Flow-responsive remodeling after angioplasty is enhanced by high cholesterol diet. Prevention with pyrrolidine dithiocarbamate

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Abstract

We examined the effects of high cholesterol diet and pyrrolidine dithiocarbamate (PDTC) on flow-dependent remodeling after angioplasty. After right common carotid balloon-injury, the right external carotid (low flow) or left common carotid artery were ligated (high flow) in rabbits fed normal diet, 1% cholesterol diet without or with the antioxidant PDTC for 7 days pre- and 7–28 days post-injury. Angiographic lumen diameter was significantly greater at 28 days in high flow than low flow normal diet animals, attributable on perfusion-fixed vessel morphometry to altered remodeling (area within the external elastic lamina: high flow 1.85 ± 0.24 vs. low flow 1.31 ± 0.04 mm², P < 0.05) rather than differences in neointima formation or vessel tone. In animals on 1% cholesterol diet high flow remodeling was significantly enhanced (area within the external elastic lamina 3.13 ± 0.17 mm², P < 0.05 vs. high flow normal diet) but low flow inward remodeling was similar (area within the external elastic lamina 1.29 ± 0.07 mm²).

Mean Doppler flow velocities (initial/post-ligation/28 day follow-up, cm/s) had almost normalized in normal diet animals (high flow 30/49/35, low flow 32/9/26) but showed overcompensation in 1% cholesterol diet animals (high flow 32/49/22, low flow 30/11/25). PDTC therapy markedly attenuated remodeling (area within the external elastic lamina: high flow 2.20 ± 0.18, and low flow 2.00 ± 0.11 both P < 0.05 vs. 1% cholesterol diet alone) and flow velocities only partially normalized (high flow 26/42/34, low flow 27/7/16). We conclude that hypercholesterolemia enhances and PDTC attenuates flow-dependent remodeling after angioplasty.

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1. Introduction

It has recently been recognized that the major determinant of lumen size and restenosis after angioplasty is inward arterial remodeling (reduction in vessel size) rather than neointimal hyperplasia [1] but the mechanisms underlying inward remodeling remain uncertain. Remodeling in normal arteries is primarily a shear-homeostatic response [2], in which the ability to remodel in response to changes in flow is dependent on shear-responsive endothelial production of nitric oxide (NO) [3], and is prevented by gentle removal of the endothelium [4]. However, low flow is also associated with increased restenosis rates after human coronary angioplasty [5,6], despite frequent endothelial denudation or damage [7]. This suggestion that flow-responsive remodeling may be endothelium-independent in injured vessels, has been further supported by a strong correlation between inward remodeling and reduced wall shear stress after porcine iliac angioplasty [8], and confirmed by the demonstration of flow-dependent remodeling after Fogarty balloon injury [9], where the vessel is typically completely deendothelialized.

The most common cause of low flow after angioplasty is microvascular endothelial dysfunction, due to hypercholesterolemia, smoking, diabetes or hypertension. All of these atherosclerotic risk factors may also have flow-independent effects on remodeling after angioplasty, particularly hypercholesterolemia. Hypercholesterolemia, appears to attenuate outward remodeling in normal or minimally diseased arteries, an effect which correlates with impairment of endothelial function [10]. However, in de novo atherosclerotic lesions compensa-
tory enlargement [11] and flow-responsive remodeling [12] are preserved and may even be encouraged by hypercholesterolemia [13]. Experimentally, hypercholesterolemia appears to enhance compensatory enlargement by increasing matrix metalloproteinase (MMP) activity [14], an effect which is redox-dependent [15]. This may have some relevance to flow-dependent remodeling after angioplasty, as MMP induction after angioplasty is flow-sensitive [16], and inward remodeling after angioplasty is attenuated by both MMP inhibitors [17] and antioxidants [18].

We, therefore, examined the effects of a high cholesterol diet on patterns of vascular remodeling after balloon injury in high and low flow states. We then assessed the influence of pyrrolidine dithiocarbamate (PDTC), on these effects.

