Human internal mammary artery organ culture model of coronary stenting: a novel investigation of smooth muscle cell response to drug-eluting stents

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ABSTRACT
Local drug delivery by coronary stents is of current research interest. Organ culture of human vascular tissue is a model of intimal hyperplasia. We report an ex vivo organ culture model of stented vessels. This allows stent–artery interactions to be studied in living tissue. The recognized anti-restenosis agent paclitaxel was chosen to test the organ culture model. Mammary artery specimens were cultured 'closed' (i.e. without opening them flat) for 72 h. Phosphocholine-coated stents, half of them loaded with the anti-restenosis drug paclitaxel, were implanted. The absorption and elution characteristics of paclitaxel were established. Artery tissue remained viable at 72 h when cultured closed, despite stent implantation. Specimens developed smooth muscle cell proliferation. The stents absorbed up to 127±29 µg of paclitaxel, with a biphasic elution curve. A mean of 13% of the absorbed paclitaxel remained after a 24 h perfusion. In mammary artery, these paclitaxel stents reduced or abolished smooth muscle cell proliferation compared with controls. This model allows the effects of stenting on human arterial tissue to be studied for at least 72 h, long enough to demonstrate effects on smooth muscle cell proliferation. Phosphocholine-coated stents absorb adequate doses of paclitaxel, which is eluted gradually, inhibiting muscle cell proliferation. Such an organ culture model of stented mammary artery will provide useful data in addition to that from animal or cell culture models of drug-eluting stents.

INTRODUCTION
Coronary artery narrowings caused by atherosclerosis are now treated routinely by the implantation of a coronary stent. Unfortunately, in-stent restenosis (re-narrowing) caused by intimal hyperplasia remains a significant clinical problem that has proven difficult to treat. Smooth muscle cell proliferation is the key event in the formation of intimal hyperplasia after stent insertion [1].

Much attention has been devoted to the development of treatments that target smooth muscle cell proliferation, including those where the treatment drug is delivered locally to the angioplasty site by the stent itself [2,3]. Several different in vivo animal models exist that attempt to reproduce the vessel wall changes seen in in-stent restenosis. These may be criticised for the inevitable variation in responses to therapies due to inter-species differences. No in vitro model yet reproduces all of the changes seen in a stented vessel. Most in vitro testing of treatments designed to inhibit smooth muscle cell proliferation depend on cell cultures. These do not allow for the interaction among cell types and cannot reproduce the typical milieu in which restenosis occurs. Co-cultures of smooth muscle and endothelial cells [4] attempt to improve the situation by modelling the interaction between the two cell types, but without the typical arterial wall structure and extracellular material.

Key words: coronary disease, histopathology, local drug delivery, restenosis, smooth muscle.
Abbreviations: IMA, internal mammary artery; PCNA, proliferating cell nuclear antigen.
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To overcome these problems, whole sections of a harvested artery have been studied *ex vivo*, i.e. organ culture. Successful organ cultures have modelled the effects of platelet-derived growth factor on the human saphenous vein [5], and have allowed gene transfer into normal and atherosclerotic vessels [6]. Human endarterectomy tissue can be cultured similarly in order to study the mechanisms involved in smooth muscle cell hyperplasia [7].

Human internal mammary artery (IMA) organ culture has been used in previously published work [8]. Intimal hyperplasia and smooth muscle cell proliferation were seen in the vessels, similar to that observed in saphenous vein organ cultures [9]. These vessels were cultured pinned, opened out in culture medium. This is useful for investigating the arterial tissue response, including when the vessel has been angioplastied prior to being cultured. However, it is not an ideal model for the study of drug-eluting stents. This is because drugs released from a stent would not be in close contact with the target tissue, since the tissue is cultured flat rather than in the normal, tubular shape.

Here we describe an IMA organ culture model of a stented vessel that allows demonstration of the effects of a stent on a human vessel still in tubular form. To demonstrate the value of this model, we further studied the effects of a known anti-proliferative agent loaded on to stents that are designed to release such drugs into the tissue.

