Available online at www.sciencedirect.com
SCIENCE DIRECT®
Acta Biomaterialia 1 (2005) 597–606
Acta BIOMATERIALIA
www.actamat-journals.com

Localized delivery of nitric oxide from hydrogels inhibits neointima formation in a rat carotid balloon injury model

Elizabeth A. Lipke [a], Jennifer L. West [a,b,*]

[a] Department of Chemical Engineering, Rice University, 6100 Main Street, Houston, TX 77005, USA
[b] Department of Bioengineering, Rice University, 6100 Main Street, Houston, TX 77005, USA

Received 16 May 2005; received in revised form 22 July 2005; accepted 25 July 2005

Abstract

Using novel nitric oxide (NO)-generating polymeric hydrogels that can be rapidly photopolymerized in situ, we can deliver NO locally at the site of vascular injury. Depending on material design, these poly(ethylene glycol) (PEG)-based hydrogels can generate NO for up to 50 d. This study demonstrates the ability of nitric oxide-generating hydrogels (PEG-Cys-NO) to influence key components of the restenosis cascade both in vitro and in vivo. PEG-Cys-NO hydrogels inhibited smooth muscle cell proliferation, increased endothelial cell proliferation, and inhibited platelet adhesion in vitro. Moreover, in vivo, PEG-Cys-NO hydrogels inhibited intimal thickening in a rat carotid balloon injury model. The perivascular application of NO-generating polymers post-injury reduced neointima formation at 14 d by approximately 80% compared to controls (intimal area/medial area (I/M): PEG-Cys-NO $= 0.20 \pm 0.17$, control $= 0.84 \pm 0.19$, $p < 0.00002$; intimal thickness: PEG-Cys-NO $= 12 \pm 10$ μm, control $= 60 \pm 18$ μm, $p < 0.00002$). Treatment with the PEG-Cys-NO hydrogels caused a significant decrease in the per cent of proliferating cell nuclear antigen positive medial cells ($29 \pm 5\%$) at 4 d as compared to treatment with the control hydrogels ($51 \pm 1\%$, $p < 0.02$). Additionally, vessel re-endothelialization at 14 d was slightly enhanced in the presence of the NO-generating hydrogels. These data indicate that localized delivery of NO from these hydrogels can significantly inhibit neointima formation in a rat carotid balloon injury model and suggest that these materials may be useful in preventing restenosis.

1. Introduction

Even after years of research, balloon angioplasty fails in 20–40% of cases due to restenosis, or re-narrowing of the vessel, within the first 6 months [1–4]; the need to improve current methods drives biomedical research in this area, including the development of new materials for next generation stents and localized drug delivery. Although delivery of NO has long been investigated for the prevention of restenosis, because of NO's short half-life in vivo and its many systemic effects, NO's potential therapeutic benefits have not yet been realized. However, by providing localized NO delivery it is possible overcome these issues. In this study, NO-generating hydrogels are used to locally deliver NO. These hydrogels can be used to achieve therapeutic levels of NO at the site of vascular injury and eliminate the possibility for systemic effects.

Restenosis is caused by a cascade events triggered by vessel injury during the balloon angioplasty procedure. Damage by the balloon to the vascular endothelium exposes the underlying thrombogenic extracellular matrix (ECM) components to blood flow, resulting in platelet adhesion, activation, and aggregation at the site of injury. The activated platelets release growth factors and cytokines, and the disruption of the ECM releases

* Corresponding author. Address: Department of Bioengineering, Rice University, 6100 Main Street, P.O. Box 1892, MS-142, Houston, TX 77251, USA. Tel.: +1 713 348 5955; fax: +1 713 348 5877.
E-mail address: jwest@rice.edu (J.L. West).

1742-7061/$ - see front matter © 2005 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.actbio.2005.07.010

additional previously sequestered growth factors, which together stimulate SMC migration, proliferation, and ECM synthesis. The thrombus provides a scaffold onto which cells can migrate and then proliferate and synthesize ECM, eventually resulting in the formation of the occlusive neointima. Over 3–6 months, constrictive vessel remodeling by adventitial myofibroblasts, leads to additional loss in lumen diameter. Complete or partial vessel re-endothelialization takes longer than 3 months; re-endothelialization is associated with suspension of SMC proliferation [5].

With the placement of a stent, acute recoil and long-term negative remodeling are limited by the force of the stent pressing against the vessel wall; however, the increased injury and inflammation caused by the stent over the long term, leads to a higher proliferative response [6,7] and more pronounced long-term endothelial dysfunction than is seen with balloon angioplasty alone [8]. To halt this excessive neointimal growth and preserve lumen diameter, drug eluting stents (DES) have been developed to provide localized drug delivery. Although DES have the potential to reduce restenosis rates, particularly among high-risk patients, such as diabetics, DES have their own drawbacks. The polymer coatings used in DES for drug delivery elicit an increased inflammatory response and, therefore, in comparison to bare stents, slower or incomplete healing has been observed [9]. In addition, the drugs being incorporated into DES, chosen for their ability to inhibit undesirable SMC proliferation and migration that cause neointimal hyperplasia, also inhibit the highly desirable EC proliferation and migration that is necessary for vessel re-endothelialization and healing. To solve the persistent problem of restenosis, we must develop new techniques and materials to control and direct vessel repair without eliciting an inflammatory response.

Nitric oxide (NO) is able to block a number of key pathways in the restenosis cascade, including inhibiting platelet activation/adhesion and smooth muscle cell (SMC) proliferation and migration. Platelets have been shown to play a major role in stimulating SMC migration into the intima following vascular injury in the rat carotid artery [10]. SMC migration, proliferation, and extracellular matrix (ECM) production at the site of balloon injury are major contributors to neointima formation. NO has been shown to inhibit platelet activation, adhesion, and aggregation both in vitro and following balloon injury [11–13]. Factors secreted by activated platelets, such as platelet-derived growth factor, serotonin and thromboxane A_2, also stimulate SMC proliferation [14–17]. NOs effects on platelets are thought to be mediated by the parallel activation of cyclic guanosine monophosphate-dependent protein kinase (PKG) and cyclic adenosine monophosphate-dependent protein kinase (PKA) [18]. NO activates soluble guanylate cyclase (sGC), which causes an increase in intracellular cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate, activating PKG and PKA [18].

