BASIC RESEARCH

Granulation encapsulated stent: a new therapeutic approach for vascular implantation

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Objectives: To determine if stents could be encapsulated with immunocompatible granulation tissue for the treatment of vascular diseases.

Methods: Bare metal stents were implanted in New Zealand white rabbits so they would be encapsulated with immunocompatible granulation tissue. The granulation encapsulated stents (GES) were then treated with either mitomycin C or saline, and implanted into rabbit iliac arteries for 4 weeks. To test whether the effect of mitomycin C was retained, we co-cultured smooth muscle cells for 3 h with subcutaneous tissue (as control) or with granulation tissue from GES treated with mitomycin C and saline.

Results: Vessels with GES treated with mitomycin C (MS) and washed with saline had significantly less neointimal area (NA) after 4 weeks (0.27 (SD 0.03) mm² than vessels containing bare metal stents (B) (1.15 (SD 0.10) mm², n=5, p<0.05) or GES treated with saline (S) (4.78 (SD 0.72) mm², n=5, p<0.05). The average vessel injury score was not significantly different among these three groups (S: 1.98 (SD 0.51), MS: 1.46 (SD 0.18) and B: 1.51 (SD 0.32)). GES treated with saline had significantly less NA than the other two groups and also blocked blood flow in the contralateral iliac artery in the abdominal aortic bifurcation immediately after implantation and 4 weeks later. Histology also showed neointimal overgrowth in the vessel wall over the contralateral iliac artery.

Conclusions: GES treated with mitomycin C can significantly inhibit neointimal formation in rabbit arteries due to the formation of granulation tissue. GES treated with saline demonstrated significantly increased NA and resisted normal rabbit artery pressures.

Artificial tissues, devices and organs (for example vessels, valves, joints, etc) are widely implanted into human bodies for various therapeutic purposes. Since these are foreign bodies, they initiate an inflammatory reaction which causes granulation formation. Metallic stents, widely used in blood vessels, the oesophagus, tracheobronchial tree, biliary tree and urethra, are among these artificial devices.^{1–5}

The widespread clinical application of the coronary stent is one of the most important advances in interventional cardiology since balloon angioplasty was introduced. Compared to balloon-based percutaneous transluminal coronary angioplasty, coronary stenting has reduced the rate of restenosis, although the rate is still relatively high.^{6,7} Percutaneous coronary revascularisation now involves a stent in about 70% of cases and over a million coronary stents are being implanted worldwide every year.^{8,9} A stent, being a foreign body in the vessel, can induce a prolonged and serious inflammatory reaction after its implantation.^{10–14} An inflammatory response with subsequent release of chemotactic and growth factors plays a very important role in the genesis of restenosis after balloon angioplasty. Therefore, decreasing this inflammatory response to stents may decrease the incidence of restenosis.

The goal of this study was to determine in a rabbit model whether the use of a granulation encapsulated stent (GES), produced by pre-implanting the stent subcutaneously over the abdomen, might decrease and increase neointimal growth within the recipient artery for different therapeutic purposes.

METHODS

Animals

All experimental protocols complied with the guidelines of the Institutional Animal Care and Use Committee. New Zealand white rabbits, 4–5 kg, were housed individually in steel mesh cages and fed rabbit chow and water for at least 1 week prior to

any operative procedures. All surgery was performed under general anaesthesia induced by the intramuscular injection of 35 mg/kg ketamine (Aveco, Fort Dodge, Iowa, USA) and 0.2 mg/kg acepromazine, after premedication with 10 mg/kg xylazine (Miles, Shawnee Mission, Kansas, USA).

Encapsulation of stents

Bare metal stents (12×3.0 mm; Medtronic AVE, Santa Rosa, California, USA) were implanted into subcutaneous tissue in the rabbit abdomen. After 3, 7 and 14 days, the stents, all of which had developed granulation capsules, were removed. In an attempt to inhibit the growth of the granulation tissue, stents encapsulated with granulation tissue at day 14 were treated with 200 µg/ml mitomycin C (M) or saline (S) at 37°C for 30 min, and then washed in 3×100 ml saline at room temperature to rinse off any residual mitomycin C. These stents were then incubated in 50 ml saline at 37°C for 5 min, in another 50 ml saline for 20 min and, finally, another 50 ml saline for 5 min. The washed encapsulated stent was then repositioned into one external iliac artery (EIA) in the same rabbit. A new, bare wire stent (control) was implanted into a similarly prepared contralateral EIA. The order of implantation was varied at random. To reduce the incidence of early occlusive thrombosis reported in stented iliac arteries in the rabbit,¹⁵ aspirin (0.07 mg/ml; Sigma, Saint Louis, MO) was added to the drinking water starting 1 day before surgery to achieve an approximate dose of 5 mg/kg/day. To test if the granulation capsule over the stent was strong enough to resist intravascular

