РАЗДЕЛ 5

СОВРЕМЕННЫЕ ИССЛЕДОВАНИЯ *IN VITRO*

PART 5

MODERN RESEARCHES *IN VITRO*

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THE BIOLOGICAL EFFECTS OF METHACRYLATE-COATED STENT ON SMOOTH MUSCLE CELLS IN VITRO

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Abstract

Synthetic polymers, like methacrylate (MA) compounds, have been clinically introduced as inert coatings to locally deliver drugs inhibiting restenosis after stenting.

The aim of this study was to evaluate the effects of MA-coating alone on vascular smooth muscle cell (VSMC) growth in vitro. Stainless steel stents were coated with MA at the following doses: 0.3 ml, 1.5 ml, and 3 ml. Uncoated/bare metal stents were used as controls. VSMCs were cultured in dishes and a MA-coated stent or an uncoated bare metal stent was gently added in each well. VSMC proliferation was assessed by bromodeoxyuridine incorporation. Apoptosis was analyzed by three distinct approaches: a) Annexin-V/propidium iodide fluorescence detection; b) DNA laddering; c) caspase-3 activation and PARP cleavage. MA-coated stents induced a significant decrease of BrdU incorporation compared to uncoated stents both at the low and high concentrations. In VSMCs incubated with MA-coated stents, Annexin-V/propidium iodide fluorescence detection show a significant increase in apoptotic cells which was corroborated by the typical DNA laddering. Apoptosis of VSMCs after incubation with MA-coated stents was characterized by caspase-3 activation and PARP cleavage. MA-coated stent induced VSMC growth arrest by inducing apoptosis in a dose-dependent manner. Thus, methacrylate is not an inert platform for eluting drugs because is biologically active *per se*. This effect should be taken in account evaluating an association of this coating with anti-proliferative agents for in-stent restenosis prevention.

Key words: Restenosis – Stents – Coating – VSMCs- Apoptosis

Introduction

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). Post balloon angioplasty restenosis is thought to involve primarily negative remodeling and, partially, VSMC proliferation and migration, forming neointima tissue (16). The only widely accepted way reducing restenosis rate has been so far the coronary stent (1, 14). However, the problem of in-stent restenosis (ISR) is becoming at least as important as restenosis after percutaneous transluminal coronary angioplasty (PTCA) (1). ISR is quite distinct from restenosis after PTCA (12). In fact, ISR is mainly a result of neointima formation alone, i.e. of VSMC proliferation (12, 14, 18), which is actually exaggerated after stent deployment due to the high pressure technique of stent deployment (8).

Since the only device able to reduce restenosis is the stent, major effort has been currently made to improve this device technology to the aim to further reduce restenosis after its deployment. Indeed, the novel concept of local drug delivery directly to the site of vascular injury via polymeric- or non-polymeric-coated stents is the most reasonable and effective approach to achieve adequate localized anti-proliferative effects preventing ISR (5, 7, 18). 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 anti-proliferative drug)-eluting stents demonstrated striking beneficial effects in terms of reduction of ISR.

In the 1990s, the development of a drug-eluting stent stumbled over the fact that the carrier polymer caused intense inflammatory reactions which ruined the anti-proliferative effect of the incorporated drugs. However, novel inert polymers such as poly-(n)-butylmethacrylate, used for drug-eluting stent manufacture, like Sirolimus, have been introduced because seem to have minimal adverse effects by the polymer itself, therefore, being an ideal inert coating to release drugs over the "stent platform" .

Although a tremendous effort is now focused to search the best agent to be used in the eluting stents (5, 7, 18), however, 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 Methacrylate coating alone on vascular smooth muscle cell proliferation and fate *in vitro*.

Methods

Preparation of Methacrylate Films on Stents

Poly-(n)-butylmethacrylate (MA) was applied in a liquid state on stainless steel ACS Multilink stents (Guidant Corporation, Indianapolis, IN) at different volumes (0.3 ml, 1.5 ml or 3 ml). The stents were rinsed 3 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 electronic balance. The highest mass (≈1800 mg) is comparable to the one used in previous studies (28). Variation in MA doses on stents was less than 10%.

Cell culture

For cell culture experiments, 1 x 10^5 rat VSMCs (A10) were plated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS). VSMCs at 70% to 90% confluence in 35-mm dishes were growth-arrested by incubation in 0.2% FCS/ DMEM for 72 hours to synchronize their cell-cycle. Then, cells were released from quiescence by replacing the low serum medium with normal growth medium (i.e., DMEM containing 10% FCS).

A MA-coated stent or an uncoated stent was gently added in each well, and other cells were grown without any stent (controls, n=8).

We examined VSMC proliferation and apoptosis at the following conditions: 1) incubation with an uncoated/bare metal stent; 2) incubation with MA (0.3 ml) -coated stent; 3) incubation with MA (1.5 ml) -coated stent; 4) incubation with MA (3 ml) -coated stent.