SMC migration, proliferation, and ECM synthesis at the site of vascular injury lead to the formation of the occlusive neointima. NO acts on smooth muscle cells to prevent this through both cGMP-dependent and cGMP-independent pathways. NO reversibly inhibits SMC migration through a cGMP dependent mechanism, independent of cell proliferation or cytotoxicity [19]. NO has been shown to inhibit SMC growth by cGMP-dependent mechanisms both in vitro and in the rat and porcine balloon injury models [18,20–22]. In addition, NO has also been shown to reversibly block SMC entry into S-phase, by a cGMP-independent mechanism [23]. Based on the ability of NO to impede critical steps in neointima formation, delivery of NO has been considered as a potential method to prevent restenosis following vascular injury.

NO donors, compounds that produce NO under physiological conditions, can be used to deliver NO in vivo. NO donors fall into a number of different chemical classes including organic nitrates, organic nitrites, ferrous nitro complexes, sydnonimines, nucleophile adducts, and *S*-nitrosothiols [24]. Although pharmacological tolerance limits the long-term usefulness of organic nitrates, this has not been shown to be a problem for other classes of NO donors [24], including the *S*-nitrosothiol NO donors that are used in these studies. In addition, unlike organic nitrates and sodium nitroprusside, *S*-nitrosothiols have been shown to induce very limited oxidative stress [25].

S-nitrosothiols are of the form RSNO, where R can be any of a wide range of chemical compounds. They are formed by the nitrosation of low-molecular-weight thiols or cysteinyl side chains [26]. In addition to being synthesized for use as NO donors, *S*-nitrosothiols are present in vivo and are thought to participate in NO storage and transport [27]. Naturally occurring *S*-nitrosothiols include *S*-nitrosoglutathione, *S*-nitrosocysteine, and *S*-nitrosoalbumin. *S*-nitrosothiols decompose by hydrolysis, forming NO and a disulfide [28]. Since NO release occurs without the need for enzymatic degradation, NO release from *S*-nitrosothiols is not dependent on cellular involvement.

Numerous studies have looked at the ability of NO donors and other NO-based interventions to prevent balloon-induced arterial occlusion, the results of over 50 of which have been summarized by Janero and Ewing [29]. The ACCORD study showed that systemic administration of NO donors caused an increase in minimal luminal diameter immediately following balloon injury, which was maintained through the final 6-month assessment; the study found, however, no significant decrease in adverse coronary events [30]. The failure of clinical outcomes to correlate with the promising results seen

in animal models can be attributed to an incomplete understanding of the biology of restenosis, drug dosages that were below the therapeutic range due to issues with systemic toxicity, and problems with the suitability of the animal models [30,31]. To achieve therapeutic levels of NO at the site of balloon-injury without inducing systemic toxicity, we propose that localized delivery is necessary. Hydrogels have been developed that are able to release NO over a period of hours to months, depending on material design [32,33]. At physiological pH and temperature, the photopolymerized *S*-nitrosocysteine (Cys-NO) hydrogels used in this study spontaneously release NO over a period of approximately 2 h [32,33]. These poly(ethylene glycol) (PEG)-based copolymers have been previously shown to inhibit smooth muscle cell growth in vitro and to significantly reduce platelet adhesion to a model thrombogenic surface [32,33]. This study demonstrates the ability of these NO-generating hydrogels to inhibit intimal thickening in a rat carotid injury model of restenosis.

2. Materials and methods

All chemicals were purchased from Sigma Aldrich unless otherwise indicated.

2.1. Poly(ethylene glycol) hydrogel precursor synthesis

Polyethylene glycol diacrylate (PEG-DA) was prepared by combining 0.1 mmol/ml dry PEG (6 kDa; Fluka), 0.4 mmol/ml acryloyl chloride, and 0.2 mmol/ml triethylamine in anhydrous dichloromethane (DCM) and reacting under argon overnight. DCM was added to bring the PEG-DA concentration to 0.04 mmol/ml and 2 mol of K_2CO_3 (2 M stock in diH_2O) were added per initial mole of acryloyl chloride. Anhydrous $MgSO_4$ was used to dry the organic phase and then removed by filtration. The PEG-DA was then precipitated with diethyl ether; the solution was allowed to mix for at least 1 h, filtered, and dried under vacuum.

2.2. PEG-Cys-NO nitric oxide donor hydrogel synthesis

PEG-Cys-NO hydrogels were formed by covalently binding cysteine to polyethylene glycol *N*-hydroxysuccinimide monoacrylate (ACRL-PEG-NHS; MW 3.4 kDa, Shearwater). PEG-Cys was then reacted with an equimolar amount of sodium nitrite ($NaNO_2$) at pH 2 and 37° for 20 min to form PEG-Cys-NO. Conversion of thiol groups to *S*-nitrosothiols was measured using Ellman's Assay. The pH was adjusted to 7.4, and the PEG-Cys-NO was incorporated into the PEG hydrogel precursor (MW 6000 g/mol), which contained 20% (w/v) PEG-diacrylate and 115 mmol/l triethanolamine in HEPES buffered saline (pH 7.4, HBS),