It is the model that was under test in this work rather than the anti-restenotic agent, and so paclitaxel was chosen as the agent used to test the model, as it has well-characterized anti-proliferative effects. Paclitaxel is a lipophilic compound derived from yew trees (*Taxus breviss*). It acts by polymerizing the tubulin components of a cell’s microtubules into defective structures. Without normal microtubule function, smooth muscle cells cannot divide or migrate [10]. Paclitaxel is used as an anti-neoplastic agent in patients with metastatic ovarian carcinoma. Neutropenia and hypersensitivity reactions are the major toxic and dose-limiting effects, mainly due to the carrier with which paclitaxel is combined for systemic use. Paclitaxel can cause asymptomatic bradycardia or bradyarrhythmia [10,11]. To avoid these effects, paclitaxel is considered an ideal agent for local (including stent-based) delivery. Its prolonged effects on proliferation after even a single, brief exposure are also attractive.

In animal models of restenosis, systemic paclitaxel has been shown to inhibit smooth muscle cell proliferation and migration after angioplasty [12]. Locally delivered paclitaxel through a balloon or infusion resulted in reduced neointima formation [13]. A biodegradable stent coating loaded with paclitaxel inhibited smooth muscle cells *in vitro* [14]. Both polymer-coated and bare stents loaded with paclitaxel lead to an encouraging reduction in intimal hyperplasia in various animal models [15,16]. Several clinical trials (ELUTES, ASPECT, TAXUS-I) have been reported recently that have used paclitaxel-eluting stents [ASPECT (S. J. Park) at the Transcatheter Cardiovascular Therapeutics meeting, Washington, September, 2001; and TAXUS-I (E. Grube) and ELUTES (A. H. Gershlick) at the American Heart Association meeting, Anaheim, California, CA, U.S.A., 11–14 November, 2001]. Significant reductions were seen in the incidence of restenosis.

The aim of the present study was to evaluate a model of a stented vessel where the vessel retains its original shape, which can be examined for pathobiological changes that lead in clinical practice to adverse outcomes. A drug with known anti-proliferative effects was delivered from the stent, and the effects on the model were studied to demonstrate proof of principle. Initial studies were performed to demonstrate that this drug could in fact be absorbed on to and released from the stent studied.

**METHODS**

**Absorption and elution studies with paclitaxel**

The stent used was the phosphocholine-coated, stainless steel BiodivYsio Drug Delivery (Biocompatibles) stent. This stent is coated with a synthetic version of a phosphocholine monomer that has been polymerized in combination with a hydrophobic monomer to create a highly hydrophobic polymer. This is dissolved in ethanol, and the stents are dipped into this solution to coat them. The polymer is cured by heat and γ-irradiation. The resulting coating is stable *in vivo*. This stent has been used in clinical practice since 1996 [17] and has been shown to absorb and release drugs intact [18]. Absorption is by diffusion, in a hydrophobic solvent such as ethanol, into the substance of the polymer, where a drug is held by hydrophobic–hydrophobic interaction between the drug and the polymer. In aqueous solvents, e.g. *in vivo*, the absorbed drug will diffuse passively out of the polymer, slowed somewhat by the hydrophobic–hydrophobic interactions between drug and polymer.

14C-radiolabelled paclitaxel (Amersham) was used as a ‘spike’ in unlabelled drug solutions to allow detection of the drugs. BiodivYsio phosphocholine Drug Delivery stents (3 mm × 15 mm) were immersed in 12 mg/ml paclitaxel solution (in alcohol) for 5 min or 1, 6 or 20 h. At each time point, stents were removed from solution and air-dried. Absorbed paclitaxel was stripped off the stents using an ultrasonic bath. The paclitaxel in the resulting solution was measured by counting the associated radioactivity in a β-counter.

