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Effect of stent coating alone on in vitro vascular smooth muscle cell proliferation and apoptosis

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Curcio, Antonio, Daniele Torella, Giovanni Cuda, Carmela Coppola, Maria Concetta Faniello, Francesco Achille, Viviana G. Russo, Massimo Chiariello, and Ciro Indolfi. Effect of stent coating alone on in vitro vascular smooth muscle cell (VSMC) proliferation and apoptosis. Am J Physiol Heart Circ Physiol 286: H902–H908, 2004. First published November 6, 2003; 10.1152/ajpheart.00130.2003.—Synthetic polymers, like methacrylate (MA) compounds, have been clinically introduced as inert coatings to locally deliver drugs that inhibit restenosis after stent. The aim of the present study was to evaluate the effects of MA coating alone on vascular smooth muscle cell (VSMC) proliferation in vitro. Stainless steel stents were coated with MA at the following doses: 0.3, 1.5, and 3 ml. Uncoated/bare metal stents were used as controls. VSMCs were cultured in dishes, and a MA-coated stent induced VSMC growth arrest by inducing apoptosis in a dose-dependent manner. Thus MA is not an inert platform for eluting drugs because it is biologically active per se. This effect should be taken into account when evaluating an association of this coating with antiproliferative agents for in-stent restenosis prevention.

RESTENOSIS, defined as “the arterial healing response after injury incurred during transluminal coronary revascularization,” has been the principal drawback of percutaneous coronary interventions (PCI) since its start in the clinical scenario (1). Postballoon angioplasty restenosis is thought to involve primarily negative remodeling and, partially, vascular smooth muscle cell (VSMC) proliferation and migration, forming neo-intima tissue (18, 19). The only widely accepted way of preventing restenosis; stents

distinct from restenosis after PTCA (13). In fact, ISR is mainly a result of neointima formation alone, i.e., of VSMC proliferation (13, 15, 16), which is actually exaggerated after stent deployment due to the high pressure technique of stent deployment (14).

Because the only device able to reduce restenosis is the stent, major effort has been currently made to improve this device technology with the aim of further reducing restenosis after its deployment. Indeed, the novel concept of local drug delivery directly to the site of vascular injury via polymeric- or nonpolymeric-coated stents is the most reasonable and effective approach to achieve adequate localized antiproliferative effects preventing ISR (6, 8, 16). Recently, eluting stents with different drugs have been introduced in the clinical scenario to improve the long-term outcome of PCI, and sirolimus (i.e., rapamycin, an antiproliferative drug)-eluting stents have demonstrated striking beneficial effects in terms of reduction of ISR (2, 8, 23, 24, 28, 29).

In the 1990s, the development of a drug-eluting stent stumbled over the fact that the carrier polymer caused intense inflammatory reactions that ruined the antiproliferative effect of the incorporated drugs (25, 26, 31). However, novel inert polymers such as poly-(n)-butyl methacrylate (MA), used for drug-eluting stent manufacture, like sirolimus, have been introduced because they seem to have minimal adverse effects by the polymer itself, therefore being an ideal inert coating to release drugs over the “stent platform” (30).

Although tremendous effort is now focused on the search for the best agent to be used in the eluting stents (6, 8, 16), no further data are yet available on the biological effect of coating per se on VSMC growth.

Accordingly, the aim of the present study was to evaluate the effect of MA coating alone on VSMC proliferation and fate in vitro.

METHODS

Preparation of MA films on stents. MA was obtained by melting the acrylic monomer in n-butoxyethanol (0.9 g/100 ml, Polysciences). Different volumes of MA (0.3, 1.5, or 3 ml) were applied in a liquid state on stainless steel ACS Multilink stents (Guidant; Indianapolis, IN) of 8-mm length and 13-mg weight. Anylin (100 µl) was added to the polymer/stent to accelerate polymer hardening on the stent surface. The stents were rinsed three times with sterile filtered water and dried overnight. Sterilization was carried out with ethylene oxide gas. The amount of MA retained on the stent was determined using an

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Table 1. Degradation of MA coating in culture

