Biodegradable Microspheres Containing a Colchicine Analogue Inhibit DNA Synthesis in Vascular Smooth Muscle Cells

K.L. March, MD, PhD; S. Mohranraj, MD; P.P.K. Ho, MD; R.L. Wilensky, MD; D.R. Hathaway, MD

**Background** Smooth muscle cell proliferation plays a major role in the genesis of restenosis after angioplasty or vascular injury. Local application of agents capable of modulating vascular responses, including smooth muscle cell proliferation, has been achieved, but difficulty in maintaining active levels locally has been a factor limiting the efficacy of such approaches. One strategy to maintain adequate levels is the local delivery of microspheres that release active agents over sustained time periods.

**Methods and Results** We incorporated a colchicine analogue into biodegradable microspheres composed of a lactic acid/glycolic acid copolymer and characterized their drug release behavior as well as their effects on bovine aortic smooth muscle cells (BASMCs) in culture. Drug release was evaluated by spectrophotometric assay. Drug effects on DNA synthesis were measured by thymidine incorporation after addition of serum to subconfluent cells synchronized by serum withdrawal as well as in asynchronous cell populations. Polymeric microspheres incorporating 10% to 17% drug by weight and averaging 6 μm in size were found to release the colchicine analog in buffered saline solutions over more than several weeks. Drug-loaded particles inhibited DNA synthesis completely, with EC₅₀ values ranging from 0.001 to 0.005 g% (wt/wt). Morphological changes suggesting microtubule depolymerization were observed after drug particle treatment, with similar EC₅₀ values. Microspheres allowed to contact the cell surface demonstrated effects similar to those seen with microspheres suspended in the nutrient medium by porous polycarbonate filters, at EC₅₀ values approximately fivefold lower. In contrast, control microspheres composed only of polymer with no incorporated active drug demonstrated no observable toxicity to BASMCs and <40% inhibition of thymidine incorporation even in suspensions containing up to 0.5 g% particles.

**Conclusions** Biodegradable microspheres were fashioned that release a colchicine analogue and inhibit DNA synthesis in smooth muscle cells. Drug-loaded polymeric particles are candidates for local delivery at sites of arterial injury to decrease restenosis. *(Circulation, 1994;89:1929-1933.)*

**Key Words** • stenosis • muscle, smooth • cells • colchicine

The localized nature of the restenotic process and the involvement of smooth muscle and other cells that are not unique to the area of restenosis make the concept of spatially specific treatment attractive. An advantage of this approach is the ability to achieve a locally high concentration of the drug while limiting unwanted systemic effects by dilution into tissue fluids and blood. A particularly exciting enhancement of local drug delivery involves the use of drug formulations designed for sustaining release of the desired agents. By controlling the release of drug in a local tissue environment, it is possible to match the properties of the medication to both the spatial and temporal features of a disease process. In the case of postangioplasty restenosis, a specific sequence of events characterizes the repair process after mechanical injury of an artery: migration of cells to the injury site and proliferation is followed by synthesis of extracellular matrix. These processes, any of which could be a target for drug therapy, occur over several weeks in a double-injury rabbit model of femoral restenosis after dilation of a focal atherosclerotic lesion (R.L.W. et al, unpublished data) and appear to occur over several months in humans. This suggests that effective treatment to modulate this response would include either protracted exposure to active agents or use of agents with extended effects.

One approach to therapy directed at the restenotic process involves the deposition of medication onto or into the arterial wall via a catheter or an indwelling stent placed by use of a catheter. Several types of catheters have been used for delivery of drug solutions or suspensions into the interior of the blood vessel wall by diffusion or as a result of an applied hydrostatic pressure. These include modified angioplasty catheters with porous balloons. Medications in an aqueous medium can be infused under pressure through the multiple side holes in these devices. A difficulty with this approach is the possibility of rapid loss of efficacy because of diffusion and convection within the wall and distribution into surrounding tissues, the vasa vasorum, or the lumen. One strategy addressing this issue of drug washout is the catheter-based administration of nondiffusible microparticles that contain the therapeutic agent. The feasibility of catheter delivery of inert microparticles to the arterial wall has recently been demonstrated, thus laying the foundation for investigations focusing on the intramural delivery of microparticles incorporating active agents. This study describes the incorporation of a colchicine analogue (CA) into...
biodegradable microspheres composed of a lactic/glycolic acid copolymer and in vitro characterization of these microspheres with regard to their CA release behavior, their suitability for catheter delivery, and their effects on bovine aortic smooth muscle cells (BASMCs) in culture.