Bromodeoxyuridine proliferation assay.

Incorporation of the thymidine analogue BrdU was measured to determine DNA synthesis (19). Starved iescent by optical density VSMCs were grown in complete media for 20 and 44 hours in the above indicated conditions. Then, 15 µM BrdU was added, and the incubation was continued for another 4 h. Cell nuclei which incorporated BrdU appeared red and were counted in four to six different high power fields (magnification of 200) per well and related to total cell number.

D*etermination of VSMC Apoptosis In Vitro*

We evaluated VSMC apoptosis by fluorescence microscopy using annexin V (Clontech) or propidium iodide (PI) as markers of early and late programmed cell death, respectively (3, 30). Starved VSMCs were plated onto 6-well plates at a density of 1.5x10^5 cells/mL, 3 mL/well, in complete DMEM growth medium and allowed to grow for a period of 12 hours; then, a bare metal stent or a MA-coated stent was gently added to each VSMC culture dish for 24 hours to evaluate cell death by apoptosis. At the end of incubation, stents were taken away and each well was washed twice in chilled (4°C) PBS. Samples were resuspended in binding buffer and fluorescein isothiocyanate–conjugated Annexin V protein (FITC Annexin V) followed by propidium iodide were sequentially added.

DNA laddering electrophoresis

DNA laddering was performed in separate dishes (using the same culture settings above described for the annexin V) to verify fluorescence microscopy findings (20). Briefly, to detect internucleosomal cleavage of the DNA, the presence of low-molecular-weight DNA fragments was determined in VSMCs cultured for 24 hours in the presence of a bare uncoated stent or a MA-coated stent. VSMCs were fixed for 24 hours at -20°C in 70% ethanol. Cells were then centrifuged at 800g for 5 minutes, and the ethanol was thoroughly removed. Pellets were resuspended in 40 µL of phosphate-citrate buffer, consisting of 192 parts of 0.2 mol/L Na_2HPO_4 and 8 parts of 0.1 mol/L citric acid (pH 7.8) at room temperature for 1 hour. Samples were centrifuged at 1000g for 5 minutes. The supernatant was transferred to new tubes and concentrated by vacuum in a SpeedVac concentrator (Savant Instruments Inc) for 15 minutes. A 3-µL aliquot of 0.25% Nonidet NP-40 (Sigma Chemical Co) in distilled water was then added, followed by 3 µL of a solution of RNase, 1 mg/mL, also in water. After 30 minutes of incubation at 37°C, 3 µL of a solution of proteinase K, 1 mg/mL (Boehringer Mannheim), was added, and the extract was incubated for an additional 1 hour at 37°C. Subsequently, 12 µL of loading buffer (0.25% bromophenol blue, 30% glycerol) was added, and samples were subjected to electrophoresis on 2% agarose gel containing 0.5 µg/mL ethidium bromide. The DNA in the gels was visualized under UV light.

Western Blot Analysis

To examine caspase-3 activation and PARP cleavage, rat aortic VSMCs (incubated for 24 hours in the above described conditions) were washed with PBS and lysis buffer. The protein content of cell lysates was quantified with Coomassie Brilliant Blue, and separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). The membranes were blocked at room temperature (24°C to 26°C) for 1 hour in PBST, incubated with a rabbit primary antibody for cleaved Caspase-3 or cleaved PARP (Cell Signaling Technology, Inc., Beverly, MA) overnight at 4°C, and then with the appropriate secondary peroxidase-conjugated antibodies (HRP-linked anti-rabbit secondary antibody for 1 hour at room temperature). Final detection was carried out with LumiGLO chemiluminescent reagent (Cell Signaling Technology, Inc., Beverly, MA) as described by the manufacturer.

Statistical Analysis

All values are expressed as the mean ± SEM. Statistical analysis of differences observed between groups was done by ANOVA comparison using a SPSS 10.0 program (15, 17). The Tukey's test was applied to compare single mean values. A p value < *0.05* was considered significant.

Results

Coating alone affects VSMC proliferation in vitro

To investigate VSMC growth in presence of an un- or MA-coated stent, the incorporation -of the thymidine analogue BrdU was measured. At 48 hours, VSMCs cultured in presence of a bare uncoated stent did not show a significant difference of DNA synthesis compared to control VSMCs. MA-coating induced a significant decrease of BrdU incorporation compared to controls and to bare stent at the low through the high doses.

Effects of stent coating on VSMC apoptosis in vitro

In order to shed lights in to the potential mechanism responsible for the decreased VSMC proliferation in presence of different concentration of Methacrylate coating, we evaluated VSMC apoptosis by three distinct approaches: a) Annexin-V/propidium iodide fluorescence detection; b) DNA laddering; c) caspase-3 activation and PARP cleavage.