<table>
<thead>
<tr>
<th>Stent Coating</th>
<th>Polymer Coating Weight, µg Before incubation</th>
<th>After incubation</th>
<th>Polymer Degradation, µg</th>
<th>MAA, µg in 3 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.3 ml</td>
<td>193 ± 17</td>
<td>173 ± 14</td>
<td>20 ± 3</td>
<td>11 ± 5</td>
</tr>
<tr>
<td>1.5 ml</td>
<td>875 ± 53</td>
<td>761 ± 51</td>
<td>114 ± 5</td>
<td>88 ± 16</td>
</tr>
<tr>
<td>3.0 ml</td>
<td>1,835 ± 50</td>
<td>1,573 ± 37</td>
<td>262 ± 13</td>
<td>209 ± 34</td>
</tr>
</tbody>
</table>

Values are means ± SE. Polymer weight coating was calculated by subtracting the constant weight of the stent from the total weight of the polymer/stent. Polymer degradation was calculated by subtracting the polymer/stent weight after the incubation to its weight before the incubation. MA, methacrylate polymer; MAA, methacrylic acid degradated from each stent into the media (3 ml) after 48 h of incubation.

phosphatase-conjugated goat anti-mouse IgG antibody (diluted 1:1,000) for 1 h at room temperature. Dako fast red substrate system (Carpinteria, CA) was used as a chromogen, and counterstaining was performed with hematoxylin. Cell nuclei that incorporated BrdU appeared red and were counted in four to six different high-power fields (magnification ×200) per well and related to the total cell number.

Table 2. Cumulative data of MA coating effects on VSMC proliferation in vitro assessed by BrdU incorporation

<table>
<thead>
<tr>
<th>MA, ml</th>
<th>Control</th>
<th>Stent 0.3</th>
<th>1.5</th>
<th>3.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>20 ± 4</td>
<td>22 ± 3</td>
<td>23 ± 4</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>24 h</td>
<td>50 ± 7.a</td>
<td>48 ± 2.a</td>
<td>31 ± 3.a</td>
<td>25 ± 4</td>
</tr>
<tr>
<td>48 h</td>
<td>78 ± 9.a</td>
<td>73 ± 6.a</td>
<td>43 ± 4.a</td>
<td>29 ± 2</td>
</tr>
</tbody>
</table>

Values are means ± SE. VSMC, vascular smooth muscle cell; BrdU, bromodeoxyuridine. *P < 0.01 vs. respective 0-h value; **P < 0.01 vs. MA (0.3, 1.5, and 3.0 ml) at 24 h; ***P < 0.01 vs. respective 0- and 24-h values; #P < 0.01 vs. MA (0.3, 1.5, and 3.0 ml) at 48 h; $P < 0.01 vs. MA (1.5 and 3.0 ml) at 48 h.

DNA laddering detection. DNA laddering was performed in separate dishes (using the same culture settings above described for the annexin V) to verify fluorescence microscopy findings (21). Briefly, to detect intranucleosomal cleavage of the DNA, the presence of low-molecular-weight DNA fragments was determined in VSMCs cultured for 24 h in the presence of a bare uncoated stent or MA-coated stent. VSMCs were fixed for 24 h at -20°C in 70% ethanol. Cells were then centrifuged at 800 g for 5 min, and the ethanol was thoroughly removed. Pellets were resuspended in 40 µl of phosphate-citrate buffer, which consisted of 192 parts of 0.2 mol/l Na 2 HPO 4 and 8 parts of 0.1 mol/l citric acid (pH 7.8) at room temperature for 1 h. Samples were centrifuged at 1,000 g for 5 min. The supernatant was transferred to new tubes and concentrated by vacuum in a SpeedVac concentrator (Savant Instruments) for 15 min. A 3-µl aliquot of 0.25% Nonidet P-40 (Sigma Chemical) in distilled water was then added, followed by 3 µl of a solution of RNase (1 mg/ml), also in water. After 30 min of incubation at 37°C, 3 µl of a solution of proteinase K (1 mg/ml, Boehringer Mannheim) were added, and the extract was
incubated for an additional 1 h at 37°C. Subsequently, 12 μl of loading buffer (0.25% bromophenol blue and 30% glycerol) were added, and the samples were subjected to electrophoresis on a 2% agarose gel containing 0.5 μg/ml ethidium bromide. DNA in the gels was visualized under ultraviolet light.