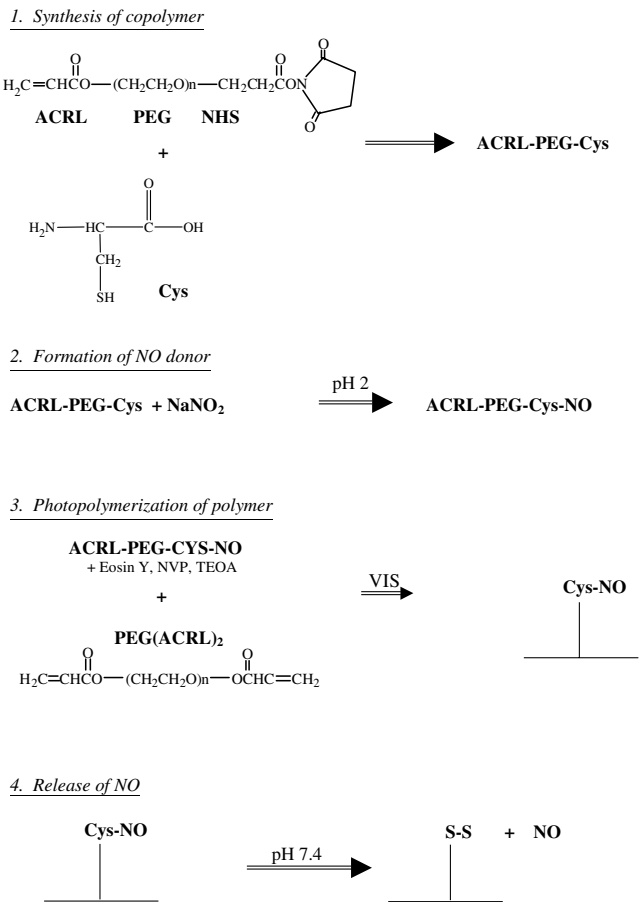

Fig. 1. Summary of PEG-Cys-NO synthesis scheme.

37 mmol/l *N*-vinylpyrrolidone, and 100 μmol/l eosin Y as a visible light photoinitiator. The PEG-Cys-NO synthesis scheme is summarized in Fig. 1. Polymer precursors were sterilized by filtration (0.8 μm pre-filter, 0.2 μm filter, Acrodisc Syringe Filter, Pall Gelman Laboratory). To assess NO release, hydrogels were formed, placed in HBS buffer, and stored at 37 °C. NO release was assessed using the Griess assay [34].

2.3. PEG-Cys-NO hydrogels impact endothelial cell and smooth muscle cell proliferation

Rat aortic smooth muscle cells (RASMCs) were obtained from Cell Applications. The cells were cultured (passage 4–6) in Dulbecco's Modified Eagle Medium supplemented with 10% FBS, 2 mM L-glutamine, 500 units penicillin, and 100 mg/l streptomycin, at 37 °C in a 5% CO_2 environment. Human umbilical vein endothelial cells (HUVECs, passage 3–4, isolated as previously described [35]) were cultured in M199 Medium with identical supplements and culture conditions to those of the SMCs. All culture flasks and wells used for HUVECs were pre-coated with 1% gelatin in PBS for 30 min at 37 °C.

To determine the effect of PEG-Cys-NO on cell proliferation, SMCs and ECs were seeded in 24-well tissue culture dishes at 10,000 cells/cm^2 and allowed to adhere for 24 h. PEG-Cys-NO hydrogels were synthesized as described above and cells were incubated for 24 h in the presence of PEG-Cys-NO hydrogels (0.5 μmol NO released per well) or PEG-DA control hydrogels.

To evaluate proliferation, three wells per condition were immunostained for proliferating cell nuclear antigen (PCNA), and the per cent of PCNA positive cells was quantified. Cells were fixed in formalin for 10 min, rinsed with PBS, and permeabilized with methanol for 2 min. The cells were then incubated with 3% H_2O_2 to block endogenous peroxidase activity and rinsed with PBS. The primary antibody, mouse IgG anti-PCNA (Dako) diluted 1:100 in PBS containing 5% fetal bovine serum (FBS), was added to each well, and incubated for 1 h at room temperature. The wells were then rinsed with PBS, the secondary antibody, anti-mouse IgG HRP diluted 1:100 in PBS containing 5% FBS, was added, and incubated for 40 min at room temperature. The wells were rinsed and the incubated with AEC (Dako) for 10 min. Mayer's hematoxylin was used as the counterstain. As a negative control, the primary antibody was omitted in a sample set.

To assess cell viability, three wells per condition were stained using a Live/Dead staining kit (Molecular Probes, Eugene, OR). Cells were treated with calcein AM (2 μmol/l), which causes viable cells to fluoresce green, and ethidium homodimer-1 (4 μmol/l), which causes dead cells to fluoresce red. The cells were examined using a fluorescence microscope (Zeiss Axiovert 135, Thornwood, NY), and micrographs were taken using a digital camera (Nikon). The number of viable cells was compared to the total cell number in four randomly chosen fields of view per well to assess viability.

2.4. Reduction of platelet adhesion to a model thrombotic surface

The ability of the PEG-Cys-NO hydrogels to inhibit platelet adhesion to a thrombotic surface was assessed. Blood was obtained from a healthy volunteer by venipuncture and anticoagulated with 10 U/ml heparin. Whole blood was incubated with mepacrine at a concentration of 10 μM for 20 min at 37 °C to fluorescently label the platelets. Glass slides were coated with a solution of 2.5 mg/ml collagen I in 3% glacial acetic acid in diH_2O, incubated for 45 min in a humidified environment at room temperature, and then gently rinsed. Mepacrine labeled whole blood was incubated with either PEG-Cys-NO hydrogels or PEG-DA hydrogels for 30 min at 37 °C. The hydrogels were then removed. The blood was transferred to collagen I-coated glass slides and incubated for 20 min at 37 °C. Slides were then rinsed with PBS. Platelets per field of view at 400× were counted under a fluorescence microscope at four randomly chosen areas per slide.

2.5. PEG-Cys-NO reduces neointima formation

Balloon denudation injury of the carotid artery of male Sprague–Dawley rats (425–450 g, Harlan) was used as an experimental model to assess the ability of PEG-Cys-NO hydrogels to inhibit intimal thickening. After induction of anesthesia with isoflurane (Henry Schein), the left carotid artery was surgically exposed. An inflated 2F Fogarty embolectomy catheter (Edwards Lifesciences) was passed three times through the common carotid to denude and injure the vessel. Either NO-generating PEG-Cys-NO hydrogel precursor solution (1.25 μmol NO) or control PEG-diacrylate hydrogel precursor solution was applied perivascularly immediately following the injury. To do this, the PEG hydrogel precursor solution was prepared as described above and frozen at −20 °C in 150 μl aliquots. At the time of application, an aliquot was thawed, and 50 μl of PEG-Cys-NO (1.25 μmol) in HBS or 50 μl of HBS alone was added. The photoinitiator, eosin Y (100 μmol/l), was then added, in addition to 37 mmol/l *N*-vinylpyrrolidone. The hydrogel solution was applied topically to the adventitial surface of the left carotid artery and the solution was polymerized using visible light (5 min) to convert the liquid precursor to a hydrogel. Previous studies have shown that neither the PEG-diacrylate hydrogel nor the photopolymerization process alters the vascular response [36]. The injured segment of the left carotid artery was explanted after 4 d or 14 d for histological analysis.