2. Methods

2.1. Study design

Fifty-four New Zealand White rabbits (mean weight $3.8 \pm 0.4$ kg) were used according to the study design shown in Fig. 1. The experimental protocols were approved by the Administrative Panel on Laboratory Animal Care of Stanford University and were performed in accordance with the recommendations of the American Association for the Accreditation of Laboratory Animal Care. Rabbits were fed for 1 week prior to and 4 weeks after balloon injury with normal diet ($n = 18$), 1% cholesterol diet ($n = 18$, Dyets, Bethlehem PA) or 1% cholesterol diet with PDTC ($n = 18$, 50 mg/kg per day dissolved in 400 ml drinking water, Sigma, St. Louis, MO). Water intake in the antioxidant group was closely monitored and mean dose of antioxidant actually ingested was calculated. After balloon injury to the right common carotid artery (RCCA), the effects of high and low flow on vessel morphology were examined at 7 days ($n = 4$ for each of high and low flow in each diet group) and 28 days ($n = 5$ per flow and diet group) and compared with uninjured control vessels. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication Number 85-23, revised 1996).

2.2. Animal surgery, angiography and flow velocity measurements

After sedation with subcutaneous ketamine 40 mg/kg and xylazine 2 mg/kg, rabbits were intubated and anesthetized with 1% inhaled isofluorane (Abbott, North Chicago, IL). A 4F arterial sheath (Cordis, Miami, FL) was inserted into the right femoral artery and a 3.0 mm angioplasty balloon (0.018 central lumen, Titan, Cordis) was passed into the RCCA using a guide wire and fluoroscopic guidance. Baseline angiography was performed (with ioxaglate, through the central lumen of the balloon, recorded on Super VHS tape) and flow velocity measured (average peak velocity (APV)) using a Doppler flow wire (WaveWire, Endosonics, Mountain View, CA). With each study the fluoroscopic image of an 8F human coronary guiding catheter was also recorded for use as a size reference. This was placed outside the body but level with the carotid arteries (for similar magnification on the fluoroscopic image). The RCCA was injured by inflating the balloon.
twice to 10 atm for 30 s in an overlapping pattern, so that the entire vessel from aorta to carotid bifurcation was injured. Low or high flow was then induced by ligation of the right external carotid artery (low flow) or left common carotid artery (high flow) and angiography and flow velocity measurements repeated. The femoral artery was ligated, the cervical and femoral incisions were closed and the animal allowed to recover. Repeat angiography and flow velocity measurements were made through a sheath in the left femoral artery prior to killing the animals at 7 or 28 days after the initial procedure. The proximal RCCA was then ligated and a 1 mm vessel ring removed for chemiluminescence. The distal portion of the vessel was cannulated and perfusion fixed with 10% phosphate buffered formalin (Fisher Scientific, Fair Lawn, NJ) at 100 mmHg for 5 min prior to a further 24 h period of immersion fixation at 4 °C.

Angiographic vessel diameter was measured offline with hand-held digital calipers from still-frame images using the 8F guiding catheter as a size reference. Angiographic vessel diameter was expressed relative to its diameter at baseline. Balloon oversizing was defined as the ratio of inflated balloon size to preinjury vessel diameter (balloon:artery ratio). Flow velocity measurements were also expressed relative to those at baseline.

2.3. Measurement of tissue oxidant stress by chemiluminescence

Freshly harvested 1 mm rings of balloon injured and uninjured vessel were immediately taken in phosphate buffered saline (PBS) and placed in 1 ml of Lucigenin (bis-N-methylacridinium nitrate, Sigma) in PBS (6.38 mg/50 ml). Ten single photon counts were performed in out-of-coincidence mode using a standard liquid scintillation analyzer (Tri-Carb 1500, Packard) and averaged. Results are expressed as a fraction of the average counts from Lucigenin solution alone.

2.4. Morphology

Perfusion fixed vessels were cut into 5 mm segments (3–4 per vessel), blocked end on in paraffin, sectioned, deparaffinized and stained with hematoxylin prior to mounting as previously described [19]. Segments with sectioning artifact affecting the measurement of vessel areas were excluded from analysis. Vessel areas (lumen, intimal and medial area and area within the external elastic lamina) were measured by computed planimetry (Image Analyst histomorphometric program, Automatix, Boston, MA) by an observer blinded to the treatment and flow, and averaged from all measurable segments in each vessel as previously described [19]. Relative vessel areas were derived from the morphometry to allow comparison of the relative changes in permanent vessel size with relative changes in angiographic lumen size, to elucidate how much of the change in angiographic lumen size was due to remodeling and how much due to change in vascular tone. Relative vessel areas were calculated by dividing vessel area in the injured vessel by that in the uninjured (control) vessel. Control vessels for the low flow group were the contralateral uninjured common carotid artery from the same animal. As the contralateral vessel had been ligated in the high flow animals, the control vessel was taken to be the uninjured vessel from a matching low flow animal (i.e. fed the same diet for the same amount of time with the weight nearest to that in the high flow animal).