Methods

Synthesis of Poly(lactide/glycolide) Microspheres Containing Colchicine Analogue at High Loading

Microspheres were produced by a modified solvent evaporation process. Briefly, the process involves emulsification followed by solvent evaporation. The polymers chosen for pilot drug release studies included polylactides as well as several lactide/glycolide copolymers (Birmingham Polymers, Inc.). Cellular studies were performed with particles incorporating a copolymer with a lactide/glycolide ratio of 50:50 and an inherent viscosity of 0.7 dL/g (hexafluoroisopropanol, 30°C). The CA was derived from colchicine by substitution of a bulky hydrophobic side chain on the colchicine heterocyclic ring. After incorporation of the CA into the polymer matrix, the drug-containing microspheres were filtered, washed extensively with sterile water, and dried.

Determination of Total Drug Loading and Release Kinetics

The total content of the CA within the microspheres was determined spectrophotometrically by ultraviolet absorption of an appropriate weight of microspheres dissolved in chloroform, using the absorbance maximum of CA (molar absorptivity, 2.11 x 10⁴ at 386 nm). Such determinations were repeated in triplicate. The release characteristics were determined in a physiological saline solution adjusted to pH 7.4, with 1% Tween 80 (Sigma Chemical Co). Particles were suspended at concentrations ranging from 0.01 to 0.4 mg/mL and sonicated, monitoring the dispersion process with microscopic inspection.

Aliquots of this homogeneous suspension were then divided into multiple 1.5-mL tubes, which were shaken at 37°C and 200 rpm until each time point was to be taken. Individual tubes were used singly for time point determinations, which were performed by determination of the supernatant absorbance at the maximum as above after centrifugation at 14 000g.

Physical Characterization of Microspheres

Microsphere morphology was examined with a Phillips 500 scanning electron microscope operated at 25 kV with an 8-nm spot size after sputter-coating with gold/palladium alloy. Size distribution was determined with a Microtrac FRX particle analyzer (Leeds and Northrup). Particle compatibility with the catheter systems was assessed by attempting to infuse suspensions of 0.1% to 10% (wt/wt) (approximately equivalent to 2 to 200 x 10⁴ particles/mL) through representative delivery catheters while monitoring volumetric flow into open collection vessels placed at the distal end and immediately inspecting balloons microscopically for occlusions of pores.

Cell Culture

BASMCs were obtained by outgrowth from medial explants of thoracic aortas of cows within 4 hours of slaughter. Initial outgrowth as well as standard maintenance growth medium was Dulbecco's modified Eagle's medium with 10% fetal bovine serum added. Media were renewed every 2 to 3 days. All growth was in a humidified incubator equilibrated with a 5% CO₂ atmosphere. Cultures were passaged immediately before full confluence by brief exposure to Hank's balanced salt solution containing trypsin (0.5 mg/mL) and EDTA (0.5 mmol/L); all experiments were performed with cells of passage 7 or less. Cells were counted and assessed for trypsin blue exclusion with a hemocytometer at each passage and at selected times during time course experiments, routinely showing >95% of the population to exclude trypsin blue. Most subcultures and all experiments were plated at a density of 10 000 cells/cm², regardless of container. These cells exhibited
typical morphological characteristics of vascular smooth muscle in vitro, including a pattern of variably multilayered growth, and demonstrated specific immunoperoxidase staining by a monoclonal antibody selective for muscle α-actin (HHF-35), which did not react with endothelial cells and typically does not stain fibroblasts.*

**[3H]Thymidine Incorporation**

Cells were seeded as above in 24-well microtiter plates and allowed to attach overnight, after which the cultures were exposed to microspheres after sonication and subsequent dilution in standard growth medium. **[3H]Thymidine** was added to a concentration of 2 μCi/mL at 42 hours after drug exposure, and cells were harvested after 6 hours of incorporation. At the end of the incubation, cells were released from the wells, incorporated precursor was removed by washing with distilled water, and cell residues were collected on glass mesh filters by an automated cell harvester. Radioactivity was measured by liquid scintillation spectroscopy. Some experiments also used polycarbonate filter inserts (Costar) immersed in the plate wells above the cells to determine the effect of loss of direct cell/bead contact on the results.