After vessels were excised, they were gently rinsed in PBS. For the 4 d studies, the vessels were immediately frozen at −80 °C. For the 14 d studies, vessels were divided into five segments. The center, distal end, and proximal end segments were immediately frozen at −80 °C. The remaining two middle segments were embedded in paraffin and sectioned to 10 μm using a rotary microtome (MICROM). To investigate intimal thickening after injury, slides were stained with van Gieson's elastin stain; digital image processing was used for analysis. Additional slides were stained with hematoxylin and eosin.

2.6. PEG-Cys-NO reduces cell proliferation in the medial layer

To examine cell proliferation, the left and right carotid vessels were excised at 4 d, gently rinsed in PBS, and immediately cryopreserved at −80 °C. The vessels were then sectioned radially to 10 μm (MICROM) and fixed for 5 min in acetone at −20 °C before immunohisto-

chemical staining for PCNA. Samples were incubated in 3% H_2O_2 in methanol for 12 min, washed three times with PBS, blocked for 20 min with 5% fetal bovine serum (FBS) in PBS, incubated overnight at 4 °C with mouse anti-PCNA (Zymed) diluted 1:100 in PBS containing 5% FBS. Samples were then washed three times with PBS and incubated for 1 h at room temperature with peroxidase-conjugated rabbit anti-mouse IgG_{2a} as the secondary antibody (Zymed) diluted 1:50 in PBS containing 5% FBS. They were then washed three times with PBS, stained with the HRP substrate diaminobenzidine (DAB; Vector Laboratories) for 7 min, and counter-stained with Mayer's hematoxylin (DAKO). Negative controls were exposed to the secondary antibody only. The number of proliferating cells and the total number of cells in the medial layer were quantified by counting three sections for each animal. Counted sections were from the distal end, middle, and proximal end of the middle region of the injured vessel. This was done for both the right (uninjured) and left (injured) vessels. The primary antibody was omitted for negative immunostaining controls.

2.7. PEG-Cys-NO influences injured vessel re-endothelialization

To assess re-endothelialization of the injured vessels at 14 d, frozen vessel segments (distal, middle, and proximal) were cryosectioned longitudinally to 10 μm and immunostained for the endothelial cell marker CD-31 in the same manner as described above with mouse anti-rat CD31 (Serotec), peroxidase-conjugated goat anti-mouse (Sigma) as the secondary antibody, and DAB as the peroxidase substrate. The sections were counterstained with Mayer's hematoxylin (DAKO). Sections were scored based on the percentage of the lumen endothelialized by an observer blinded to the treatment groups.

Control injuries were done on two rats to determine whether the vessel had been completely de-endothelialized during the procedure; these vessels were excised after 24 h, cryosectioned longitudinally, and immunostained for CD31 as described above. There was no positive staining for CD31 along the entire length of the control injury vessels, thus indicating that the vessels were completely de-endothelialized by the balloon injury. Uninjured right carotid vessels were used as positive controls.

2.8. Statistical analysis

All experiments were performed minimally in triplicate. Error bars reflect standard deviations, and p-values were assessed using two-tailed, unpaired t-tests. p-Values less than 0.05 were determined to be significant.

3. Results

3.1. PEG-Cys-NO hydrogels impact endothelial cell and smooth muscle cell proliferation

NO release from PEG-Cys-NO hydrogels significantly decreased SMC proliferation and significantly increased EC proliferation in vitro. When RASMCs were cultured in the presence of the PEG-Cys-NO hydrogels, there was a significant decrease in the per cent of cells that stained positively for the proliferation marker PCNA ($49 \pm 7\%$) as compared to the per cent of cells that stained positively for PCNA when treated with PEG-DA control hydrogels ($83 \pm 6\%$, $p < 0.0003$). In contrast, HUVECs cultured in the presence of PEG-Cys-NO hydrogels showed a significant increase in the per cent of PCNA-positive cells ($77 \pm 5\%$) as compared to the per cent PCNA-positive cells when cultured in the presence of PEG-DA control hydrogels ($35 \pm 17\%$, $p < 0.02$). Based on the ratios of viable cells to total cell number found using on the live/dead assay, there was no difference in cell viability between cells cultured in the presence of controls and cells cultured in the presence of PEG-Cys-NO hydrogels for either the RASMCs or the HUVECs.

3.2. Reduction of platelet adhesion to a model thrombotic surface

Treatment with PEG-Cys-NO hydrogels significantly reduced platelet adhesion to a model thrombotic surface (collagen I). Significantly fewer platelets adhered to collagen I-coated glass slides when the mepacrine-labeled whole blood was incubated with PEG-Cys-NO hydrogels (70 ± 10 platelets/field of view) as compared to when the blood had been incubated with PEG-DA control hydrogels (451 ± 32 platelets/field of view, $p < 0.003$).

3.3. PEG-Cys-NO reduces neointima formation

Neointima formation was significantly reduced by the perivascular application of PEG-Cys-NO hydrogels following balloon injury. At 14 d post-injury, the intimal thickness of vessels treated with NO-generating PEG-Cys-NO hydrogels was 80% less than vessels treated with the PEG-DA control hydrogel ($p < 0.00003$), and the intimal area to medial area ratio was 77% lower for vessels treated with NO-generating PEG-Cys-NO hydrogels than for vessels treated with the PEG-DA control hydrogel ($p < 0.00002$). Medial thickness and medial area were not affected by the PEG-Cys-NO treatment. Photomicrographs of representative left carotid artery cross-sections and numerical results are presented in Fig. 2. PEG-Cys-NO treatment also impacted intimal