2.5. Statistics

All results are presented as mean ± S.E.M. Within diet group comparisons of high flow to low flow were made using an unpaired t-test or Rank Sum test where appropriate. Comparisons between diet groups were made using analysis of variance (ANOVA) with post-hoc pairwise testing using Student–Neuman–Keuls test.

3. Results

3.1. Balloon injury and diet tolerance

All animals tolerated the balloon injury well, and weight gain was consistent and very similar between the diet groups (not shown). Water (and drug) consumption in the PDTC-treated group was less than expected so that mean consumption of PDTC was 19.9 ± 0.4 mg/kg per day with no significant differences between high and low flow groups. Mean balloon:artery ratio in the high and low flow groups, respectively, were 1.36 ± 0.06 and 1.34 ± 0.04 on normal diet, 1.40 ± 0.05 and 1.35 ± 0.02 on 1% cholesterol diet, and 1.35 ± 0.05 and 1.28 ± 0.04 on 1% cholesterol with PDTC diet (P = NS).

3.2. Animals on normal diet: responses to balloon injury and altered flow

Ligation of the right external carotid artery (low flow model) consistently resulted in an immediate 60–70% reduction in APV in the RCCA, while ligation of the left common carotid artery resulted in a mean increase of 75% in APV in the RCCA (Fig. 2). Angiographic lumen diameter increased after balloon dilatation and did not differ between high and low flow vessels (Fig. 2), suggesting that acute flow-responsive changes in vascular tone were blunted by balloon injury. However, by 7 days after injury angiographic vessel diameter was increased in the high flow group, and reduced in the low flow group (Fig. 2), and was associated with near-normalization of flow velocity in both groups (Fig. 2).
While the difference in vessel diameter between the high and low flow groups was preserved at 28 days, both groups demonstrated a reduction in vessel diameter between 7 and 28 days after injury (Fig. 2). Despite this uniform reduction in vessel size in both the high and low flow groups, the flow velocities were not greatly changed (Fig. 2) implying that the volume of flow through the vessels decreased over this time.

Vessel morphometry at 28 days showed that, although flow had some effect on neointima formation, the majority of the flow-dependent difference in lumen size was due to change in area within the external elastic lamina (remodeling) (Table 1). Comparison of the time course of relative changes in histological vessel area and angiographic lumen diameter demonstrated very similar patterns, implying that flow-dependent changes in angiographic lumen size were largely due to permanent changes in vessel structure (remodeling) rather than change in vessel tone (Fig. 3).

Vessel wall oxidant stress, as measured by chemiluminescence in Lucigenin solution, was significantly increased in both high and low flow injured vessels relative to uninjured controls (Fig. 4). These elevations were evident at 7 days, persisted essentially unchanged at 28 days and were similar to those that have been reported with balloon injury alone [20]. Elevations in oxidant stress tended to be more pronounced in vessels with low flow than in those with high flow, an effect that was statistically significant at 28 days.

3.3. Animals on 1% cholesterol diet: responses to balloon injury and altered flow

Balloon dilatation and flow manipulation resulted in similar immediate changes in vessel diameter and flow velocity as in animals on normal diet (Fig. 2). Angiographic follow-up of the animals on 1% cholesterol diet showed that early (7 day) flow-dependent divergence in vessel size was similar to that seen in animals on normal diet (Fig. 2). However, the late flow-independent loss of lumen size was not apparent in this group—the difference in mean vessel diameter between vessels with high and low flow continued to increase between 7 and 28 days. While mean flow velocities in high and low flow vessels were still widely different at 7 days, by 28 days they had normalized (Fig. 2).

Morphological evaluation of these vessels showed that most of the observed differences in angiographic lumen size were accounted for by permanent changes in area within the external elastic lamina (remodeling), and
were not merely flow-responsive changes in vascular tone (Table 1, Fig. 3). While 1% cholesterol diet had little effect on inward remodeling in the low flow vessels it significantly enhanced outward remodeling in the presence of high flow velocities. Consequent to the larger vessel size, medial area was also increased (while medial thickness was relatively constant). There was a trend towards an increase in intimal area in both high and low flow vessels relative to their normal diet counterparts but this was not statistically significant.

