Role of GADD153 (growth arrest- and DNA damage-inducible gene 153) in vascular smooth muscle cell apoptosis

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ABSTRACT
GADD153 (growth arrest- and DNA damage-inducible gene 153) is expressed at very low levels in growing cells, but is markedly induced in response to a variety of cellular stresses, including glucose deprivation, exposure to genotoxic agents and other growth-arresting situations. Forced expression of GADD153 induces cell cycle arrest in many types of cells. It is also reported that GADD153 is directly associated with apoptosis. Recently we have reported that platelet-derived growth factor (PDGF)-BB induces apoptosis in cultured vascular smooth muscle cells (VSMC), but only when 100% confluency is reached. These results suggested that cell–cell contact inhibition (cell growth arrest) may be a critical factor for induction of VSMC apoptosis by PDGF-BB. In the present study, we explored the role of GADD153, one of a number of growth-arrest-related gene products, in the molecular mechanisms of VSMC apoptosis in vitro and in vivo. GADD153 was markedly induced at both the mRNA and protein levels, in parallel with the induction of VSMC apoptosis, after treatment with PDGF-BB. Moreover, overexpression of GADD153 in VSMC significantly reduced cell viability and induced apoptosis. In the carotid artery balloon injury model in rats, GADD153 protein was expressed in apoptotic VSMC which were positively stained by in situ DNA labelling. These results demonstrate an important role for GADD153 in the molecular mechanisms of VSMC apoptosis.

INTRODUCTION
GADD153 (growth arrest- and DNA damage-inducible gene 153) is a member of the CCAAT/enhancer-binding protein (C/EBP) family of transcriptional factors [1–12]. The expression of GADD153 is low during normal cell growth, but is markedly induced in response to a variety of cellular stresses, including glucose deprivation, exposure to genotoxic agents and other growth-arresting situations [13–15]. Microinjection of GADD153 into NIH-3T3 fibroblasts induces G1 arrest [16]. Transient expression of GADD153 in several different human tumour cell lines leads to growth arrest [17]. Genes involved in cell growth arrest in response to various stimuli are also potential candidates for mediation of apoptosis [18].

In atherosclerotic lesions and in restenotic lesions after injury, the major cause of disease progression is believed to be excessive accumulation of cells in the intima [19,20]. This accumulation is attributed to increased migration and/or proliferation of cells, including vascular smooth muscle cells (VSMC), monocytes/macrophages and T

Key words: balloon injury, CCAAT/enhancer-binding protein, growth arrest- and DNA damage-inducible gene 153, platelet-derived growth factor, vascular smooth muscle cell.

Abbreviations: C/EBP, CCAAT/enhancer-binding protein; CHOP, C/EBP homologous protein; GADD153, growth arrest- and DNA damage-inducible gene 153; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PDGF, platelet-derived growth factor; RT-PCR, reverse transcriptase–PCR; VSMC, vascular smooth muscle cells.

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Recent studies have demonstrated that dysregulated apoptosis also plays an important role in the pathogenesis and progression of cardiovascular disease [23]. These observations indicate that cell growth and apoptosis are tightly linked processes in cardiovascular diseases, including atherosclerosis and restenosis.

Platelet-derived growth factor (PDGF) is a major mitogen in serum, and is responsible for the proliferation of VSMC [24]. PDGF also induces other important cellular responses, including transformation and survival [25,26]. Against this, however, we recently reported that rat cultured VSMC showed typical apoptotic changes after treatment with PDGF-BB [27]. This VSMC apoptosis was induced by PDGF-BB only when 100% confluency was reached. Given these observations, we hypothesized that cell–cell contact inhibition, also known as cell growth arrest, may be one of the critical factors in the induction of VSMC apoptosis after treatment with PDGF-BB. Recent studies have indicated that both cell growth arrest and cell proliferation are regulated by specific genes encoding cell-cycle-related proteins and transcriptional nuclear factors [28,29].

In the present study we focused on GADD153, one of the cell growth arrest-related genes, and investigated the roles of GADD153 in the cellular functions of VSMC apoptosis in vitro and in vivo situations.

METHODS

Cell culture and induction of apoptosis

All surgical treatments conformed to the Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health (NIH Publication No. 85-23; revised 1985). VSMC were isolated from the thoracic aortas of 10-week-old male Sprague–Dawley rats (Charles River Japan Inc., Kanagawa, Japan) by enzyme dispersal [30]. Cells (passages 3–10) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) heat-inactivated fetal calf serum and maintained at 37 °C in a humidified atmosphere of 95% air/5% CO₂. After 100% confluency was reached, the culture medium was changed to Dulbecco’s modified Eagle’s medium/0.1% fetal calf serum, and cells were incubated for an additional 24 h. Apoptosis was then induced in 100% confluent cells by incubation with PDGF-BB (Upstate Biotechnology, Lake Placid, NY, U.S.A.) at a final concentration of 100 ng/ml.