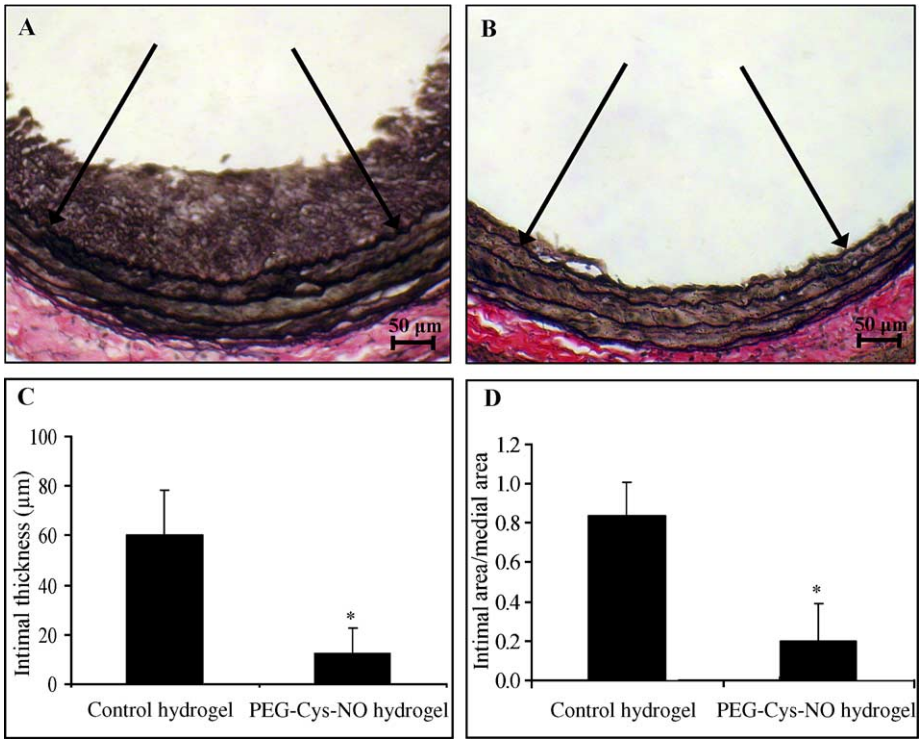

Fig. 2. Treatment with PEG-Cys-NO hydrogels significantly inhibited neointimal formation in a rat model of balloon angioplasty at $t = 14$ d. Histological sections of injured arteries treated with (A) PEG-DA control hydrogel and (B) PEG-Cys-NO hydrogel. Arrows indicate the internal elastic lamina. Sections stained with van Geison's elastin stain. (C) Intimal thickness was approximately 80% less and (D) the intimal area to medial area ratio was approximately 77% lower in injured vessels treated by PEG-Cys-NO hydrogels as compared to vessels treated with PEG-DA control hydrogels (*$p < 0.00003$, $n = 7$).

layer cellularity. Vessels treated with PEG-Cys-NO hydrogels had a significantly higher number of cells per intimal area than vessels that had been treated with PEG-DA control hydrogels, signaling a decrease in ECM synthesis. Cellularity of the medial layer was not affected. Numerical results are given in Table 1.

3.4. PEG-Cys-NO reduces cell proliferation in the medial layer

Treatment with the PEG-Cys-NO hydrogels caused a significant decrease in the per cent of medial cells that stained positive for the proliferation marker PCNA ($29 \pm 5\%$) as compared to treatment with the PEG-DA control hydrogels ($51 \pm 1\%$, $p < 0.02$) at 4 d post-injury (Fig. 3). In uninjured contralateral vessels, $5 \pm 1\%$ of cells in the medial layer stained positive for PCNA.

Table 1
Summary of the quantitative analysis of injured vessel morphology

	Control hydrogel	PEG-Cys-NO hydrogel
Intimal area (mm^2)	0.151 ± 0.052	0.034 ± 0.028
Intimal thickness (μm)	60 ± 18	12 ± 10
Medial area (mm^2)	0.176 ± 0.027	0.178 ± 0.019
Medial thickness (μm)	57 ± 8	61 ± 7
I/M	0.838 ± 0.194	0.197 ± 0.168
Intimal cellularity (cells/mm^2)	9320 ± 3750	$15{,}940 \pm 6170$
Medial cellularity (cells/mm^2)	3670 ± 1570	4470 ± 700

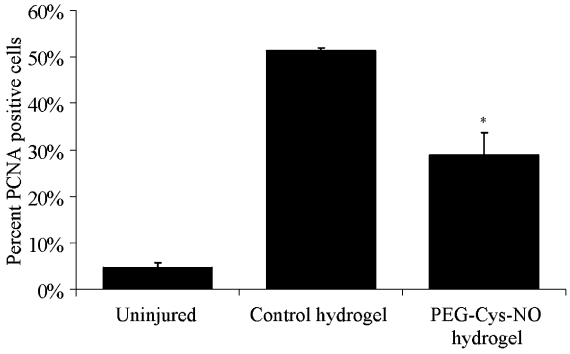

Fig. 3. SMC proliferation in the medial layer of injured and uninjured vessels at $t = 4$ d. The per cent of PCNA-positive medial cells in injured vessels treated with the PEG-Cys-NO hydrogel was significantly lower than in injured vessels treated with the PEG-DA control hydrogel (*$p < 0.02$, $n = 3$).

3.5. PEG-Cys-NO influences vessel re-endothelialization

Vessels treated with the PEG-Cys-NO hydrogels showed a trend of enhanced re-endothelialization as compared to vessels treated with the PEG-DA control