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EIA, external iliac artery; GES, granulation encapsulated stent; GT, granulation tissue; NA, neointimal area; VIS, vessel injury scores; VSMC, vascular smooth muscle cells

Figure 1 (A) Representative GES at day 14, which was dilated by an angioplasty balloon using a pressure of 10 atm after it was treated with 200 µg/ml mitomycin C for 1 h and washed in saline for 30 min. (B) The GES after the balloon was removed. (C) The granulation capsule inside the stent encapsulated at day 14 after dilation. Noted that in all three pictures, the granuloma capsules were entirely intact. (D) Histology of a bare metal stent after 30 h in vessel. (E) Granulation encapsulated stent at day 14 after 30 h in vessel.

pressure, the GES was implanted in the abdominal aortic bifurcation to block blood flow in the contralateral EIA.

Intactness of GES

When stents were removed from subcutaneous tissue over the abdomen after 3, 7 and 14 days, they had become encapsulated with granulation tissue. After being treated with 200 µg/ml mitomycin C (MS) or saline (S) for 30 min, and then washed in saline, GES were dilated by balloons using a pressure of 10 atm. The granulation capsules within and outside the stents from days 3 to 14 in both the MS and S groups were completely intact without any visible fractures (fig 1A–C). Another group of GES, which had been treated as noted above, were then implanted with a pressure of 10 atm into the rabbit iliac arteries for 30 h before harvesting. Histology showed that granulation capsules over the stents were completely intact in the iliac artery (fig 1D,E) 30 h after implantation.

Stenting in EIA

The stenting process has been described previously.¹⁶ Briefly, under anaesthesia, a 5F sheath (Cordis, Miami Lakes, Florida, USA) was placed in the right carotid artery. Heparin sulfate (1000 IU) and acetylsalicylic acid (50 mg) were injected intra-arterially to prevent early arterial occlusion. Under fluoroscopic guidance using an over-the-wire system, a 3F angioplasty balloon was passed into both EIAs. The initial 4 cm of the right EIA immediately distal to the aortic bifurcation underwent dilatation for 45 s using a pressure of 8 atm. Thereafter, a premounted stent was deployed randomly into either the left or

right EIA using two 40-s inflations, initially at 8 atm and then at 10 atm. The contralateral EIA served as an internal control, being dilated with the same pressure and implanted with the same size bare stent as the GES in the initial EIA. EIAs with implanted stents were harvested at day 28.

Stent harvesting and processing

The stented arteries were fixed in situ with 10% neutral buffered formalin. The arteries were removed, dehydrated in ascending grades of acetone and processed in ascending grades of Spurr (resin)/acetone before being embedded and polymerised in Spurr resin at 500°C. Cross-sections (5 µm) were cut through the middle of the stented artery using a Leica RM 2165 rotary microtome (Leica, Richmond Hill, Canada) equipped with a tungsten carbide knife. The 5-µm sections were stained with H&E and Verhoeff's tissue elastin stain for neointimal area (NA) and vessel injury scores (VIS). The NA and VIS of each stent were derived from three contiguous sections (5 µm/each section) in the middle of each stent. Each group consisted of five stents.

Stent analysis

As described previously,^{17–19} NA and VIS were quantified using Verhoeff's tissue elastin stain by using computer-assisted digital planimetry. Briefly, the original lumen was measured as that area subtended within the internal elastic lamina and the stenotic lumen as the remaining lumen of the artery. The total NA of each section was calculated as the difference between the original and stenotic lumens (original area minus

stenotic area). Quantitative injury severities were measured from the elastic van Gieson-stained sections. The vessel injury at every strut of the stent in the vessel was determined by the anatomic vessel structures penetrated by each strut. A numeric value, from 0 to 3, was assigned according to injury severity defined as follows: 0: internal elastic lamina intact, endothelium typically denuded, media compressed but not lacerated; 1: internal elastic lamina lacerated, media typically compressed but not lacerated; 2: internal elastic lamina lacerated, media visibly lacerated, external elastic lamina intact but compressed; 3: external elastic lamina lacerated, typically large lacerations of media extending through the external elastic lamina, strut sometimes residing in the adventitia. Mean VIS for each section was calculated as the sum of scores for each strut/number of struts present in the vessel.

