Inhibitor of apoptosis protein survivin regulates vascular injury

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Survivin (also termed *Birc5*) belongs to the family of genes known as inhibitors of apoptosis, and it has been implicated in both prevention of cell death and control of mitosis. The survivin pathway is exploited in cancer, but its potential role in vascular injury is unknown. Here, we show that balloon-mediated arterial injury in rabbits resulted in expression of survivin in vascular cells. Serum or PDGF-AB stimulated survivin expression in cultured smooth-muscle cells (SMCs), which suppressed apoptosis and prevented caspase activation. Adenoviral delivery of a phosphorylation-defective survivin mutant reversed the cytoprotective effect of PDGF in SMCs without affecting mitotic progression, suppressed neointimal formation in wire-injured mouse femoral arteries, and induced vascular cell apoptosis *in vivo*. These data identify survivin as a critical regulator of SMC apoptosis after acute vascular injury. Disrupting the survivin pathway may provide a novel therapy to limit pathological vessel-wall remodeling.

Unbalanced apoptosis, or programmed cell death[1] is thought to contribute to various human diseases, including cancer[2], as well as acute[3] and chronic vascular disorders[4]. Changes in cell death or viability of endothelial[5], or vascular smooth-muscle cells (SMCs)[3] have been implicated in developmental and pathological pathways of vascular remodeling. Increase in SMC apoptosis initiated by trauma[6], ligation of death receptors[7,8] or exposure to oxidized low-density lipoproteins[9] has been documented in vascular injury, and tentatively associated with plaque instability in atherosclerosis[10]. Conversely, cytokines released in the vascular microenvironment, including platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF)[11] or epidermal growth factor (EGF)[12] provide mitogenic and survival signals that favor SMC accumulation and pathological neointima formation[4].

The impact of SMC survival in pathological remodeling of the vascular wall is only beginning to emerge. Although a role for Bcl-2 family members in vascular injury has been postulated[13–16], it is unclear if other mechanisms of apoptosis control are operative in SMCs. Recently, a member of the inhibitor of apoptosis (IAP) gene family[17], survivin[18] (also known as *Birc5*), has been implicated in both preservation of cell viability and control of mitosis[18]. Largely undetectable in normal adult tissues, deregulated expression of the survivin gene[19] has been described in most human cancers, and associated with apoptosis inhibition and unfavorable prognosis[18]. However, recent evidence suggests that re-activation of the survivin pathway may also occur in vascular responses. Endothelial cell (EC)-stimulation with vascular endothelial-cell growth factor (VEGF)[20,21] or angiopoietin-1 (ref. 22) resulted in an up to 20-fold increase in survivin expression, and antisense ablation of survivin in VEGF-stimulated ECs caused apoptosis and regression of newly formed capillaries[23].

Here we investigated a potential role of survivin in acute vascular injury. We found that survivin is a critical downstream effector of PDGF-mediated SMC viability, and that disruption of the survivin pathway prevented neointimal formation after injury, *in vivo*.

Induced survivin expression in acute vascular injury

The rabbit balloon injury model produces a proliferative neointimal lesion that has been well characterized. The neointima, composed primarily of α-actin-positive SMCs, is visible by 7 days, and increases markedly in area over the ensuing 4–6 weeks. Ki-67 expression, a marker of cell proliferation, is increased within the first several days, peaks at approximately one week in the neointima, and then rapidly declines to control levels beyond one month (Fig. 1*a–c*). Survivin staining was negligible in normal, uninjured artery sections (data not shown). At 4–7 days after injury, survivin expression was noted in medial SMCs, particularly those adjacent to the internal elastic lamina, and adventitial cells (Fig. 1*a* and *b*). Strong expression of survivin was observed in the developing neointima and media with a peak at approximately 14 days, and persisting out to beyond 6 weeks (Fig. 1*a–c*). Although double-staining methods were not used, the vast majority of survivin-positive cells in the neointima and media appeared to be α-actin-positive SMCs. Quantitative scoring of stained sections revealed that the temporal and spatial patterns of expression for Ki-67 and survivin were distinct (Fig. 1*c* and *d*). Similar results were obtained in a mouse model of wire arterial injury (Fig. 1*e*). In these experiments, increased expression of