Northern blot analysis and cDNA probe

Isolation of total RNA, Northern blotting, autoradiography and densitometric analyses were carried out as described previously [30,31]. Total RNA was isolated from VSMC after 0, 1, 2 and 3 days of incubation with or without PDGF-BB. A 0.8 kb portion of the rat GADD153 cDNA clone, which contained the entire sequence of the coding region, was isolated by screening of a cDNA library prepared from VSMC after 3 days of incubation with PDGF-BB. After the entire sequence of this clone was determined, a full-length fragment of the cDNA was used as a probe for Northern blotting.

Protein extraction and Western blot analysis

After 0, 1, 2, 3 and 4 days of incubation with PDGF-BB, VSMC were homogenized with cell lysis buffer (50 mmol/l Tris/HCl, pH 8.0, 150 mmol/l NaCl, 2 mmol/l PMSF, 1 μg/l aprotinin and 1% Triton X-100). Supernatants obtained after centrifugation at 20000 g for 10 min were used as protein extracts. Western blotting and detection of immunoreactive proteins were carried out as described previously [32]. For detection of GADD153 protein, anti-(human GADD153) antibodies (Santa Cruz Biotechnology), which cross-react readily with rat GADD153, were used as the primary antibody.

Plasmid constructs and transfection experiments

The rat GADD153 expression vector was prepared from ligation of the 0.8 kb GADD153 cDNA fragment into expression vector pcDNA3 (Invitrogen Corp., Carlsberg, CA, U.S.A.); the resulting plasmid was designated pcDNA/GADD. Using LIPOFECTAMINE Plus™ (GIBCO BRL, Gaithersburg, MD, U.S.A.), transient transfection experiments were carried out according to the manufacturer’s specifications. pcDNA3 was used as a mock DNA vector, and a pSV β-galactosidase vector (pSV-β-Gal; Promega, Madison, WI, U.S.A.) was used as a control vector for visualization of the transfected cells. At 1 day before transfection, VSMC were seeded on to 96-well culture plates (2000 cells per well) for measurement of cell viability, or on to 60 mm culture dishes (5 × 10⁴ cells per dish) for morphological evaluation. For measurement of cell viability, pcDNA/GADD or pcDNA3 (0.1 μg per well each) was used for transfection of VSMC. Cell viability was determined using cell proliferation agent WST-1 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzenedisulphonate; Boehringer Mannheim, Mannheim, Germany; cleavage of this tetrazolium salt by mitochondrial dehydrogenases results in formazan formation in viable cells, which was used for the quantification of cell viability by a colorimetric assay. At 1 day after transfection, a 10 μl aliquot of WST-1 was added directly to each well, and cell viability was determined according to the manufacturer’s specifications.

To evaluate the morphological changes of gadd153-transfected cells, pcDNA/GADD or pcDNA3 (2 μg per dish each) was co-transfected with pSV-β-Gal.
(2 μg per dish). At 1 day after transfection, cells were washed with PBS and fixed with 0.25% glutaraldehyde, and the transfected cells were visualized by incubation with substrate solution (0.2% 5-bromo-4-chloro-indol-3-yl β-d-galactopyranoside, 1 mmol/l MgCl₂, 150 mmol/l NaCl, 3.3 mmol/l K₃[Fe(CN)]₆·3H₂O, 3.3 mmol/l K₃[Fe(CN)]₆, 60 mmol/l Na₂HPO₄ and 40 mmol/l NaH₂PO₄) for 12 h. On the same day, levels of expression of GADD153 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs were measured by reverse transcriptase–PCR (RT-PCR) as described previously [33]. Nucleotide sequences of primers used for PCR were, for GADD153: forward primer, 5'-TCATGCTTGGTGATGCGACGTGACTCTGCTCTTTTC-3'; reverse primer, 5'-TCATGCTTGGTGACTGACCCTGACATC-3'; and for GAPDH: forward primer, 5'-TGGAGTCTACTGGCGTCTTC-3'; reverse primer, 5'-CAAAGGTGGAGGGAATGGGAG-3'. PCR conditions were: denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s and elongation at 72 °C for 60 s (25 cycles).