Western blot analysis. To examine caspase-3 activation and PARP cleavage, rat aortic VSMCs (incubated for 24 h in the above-described conditions) were washed with PBS and lysis buffer (which contained 50 mmol/l Tris·Cl, 150 mmol/l NaCl, 0.02% NaN₃, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 5 μg/ml leupeptin, and 1 μg/ml aprotinin). The protein content of cell lysates was quantified with Coomassie brilliant blue, separated by SDS-PAGE, and transferred to nitrocellulose membranes (Schleicher & Schuell). The membranes were blocked at room temperature (24–26°C) for 1 h in PBS-Tween [which contained (in mmol/l) 130 NaCl, 2.5 KCl, 10 Na₂HPO₄, 1.5 KH₂PO₄, 0.1% Tween 20, and 5% BSA (pH 7.4)], incubated with a rabbit primary antibody for cleaved caspase-3 or cleaved PARP (Cell Signaling Technology; Beverly, MA) overnight at 4°C, and then incubated with the appropriate secondary peroxidase-conjugated antibodies (horseradish peroxidase-linked anti-rabbit secondary antibody for 1 h at room temperature). Final detection was carried out with LumiGLO chemiluminescent reagent (Cell Signaling Technology) as described by the manufacturer.

Degradation of MA coating in DMEM complete medium. Polymers like MA are delivery systems that release drugs according to the diffusion of these compounds and/or the degradation of the polymer itself (3). On this basis, we evaluated the presence in the culture medium of the major breakdown product of MA polymer, methacrylic acid (MAA), presumably leached out/degraded into the media. Therefore, 48 h after the incubation with the three different coated stents (MA at 0.3, 1.5 and 3.0 ml), the media of VSMC culture dishes were recovered, and a 500-μl sample was mixed with 500 μl [6% (wt/vol)] trichloroacetic acid in water. The precipitated proteins were removed by centrifugation, and the amount of MAA in the supernatant was quantified with HPLC, as previously described (3).

![Fig. 1. Effects of uncoated stent (bare metal) and low (0.3 ml), intermediate (1.5 ml), and high (3 ml) doses of poly-(n)-butyl methacrylate (MA) coating on DNA synthesis addressed by bromodeoxyuridine (BrdU) incorporation. A significant reduction in cell proliferation was observed with MA-coated stents. VSMCs, vascular smooth muscle cells. Proliferation activities are means ± SD of 6 measurements. *P < 0.01 vs. respective 0-h value; †P < 0.01 vs. MA (0.3, 1.5, and 3.0 ml) at 24 h; **P < 0.01 vs. respective 0- and 24-h values; ‡P < 0.01 vs. MA (0.3, 1.5, and 3.0 ml) at 48 h; #P < 0.01 vs. MA (1.5 and 3.0 ml) at 48 h.]

![Fig. 2. A: VSMCs cultured in the absence and presence of uncoated/bare metal stents or MA (Metha)-coated stents at a growing dose. Apoptotic cells are shown by annexin V (green) and propidium iodide (PI; red) staining. Magnification: ×10. B: cumulative percent data of apoptotic VSMCs. MA-coated stents induced dose-dependent VSMC apoptosis. Apoptotic VSMC data are means ± SD of 6 measurements. *P < 0.01 vs. control (Con) and uncoated stents; **P < 0.01 vs. MA (0.3 ml); ***P < 0.01 vs. MA (1.5 ml).]
Statistical analysis. All values are expressed as means ± SE. Statistical analysis of differences observed between groups was done by ANOVA comparison using the SPSS 10.0 program (17, 18). Tukey’s test was applied to compare single mean values. A P value of <0.05 was considered significant.

RESULTS

Coating alone affects VSMC proliferation in vitro. To investigate VSMC growth in the presence of a bare metal stent or MA-coated stent, the incorporation of the thymidine analog BrdU was measured. At 48 h, VSMCs cultured in presence of a bare uncoated stent did not show a significant difference of DNA synthesis compared with control VSMCs (Table 2 and Fig. 1). MA coating induced a significant decrease of BrdU incorporation compared with controls (Table 2 and Fig. 1) and compared with the bare stent at the low through the high doses (Table 2 and Fig. 1).