Effect of mitomycin C

Vascular smooth muscle cells (VSMCs) were obtained by explantation of rabbit aortae, the tissue was placed in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, California, USA) and the media was dissected out and grown as explants in 24-well plates containing DMEM supplemented with 10% fetal calf serum (FCS; Invitrogen). Cells were cultured at 37°C in a humidified 5% CO₂ atmosphere. At confluence, cells were trypsinised using 0.125% trypsin in 4 mM EDTA and subcultured, cells from passages 6 to 9 being used in the studies.

Cultured VSMCs were seeded into 24-well plates (5×10^4 /ml) and then incubated at 37°C in DMEM with 10% FCS containing a range of concentrations of mitomycin C (Sigma) from 0 (no PDGF-AB as a control), 10^{-4} , 10^{-3} , 10^{-2} , 10^{-1} , 1 to 10 µg/ml for 2 h. Thereafter, the culture medium above with different concentrations of mitomycin C was replaced with DMEM in the absence of FCS to arrest growth for 6 h and then DMEM with methyl-³H-thymidine (1 Ci/well) containing 0.5% FCS alone in the 0 µg/ml mitomycin C group as a control and 0.5% FCS plus 20 ng/ml human PDGF-AB (Sigma) in all other groups for a further 24 h. The cells were then trypsinised in 150 µl 0.125% trypsin in 4 mM EDTA, transferred to 96-well plates and harvested using a cell harvester (Skatron Instruments, Tranby, Norway). The filters (Whatman, Maidstone, UK) containing the cells were placed into 5-ml scintillation fluid (Beckman, Fullerton, California, USA) and shaken before counting in a liquid scintillation counter. Each value was obtained from three wells in each of two separate experiments.

VSMCs were then cultured under the same conditions as above in 16-mm, 24-well plates containing 10% FCS DMEM in the presence of 30 mg subcutaneous granulation tissues (as a

control) and 30 mg granulation tissue from the encapsulated stents treated with mitomycin C (after being washed by saline) and saline in 12-mm 0.4-µm pore transwell dishes (Costar, Cambridge, MA) for 3 h. Subsequently, these subcutaneous granulation tissues were cut into five to eight pieces. VSMCs were then cultured in FCS-free DMEM to arrest growth for another 6 h. DMEM with methyl-³H-thymidine (1 Ci/well) containing 0.5% FCS plus 20 ng/ml human PDGF-AB then replaced the FCS-free DMEM for another 24 h. Thereafter, VSMCs were harvested and counted in a liquid scintillation counter in the same way as described above.

Statistics

All data are presented as mean values (SD). Student's *t* test was used (as appropriate) to determine whether there was any significant difference among the groups of rabbits with the GES treated with mitomycin C and saline, and those with bare stents. A *p* value ≤ 0.05 was considered significant.

RESULTS

Effect of GES upon neointimal formation in the EIA

The vessels with GES implanted subcutaneously for 14 days, treated with mitomycin C (MS) and thereafter washed with saline had significantly less NA after 4 weeks (0.27 (SD 0.03) mm²) than did the vessels containing the bare metal stents (B) (1.15 (SD 0.10) mm², *n* = 5, *p* < 0.05) (fig 2A,B) and GES treated with saline (S) (4.78 (SD 0.72) mm², *n* = 5, *p* < 0.05) (fig 2C). GES treated with saline had significantly increased NA compared with the other two groups. The average VIS showed no significant difference among these three groups (S: 1.98 (SD 0.51), MS: 1.46 (SD 0.18) and B: 1.51 (SD 0.32), *n* = 5, *p* > 0.05).