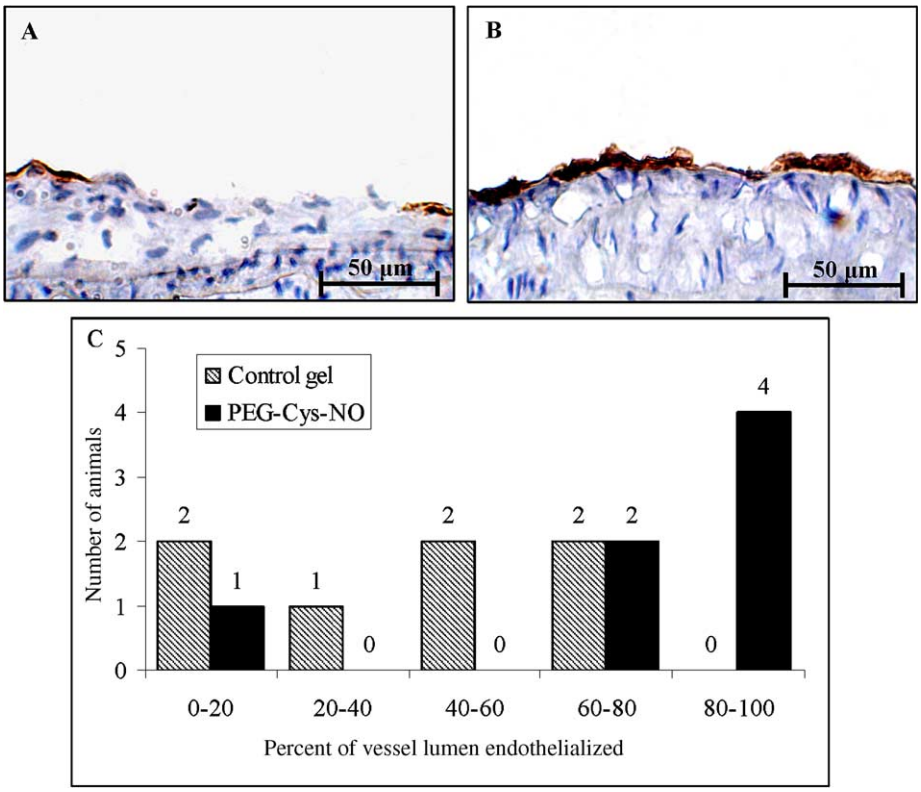

Fig. 4. Re-endothelialization of injured vessel. Representative histological sections of the proximal segments of injured vessels treated with (A) PEG-DA control hydrogel and (B) PEG-Cys-NO hydrogel at $t = 14$ d. Vessels have been cryosectioned lengthwise and immunostained for the endothelial cell marker CD31. (C) Per cent of vessel lumen endothelialized. Histogram shows number of animals per bin. $n = 7$ per group.

hydrogels at 14 d post-injury. Although none of the middle segments of vessel showed substantial re-endothelialization, the proximal segments of vessel tended to have a greater percentage of the luminal surface endothelialized when they had been treated with the NO-generating PEG-Cys-NO hydrogel as compared to having been treated with the PEG-DA control hydrogel (Fig. 4). No conclusions could be drawn about the distal vessel segments; the proximity of these segments to the ligated external carotid created inconsistencies between the samples.

4. Discussion

Platelet activation, adhesion, and aggregation at the site of balloon injury and subsequent SMC migration, proliferation, and extracellular matrix (ECM) production are key factors contributing to neointimal hyperplasia; impeding these steps in the restenosis cascade may be the principal mechanism by which the PEG-Cys-NO hydrogels inhibited neointima formation in this study. Treatment with PEG-Cys-NO hydrogels significantly decreased medial SMC proliferation at 4 d, as compared to proliferation with control hydrogel treatment. This decrease in proliferation alone does not completely account for the substantial restriction of neointima formation in the PEG-Cys-NO treated vessels. Decreased ECM production and increased vessel re-endothelialization in the presence of the PEG-Cys-NO hydrogels may have also played a role in limiting neointimal hyperplasia.

The results of this study suggest that local delivery of NO may potentially enhance the rate of vessel re-endothelialization following vascular injury. During vascular intervention, the normal protective endothelial cell lining of the vessel is often damaged or destroyed, exposing the medial SMCs and ECM components to blood. In addition to serving as a barrier, the endothelium secretes a number of protective substances, including NO, that help ECs maintain vascular homeostasis. Even in the absence of medial damage, denudation of the vascular endothelium has been shown to be followed by smooth muscle cell proliferation and migration, leaving to intimal thickening [37]. Re-growth of the endothelium has, in turn, been shown to down regulate intimal smooth muscle cell proliferation in the rat carotid artery model [38]. NO may enhance vessel re-endothelialization by influencing EC proliferation and migration. NO has been shown to enhance EC growth [18]. NO supports EC migration by maintaining the expression of integrin $\alpha_v\beta_3$ [39]; there are conflicting conclusions about

whether NO enhances or inhibits EC migration overall [40]. NO also seems to be critical in sustaining the endothelial barrier function [18]. Local delivery of NO, such as in this study, may aid in the prevention of restenosis by enhancing the rate of vessel re-endothelialization following vascular injury and by replacing some of the damaged endothelium's natural protective function, until re-endothelialization occurs.

These results suggest that these PEG-Cys-NO hydrogels may be useful in the prevention of restenosis. Several other types of NO-releasing materials have been used for localized NO delivery studies. NO-releasing polyethyleneimine microspheres have been incorporated into vascular grafts for localized NO delivery [41]. NO-releasing diazeniumdiolate donors have been incorporated into polyurethane and poly(vinyl chloride) to improve their thromboresistivity for use in biomedical applications [42]. Polyurethane and polycaprolactone-derived NO-releasing coronary stent coatings have been investigated in the porcine coronary injury model; success of these treatments was limited by inflammatory response to the base material [43,44].

Combination NO donor and paclitaxel-NO donor conjugate polymethacrylate-based stent coatings have been shown to reduce stenosis in a rabbit model compared to coatings containing paclitaxel alone. The success of these materials was, however, also limited by inflammation; the NO and paclitaxel combination coating only reduced the degree of stenosis to the same level as seen with a bare stent [45].

The PEG-based copolymers used in this study offer advantages over the polymers previously used. PEG is widely considered one of the most biocompatible synthetic polymers known [46–48]. Due to the high motility of hydrated PEG chains and the hydrogen bonding of PEG with water, volume exclusion and steric repulsion inhibit protein adsorption and cellular and bacterial adhesion to surfaces coated with PEG and to PEG hydrogels. This lack of protein and cellular interactions makes PEG hydrogels non-thrombogenic and allows them to invoke only a minimal inflammatory response [46]. In addition, these hydrogels have the ability to be polymerized in situ as thin films [36,49], making it possible to use these materials as a tissue or stent coating for highly localized NO delivery. Hydrogel barriers formed on the inside of balloon-denuded vessels have been shown to inhibit thrombosis and reduce intimal thickening by approximately 80% in both a rat and rabbit injury model [49,50]. When formed over a stent immediately after deployment, endoluminal hydrogels decreased thrombosis at 1 h and 3 d post-stenting and reduced the degree of neointimal hyperplasia development by 34% at 30 d in porcine models [51,52]. In the current study, the hydrogels were applied periadventitially rather than intraluminally to allow examination of the effects of the locally delivered NO. The use of NO-generating intraluminal hydrogels should provide optimal results by combing the passive non-thrombogenic barrier of the hydrogel coating and the active delivery of NO; this system will be tested in more challenging animal models in the future.