Effects of stent coating on VSMC apoptosis in vitro. To shed light on the potential mechanism responsible for the decreased VSMC proliferation in the presence of different concentration of MA coating, we evaluated VSMC apoptosis by three distinct approaches: 1) annexin V/PI fluorescence detection; 2) DNA laddering; and 3) caspase-3 activation and PARP cleavage.

Apoptosis assays were performed in six independent experiments for each of the three approaches.

For annexin V/PI detection, each well was analyzed with fluorescence microscopy after a 24-h incubation with uncoated and MA-coated stents (Fig. 2). Compared with control VSMC dishes (cultured without any kind of stents), bare metal stents induced a slight but not significant increase in cell apoptosis (4.4 ± 1.2% in control wells vs. 9.3 ± 1.2% in bare metal incubated wells, P = not significant). In contrast, incubation with MA-coated stents induced increased VSMC apoptosis in a dose-dependent manner compared with control VSMCs and compared with VSMCs cultured in presence of an uncoated stent (21.9 ± 9.0% at 0.3 ml, 38.5 ± 12.1% at 1.5 ml, and 77.6 ± 8.7% at 3 ml of MA coating, all P < 0.05 vs. controls and bare metal stents; see Fig. 2).

To further confirm these findings, we detected and evaluated the DNA fragmentation, examining whether genomic DNA isolated from VSMCs, cultured in presence of a MA-coated stent, produced a typical “ladder” pattern (180-bp multiples) when analyzed on an agarose gel. Figure 3 illustrates that DNA fragments of size equivalent to the mononucleosomes and oligonucleosomes were detected in VSMCs incubated with MA-coated stents. This pattern of DNA damage was barely visible in control VSMCs (Fig. 3). Therefore, MA coating was associated with DNA laddering in vitro VSMCs.

Finally, the change in expression of caspase-3 and its activity (measured by PARP cleavage) was analyzed by Western blot (Fig. 4). Caspase-3 is synthesized as a precursor that has little, if any, catalytic activity. Activation of caspase-3 requires proteolytic processing of its inactive zymogen into activated p17 subunits. Therefore, we evaluated the expression of active caspase-3 using a primary antibody that specifically detects endogenous levels ofcleaved caspase-3. Moreover, we determined the activity of caspase-3 by assessing the cleavage of PARP into its 89-kDa form, which is the target of active caspase 3 and is considered a hallmark of apoptosis (27). Activated caspase-3 was absent in VSMCs of the control group, without any significant difference in the expression level between untreated plates or those incubated with a bare metal stent. In contrast, VSMCs incubated in presence of stents coated with increasing doses of MA showed a dose-dependent increase in caspase-3 activation compared with both VSMCs cultured in the absence or presence of an uncoated stent (Fig. 4). The activation of caspase-3 was followed by similar cleavage of PARP, confirming the specific activation of this caspase by MA coating (Fig. 4).

Degradation of MA polymer in vitro. Polymers like MA are delivery systems that release drugs, proteins, and active peptides according to the diffusion of these compounds and/or the degradation of the polymer itself (3). The results described above prompted us to assess whether the effects of the MA-coated stents on VSMC proliferation and death could be correlated to the major breakdown product of the polymer MAA. The amount of MAA leached out/degraded into the media was quantified with HPLC (3). First, to assess empirically if polymer degradation occurred, the weight of each polymer/stent was calculated at the end of the incubation (Table 1). From these measures, we observed that the MA-coated stent lost weight at the end of the incubation, indicating a partial degradation of the polymer (assuming a constant weight of the stent) (Table 1). Interestingly, the drop in weight of MA-coated stents was parallel with an increase in MAA
concentration in the culture media (Table 1 and Fig. 5), demonstrating minimal degradation of the polymer in culture.

Whereas many stents have significant alterations in the properties of the coating material once they are expanded, to mimic the real clinical procedure the coated stents were expanded on balloons before placement into the media. This procedure did not change the weight of the polymer/stent (data not shown), making unlikely the possibility that stent expansion could affect per se the coating, biasing the baseline condition. However, we assessed the degradation of the MA (3.0 ml) coating in the culture media from expanded compared with not expanded stents. The amount of MAA (measured by HPLC) leached out in the culture media after a 48-h incubation from expanded MA-coated stent (209 ± 34 µg in 3 ml of media) was not different from nonexpanded stents (200 ± 29 µg in 3 ml of media), supporting the concept that the degradation of the coating was not acutely affected by the stent expansion.

