Akishita M, Kozaki K, Toba K, Namiki A, Yamaguchi T, Orimo H: Immunocytochemical detection of parathyroid hormone-related protein in vascular endothelial cells. *Biochem Biophys Res Commun* 1994;199:547-551.
- 18 Rian E, Jemtland R, Olstad OK, Endresen MJ, Grasser WA, Thiede MA, Henriksen T, Bucht E, Gautvik EM: Parathyroid hormone-related protein is produced by cultured endothelial cells: a possible role in angiogenesis. *Biochem Biophys Res Commun* 1994;198:740-747.
- 19 Wenzel S, Schorr K, Degenhardt H, Frischkopf K, Kojda G, Wiesner RJ, Rosenkranz S, Piper HM, Schlüter K-D: TGF- β_1 downregulates PTHrP in coronary endothelial cells. *J Mol Cell Cardiol* 2001;33:1181-1190.
- 20 Kobayashi N, DeLano FA, Schmid-Schönbein GW: Oxidative stress promotes endothelial cell apoptosis and loss of microvessels in the spontaneously hypertensive rats. *Arterioscler Thromb Vasc Biol* 2005;25:2114-2121.
- 21 Noll T, Muhs A, Besselmann M, Watanabe H, Piper HM: Initiation of hyperpermeability in energy-depleted coronary endothelial monolayers. *Am J Physiol* 1995; 268:H1462-H1470.
- 22 Wang J-L, Zhang Z-J, Choski S, Shan S, Luz U, Croce CM, Alnemri ES, Korngold R, Huang Z: Cell permeable bcl-2 binding peptides: A chemical approach to apoptosis induction in tumor cells. *Cancer Res* 2000;60:1498-1502.
- 23 Schlüter K-D, Katzer C, Frischkopf K, Wenzel S, Taimor G, Piper HM: Expression, release and biological activity of parathyroid hormone-related peptide from coronary endothelial cells. *Circ Res* 2000;86:946-951.
- 24 Taimor G, Schlüter K-D, Piper HM: Hypertrophy-associated gene induction after β -adrenergic stimulation in adult cardiomyocytes. *J Mol Cell Cardiol* 2001;33:503-511.
- 25 Razani B, Wang XB, Engelman JA, Battista M, Lagaud G, Zhang XL, Kneitz B, Hou H Jr, Christ GJ, Engemann W, Lisanti MP: Caveolin-2-deficient mice show evidence of severe pulmonary dysfunction without disruption of caveolae. *Mol Cell Biol* 2002; 22:2329-2344.
- 26 Taimor G, Lorenz H, Hofstaetter B, Schlüter K-D, Piper HM: Induction of necrosis but not apoptosis after anoxia and reoxygenation in isolated adult cardiomyocytes of rat. *Cardiovasc Res* 1999;41:147-156.
- 27 Okoumassoun L, Averill-Bates D, Denizuea F, Henderson JE: Parathyroid hormone related protein (PTHrP) inhibits TNF α -induced apoptosis by blocking the extrinsic and intrinsic pathways. *J Cell Physiol* 2007;210:507-516.
- 28 Tovar VA, Sepulveda T, Shen X, Falzon M: Intracrine PTHrP protects against serum starvation-induced apoptosis and regulates cell cycles in MCF7 breast cancer cells. *Endocrinology* 2002;143:596-606.
- 29 Henderson JE, Amizuka N, Warshawsky H, Biasotto D, Lanske BM, Goltzman D, Karaplis AC: Nucleolar localization of parathyroid hormone-related peptide enhances survival of chondrocytes under conditions that promote apoptotic cell death. *Mol Cell Biol* 1995;15:4064-4075.
- 30 Majid MA, Smith VA, Easty DL, Baker AH, Newby AC: Adenovirus mediated gene delivery of tissue inhibitor of metalloproteinases-3 induces death in retinal pigment epithelial cells. *Br J Ophthalmol* 2002;86:97-102.
- 31 Majid MA, Smith VA, Easty DL, Baker AH, Newby AC: Sorsby's fundus dystrophy mutant tissue inhibitor of metalloproteinase-3 induce apoptosis of retinal pigment epithelial and MCF-7 cells. *FEBS Lett* 2002;529:281-285.
- 32 Smith MR, Kung H, Durum SK, Colburn NH, Sun Y: TIMP-3 induces cell death by stabilizing TNF- α receptors on the surface of human colon carcinoma cells. *Cytokine* 1997;9:770-780.
- 33 Baker AH, Zaltsman AB, George SJ, Newby AC: Divergent effects of tissue inhibitor of metalloproteinases-1, -2, or -3 overexpression on rat vascular smooth muscle invasion, proliferation, and death in vitro. TIMP-3 promotes apoptosis. *J Clin Invest* 1998;101:1478-1487.
- 34 Lim MS, Guede L, Stetler-Stevenson WG, Stetler-Stevenson M: Tissue inhibitor of metalloproteinase-2 induces apoptosis in human T-lymphocytes. *Ann N Y Acad Sci* 1999;878:522-523.
- 35 Barasch J, Yang J, Quia J, Tempst P, Erdjument-Bornage H, Leung W, Oliver JA: Tissue inhibitor of metalloproteinase-2 stimulates mesenchymal growth and regulates epithelial branching during morphogenesis of the rat metanephros. *J Clin Invest* 1999;103:1299-1307.
- 36 Valente P, Fassina G, Melchiorri A, Masiello L, Cilli M, Vacca A, Onisto M, Santi L, Stetler-Stevenson GW, Albini A: TIMP-2 overexpression reduces invasion and angiogenesis and protects B16F10 melanoma cells from apoptosis. *Int J Cancer* 1998;75:246-253.
- 37 Abdallah Y, Ross G, Dolf A, Heinemann MP, Schlüter K-D: N-terminal parathyroid hormone-related peptide hyperpolarizes endothelial cells and causes a reduction of the coronary resistance of the rat heart via endothelial hyperpolarization. *Peptides* 2006; 27:2927-2934.
- 38 Meyer R, Schreckenberger R, Kretschmer F, Bittig A, Conzelmann C, Grohé C, Schlüter K-D: Parathyroid hormone-related protein (PTHrP) signal cascade modulates myocardial dysfunction in the pressure overloaded heart. *Eur J Heart Fail* 2007;9:1156-1162.
- 39 Degenhardt H, Jansen J, Schulz R, Sedding D, Braun-Dulläus R, Schlüter K-D: Mechanosensitive release of parathyroid hormone-related peptide from coronary endothelial cells. *Am J Physiol* 2002;283:H1489-H1496.
- 40 Wenzel S, Taimor G, Piper HM, Schlüter K-D: Redox-sensitive intermediates mediate angiotensin II-induced p38 MAP kinase activation, AP-1 binding activity, and TGF- β expression in adult ventricular cardiomyocytes. *FASEB J* 2001;15:2291-2293.
- 41 Boluyt MO, O'Neill I, Meredith AL, Bing OH, Brooks WW, Conrad CH, Crow MT, Lakatta EG: Alterations in cardiac gene expression during the transition from stable hypertrophy to heart failure. *Circ Res* 1994;75:23-32.
- 42 Tsukada T, Eguchi K, Migita K, Kawabe Y, Kawakami A, Matsuoka M, Takashima H, Mizokami A, Nagataki S: Transforming growth factor β 1 induces apoptotic cell death in cultured human umbilical vein endothelial cells with down-regulated expression of bcl-2. *Biochem Biophys Res Commun* 1995; 210:1076-1082.