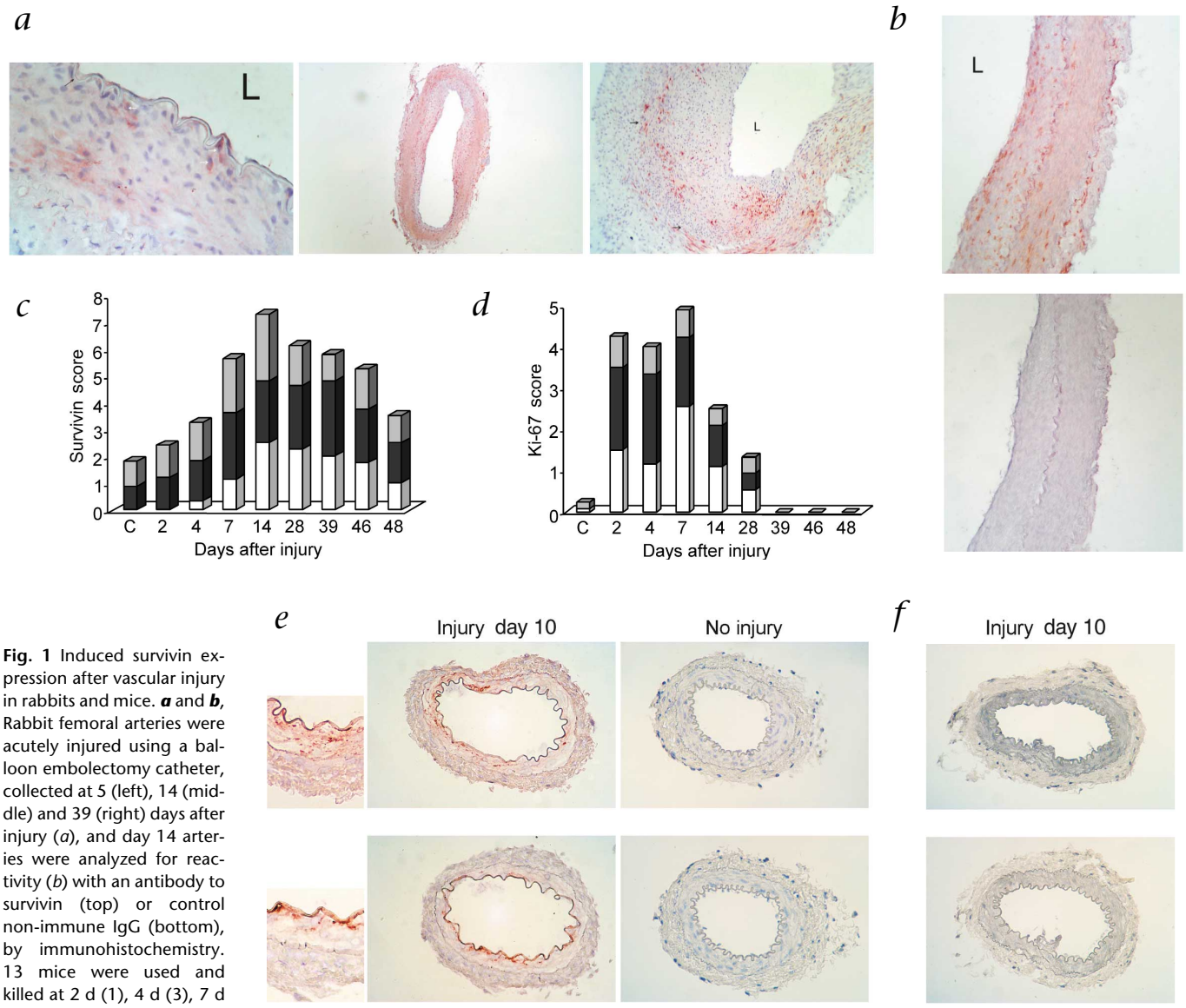

Fig. 1 Induced survivin expression after vascular injury in rabbits and mice. ***a*** and ***b***, Rabbit femoral arteries were acutely injured using a balloon embolectomy catheter, collected at 5 (left), 14 (middle) and 39 (right) days after injury (*a*), and day 14 arteries were analyzed for reactivity (*b*) with an antibody to survivin (top) or control non-immune IgG (bottom), by immunohistochemistry. 13 mice were used and killed at 2 d (1), 4 d (3), 7 d (3), 14 d (2) and > 28 d (28–48 days; 4). 2 animals were used as uninjured controls. Magnification in *a*, x400 (left), x40 (middle), x100 (right), and in *b*, x100. ***c*** and ***d***, A normalized score for survivin (c) or Ki-67 (d) expression was calculated at indicated time after injury, by immunohistochemistry. L, lumen. ■, media; ■, adventitia; □, neo-intima. ***e***, Expression of survivin and PDGF-B in wire-injured mouse arteries.Cryosections of wire-injured (right and middle panels) or uninjured (left panels) femoral arteries in mice were collected after 10 d and analyzed for expression of survivin (top panels) or PDGF-B chain (bottom panels) by immunohistochemistry. ***f***, Naive normal rabbit (top, right) and goat (bottom, right) antisera wereused as negative controls. Magnification, x200. Enlargement of left panels are shown at far left. Magnification, x400.

survivin 10 days after injury paralleled an upregulation of PDGF-B in the media of injured vessels (Fig. 1 *e*).

PDGF regulation of survivin expression

Endogenous survivin was expressed at negligible levels in quiescent SMCs in culture (Fig. 2*a*). However, SMC stimulation with serum, PDGF-AB or HB-EGF increased survivin expression (by 16-, 13- and 9-fold, respectively) in a concentration-dependent manner, with peak expression occuring 20–24 hours after stimulation (Fig. 2*a* and data not shown). In contrast, EGF (25 ng/ml), bFGF (25 ng/ml) or TGF-β1 (2 ng/ml) did not influence survivin expression in SMCs (Fig. 2*a* and data not shown). In addition, PDGF-AB did not affect the levels of anti-apoptotic Bcl-xL (refs. 6,15), whereas TGF-β1 reduced Bcl-xL expression in SMCs (Fig. 2*a*). In subcellular fractionation experiments, increased survivin expression in PDGF-AB-stimulated cultures accumulated in the cytosolic and cytoskeletal fractions and was associated with microtubules of midbodies at telophase (Fig. 2*b*). Preincubation of SMCs with the transcriptional inhibitor actinomycin D (Fig. 2*c*) or antagonists of MEK (PD098059) or PI3 kinase (LY294002) suppressed PDGF-AB-induced survivin expression in SMCs (Fig. 2*c* and *d*). In contrast, the p38 MAPK inhibitor SB203580 was without effect (Fig. 2*d*). No changes in SMC viability occurred in the presence of the various pharmacological inhibitors (Fig. 2*d*).