5. Conclusions

Localized NO release from biomaterials used in the vascular system provides potential benefits for both long-term and short-term applications. In the rat carotid balloon angioplasty model, perivascular application of PEG-Cys-NO hydrogels following vessel injury significantly inhibited neointima formation. Using the PEG-Cys-NO hydrogels either as a stent coating or as an endoluminal paving, may inhibit vascular smooth muscle cell proliferation and enhance vessel re-endothelialization, thereby promoting vessel healing and reducing restenosis.

Acknowledgments

We would like to thank Heather A. Myler, Marcella E. Estrella, Candace K. Chan, Meghan S. Liel, and Elizabeth E.H. Rice for their assistance. Funding was provided by NSF-IGERT Grant DGE-0114264 and NSF-CAREER Award BES-9875607.

References

[1] Libby P, Schwartz D, Brogi E, Tanaka H, Clinton SK. A cascade model for restenosis. A special case of atherosclerosis progression. Circulation 1992;86:47–52.
[2] Schwartz RS, Holmes Jr DR, Topol EJ. The restenosis paradigm revisited: an alternative proposal for cellular mechanisms. J Am Coll Cardiol 1992;20:1284–93.
[3] Bhatia V, Bhatia R, Dhindsa M. Drug-eluting stents: new era and new concerns. Postgrad Med J 2004;80:13–8.
[4] Eltchaninoff H, Koning R, Tron C, Gupta V, Cribier A. Balloon angioplasty for the treatment of coronary in-stent restenosis: immediate results and 6-month angiographic recurrent restenosis rate. J Am Coll Cardiol 1998;32:980–4.
[5] Allaire E, Clowes AW. Endothelial cell injury in cardiovascular surgery: the intimal hyperplastic response. Ann Thorac Surg 1997;63:582–91.
[6] Beyar R. Novel approaches to reduce restenosis. Ann NY Acad Sci 2004;1015:367–78.
[7] Fattori R, Piva T. Drug-eluting stents in vascular intervention. Lancet 2003;361:247–9.
[8] van Beusekom HM, Whelan DM, Hofma SH, Krabbendam SC, van Hinsbergh VW, Verdouw PD, et al. Long-term endothelial dysfunction is more pronounced after stenting than after balloon angioplasty in porcine coronary arteries. J Am Coll Cardiol 1998;32:1109–17.
[9] Virmani R, Kolodgie FD, Farb A, Lafont A. Drug eluting stents: are human and animal studies comparable? Heart 2003;89:133–8.
[10] Fingerle J, Johnson R, Clowes AW, Majesky MW, Reidy MA. Role of platelets in smooth muscle cell proliferation and migration