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

For Annexin-V/propidium iodide detection, each well was analyzed at fluorescence microscopy after 24 hours incubation with un- and MA-coated stents. Compared to 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 well vs. 9,3%±1.2 in bare

metal incubated wells, p=NS). In contrast, incubation with MA-coated stents induced an increased VSMC apoptosis in a dose-dependent manner compared to control VSMCs and to 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.

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 analysed on an agarose gel. 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. Therefore, MA-coating was associated with DNA laddering in *in vitro* VSMCs.

Finally, the change in expression of caspase-3 and its activity (measured by PARP cleavage) was analysed by Western blotting (figure 1). Caspase-3 is synthesized as 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 the active caspase-3 using a primary antibody which specifically detects endogenous levels of cleaved caspase-3. Moreover, we determined the activity of caspase-3 by assessing cleavage of poly-(ADP-ribose) polymerase (PARP) into its 89 kDa form, which is the target of active caspase 3 and is considered a hallmark of apoptosis. 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 to both VSMCs cultured in absence or in presence of an uncoated stent (figure 1). The activation of caspase 3 was followed by similar cleavage of PARP, confirming the specific activation of this caspase by MA coating (figure 1).

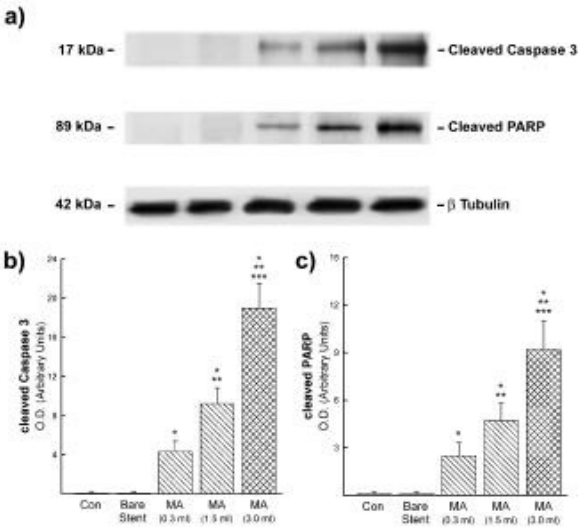

Figure 3.
Panel a Representative Western blotting for caspase-3 activation.
Thirty to fifty µg of total cell extract were electrophoresed onto a 12.5% SDS-PAGE and transferred to PVDF membrane. The blot was incubated with a monoclonal anti-active caspase 3 antibody and the presence of caspase was revealed by chemiluminescence.
A rabbit polyclonal antibody against ERK1/2 (Cell Signaling Technology, Inc., Beverly, MA) was used to normalize the amount of protein loaded.
Panel b Arbitrary optical density (OD) units of activated caspase 3 expression.
$p<0.01$ vs. all
The graphs represent Mean±SD of 6 measurements.
1= Control VSMCs
3= VSMCs incubated with bare metal stent
3= VSMCs incubated with stent coated with 0.3 ml Methacrylate
4= VSMCs incubated with stent coated with 1.5 ml Methacrylate
5= VSMCs incubated with stent coated with 3.0 ml Methacrylate

Discussion

The major finding of the present study is that methacrylate stent coating affects VSMC growth inducing apoptotic cell death *in vitro*. Therefore, although the platform used to delivery specific agents was retained to be highly biocompatible, these polymers may have some important biological effect 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 (16). On the other hand, whereas stent deployment abolished inward vascular remodeling, in-stent restenosis (ISR) is mainly determined by VSMC proliferation generating neointimal formation (1, 12, 14, 18).

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 (8-18). 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 a favorable anti-proliferative effects (5, 7, 18).

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 . In fact, polymer coatings by their nature typically induce inflammatory responses and fibrinoid deposits (5, 7, 18). Moreover histological studies show 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.

It is remarkable that our data, using well-controlled experimental settings, demonstrated that Methacrylate shows specific apoptotic properties. Indeed, MA-coated stents inhibits, in a dose-dependent manner, VSMC proliferation inducing apoptotic deat. Cell apoptosis assessed by annexin-V and PI was 9.3% in bare metal stents and 21.9%, 38.5% and 77.6% in MA-coated stent, respectively. This finding was qualitatively confirmed by typical DNA laddering and could be partially explained by the increase in caspase-3 protein activation and PARP cleavage.

The fact that Methacrylate polymer induces apoptosis in VSMC could lead to a cumulative effect on VSMC apoptosis together with 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. Then, it could not be excluded that Methacrylate apoptotic properties on VSMCs, 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. Nevertheless, at least in the clinical setting, this was not the case of rapamycin eluting-stents because even the follow-up at two years excluded any significant late thrombosis in patients implanted with these stents (2, 22).

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

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