Inhibition of SMC apoptosis by PDGF or survivin

Exposure of SMCs to C_2 ceramide or the combination of

tumor necrosis factor-α (TNF-α) plus cycloheximide (CHX) resulted in nuclear morphology of apoptosis with chromatin condensation and DNA fragmentation (Fig. 3*a*). Pretreatment with PDGF-AB inhibited C_2 ceramide- or TNF-α/CHX-induced apoptosis in SMCs ($P < 0.015$ and $P < 0.01$, respectively) (Fig. 3*a* and *b*), and attenuated the generation of active caspase-3 (Fig. 3*b*, insert). Transduction of SMCs with a replication-deficient adenovirus encoding survivin (pAd-Survivin)[24] resulted in strong cytosolic expression of a 16.5-kD survivin band, whereas the endogenous levels of anti-apoptotic XIAP were unchanged (Fig. 3*c*). Forced survivin expression in SMCs reproduced the cytoprotective effect of PDGF-AB, and counteracted apoptosis induced by C_2 ceramide or TNF-α/CHX ($P < 0.015$ and $P < 0.02$, respectively) (Fig. 3*d*), and suppressed the generation of active caspase-3 and -7 (Fig. 3*e*).

Survivin targeting induces apoptosis of SMCs

To target the survivin pathway in SMCs, we used adenoviral delivery of a phosphorylation-defective survivin $Thr^{34}\rightarrow Ala$ mutant[25], which prevents phosphorylation of endogenous survivin by the mitotic kinase $p34^{cdc2}$-cyclin B1, and results in apoptosis[25,26]. Transduction of SMCs with a replication-deficient adenovirus encoding survivin $Thr^{34}\rightarrow Ala$ (pAd-T34A)[24] resulted in induction of a 16.5-kD survivin band (Fig. 4*d*). Adenoviral expression of pAd-T34A in SMCs abrogated the cytoprotective effect of PDGF-AB against ceramide-induced apoptosis (Fig. 4*a*), whereas pAd-GFP was ineffective (Fig. 4*a*). Consistent with the absence of survivin in quiescent SMCs (Fig. 2) and previous observations[24], expression of pAd-T34A in SMCs grown in 2% serum did not result in loss of cell viability (Fig. 4*b*). In contrast, transduction of serum-stimulated SMCs (20% FBS) with pAd-T34A resulted in time-dependent induction of apoptosis (Fig. 4*b* and *c*). In these experiments, apoptosis induced by pAd-T34A was not associated with defects of mitotic progression, and resulted in selective decrease in the fraction of proliferating cells with G2/M DNA content, as compared with pAd-GFP-transduced cultures (Fig. 4*c*). SMC apoptosis induced by expression of pAd-T34A was associated with increased processing of proform caspase-9, and generation of active caspase-3 (Fig. 4*d*). In control experiments, infection with pAd-GFP did not cause SMC apoptosis in 2% or 20% serum (Fig. 4*b* and *c*), and was not associated with processing of caspase-9 or generation of active caspase-3 (Fig. 4*d*).

Survivin targeting suppresses neointimal formation *in vivo*

Wire injury of mouse femoral arteries resulted in *de novo* neointimal expression of survivin (Fig. 1*d*), similar to the results obtained after balloon-mediated injury (Fig. 1*a*). Wire-injured femoral arteries transduced with pluronic gel containing pAd-GFP exhibited neointimal formation 3 weeks after injury (Fig. 5*a*). In contrast, arteries transduced with pAd-T34A under the same experimental conditions revealed a marked reduction in neointimal formation (Fig. 5*a*). By morphometric analysis of elastic-stained sections, expression of pAd-T34A suppressed neointimal formation by >63% ($P < 0.005$), as compared with pAd-GFP-transduced vessels (Fig. 5*b*). Histological analysis of non-injured or injured vessels in the presence or absence of adenoviral transduction did not reveal detectable changes in accumulation of inflammatory cells, by H&E staining (Fig. 5*c*). Strong expression of adenovirus-encoded GFP protein was detectable 10 days after vessel transduction, and localized to the adventitia, as well as to the media and the forming neointima (Fig. 5*d*). In addition, arteries transduced with pAd-T34A and collected 10 days after injury showed a significant increase in the number of apoptotic cells in the media ($P < 0.001$) and adventitia, as compared with pAd-GFP-treated vessels (Fig. 6*a* and *b*).