In situ DNA labelling and immunohistochemistry in balloon-injured carotid arteries

Male Sprague–Dawley rats weighing 380–400 g were used for these experiments. Balloon injury of the right carotid artery, tissue fixation, tissue excision and embedding were carried out as described previously [34]. At 2 weeks after injury, the whole length of each carotid artery was excised, and in situ DNA labelling and immunohistochemistry of tissue sections were carried out as described previously [34]. Positively staining cells were visualized with a substrate solution of 3,3'-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Osaka, Japan), and counter-staining was performed with haematoxylin. Anti-(human GADD153) antibodies, as described above for Western blotting, were used as the primary antibody for immunohistochemistry.

Statistical analysis

All data are expressed as means±S.E.M. Statistical evaluation was performed by ANOVA (Kruskal–Wallis H-test). Comparison of means between two treatment groups was carried out by the Mann–Whitney U-test. Statistical significance was defined as P < 0.05.

RESULTS

GADD153 expression in apoptotic VSMC induced by PDGF-BB

No obvious changes were observed in control cells during 4 days of incubation. In PDGF-BB-treated cells, although no morphological changes were observed after 1 day of incubation, apoptotic changes were induced in a time-dependent manner between 2 and 4 days of incubation, as described previously [27].

In control cells, basal levels of GADD153 mRNA were very low or almost negligible; these were increased slightly following longer incubations (Figure 1A). In contrast, in PDGF-BB-treated VSMC, GADD153 mRNA levels were markedly increased on days 2–3 of incubation (Figure 1B). Western blotting also indicated that GADD153 protein levels were drastically increased on days 2–4 of incubation with PDGF-BB (Figure 2). GADD153 expression at the mRNA and protein levels was induced in parallel with the induction of VSMC apoptosis.

Cell viability and morphological evaluation of GADD153-transfected VSMC

To examine further the direct effects of GADD153 on the viability and morphology of VSMC, GADD153 was overexpressed by transient transfection of the pcDNA/GADD vector (Figures 3 and 4). The viability
Figure 3  Effect of GADD153 overexpression in VSMC on cell viability
VSMC were seeded on to 96-well plates for measurement of cell viability, or on to 60 mm dishes for measurement of GADD153 mRNA expression levels. For measurement of cell viability (upper panel), pcDNA/GADD or mock vector (0.1 μg/well each) was used for transient transfection of VSMC. At 1 day after transfection, cell viability was determined spectrophotometrically as arbitrary absorbance (OD) units in pcDNA/GADD-transfected (closed column) or mock-vector-transfected (open column) cells. Significant difference between treatment groups: **P < 0.01. All data are expressed as means ± S.E.M. of six separate assays. For measurement of GADD153 mRNA levels (lower panels), pcDNA/GADD (gadd153) or mock vector (control) (2 μg/dish each) was used for transient transfection of VSMC. At 1 day after transfection, total RNA was extracted from VSMC, and levels of GADD153 and GAPDH mRNAs were determined by RT-PCR. GAPDH mRNA was used as a loading control.

of pcDNA/GADD-transfected cells was significantly lower (approx. 60%) than that of mock-vector-transfected cells (Figure 3, upper panel), indicating that GADD153 overexpression induced cell death in VSMC. To confirm whether the transfected gene was actually expressed, GADD153 mRNA levels were assessed by RT-PCR (Figure 3, lower panels). Higher levels of RT-PCR products from GADD153 mRNA were detected in pcDNA/GADD-transfected VSMC than in mock vector-transfected ones. Transfection efficiency is well known to be very low in VSMC, even when using LIPOFECTAMINE Plus, but still resulted in significant expression of the transfected gadd153 gene. In addition, the morphological appearance of the transfected VSMC was evaluated by phase-contrast microscopy (Figure 4). Although apoptotic changes were not observed in mock-transfected VSMC (Figure 4A), pcDNA/GADD-vector-transfected VSMC, which were identified by blue staining, exhibited the apoptotic features of cell shrinkage, membrane blebbing and rounding (Figure 4B), indicating that GADD153 overexpression induced apoptosis in VSMC.