### Table 1

<table>
<thead>
<tr>
<th>Diet</th>
<th>Vessel</th>
<th>Lumen area</th>
<th>Intimal area</th>
<th>Medial area</th>
<th>Vessel area</th>
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<td>0.39 ± 0.02</td>
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<td>1.31 ± 0.04*</td>
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<tr>
<td></td>
<td>H</td>
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<td>1.85 ± 0.24†</td>
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<tr>
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<td>–</td>
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<td>2.11 ± 0.24</td>
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<tr>
<td></td>
<td>L</td>
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<tr>
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<td>0.59 ± 0.02†</td>
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<td>1% Chol + PDTC</td>
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<td>0.40 ± 0.07</td>
<td>1.84 ± 0.23</td>
</tr>
<tr>
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</tr>
<tr>
<td></td>
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<td>0.10 ± 0.03</td>
<td>0.54 ± 0.03§</td>
<td>2.20 ± 0.18§</td>
</tr>
</tbody>
</table>

Mean ± S.E.M., all areas in mm²; Chol, cholesterol; PDTC, pyrrolidine dithiocarbamate; U, uninjured control; L, injured vessel with low flow; H, injured vessel with high flow. * P < 0.05 vs. U (same diet), † P < 0.05 vs. low flow, ‡ P < 0.05 vs. normal diet, § P < 0.05 vs. 1% cholesterol diet.

3.4. Animals on 1% cholesterol diet and PDTC: responses to balloon injury and altered flow

Treatment of hypercholesterolemic animals with PDTC markedly attenuated flow-dependent divergence in angiographic vessel size (Fig. 2). Flow velocities remained persistently high in the high flow group and low in the low flow group and were significantly
different from those in the 1% cholesterol group at 28 days (Fig. 2).

Morphometry at 28 days revealed that area within the external elastic lamina (vessel area) was very similar in the high and low flow vessels (Table 1). When expressed relative to those in control (uninjured) arteries, vessel areas in the high and low flow group were minimally different, suggesting that the small but significant differences in angiographic lumen diameter were due to flow-responsive differences in tone (Fig. 3). Although antioxidant therapy has been previously shown to reduce neointima formation after injury in the presence of normal flow [21], antioxidant treatment had no significant effect on neointima formation in the present study, perhaps because of the persistence of abnormal flow velocities in the absence of remodeling.

Lucigenin chemiluminescence of vascular rings was reduced by PDTC treatment in uninjured vessels of animals on 1% cholesterol diet and was similar to that in animals on normal diet (Fig. 4). Similarly, oxidant stress levels in injured vessels in this group were lower than those in injured vessels in the other two groups. However, treatment with PDTC was not sufficient to reduce chemiluminescence levels to those observed in uninjured vessels.

4. Discussion

This study demonstrates that flow-dependent remodeling after angioplasty is enhanced by high cholesterol diet. The ability of PDTC to prevent flow-dependent remodeling, as well as quench the elevations in oxidant stress due to hypercholesterolemia and angioplasty, suggests that flow-responsive remodeling after angioplasty may be due to the ability of PDTC at low doses to scavenge superoxide.

Consistent with our previous study [9] and the data of others [8,22], we found that remodeling after angioplasty was flow-responsive. In addition, sequential angiographic measurements of vessel diameter allowed these changes to be expressed relative to baseline vessel size, demonstrating that flow-dependent divergence in remodeling starts early after balloon injury, when any remaining endothelium is most dysfunctional, providing further support for the endothelium-independent nature of this remodeling.

The effects of hypercholesterolemia on remodeling appear dependent on the presence or absence of functioning endothelium. In minimally diseased vessels, with intact endothelium in close proximity to underlying media and adventitia, hypercholesterolemia impairs endothelial function and attenuates outward remodeling [10], and attenuates flow-responsive remodeling in normal vessels (personal communication, Dr Alain Tedgui). However, in de novo atherosclerosis, when the endothelium is significantly displaced from the media and adventitia, hypercholesterolemia does not appear to impair compensatory [13] or flow-responsive outward remodeling [12]. In contrast, outward remodeling in severe hyperlipidemia may ‘overcompensate’, resulting in ectasia in de novo coronary lesions and enhancing enlargement of aortic aneurysms, effects that have been linked to persistent redox-dependent activation of metalloproteinases by oxidized LDL in the vessel wall [14,15,23]. Consistent with this mechanistic hypothesis, we have shown that after angioplasty, when the endothelium is denuded or damaged, a high cholesterol diet enhances flow-responsive outward remodeling, overcompensating for the initial high flow velocities,
so that mean flow velocity at follow-up was lower than that at baseline. MMP-9 activity, which appears specifically linked to outward remodeling after angioplasty [24], was recently shown to be specifically enhanced in macrophage foam cells in hypercholesterolemia an effect reduced by the antioxidant N-acetyl cysteine [15], providing a potential explanation for the pronounced effects of high cholesterol diet on outward remodeling.