**Results**

**Physical Properties and Release Kinetics of Microspheres**

Multiple sets of microspheres were produced with a variety of polymer-to-drug ratios and reaction conditions until an optimal set of conditions was determined that allowed the reproducible formation of both colchicine- and CA-containing particles fulfilling three criteria: (1) average diameter range from 5 to 10 μm; (2) total drug load of 15% to 17% (wt/wt); and (3) release of drug content over more than 3 to 4 weeks in sink conditions in vitro. Three consecutive reactions then yielded particles with these characteristics. Scanning electron microscopy of a representative batch of drug-loaded particles is shown in Fig 1A. These particles are spherical, with approximately 80% of total mass contributed by particles ranging in diameter from 3 to 8 μm, as shown in the histogram (Fig 1B).

During passage of several lots of particles through porous infusion catheters (Advanced Cardiovascular Systems, Inc), flow was not routinely seen to fall by more than 20% over the increasing concentration range tested (10⁴ to 10⁶ particles/mL) under an infusion pressure of 6 atm; pore occlusion was not noted in any of these studies (data not shown).

The total drug loads for three particle lots containing CA were 16%, 16%, and 17% (wt/wt). Release of the CA from these particles in vitro over the course of 1 month featured an initial burst of release of approximately 4% of the total drug load. Subsequent determinations of drug levels showed a release of 16% of the incorporated drug over the ensuing month with nearly zero-order kinetics (Fig 1C). This release, if sustained, would extrapolate to completed drug release by approximately 5 to 6 months. Drug solubility was not rate limiting in these experiments, since the CA in similar amounts dissolved from free powder within minutes of solvent contact (data not shown).

**Effects on Smooth Muscle Proliferation of Exposure to Microspheres**

Exposure to CA-loaded particles was found to be inhibitory to DNA synthesis in asynchronously dividing smooth muscle cells as measured by **[3H]thymidine incorporation**, with EC₅₀ values of 0.001 g% (wt/wt) (Fig 2A). Microspheres allowed to contact the cell surface demonstrated effects similar to those seen with microspheres suspended in the nutrient medium by porous polycarbonate filters, at EC₅₀ values approximately five-fold lower (Fig 2A). In contrast, control microspheres composed only of polymer with no incorporated active drug demonstrated no observable toxicity to BASMCs and <40% inhibition of thymidine incorporation even in suspensions containing up to 0.5 g% particles. The EC₅₀ values observed for both loaded microparticles were very similar in experiments using serum-stimulated cells (data not shown). Cell counting was used to evaluate the effects on proliferation of extended exposure to the active microspheres. This revealed substantial stasis of growth at subconfluent cell densities at particle concentrations as low as 0.0001% (wt/wt) (Fig 2D). Cell viability as assessed by trypan blue staining was approximately 80% after 7 days of incubation with microparticles at this concentration. There was no apparent effect on cell growth after 7 days of incubation with control (microparticles at any concentration up to 0.001%); cell viability in these experiments was approximately 95%.

Morphological changes suggesting microtubule depolymerization were observed after both CA particle treatment (Fig 2B) and colchicine-particle treatment (not shown) at similar EC₅₀ values, whereas cellular morphology appeared essentially unperturbed in the presence of polymer-only particles (Fig 2C). Fig 2B is taken under combination Hoffman modulation and epifluorescence excitation in the range of 480 nm with emission detection in the range of 510 nm. This additionally reveals the fluorescence of the CA incorporated within the polymeric matrix and shows it to have a microaggregated distribution, creating a substrate in the polymeric particles. Particles without drug did not demonstrate epifluorescence (not shown).