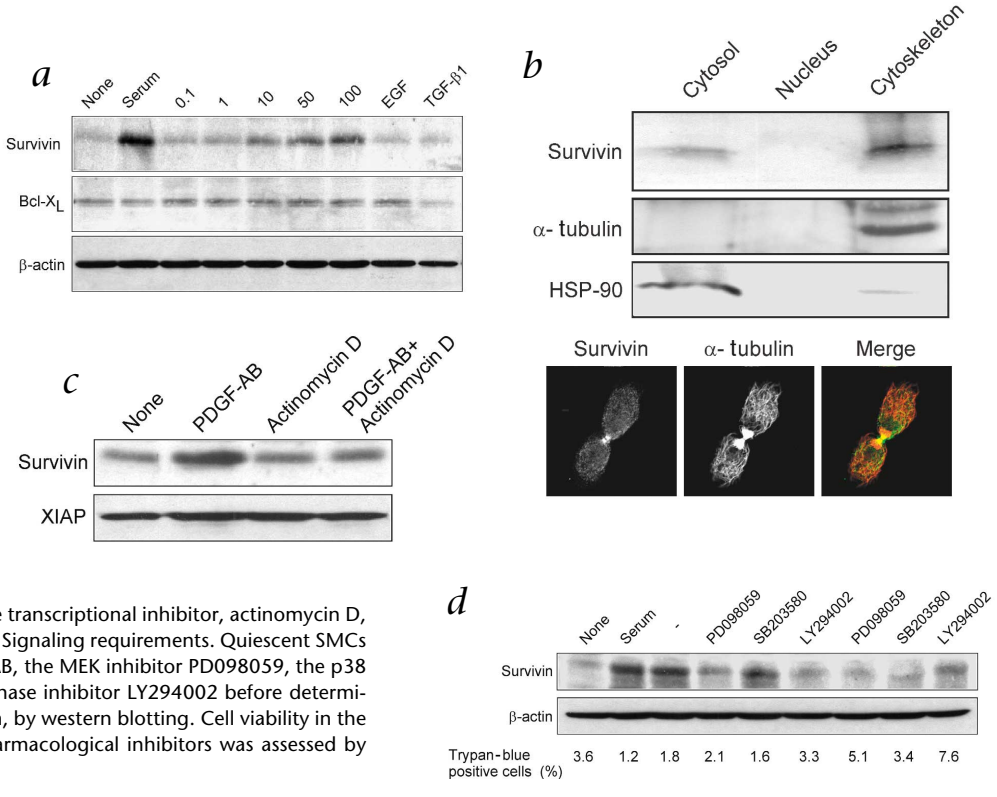

Fig. 2 PDGF regulates survivin expression in SMCs. ***a***, Concentration-dependence. Quiescent SMCs were incubated with medium (none), serum EGF, TGF-β1 or increasing concentrations of PDGF-AB and analyzed for expression of survivin, Bcl-X_L or actin by western blotting. ***b***, Subcellular localization. PDGF-AB-stimulated SMC cultures were analyzed for survivin expression in the indicated isolated subcellular fractions by western blotting (top) or by immunofluorescence and confocal microscopy (bottom). HSP-90 and α-tubulin were also immunodetected as specific markers of the cytosolic and cytoskeletal fractions, respectively. Relative height of panel is 60 μm. ***c***, Transcriptional requirements. Quiescent SMCs were incubated with PDGF-AB with or without the transcriptional inhibitor, actinomycin D, before analysis of survivin expression. ***d***, Signaling requirements. Quiescent SMCs were incubated with or without PDGF-AB, the MEK inhibitor PD098059, the p38 MAPK inhibitor SB203580, or the PI_3 kinase inhibitor LY294002 before determination of survivin and β-actin expression, by western blotting. Cell viability in the presence or absence of the various pharmacological inhibitors was assessed by Trypan-blue exclusion.