Apoptotic changes and GADD153 expression in injured carotid arteries
The maximum thickness of neointimal formation was observed in the right carotid arteries 2 weeks after balloon injury, as reported previously [34]. In situ DNA labelling of injured arteries clearly demonstrated positive staining of nuclei (brown colour) in the neointimal lesion, exclusively in the innermost layer (Figure 5A). Similarly, GADD153 protein expression was observed in injured arteries by immunohistochemistry (Figure 5B). Positive staining of cells with immunoreactive GADD153 (brown colour) was also detected mainly in the innermost layer of the neointima, and was co-localized with that of in situ DNA labelling. No positive staining with in situ DNA labelling or immunoreactive GADD153 were observed in non-injured control carotid arteries (results not shown).
DISCUSSION

The results of the present study indicate that GADD153 contributes to the molecular mechanisms of apoptosis of VSMC, and plays an important role in the pathogenesis of neointimal formation after balloon injury.

GADD153, also known as C/EBP homologous protein-10 (CHOP-10), belongs to the family of basic-region/leucine-zipper transcriptional factors, the C/EBP family [1–12]. In mammals, the C/EBP family currently contains at least six unique members: C/EBPα, C/EBPβ, C/EBPδ, C/EBPγ, C/EBPε, and GADD153/CHOP-10. All C/EBP members, except GADD153, can form homo- or hetero-dimers, and can recognize and bind to the same DNA consensus sequence [T(T/G)-NNG(T/C)AA(T/G)]. GADD153 forms only hetero-dimers with other C/EBP members. The heterodimers so formed show a reduced ability to bind to the DNA consensus sequence, due to several amino acid substitutions in the DNA-binding domain [35]. On the basis of this unique structure, GADD153 usually acts as a dominant-negative regulatory factor for the transcriptional activities of other C/EBP members, and controls much gene expression in this manner.

Originally, GADD153 was acknowledged as a growth-inhibitory factor in certain types of cells, including fibroblasts [36], hepatocytes [37] and haematopoietic cells [38]. In these cells, GADD153 expression is induced by DNA-damaging stresses, such as UV radiation and alkylating agents, or under specific culture conditions such as 100% confluency and serum deprivation [13–15]. In the present study, although VSMC were cultured under conditions of both 100% confluency and serum deprivation (0.1% fetal calf serum), GADD153 mRNA expression was not induced following 3 days of incubation without PDGF-BB (results not shown). This discrepancy may be due to differences in cell-type specificity or in sensitivity to cell confluency and serum deprivation, or both. In contrast, VSMC highly expressed both the mRNA and protein of GADD153 after 2–4 days of incubation with PDGF-BB, and simultaneously showed morphological changes characteristic of apoptosis. At first sight, this result seems paradoxical, because PDGF is known to be a potent mitogen [39] or survival factor [40] in VSMC, and is usually considered to down-regulate gadd153 gene expression [41]. We now believe, however, that these findings are not contradictory. Recent studies have demonstrated that VSMC death, mainly apoptosis, occurs in atherosclerotic or restenotic lesions even in the presence of excessive accumulation of VSMC [42]. Recently we obtained the same result, namely that VSMC apoptosis co-exists with VSMC migration or proliferation in the neointima of carotid arteries after balloon injury [34]. These observations indicate that the pathogenesis of atherosclerotic or hypertrophic vascular disorders is based on the balance between cell growth and cell apoptosis.

Several studies have suggested that GADD153 is directly associated with apoptosis induced by anti-cancer agents in many types of cancer cell lines, including ovarian cancer [43], leukaemia [44] and prostate cancer [45]. Further evidence for the direct relationship between GADD153 expression and apoptosis was obtained from a study in mice carrying null mutations in the gadd153/chop-10 gene. Compared with the wild type, mouse embryonic fibroblasts derived from chop-10−/− animals exhibited significantly less apoptosis on exposure to agents that perturb the cellular functions of the endoplasmic reticulum, such as tunicamycin and the calcium ionophore A23187 [46]. The critical importance of GADD153 was also revealed in the signalling cascade of apoptotic cells exposed to endoplasmic reticulum stress. In response to such stress, GADD153 is induced and phosphorylated via a pathway involving p38 mitogen-activated protein kinase; this phosphorylated protein can enhance the function or gene transcription of GADD153 itself [47]. Furthermore, Brenner et al. [48] reported that Fas-induced apoptosis in human leukemic
Jurkat cells is mediated directly by phosphorylation of GADD153 via p38 mitogen-activated protein kinase. Taking these and the present findings together, we propose the existence of a signalling pathway mediated by GADD153 as an underlying mechanism of apoptosis in VSMC.

In conclusion, our study has identified GADD153 as an important specific regulatory factor in VSMC apoptosis in vitro and in vivo. Furthermore, our results may also provide important information with regard to our understanding of the mechanisms underlying atherosclerosis and restenosis.

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