DISCUSSION

The major finding of the present study is that MA stent coating per se affects VSMC growth, inducing apoptotic cell death in vitro. Therefore, although the platform used to deliver specific agents was retained to be highly biocompatible (30), these polymers may have some important biological effects that can amplify or interact with the drug used to prevent in-stent restenosis.

Restenosis after balloon angioplasty is primarily due to negative vascular remodeling and only partially to VSMC proliferation (18, 19). On the other hand, whereas stent deployment abolished inward vascular remodeling, ISR is mainly determined by VSMC proliferation generating neointimal formation (1, 13, 15, 16).
The mechanisms of VSMC proliferation after vascular injury have been extensively studied in our laboratory, and many candidate agents or molecules have been identified to inhibit VSMC proliferation (9–19). However, a good candidate agent should be locally delivered (at the site of vascular injury) in adequate concentrations without toxic effects and over an appropriate period of time to achieve favorable antiproliferative effects (6, 8, 16).

Numerous copolymers have been applied at the surface of metallic stents to serve as a matrix for drug loading. However, animal data demonstrated possible bulk effects of the polymers including strut-associated inflammation (25, 26, 31). In fact, polymer coatings by their nature typically induce inflammatory responses and fibrinoid deposits (6, 8, 19). Moreover, histological studies have shown that the stability of polymeric material may degrade over time, bringing the risk of delayed intimal hyperplasia. Therefore, the perfect carrier is still being searched for.

Recent clinical data comparing a sirolimus-eluting stent using MA coating with a standard stent in patients with single coronary lesions amenable to stenting demonstrated striking results for ISR prevention in different trials (6, 8, 23, 24, 28, 29).

Taxol, an antineoplastic agent, is (along with rapamycin) the most promising among all the agents that are now undergoing clinical trials. Indeed, taxol-eluting stents reduced the restenosis rate even when different adverse events, such as late thrombosis, delayed restenosis, and aneurysm formation (6–8), were shown. However, the first clinical experience with a paclitaxel derivate-eluting stent coated by multiple polyacrylic acid were shown. However, the effect of the specific drug loaded in the polymer (like in the case of sirolimus), increasing its potential to reduce restenosis. On the other hand, it should be pointed out that all the tools used to prevent restenosis, including stent-based drug delivery, might delay maturation and normal endothelial function, thus increasing the potential for a late thrombotic event. It can not be excluded that the apoptotic properties of MA on VSMCs, as described in this study, could also interfere with in vivo endothelial cell proliferation, forming the new endothelium layer of the stented-denuded vessel, consequently impairing the complete healing process and yielding to a late negative event.

Thus the overall success of any drug-eluting stents might be dependent not only on the drug alone but also on polymer properties.

Limitations. The most used polymer in the manufacture of the eluting stents is poly-MA. Therefore, we decided to assess the effects of MA alone on VSMC proliferation. However, slight differences are present between our model (designed to assess the role of MA alone) and the commercially available eluting stents. For instance, Cypher sirolimus stents (Cordis) are coated with a layer of MA plus polyethylene vinyl acetate as copolymer (28).

Furthermore, the compound anylin has been added to the stent/polymer as probably in the commercially available stents (although this is not specifically specified from the relative companies). Anylin has been used for hardening the polymer on stent surface. It should be noted that the dose used was actually minimal and that it is almost completely consumed during the hardening reaction. It is also important to underline that anylin was added to the different volumes (0.3, 1.5, and 3.0 ml) of MA polymer at a constant dose of 100 μl. Therefore, we believe that anylin per se could not explain the dose-dependent effect of MA coating on VSMC proliferation and apoptotic death. However, as never tested before on VSMC in the experiments in which we exposed starved VSMC to anylin at the above-mentioned dose. At this concentration, we did not observe any toxic effects by anylin on VSMC growth (BrDU incorporation was not different after 48 h of anylin treatment compared with control cells; data not shown).

In summary, the data of the present study strongly suggest that MA coating induces VSMC apoptosis. Therefore, MA is biologically active per se and not an inert platform to release anti-proliferative drugs for in-stent restenosis prevention. This apoptotic property of MA coating is a novel finding that may be useful for planning future projects with drug eluting stents.

GRANTS

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