In contrast, hypercholesterolemia had no significant effect on inward remodeling in the presence of low flow. In both the normal and high cholesterol diet groups inward remodeling was appropriate in returning flow velocity within the vessel to that at baseline. Although hypercholesterolemia would be expected to heighten activity of growth factors and proteases linked with inward remodeling (platelet-derived growth factor (PDGF), TGF-β, FGF and matrix metalloproteinase-2 (MMP-2)), it is possible that they may be maximally activated by angioplasty and low flow. For example, MMP-2 activity is increased after injury and is further increased by low flow [16] and enhancement by hypercholesterolemia [25] may not be additive. Alternately flow-independent stimulation of outward remodeling by hypercholesterolemia may counterbalance the effects on inward remodeling.

Addition of PDTC to a high cholesterol diet markedly attenuated flow-dependent remodeling. Flow velocities at 28 days were persistently abnormal indicating that this was not due to an effect on cerebral autoregulation of blood flow. Treatment with PDTC inhibited both inward and outward flow-dependent remodeling and was associated with a reduction in oxidant stress as measured by lucigenin chemiluminescence levels. Oxidant stress activates MMP-2 [26] and MMP-9 [15], proteases necessary for initiation of bidirectional flow-dependent remodeling in uninjured vessels [27–29]. Similarly, flow-dependent inward remodeling is associated with overexpression of PDGF [30], and basic fibroblast growth factor (FGF-2) [31], whose activity is increased by oxidant stress [32,33]. The mechanism by which altered shear stress may regulate superoxide production and remodeling is unclear but may involve NAD(P)H oxidase as this membrane bound enzyme is the chief source of the superoxide surge after balloon injury [34], and its activity is shear-sensitive [35].

This study has two limitations which should be mentioned. PDTC has many effects that have been reported in the literature: at low concentrations (1–25 μM) it has antioxidant effects related to stimulation of glutathione production, but as the dose gets higher (50–250 μM) there may be pro-oxidant effects [36]. The pro-oxidant effects are dependent on influxes of redox active copper, but these pro-oxidant effects are highly variable and dependent on cell type and culture density [37]. Measurement of serum levels of PDTC would also be unlikely to be helpful due to the differences in serum and tissue levels brought about by its lipophilicity [38]. However, PDTC is likely acting as an antioxidant rather than a pro-oxidant in these experiments as the lucigenin chemiluminescence measurements showed a significant attenuation of hypercholesterolemia and injury-induced oxidant stress. In addition, the ability of PDTC to specifically prevent many of the cellular effects of oxidized LDL is mediated through inhibition of pathways activated by oxidant stress [39,40], providing a link between the enhanced remodeling seen in hypercholesterolemic animals and the marked attenuation seen in these animals when treated with PDTC. The ability of PDTC to promote influx of zinc and copper is important to many of its actions including its antioxidant actions and these actions may be significantly affected by extracellular metal ion concentrations. These factors prevent generalizability of these effects of on flow-dependent remodeling to all antioxidants, particularly when most other antioxidants also have other actions.

Secondly, the use of the lucigenin chemiluminescence assay has recently been the subject of some controversy. At the higher concentrations used in this study (250 μmol/l) there has been some evidence that lucigenin may cause redox cycling with flavin-containing enzymes and artifically overestimate superoxide production leading some to say that this assay should not be used, and others only to advocate use at low dose (5 μmol/l) [41]. However, this does not appear to be important in estimation of relative differences in superoxide, as the relative increases induced by hypercholesterolemia in vivo appear to be constant whether assessed by high dose lucigenin chemiluminescence, low dose chemiluminescence, cypridina luciferin analogue (CLA) chemiluminescence or electron spin resonance methodology [42]. In our study we merely aimed to demonstrate that the animals were consuming enough PDTC to have an antioxidant effect. It is possible that the noise inherent in the high dose assay may have hidden some true differences between groups and, therefore, the negative results here should be interpreted cautiously.

In summary, we have shown that high cholesterol diet enhances flow-dependent outward remodeling after angioplasty, while PDTC inhibited both inward and outward flow-responsive remodeling and reduced vascular oxidant stress as measured by high dose lucigenin chemiluminescence. It would be interesting to know whether inhibition of inward remodeling and restenosis after human angioplasty with antioxidant therapy [43] is more pronounced in patients with low flow. Further studies to precisely define the different molecular mechanisms in each type of remodeling may lead to new therapeutic approaches to restenosis after angioplasty.
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