Effect of mitomycin C retained in the GES

The inhibitory effect of mitomycin C retained in GES was explored. First we determined the inhibitory effect of the mitomycin C concentrations in our system as a positive control. As shown in fig 3, we repeated other investigators' work²⁰⁻²² and established the inhibitory effect of a range of concentrations of mitomycin C on the DNA synthesis induced by PDGF-AB. PDGF-AB (20 ng/ml) can significantly increase DNA synthesis (96.30% (SD 5.31%)) compared with control (0.5% FCS DMEM without PDGF-AB, *n* = 5, *p* < 0.05; fig 3A). At a concentration of >1 µg/ml, mitomycin C significantly inhibited the DNA synthesis induced by 20 ng/ml PDGF-AB.

Both granulation tissues treated with mitomycin C only and then washed with saline (GT-MS: 98% (SD 2.6%) of control, *n* = 5, *p* > 0.05, fig 3B) and granulation tissue from GES

Figure 2 (A) Representative neointimal formation in the iliac artery at 4 weeks after the implantation of the GES treated with mitomycin C. (B) Representative neointimal formation in the iliac artery at 4 weeks after the implantation of the bare stent. (C) Representative neointimal formation in the iliac artery at 4 weeks after the implantation of the GES treated with saline.

Figure 3 (A) Inhibitory effect of a range of concentrations of mitomycin C on the DNA synthesis induced by PDGF-AB. PDGF-AB (20 ng/ml) can significantly increase DNA synthesis (96.30% (SD 5.31%), $n = 5$, $p < 0.05$) compared to control (0.5% FCS DMEM without PDGF-AB). At a concentration of $> 1 \mu\text{g/ml}$, mitomycin C significantly inhibited the DNA synthesis induced by 20 ng/ml PDGF-AB. (B) Granulation tissues (GT-MS) from GES treated with mitomycin C and washed with saline did not show any significant inhibitory effect compared to GT-S of GES treated with saline on DNA synthesis induced by 20 ng/ml PDGF-AB. However, granulation tissue (GT-M) from GES treated with mitomycin C alone did show a significant inhibitory effect. Control is the subcutaneous tissues.

implanted subcutaneously for 14 days treated with saline (GT-S: 3% (SD 2.9%) of control, $n = 5$, $p > 0.05$, fig 3B) did not show any significant inhibitory effect on DNA synthesis, but GT-M did significantly inhibit the DNA synthesis of VSMC (5.27% (SD 1.81%) of control, $n = 5$, $p < 0.05$). Therefore, the saline washed away any mitomycin C retained in the GES, and so any effect of GES on decreased neointimal formation was not due to the mitomycin retained in the GES.

GES resistance to normal intra-vascular pressure

The granulation capsule over the stent was strong enough to resist intra-vascular pressure and had the potential to fill the enlarged vessel space in aneurysms. Angiography showed that the contralateral iliac artery was totally blocked immediately after stent implantation and 4 weeks later. As shown in fig 4A, the contralateral iliac artery has shrunk, presumably because of no blood flow for 4 weeks. Histologically, there was neointimal overgrowth in the wall of the contralateral iliac artery (fig 4B).

DISCUSSION

We have investigated a new approach to the interventional treatment of vascular diseases. Our study has demonstrated that stents are capable of being encapsulated by granulation tissue after 3, 7 and 14 days of subcutaneous abdominal

implantation. The new granulation tissue over the stents was intact after the stents were dilated with pressures of 8 and 10 atm inside and outside the vessels. Compared to bare stents, GES at day 14 treated with mitomycin C demonstrated significantly reduced neointimal formation, but this effect apparently not related to the mitomycin C retained in GES. However, GES at day 14 treated with saline demonstrated significantly increased neointimal formation which could help fill the aneurysmal space and thus help resist normal intra-arterial blood pressure.

Our work has shown that the granulation capsules at days 3, 7 and 14 were intact both inside and outside the arteries after dilatation with pressures of 8 and 10 atm. Several investigators have demonstrated that a foreign body introduced into the body of a rat, rabbit or mouse for 2 weeks initiates an inflammatory response with a resultant capsule of granulation tissue.^{23–25} These granulation capsules were so strong that they could be used as arteries to resist normal arterial pressure.²³ Our work has further proved that the granulation capsule over the stent can resist normal blood pressure in rabbit arteries. Moreover, we showed that this granulation tissue could further grow in the vessel to fill the enlarged space of an aneurysm. Inflammatory cells from subcutaneous tissue in the granulation capsule over the stents still act as cells derived locally from the