The elution characteristics of the stents were determined by placing similarly loaded stents in a perfusion circuit. IMA tissue was not used at this stage. The circuit contained an albumin-buffer solution of the same protein.
content, pH, temperature and perfusion rate as blood in vivo (Figure 1). Drug-coated stents were removed from this circuit at set time points and residual drug was quantified as before.

Organ culture

Tissue preparation

During bypass surgery, the IMA was mobilized and all minor side branches were clipped. The vessel was injected with a heparin solution. Surplus tissue destined for waste disposal was taken for this research under the authority of the Local Ethics Committee and with the prior informed consent of patients. Under sterile conditions, the adventitia and any other adhering tissue were gently removed. The sections of artery were trimmed to a length of approx. 15–20 mm.

Treatment of IMA sections

Pieces of IMA were divided into three groups: group 1, IMA cultured open, uninjured \((n = 6)\); group 2, IMA cultured closed after angioplasty/stent injury with no drug on stent \((n = 6)\); group 3, IMA cultured closed after angioplasty/stent injury with paclitaxel-loaded stent \((n = 6)\).

Stented segments of artery were pre-balloonied to 810 kPa (8 atm) for three inflations of 1 min each with a sterile angioplasty balloon (Penant 20 mm coronary stent delivery system). The balloon was withdrawn and a sterile 3 mm × 15 mm BiodivYsio Drug Delivery stent was deployed at 810 kPa. In group 3, paclitaxel had been absorbed on to the stents under sterile conditions, as in the absorption studies. Baseline specimens were taken from each group. All cultured segments of artery were cultured in RPMI 1640-based organ culture medium containing 30% (v/v) foetal calf serum (Gibco BRL) at 37 °C in separate compartments of a Petri dish. This was intermittently tipped to and fro to encourage circulation of medium through the lumen of the vessel, or around the specimen. Perfusion of the stented vessels was thus entirely passive, without the pulsatile flow seen in the initial experiments. After 72 h, these sections were divided lengthways and the stent (in groups 2 and 3) was removed. Both these samples and the baseline samples were processed according to a method of wax-embedding used in previously published work [7]. Sections of 6 \(\mu\)m thickness were cut and mounted for staining. Haematoxylin and eosin staining was performed to demonstrate cell morphology (nuclear morphology/fragmentation, tissue cohesion), which gave an indication of tissue viability.

Smooth muscle cell \(\alpha\)-actin and proliferating cell nuclear antigen (PCNA), both markers of vascular smooth muscle cell proliferation, were also stained (DAKO and Sigma respectively) as in previous work [7,19]. Briefly, this involved initial staining with monoclonal antibodies to these antigens, and then a streptavidin–biotin complex/horseradish peroxidase technique was used. Negative controls without primary antibody were also performed. Assessment of the extent of staining was done in a blinded fashion. Staining was graded as absent, slight, moderate or strong. Typical slides were photographed.

RESULTS

Absorption and elution studies with paclitaxel

Absorption

A maximum of 127 ± 29 \(\mu\)g of paclitaxel was absorbed on to the stents when they were immersed in 12 mg/ml drug solution (Table 1). No increase was seen with absorption times longer than 5 min (results not shown).

<table>
<thead>
<tr>
<th>Concentration of paclitaxel solution (mg/ml)</th>
<th>Absorption of paclitaxel ((\mu)g)</th>
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<tbody>
<tr>
<td>3</td>
<td>72.8 ± 13.7</td>
</tr>
<tr>
<td>6</td>
<td>124.5 ± 4.8</td>
</tr>
<tr>
<td>12</td>
<td>127.1 ± 29.0</td>
</tr>
</tbody>
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Table 1  Absorption of paclitaxel on to the BiodivYsio stent after a 5 min immersion and air-drying

Values are means ± S.D.
Figure 2  Elution of paclitaxel from the BiodivYsio stent

Note that a logarithmic scale is used for the time axis. This is because much of the elution occurs very early, followed by a slower phase of loss. This pattern has been seen previously in similar work on drug elution from polymer-coated stents.