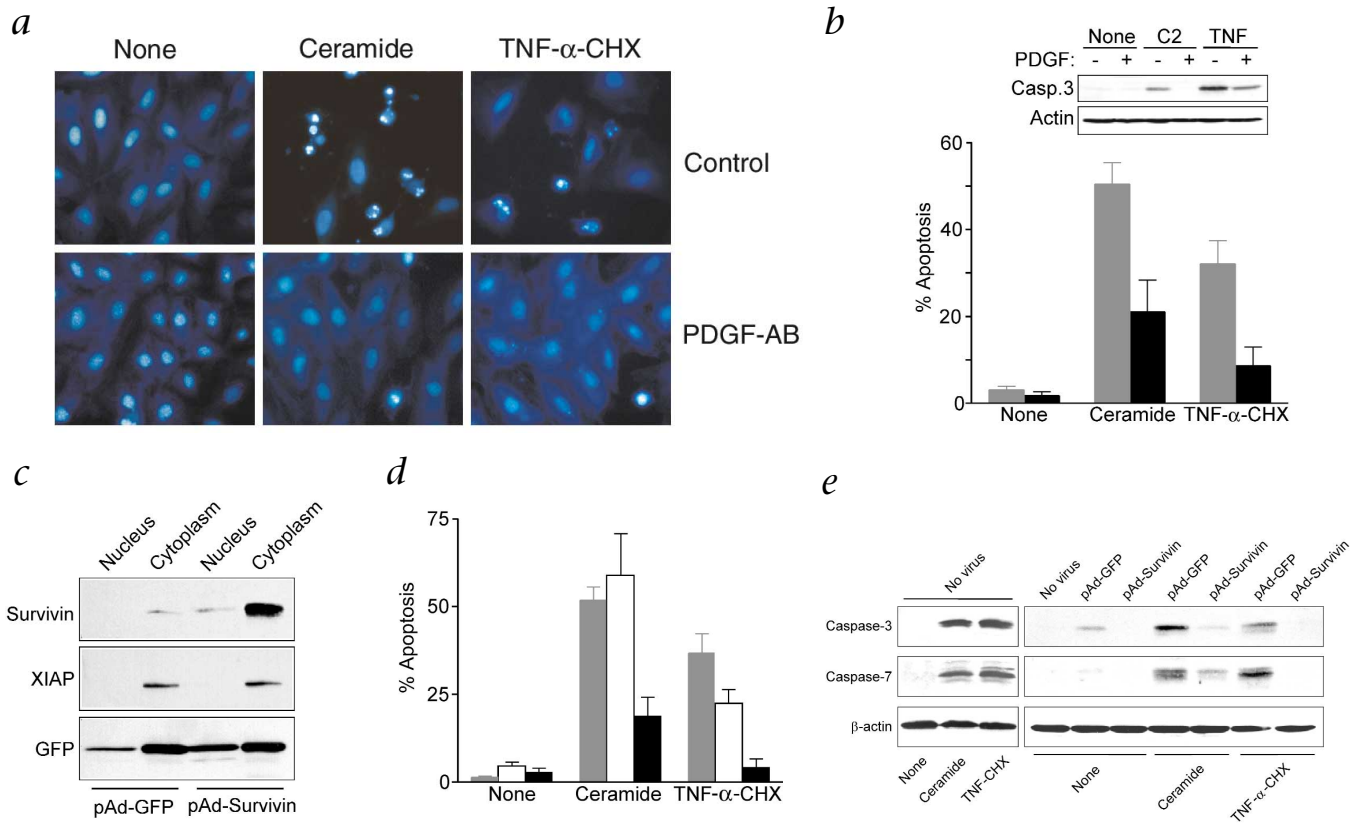

Fig. 3 SMC cytoprotection by PDGF or survivin. ***a*** and ***b***, PDGF-AB-mediated SMC survival. Quiescent SMCs were incubated with medium (control) or PDGF-AB and subsequently treated with C_2 ceramide or the combination of TNF-α/CHX (*a*) before analysis of nuclear morphology of apoptosis (chromatin condensation and nuclear fragmentation) by DAPI staining and fluorescence microscopy (*b*). ■, control; ■, PDGF-AB pre-treatment. Magnification in *a*, × 500. *Insert*, Suppression of active caspase-3 generation by PDGF. The experimental conditions are the same as in *a*, except that SMCs treated with C_2 ceramide (C2) or TNF-α/CHX with or without PDGF-AB preincubation were collected and analyzed for expression of active caspase-3 by western blotting. ***c***, Adenoviral expression of survivin. SMCs were infected with a replication-deficient adenovirus encoding wild-type survivin (pAd-Survivin) or control pAd-GFP and subsequently analyzed for survivin, XIAP and GFP expression by western blotting. ***d***, Apoptosis inhibition by survivin expression. Uninfected or SMCs infected with pAd-GFP or pAd-Survivin, were treated with medium (control), C_2 ceramide or the combination TNF-α/CHX and analyzed for nuclear morphology of apoptosis by DAPI-staining. For *b* and *d*, data are the mean ± s.e.m. of at least 3 independent experiments. ■, control; □, pAd-GFP; ■, pAd-Survivin. ***e***, Inhibition of caspase generation. Uninfected or SMCs infected with pAd-GFP or pAd-Survivin and exposed to the various indicated conditions were analyzed with antibodies to the active, processed forms of caspase-3 or -7, by western blotting. Protein levels were normalized by reprobing the membrane with an antibody to β-actin.

Discussion

Here we show that survivin[18] is a critical regulator of SMC viability mediated by PDGF-AB. Furthermore, adenoviral expression of a phosphorylation-defective survivin Thr[34]→Ala mutant[25,26] reversed the cytoprotective effect of PDGF against cell-death stimuli, caused vascular-cell apoptosis independently of defects of mitotic progression, and suppressed neointimal formation after acute vascular injury, *in vivo*.

In addition to the deregulated increased expression of survivin in most human cancers, which results from oncogenic transformation[19], re-activation of the survivin pathway may also occur in normal cell types, particularly during vascular responses[21]. Here, the induction of survivin in SMCs stimulated by serum mitogens or PDGF is consistent with the cell cycle-dependent expression of the *survivin* gene at mitosis[27]. In addition, the role of MEK and PI-3 kinase signaling in survivin induction in SMCs is consistent with similar requirements in leukemic cells[28], or ECs (ref. 22). On the other hand, the persistent expression of survivin in the neointima of injured vessels, which contrasted with the rapid decline of cell proliferation, suggests that additional mechanisms independent of mitotic progression may contribute to the sustained SMC expression of survivin. A similar model has been proposed for ECs, where stimulation with non-mitogenic cytokines IL-11 (ref. 29), or angiopoietin-1 (ref. 22) resulted in survivin expression independent of cell-cycle progression.

When expressed in SMCs either following PDGF stimulation or by adenoviral delivery, survivin effectively counteracted apoptosis and attenuated the generation of active caspases, consistent with its role as a genuine cell death inhibitor, *in vivo*[18]. Although the precise mechanism(s) by which survivin inhibits apoptosis has been debated, recent evidence points to a direct interference with the intrinsic (mitochondrial) initiation of the caspase cascade[30]. Accordingly, survivin suppressed a typical mitochondrial-dependent apoptotic pathway mediated by ceramide in ECs (ref. 23), or SMCs (this study). In addition, the ability of survivin to antagonize partly TNF-α-induced apoptosis in both cell types may reflect the involvement of a mitochondrial amplification loop[1] in death-receptor signaling[23]. Consistent with current models of