**Discussion**

Since the report by Wolinsky and Thung describing the porous balloon catheter, numerous reports using such catheters to deliver medications into the vascular wall have appeared in the literature. Despite the initial promise of this balloon, it has become apparent that successful delivery into the vascular wall is not synonymous with retention in the wall. Studies with several agents have demonstrated drug disappearance from the arterial wall within several hours to several days. Given the time course of the postangioplasty response as described above, this may explain the lack of any measurable advantage of administration of heparin, methtretaxne, or colchicine via porous balloon application in animal models of angioplasty restenosis. The intravascular implantation of stents coated with drug-eluting polymers at the time of vascular injury has likewise not yet been fruitful, probably because of elution of most of the loaded drug within hours after implantation, perhaps in conjunction with inadequate total drug loading. Conversely, some studies involving the surgical implantation of drug-eluting polymer matrices near injured blood vessels have yielded encouraging results with respect to both thrombosis and intimal proliferation. Catheter-based microparticle delivery...
offers an attractive nonsurgical alternative. These microparticles would preferably be composed of biocompatible and biodegradable materials so as to avoid inflammation and allow for gradual reabsorption. The amount of drug released over a specified time interval would be tailored to the evolving pathology by formulation of various polymer/drug combinations to yield desired total doses of medication and release kinetics. Microparticle-based, controlled-release technology of this sort is currently used clinically as a strategy to deliver a subcutaneous luteinizing hormone-releasing hormone agonist for treatment of endometriosis. As applied to angioplasty restenosis, therapy might eventually include release of drug combinations designed to take advantage of the temporal sequence of events (i.e., migration, proliferation, and extracellular matrix synthesis of vascular smooth muscle cells). In this way, different drugs targeting each step could be released at various times and rates by use of a simple mixture of the appropriate microparticles for catheter injection.

The microparticles described in this study fit initial criteria for delivery into the arterial wall in an effort to combat restenosis: the polymer matrix itself has been found not to evidence substantial toxicity with respect to smooth muscle cells, whereas the matrix/drug combination is a potent inhibitor of smooth muscle cell replication under several conditions of culture. Additionally, they are of an appropriate size to pass through the delivery balloon without undue clogging while releasing either colchicine or the CA over a time sufficient to correlate with the anticipated biological response. Few studies have described the production and activity of loaded oligodisperse microparticles in this size range; the most notable characteristic of these particles is the length of the observed time course of release. Although similar rates have been documented in larger microspheres (diameter, 40 to 250 μm), the demonstration of such protracted release in particles of the size described here is remarkable. The relatively low initial burst release and subsequent release rate observed in

Fig. 2. A, Graph showing concentration dependence of effects of drug-loaded or control (unloaded) poly(lactic acid/glycolic acid) microparticles on [3H]thymidine incorporation by subconfluent bovine aortic smooth muscle cells. Cells were exposed to particles at the indicated concentrations 42 hours before the start of a 6-hour thymidine pulse. Microspheres were either allowed to contact the cell surface or suspended in the nutrient medium by porous polycarbonate filters, as indicated. The data points represent the mean of four observations each, and the error bars the SEM. The control value in the absence of particles was 100±0.3%. Absolute numbers were typically 40 000 cpm per well for serum-stimulated controls and 3500 cpm per well for growth-arrested cells. B, Photomicrograph of subconfluent smooth muscle cell cultures exposed to microparticles containing the colchicine analogue, taken under combination Hoffman modulation and epifluorescence excitation in the range of 480 nm with emission filters passing signal in the range of 510 nm. C, Photomicrograph of subconfluent smooth muscle cell cultures in the presence of polymer-only particles. D, Graph showing concentration dependence of effect of prolonged incubation with drug-loaded vs unloaded microparticles on proliferation of bovine aortic smooth muscle cells. Cells were seeded at 10 000 cells/cm² and allowed to attach for 12 to 24 hours. Cells were then exposed to direct contact with colchicine analogue-loaded microparticles at the indicated concentrations in standard medium. Cell counts were performed at the depicted intervals. Cells incubated with unloaded microparticles at concentrations from 0.00001% to 0.001% (not shown) grew with profiles similar to those of cells incubated in the absence of microparticles (control).
these particles is consistent with results found in studies of larger microparticles demonstrating slowest release rates for compounds with low hydrophilicity.21,26,27

Given microparticles with these characteristics, the local drug concentration and thus effectiveness in an in vivo setting will be dependent on a balance between the rate of drug release and the parameters of distribution from the arterial wall after release. Local drug distribution will in turn be determined by intramural diffusion and convection. Convective fluid flux through an arterial wall increases with endothelial denudation28 or even hypercholesterolemia.29 The presence of extensive atherosclerosis, which also leads to considerable plaque neovascularization,30 may substantially change the dynamics of drug washout as well. For these reasons, extension of the studies in cell culture into well-described animal models will be critical as the next step in assessing the feasibility of local microparticle-based therapeutics of the vascular wall.

In conclusion, biodegradable microspheres have been fashioned that release a CA and inhibit DNA synthesis in smooth muscle cells. Such particles, containing agents such as these antimitotics, heparin, or possibly antisense oligonucleotides, are candidates for local delivery at sites of arterial injury to decrease restenosis.

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