Figure 3  Characteristic staining after a 3-day culture of IMA tissue that had been cultured closed with a BiodivYsio Drug Delivery stent in situ

Haematoxylin and eosin staining (top panels) shows merely that the tissue survived the stenting and culture process. Normal tissue morphology is seen. α-Actin (middle panels) and PCNA (bottom panels) staining shows a reduction in cell-specific staining in the tissue that was stented with a paclitaxel-absorbed stent.

Elution

Elution of paclitaxel from phosphocholine stents is shown in Figure 2. After 24 h, 13% of the paclitaxel remained on the stent; by 48 h, almost all (96%) of the original amount had eluted from the stent. This showed a very rapid initial release of paclitaxel, followed by a slower elution phase.

Organ culture

In the group 1 experiments, the viability of the IMA tissue cultured for up to 14 days was studied. The day 14 post-culture tissue showed a marked increase in the positivity of PCNA staining of vascular smooth muscle cells in paraffin-embedded sections compared with fresh
vessels (results not shown). The IMA organ cultures showed a qualitative increase in PCNA staining similar to that seen in previous work using saphenous vein and endarterectomy tissue cultured for 10 and 14 days respectively [20], demonstrating an increase in the proliferation of vascular smooth muscle cells in these cultures.

Staining for smooth muscle cell actin and PCNA in paraffin-embedded IMA tissue sections is shown in Figure 3. This demonstrates the differences seen between segments treated with normal phosphocholine stents and those treated with stents that had been absorbed with paclitaxel. Marked PCNA staining was seen in stented vessels at 3 days (three out of the five vessels), similar to that seen in the arteries cultured opened flat. By contrast, vessels stented with paclitaxel-eluting stents showed either no PCNA staining (three out of the four vessels) or very slight staining (one vessel). Vessels processed at baseline also showed no staining for PCNA.

**DISCUSSION**

**Absorption and elution studies**

The results showed that paclitaxel could be absorbed into the phosphocholine polymer that coated these stents. This absorption occurred very quickly. This result is similar to that from work done with a phosphocholine-coated stent absorbed with oligonucleotides [21].

The elution of the drug from the stent occurred continuously over the period of study of perfusion in our model. Paclitaxel has an irreversible effect on the microtubules of the cells it encounters. The time course of drug release and the exposure of target cells to the drug in this model appears to be effective, in 3-day organ culture at least.

A biphasic elution curve is characteristic of the release of drugs from many polymer-coated stents, including this polymer-coated stent. This is thought to be due to a very rapid wash-off of very lightly adherent drug molecules on or near the surface of the polymer, followed by a slower release of drug from within the substance of the polymer. Elution seen with other drug/stent combinations, including the anti-thrombotic medications aPC and abciximab, also follows a similar rapid initial release and then a slower, more sustained release over several days [2]. The 4% of drug remaining adherent to the stent at 48 h may be compared with the delivery efficiency seen using delivery balloons, where drug delivery may be lower than 1% [22]. Since paclitaxel has an irreversible and immediate action on the proliferation of smooth muscle cells, its relatively quick release is compatible with a sustained effect on nearby cells.

The elution characteristics seen can only be considered to be an approximation of those that may occur in vivo, since the model used is clearly not identical to the conditions found in an atherosclerotic human coronary artery. In particular, the stent is not perfused with blood, as would be the case in vivo. This model has, however, been used previously to predict in vivo effectiveness [2].

It is assumed in this work that the phosphocholine polymer itself has no chemical interaction with the paclitaxel that is absorbed, and that the phenomenon is entirely passive. Phosphocholine was chosen for use in these stents particularly because it is an inert substance. Phosphocholine-coated stents have entered clinical use without adverse effects, and are licensed on the basis that they do not interact with the tissues that they are exposed to.