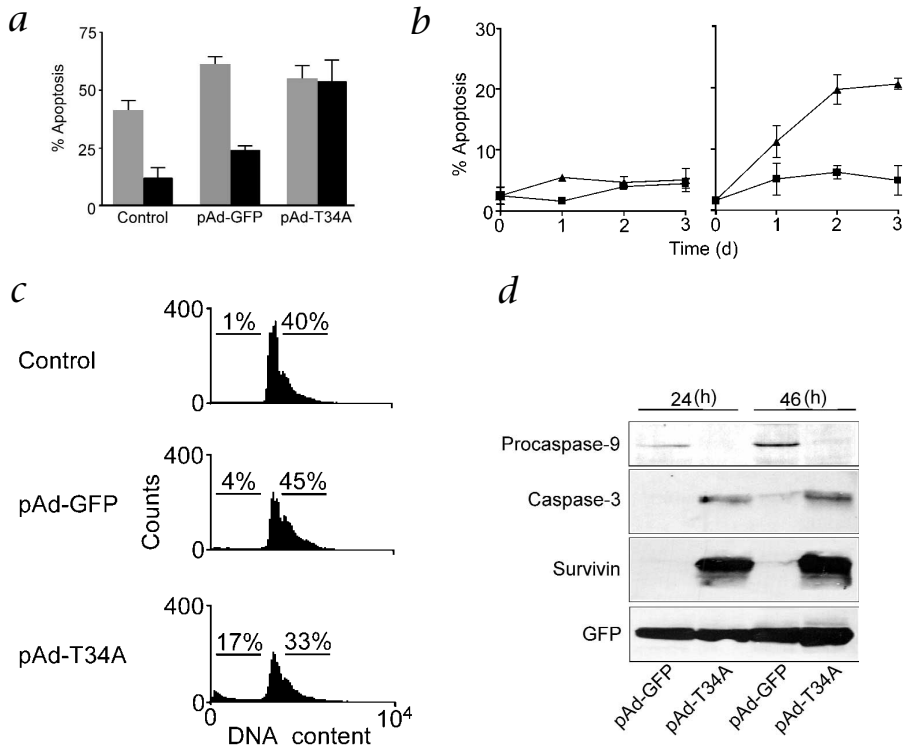

Fig. 4 Survivin targeting induces apoptosis in serum-stimulated SMCs. ***a***, Role of survivin in PDGF-mediated SMC survival. Uninfected or SMCs infected with pAd-GFP or pAd-T34A, treated subsequently with medium (■) or PDGF-AB (■), and incubated later with C_2 ceramide before analysis of nuclear morphology of apoptosis by DAPI staining. ***b***, SMCs were maintained in 2% (left) or 20% serum (right), infected with pAd-GFP (■) or pAd-T34A (▲) and analyzed at the various time intervals for nuclear morphology of apoptosis by DAPI staining. ***c***, Cell-cycle analysis. SMCs in 20% FBS were infected with or without pAd-GFP or pAd-T34A and analyzed for DNA content by propidium iodide staining and flow cytometry. The percentages of cells with hypodiploid (sub-G1, apoptotic) or mitotic (G2/M) DNA content are indicated per each condition tested. ***d***, Caspase activation. Detergent-solubilized extracts of SMCs infected with pAd-GFP or pAd-T34A were analyzed with antibodies to GFP, survivin, caspase-9 or the processed form of caspase-3, by western blotting.

apoptosis regulation[1], survivin cytoprotection involved a predominant cytosolic localization of endogenous (PDGF-induced) or adenovirally expressed survivin. Although earlier claims obtained in preliminary over-expression experiments[31] suggested an exclusively nuclear localization of survivin, our data agree with more in-depth subcellular fractionation and immunofluorescence studies demonstrating that human survivin is predominantly cytoplasmic and prominently associated with microtubules of the mitotic apparatus[32] after nuclear envelope breakdown.

Compelling experimental evidence underscores a role of SMC apoptosis in pathological mechanisms of vascular remodeling[3]. This has been exploited therapeutically to preserve vascular function, and antagonists of the PDGF receptor[33,34], antisense or ribozyme ablation of anti-apoptotic Bcl-2 family members[13,15] or topical application of pro-apoptotic stimuli[35] have been reported to reduce neointimal formation following acute vascular injury. Despite the redundancy of cell-death pathways, current evidence suggests that over-expression of survivin—whether as a result of oncogenic transformation or angiogenesis—becomes a requirement to preserve cell viability[18]. This has been exploited in novel anti-cancer strategies, and ablation of endogenous survivin by antisense[36] or hammerhead ribozyme[37], or expression of dominant-negative survivin mutants[26,38] directly triggered tumor-cell apoptosis, enhanced chemotherapy-induced cell death, and suppressed tumor growth, *in vivo*. Based on two arterial injury models in mice and rabbits, a similar paradigm can now be extended to pathological mechanisms of SMC proliferation. Adenoviral expression of a phosphorylation-defective survivin mutant (pAd-T34A) that exhibited strong anti-tumor activity *in vivo*[24], did not reduce SMC viability under quiescent conditions, in agreement with previous observations[24]. In contrast, serum-stimulated SMCs that over-express survivin became exquisitely sensitive to pAd-T34A targeting, and exhibited processing of proform caspase-9, activation of caspase-3 and apoptosis independently of defects of mitotic progression, and similarly to the response of cancer cells transduced with pAd-T34A (ref. 24). When tested in an acute arterial injury model, expression of pAd-T34A resulted in medial-cell apoptosis and marked reduction in neointimal formation. In the absence of endogenous survivin in neighboring, non-injured cells, vascular targeting with pAd-T34A may provide a high degree of selectivity, and interfere exclusively with pathological remodeling without affecting normal vessel-wall function. Notably, inhibition of neointimal formation was recently observed in response to taxol[39,40], a chemotherapeutic agent that, similarly to pAd-T34A, is expected to target the mitotic transition of proliferating SMCs.

In summary, these data identify survivin as a novel interventional target to prevent pathological vascular remodeling. This may be relevant to angioplasty, where a high incidence of restenosis attributable to neointimal formation hampers long-term preservation of lumen diameter and vessel function[41].

Methods

Cells and cell culture. Rat aortic SMCs (A-10, CRL 1476; ATCC, Manassas, Virginia) were cultured in DMEM containing 20% FBS in 5% CO_2 at 37 °C. Trypsinized cells were seeded in 6-well plates (Costar, New Bedford, Massachusetts) and used below passage 25 when 40% confluent.

Survivin expression in SMCs. SMCs were quiesced (DMEM plus 0% FBS, 24 h) and stimulated with PDGF-AB (0.1–100 ng/ml), EGF (25 ng/ml), bFGF (25 ng/ml), HB-EGF (25 ng/ml), TGF-β_1 (2 ng/ml) (all from Calbiochem, San Diego, California) or 0% FBS for up to 48 h. Western blotting was performed with antibodies to survivin (1.5 µg/ml; NOVUS Biologicals, Littleton, Colorado), XIAP (1:250; Transduction Laboratories, Lexington, Kentucky), Bcl-xL (1:500; Transduction Laboratories), HSP-90 (1:1,000; Transduction Laboratories), α-Tubulin