after vascular injury in rat carotid artery. Proc Natl Acad Sci USA 1989;86:8412–6.
[11] Sogo N, Magid KS, Shaw CA, Webb DJ, Megson IL. Inhibition of human platelet aggregation by nitric oxide donor drugs: relative contribution of cGMP-independent mechanisms. Biochem Biophys Res Commun 2000;279:412–9.
[12] Groves PH, Penny WJ, Cheadle HA, Lewis MJ. Exogenous nitric oxide inhibits in vivo platelet adhesion following balloon angioplasty. Cardiovasc Res 1992;26:615–9.
[13] Folts JD, Stamler J, Loscalzo J. Intravenous nitroglycerin infusion inhibits cyclic blood flow responses caused by periodic platelet thrombus formation in stenosed canine coronary arteries. Circulation 1991;83:2122–7.
[14] Jawien A, Bowen-Pope DF, Lindner V, Schwartz SM, Clowes AW. Platelet-derived growth factor promotes smooth muscle migration and intimal thickening in a rat model of balloon angioplasty. J Clin Invest 1992;89:507–11.
[15] Le Breton H, Plow EF, Topol EJ. Role of platelets in restenosis after percutaneous coronary revascularization. J Am Coll Cardiol 1996;28:1643–51.
[16] Chandrasekar B, Tanguay JF. Platelets and restenosis. J Am Coll Cardiol 2000;35:555–62.
[17] Pakala R, Willerson JT, Benedict CR. Effect of serotonin, thromboxane A2, and specific receptor antagonists on vascular smooth muscle cell proliferation. Circulation 1997;96:2280–6.
[18] Heydrick S. Cellular signal transduction and nitric oxide. In: Loscalzo J, Vita JA, editors. Nitric oxide and the cardiovascular system. Totowa, NJ: Humana Press; 2000. p. 33–49 [Chapter 3].
[19] Sarkar R, Meinberg EG, Stanley JC, Gordon D, Webb RC. Nitric oxide reversibly inhibits the migration of cultured vascular smooth muscle cells. Circ Res 1996;78:225–30.
[20] Garg UC, Hassid A. Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. J Clin Invest 1989;83:1774–7.
[21] Seki J, Nishio M, Kato Y, Motoyama Y, Yoshida K. FK409, a new nitric-oxide donor, suppresses smooth muscle proliferation in the rat model of balloon angioplasty. Atherosclerosis 1995;117:97–106.
[22] Groves PH, Banning AP, Penny WJ, Newby AC, Cheadle HA, Lewis MJ. The effects of exogenous nitric oxide on smooth muscle cell proliferation following porcine carotid angioplasty. Cardiovasc Res 1995;30:87–96.
[23] Sarkar R, Gordon D, Stanley JC, Webb RC. Cell cycle effects of nitric oxide on vascular smooth muscle cells. Am J Physiol 1997;272:H1810–8.
[24] Bauer JA, Booth BP, Fung HL. Nitric oxide donors: biochemical pharmacology and therapeutics. Adv Pharmacol 1995;34:361–81.
[25] Jaworski K, Kinard F, Goldstein D, Holvoet P, Trouet A, Schneider YJ, et al. S-nitrosothiols do not induce oxidative stress, contrary to other nitric oxide donors, in cultures of vascular endothelial or smooth muscle cells. Eur J Pharmacol 2001;425:11–9.
[26] Leopold J, Loscalzo J. S-nitrosothiols. In: Loscalzo J, Vita JA, editors. Nitric oxide and the cardiovascular system. Totowa, NJ: Humana Press; 2000. p. 411–29 [Chapter 23].
[27] Williams DLH. The chemistry of S-nitrosothiols. Accounts Chem Res 1999;32:869–76.
[28] Butler AR, Rhodes P. Chemistry, analysis, and biological roles of S-nitrosothiols. Anal Biochem 1997;249:1–9.
[29] Janero DR, Ewing JF. Nitric oxide and postangioplasty restenosis: pathological correlates and therapeutic potential. Free Radic Biol Med 2000;29:1199–221.
[30] Faxon DP, Currier JW. Prevention of post-PTCA restenosis. Ann NY Acad Sci 1995;748:419–27, discussion 427–418.
[31] Bult H. Restenosis: a challenge for pharmacology. Trends Pharmacol Sci 2000;21:274–9.
[32] Bohl KS, West JL. Nitric oxide-generating polymers reduce platelet adhesion and smooth muscle cell proliferation. Biomaterials 2000;21:2273–8.
[33] Masters KS, Lipke EA, Rice EE, Liel MS, Myler HA, Zygourakis C, et al. Nitric oxide-generating hydrogels inhibit neointima formation. J Biomater Sci Polym Ed 2005;16:659–72.
[34] Scott-Burden T, Schini VB, Elizondo E, Junquero DC, Vanhoutte PM. Platelet-derived growth factor suppresses and fibroblast growth factor enhances cytokine-induced production of nitric oxide by cultured smooth muscle cells. Effects on cell proliferation. Circ Res 1992;71:1088–100.
[35] McCormick SM, Eskin SG, McIntire LV, Teng CL, Lu CM, Russell CG, et al. DNA microarray reveals changes in gene expression of shear stressed human umbilical vein endothelial cells. Proc Natl Acad Sci USA 2001;98:8955–60.
[36] West JL, Hubbell JA. Separation of the arterial wall from blood contact using hydrogel barriers reduces intimal thickening after balloon injury in the rat: the roles of medial and luminal factors in arterial healing. Proc Natl Acad Sci USA 1996;93:13188–93.
[37] Fingerle J, Au YP, Clowes AW, Reidy MA. Intimal lesion formation in rat carotid arteries after endothelial denudation in absence of medial injury. Arteriosclerosis 1990;10:1082–7.
[38] Clowes AW, Clowes MM, Reidy MA. Kinetics of cellular proliferation after arterial injury. III. Endothelial and smooth muscle growth in chronically denuded vessels. Lab Invest 1986;54:295–303.
[39] Murohara T, Witzenbichler B, Spyridopoulos I, Asahara T, Ding B, Sullivan A, et al. Role of endothelial nitric oxide synthase in endothelial cell migration. Arterioscler Thromb Vasc Biol 1999;19:1156–61.
[40] Lau YT, Ma WC. Nitric oxide inhibits migration of cultured endothelial cells. Biochem Biophys Res Commun 1996;221:670–4.
[41] Pulfer SK, Ott D, Smith DJ. Incorporation of nitric oxide-releasing crosslinked polyethyleneimine microspheres into vascular grafts. J Biomed Mater Res 1997;37:182–9.
[42] Mowery KA, Schoenfisch MH, Saavedra JE, Keefer LK, Meyerhoff ME. Preparation and characterization of hydrophobic polymeric films that are thromboresistant via nitric oxide release. Biomaterials 2000;21:9–21.
[43] Buergler JM, Tio FO, Schulz DG, Khan MM, Mazur W, French BA, et al. Use of nitric-oxide-eluting polymer-coated coronary stents for prevention of restenosis in pigs. Coron Artery Dis 2000;11:351–7.
[44] Yoon JH, Wu CJ, Homme J, Tuch RJ, Wolff RG, Topol EJ, et al. Local delivery of nitric oxide from an eluting stent to inhibit neointimal thickening in a porcine coronary injury model. Yonsei Med J 2002;43:242–51.
[45] Lin CE, Garvey DS, Janero DR, Letts LG, Marek P, Richardson SK, et al. Combination of paclitaxel and nitric oxide as a novel treatment for the reduction of restenosis. J Med Chem 2004;47:2276–82.
[46] Hill-West JL, Chowdhury SM, Sawhney AS, Pathak CP, Dunn RC, Hubbell JA. Prevention of postoperative adhesions in the rat by in situ photopolymerization of bioresorbable hydrogel barriers. Obstet Gynecol 1994;83:59–64.
[47] Graham NB. Hydrogels: their future, Part I. Med Device Technol 1998;9:18–22.
[48] Graham NB. Hydrogels: their future, Part II. Med Device Technol 1998;9:22–5.
[49] Hill-West JL, Chowdhury SM, Slepian MJ, Hubbell JA. Inhibition of thrombosis and intimal thickening by in situ photopolymerization of thin hydrogel barriers. Proc Natl Acad Sci USA 1994;91:5967–71.
[50] West JL, Chowdhury SM, Sawhney AS, Pathak CP, Dunn RC, Hubbell JA. Efficacy of adhesion barriers. Resorbable hydrogel, oxidized regenerated cellulose and hyaluronic acid. J Reprod Med 1996;41:149–54.

[51] Slepian MJ, Massia SP, Weselcouch E, Khosravi F, Roth L. In situ photopolymerized hydrogel barriers applied following porcine arterial stenting reduce intra-stent neointimal thickening. Circulation 1995;92:1823.

[52] Slepian MJ, Massia SP, Weselcouch E, Khosravi F, Roth L. Photopolymerization of hydrogel barriers on endoluminal surfaces of porcine stented arteries reduces stent and adjacent arterial-wall thrombogenicity. Circulation 1995;92:3297.