**Organ culture studies**

This model for the stented human artery can be kept viable for at least 3 days, which is long enough to demonstrate smooth muscle cell proliferation, detected using PCNA staining. PCNA is a recognized marker for the proliferation of cell lines, including in the restenosis process [23]. Proliferation was seen in unstented and stented controls, similar to that in specimens that had been cultured opened flat. This result establishes the model as one that behaves similarly to existing techniques. However, the responses seen were obtained in a vessel still containing a stent. The model can therefore be used as a research tool to study the effects of stenting on human arterial tissue, which otherwise relies on necropsy specimens [24].

Furthermore, the model has been tested as a model for the effects on human vascular tissue of anti-restenotic drugs carried on the stents. We have seen that, while vessels containing unloaded stents developed smooth muscle cell proliferation, stents loaded with the drug paclitaxel inhibited this response. This effect is seen despite the rapid release of paclitaxel from the stent polymer. The actions of paclitaxel on tubulin are irreversible, making a single exposure sufficient to prevent cell replication.

**Limitations of the study**

Some of the stented pieces of artery did not survive for 3 days. These pieces of tissue showed typical changes characteristic of necrosis and took up no stain. This occurred in each injured group (one in group 2 and two in group 3), irrespective of whether the vessel was a control or paclitaxel stent. It was surmised that these vessels may have been too small in calibre to tolerate the angioplasty/stent injury, which was carried out with a uniform inflation pressure at a diameter of 3 mm. IMA tissue is of variable internal diameter, as are the coronary vessels. Thus this model is reliable only with pieces of tissue large enough to tolerate the admission of a 3 mm angioplasty/stent. The size of the IMA segments used in this work was not measured before use, as this is difficult to do in living tissue other than by angiography, which
would have constituted an additional procedure for the patient that could not be justified.

Stented tissue was studied for only 72 h. While this was long enough to demonstrate a qualitative difference in the smooth muscle cell response with or without paclitaxel, longer periods of culture may be required to demonstrate other changes that occur as a result of the restenosis process. Longer periods of study were not attempted in this research, although IMA culture has been maintained for up to 14 days.

Only qualitative data suggesting reduced smooth muscle cell proliferation are presented. No quantitative measurements were made of the degree to which paclitaxel had inhibited smooth muscle cell growth. The aim of the present study was not to judge the effectiveness of paclitaxel as an anti-restenotic agent, as this has been done extensively elsewhere, but to determine whether the model of viable, stented vessels was valid, using paclitaxel to illustrate the model’s use with drug-eluting stents.

Conclusions

The model that we have described will allow the effectiveness of other anti-restenotic agents to be tested in human tissue before they are tested in clinical trials. This may be important, as anti-restenosis agents can show promise in animal models only to prove disappointing after the conclusion of expensive and time-consuming randomized controlled clinical trials in humans [25,26]. With societal pressures on the use of animals in research, it may be helpful to use this model before progressing to animal studies to test new treatments. The advantage of using this model of stented arterial tissue is that it allows the interaction of stent and vessel to be studied. This cannot be done with existing in vitro models. It also allows evaluation of a stent delivering any anti-restenosis drug, and may give a useful indication of the likelihood that a specific drug may be beneficial in humans, since the tissue used is human arterial tissue of similar calibre to the coronary artery.

The model cannot be held up as an exact model of the in vivo situation, since it does not reproduce the effects of pre-existing atheroma or of humoral factors that affect restenosis in the blood. IMA tissue in culture does, however, undergo the smooth muscle cell changes that are typical of the restenotic process. In conjunction with animal models, the data from an IMA stent organ culture model may together prove a better indicator of what is likely to be of benefit in clinical studies of drug-eluting stents.

ACKNOWLEDGMENTS

We thank Mr T. Spyt for his kind co-operation in allowing us to approach his patients to request the use of the tissue required for this study. This work was supported by a grant from the British Heart Foundation and Glenfield Hospital NHS Trust.

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Received 18 January 2002; accepted 11 June 2002