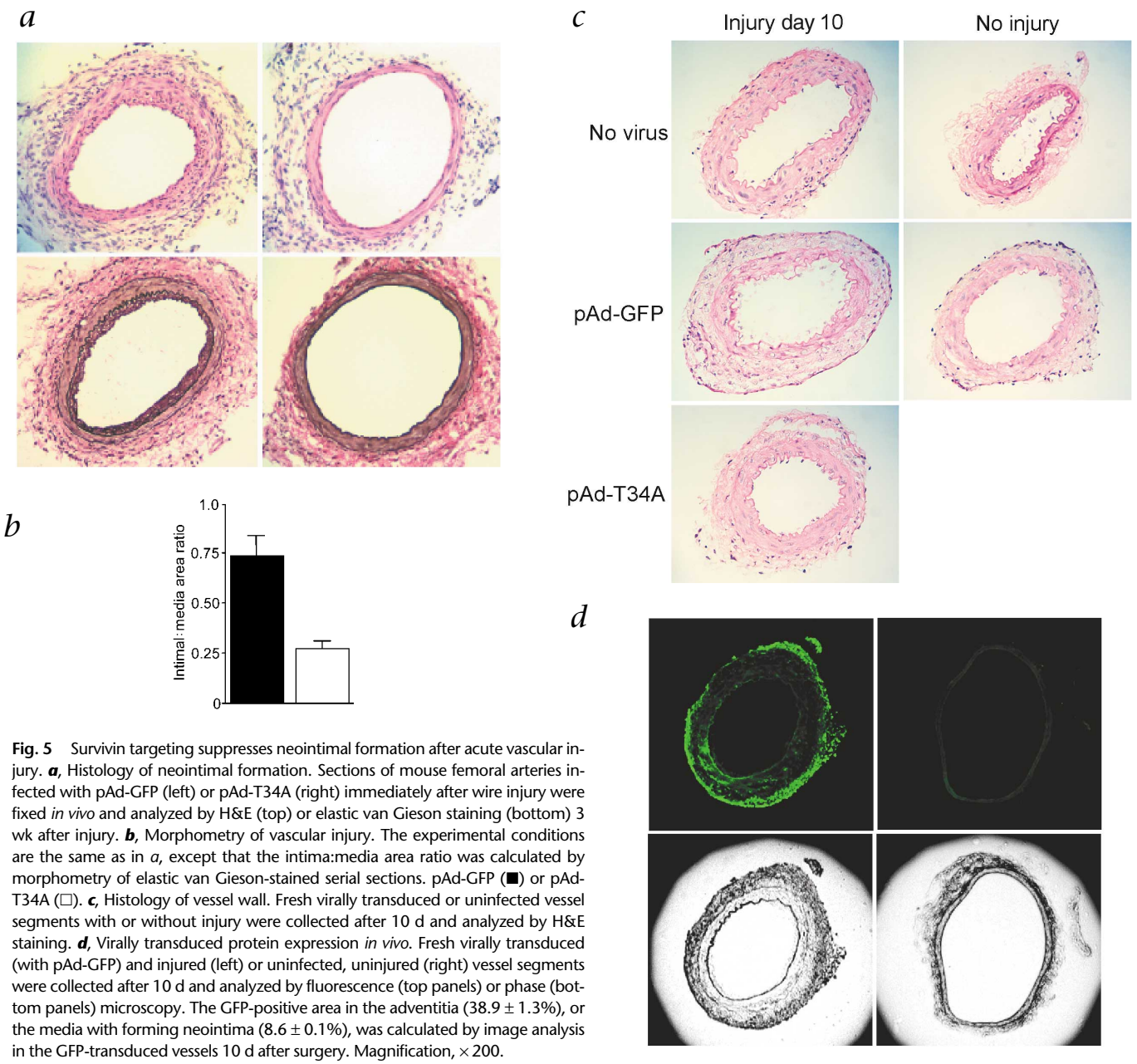

Fig. 5 Survivin targeting suppresses neointimal formation after acute vascular injury. ***a***, Histology of neointimal formation. Sections of mouse femoral arteries infected with pAd-GFP (left) or pAd-T34A (right) immediately after wire injury were fixed *in vivo* and analyzed by H&E (top) or elastic van Gieson staining (bottom) 3 wk after injury. ***b***, Morphometry of vascular injury. The experimental conditions are the same as in *a*, except that the intima:media area ratio was calculated by morphometry of elastic van Gieson-stained serial sections. pAd-GFP (■) or pAd-T34A (□). ***c***, Histology of vessel wall. Fresh virally transduced or uninfected vessel segments with or without injury were collected after 10 d and analyzed by H&E staining. ***d***, Virally transduced protein expression *in vivo*. Fresh virally transduced (with pAd-GFP) and injured (left) or uninfected, uninjured (right) vessel segments were collected after 10 d and analyzed by fluorescence (top panels) or phase (bottom panels) microscopy. The GFP-positive area in the adventitia (38.9 ± 1.3%), or the media with forming neointima (8.6 ± 0.1%), was calculated by image analysis in the GFP-transduced vessels 10 d after surgery. Magnification, × 200.

(1:5,000; Oncogene Research Products, Boston, Massachusetts), or β-actin (1:10000; Sigma, St. Louis, Missouri). In some experiments, SMCs were pre-incubated for 1 h with inhibitors of p42/44 MAPK (PD98059, 80 μM), p38 MAPK (SB203580, 20 μM), or PI3-kinase (LY294002, 50 μM), (all from Calbiochem), and stimulated with 25 ng/ml PDGF-AB for 24 h, before western-blot analysis. Cell viability was determined by Trypan blue exclusion. Survivin distribution in PDGF-stimulated SMCs was analyzed by subcellular fractionation (cytosol, nucleus and cytoskeleton) and western blotting, or confocal microscopy[32].

Adenoviral expression of survivin. Replication-deficient adenoviruses expressing wild-type survivin (pAd-Survivin) or the phosphorylation-defective Thr^{34}→Ala survivin mutant (pAd-T34A)[25] were as described[24]. Viruses were used at multiplicity of infection (m.o.i.) of 50 in 20% FBS for 10 h at 37 °C, followed by 12 h in 2% serum.