Figure 4 (A) The GES has blocked the right iliac artery at 4 weeks after implantation. (B) Histology of the tissue with red line in the black rectangle in A demonstrating neointimal formation over the contralateral iliac artery which had overgrowth, and which could resist normal intra-vascular pressure.

arteries and cause increased neointimal formation. This effect suggests GES may be very promising in the treatment of arterial aneurysm. Moreover, the granulation capsule over the stents might be amenable to molecular engineering using different genes for the treatment of various types of arterial aneurysms. For instance, it may be possible to modify the granulation capsule by inserting the fibrillin gene to treat Marfan's syndrome in which the lack of fibrin in the vessel wall can result in a vascular aneurysm.

Implantation of GES also appears very promising as a technique to prevent in-stent restenosis. There are two possible reasons for the decreased neointimal formation from GES: i) mitomycin C which could not be washed away by saline is retained in granulation tissue over the stent; and ii) the effect of GES could be related to immunocompatible granulation tissue over the metal stent. Our work suggests that there is no effect from mitomycin C retained in granulation tissue on the stents in our system. Moreover, it has previously been shown that local delivery of mitomycin C had no favourable effect on the inhibition of intimal hyperplasia compared with control saline-treated arteries.¹⁶ As a result, the inhibition by GES of neointimal formation in the vessel could be related to immunocompatible granulation tissue over the metal stents, which could decrease the local inflammatory reaction in the vessels. This reaction has been proved to be a major contributor to in-stent restenosis.^{14 26–31} Mitomycin C, an alkylating agent, can inhibit these cells from dividing. It is widely used in preparing the feeder layer for stem cell cultures. Cells treated with mitomycin C do not die but are mitotically arrested.^{32–33} However, the effect of other drugs retained in granulation tissues over the stents may produce a totally new form of “drug eluting stent”. By regulating the wash time, one could control the concentration of drug combined with the granulation capsules. Therefore, drugs combined with granulation tissue would not be totally washed away, but could be slowly released after a GES was implanted. The GES, which is perfectly biocompatible and haemocompatible, has advantages over currently-available drug eluting stents. For instance, commercially-available drug eluting stents have a non-erodable polymer matrix, which itself can cause an inflammatory response and neointimal formation in vessels.^{34–37}

Decreasing the incidence of in-stent restenosis is one of the most challenging tasks in the treatment of cardiovascular diseases. Efforts in the past 20 years to prevent restenosis have resulted in much progress. For example, β -brachytherapy^{38–40} has achieved a very low incidence of in-stent restenosis. However, this benefit has come at the cost of increased late in-stent thrombosis, late in-stent restenosis and a relatively high myocardial infarction rate. Sirolimus-eluting stents have achieved very low restenosis rates. However, some investigators have reported that the

incidence of restenosis was about 10%. Moreover, there is still debate over their safety and efficacy. The occurrence of subacute in-stent thrombosis has been reported.^{41–43} It is still too early to determine the incidence of late in-stent restenosis and late myocardial infarction in this drug-eluting stent. Our findings provide a new approach, which may significantly decrease the incidence of restenosis and simultaneously decrease the incidence of thrombosis and myocardial infarction.

Limitations

Although our findings have demonstrated a proof of concept for the feasibility of creating GES, the question remains regarding the practicality of this approach for clinical medicine. The necessity of implanting a metal stent in the subcutaneous tissue of a human, waiting 3 days to 2 weeks, harvesting the material in a sterile fashion and subsequently implanting it in a vascular location excludes its use in urgent situations. However, most stenting (over half) is typically done in an elective and subacute situation.⁴⁴ In addition, the presence of granulation tissue on the stent would make a catheter-based delivery system difficult with the current design. Nonetheless, we delivered all the GES with catheters.

In summary, we report a new, promising method for preparing intra-vascular stents that appears applicable to the prevention of in-stent restenosis and treatment of arterial aneurysms. With appropriate treatment, for example mitomycin C, these GES can decrease neointimal formation. Therefore, they can potentially be used in the prevention of in-stent restenosis. In the absence of such treatment, these GES increase neointimal formation in the arteries after implantation and appear to resist the normal intra-arterial blood pressure. This method may also be widely applicable to modify artificial devices, tissue and organs for implantation.

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