SMC apoptosis and cell cycle analysis. SMCs incubated with 100 μM C_2 ceramide (Calbiochem), or 10 ng/ml TNF-α (Endogen, Woburn, Massachusetts) plus 10 μg/ml CHX (Sigma) for 4 to 6 h at 37 °C were stained with DAPI for apoptotic nuclear morphology. Caspase activation was monitored by western blotting. SMCs transduced with pAd-GFP or pAd-T34A in 20% FBS were analyzed after 48 h for DNA content by propidium iodide staining and flow cytometry[24,32].

Acute vascular injury models. All experiments were approved by the institutional animal care and use committees of Yale University and Harvard Medical Schools. 8–10-wk-old male C57/BL6J mice (Taconic Farms, Germantown, New York) were anesthetized with ketamine/xylazine. Left common femoral arteries were blunt-dissected. The great saphenous artery's proximal part, where arterioctomy was performed, was separated and ligated distally for reverse blood control. Proximal clamping of the femoral artery stopped blood flow and allowed the insertion of a 0.25-mm diameter angioplasty guidewire into the saphenous artery. After clamp removal, endoluminal injury was inflicted by advancing and withdrawing the wire 3 times into the femoral artery, with rotation. A 9-0 nylon suture was tied around the proximal portion of the saphenous artery. Blood flow was restored in the femoral artery. pAd-GFP or pAd-T34A (3×10^8 GFU) was delivered by painting the cir-

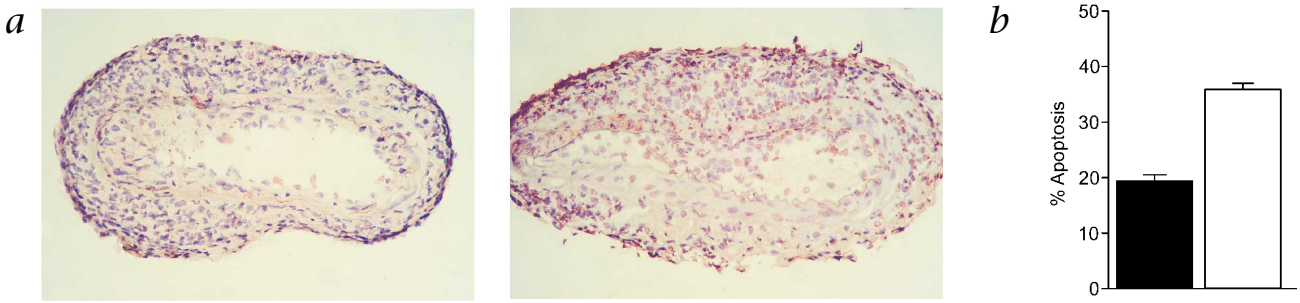

Fig. 6 Induction of vascular cell apoptosis by survivin targeting with pAd-T34A. ***a***, TUNEL staining. The experimental conditions are the same as in Fig. 5*a*, except that fresh-frozen samples of mouse femoral arteries locally infected with pAd-GFP (left) or pAd-T34A (right) were analyzed 10 d after wire injury by TUNEL staining. ***b***, Apoptotic index. Percentage of apoptotic cells in the media of wire-injured vessels transduced with pAd-GFP (■) or pAd-T34A (□). Maginfication, × 300.

cumference of the femoral artery with 50 μl of an adenovirus (15 μl)/Pluronic-127 gel (35 μl) mixture immediately after injury. Injured femoral arteries were collected 10 days and 3 wk after surgery. A rabbit model of balloon injury was carried out as described[42]. Iliofemoral arteries were collected 2–48 days after injury, divided into proximal, central, distal segments, and frozen in OCT (Sakura Finetek, Torrance, California).

Histology and immunohistochemistry. Cryosections (5-μm thick) of injured arteries were quenched for endogenous peroxidase, blocked in 10% goat, donkey or horse serum and incubated with antibodies to survivin (60.1, NOVUS, 1:500 or 0.004 μg/μl)[32], Ki-67 (MIB-1, Cell Marque Corp., 1:100 Hot Springs, Arkansas), or PDGF-B chain (R&D Systems, Minneapolis, Minnesota, 1:400). Isotype-matched primary antibodies and naïve normal antisera were used at equivalent concentrations. Bound primary antibodies were detected using avidin-biotin-peroxidase (NovaRed Peroxidase Substrate Kit, Vector Laboratories, Burlingame, California) and 3-amino-9-ethyl carbazole as chromophore (AEC, Vector). Positive staining in the intima, media and adventitia was graded (0: no expression, to 5: 81-100% positive). 3 sections (proximal, central, distal segments) from 14 injured rabbit arteries distributed between 8 time points (3–9 sections per time point) were scored twice by 2 independent investigators. *In vivo* apoptosis was determined by TUNEL analysis (Zymed, San Francisco, California), as described[24]. About 700 cells were counted in 3–4 sections from 4 individual mouse arteries (14 sections per treatment). Percentage of $TUNEL^+$ cells was determined in the media and forming neointima.

Morphometry. 10 cross-sections from 5 wire-injured *in vivo*-fixed mouse vessels (50 sections per treatment) were stained with a hematoxylin-iodine-ferric chloride–based elastic staining (Sigma). Cross-section images were collected using a Zeiss microscope and on line CCD camera (DAGE-MTI, Michigan City, Indiana). The circumference of the lumen, internal elastic lamina (IEL) and external elastic lamina (EEL) were measured by image analysis (Scion Softwares, Frederick, Maryland). Statistical analysis was performed using Student's *t*-test. A $P < 0.05$ was considered statistically significant.

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Competing interests statement

The authors declare that they have no competing financial interests.

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