Single Perivascular Delivery of Mitomycin C Stimulates p21 Expression and Inhibits Neointima Formation in Rat Arteries

Juan F. Granada, Diana Ensenat, Amit N. Keswani, Grzegorz L. Kaluza, Albert E. Raizner, Xiao-ming Liu, Kelly J. Peyton, Mohammed A. Azam, Hong Wang, William Durante

Objective—Mitomycin C (MMc) is an antibiotic that exerts a potent antiproliferative effect in tumor cells. Because the proliferation of vascular smooth muscle cells (VSMCs) plays a prominent role in the development of restenosis after percutaneous coronary interventions, the present study examined the effect of MMc on VSMC proliferation and on neointima formation after arterial balloon injury.

Methods and Results—Treatment of cultured rat aortic VSMCs with MMc (1 nmol to 30 μmol/L) inhibited smooth muscle cell (SMC) proliferation in a concentration-dependent manner. Whereas high concentrations of MMc (1 to 30 μmol/L) induced SMC apoptosis, as reflected by DNA laddering and caspase-3 activation, lower concentrations of MMc (1 to 300 nmol) directly inhibited SMC growth by arresting cells in the G2/M phase of the cell cycle. The antiproliferative action of MMc was associated with a selective increase in the expression of the cyclin-dependent kinase inhibitor p21, and with a decrease in cyclin B1-cyclin–dependent kinase-1 complex activity. Finally, the local perivascular delivery of MMc immediately after balloon injury of rat carotid arteries induced p21 expression and markedly attenuated neointima formation.

Conclusion—These studies demonstrate that MMc exerts a potent inhibitory effect on VSMC proliferation and neointima formation after arterial injury. MMc represents a potentially new therapeutic agent in treating and preventing vasculoproliferative disease.

Key Words: angioplasty ■ arteries ■ mitomycin C ■ restenosis ■ smooth muscle cells

The healing response to stent implantation starts immediately after injury, generally peaks at 2 weeks after the initial insult has occurred, and is mainly regulated by vascular smooth muscle cells (VSMCs).1 Drug-eluting stents reduce restenosis by decreasing the formation of a neointima caused by excessive cellular activation after arterial injury.4 The successful development of drug-eluting stents has relied on the controlled release of antiproliferative agents over several weeks to regulate the activation and proliferation of VSMCs.4 Accordingly, potent antiproliferative agents have the potential of decreasing the degree of neointimal formation after stent implantation of cytostatic rather than cytotoxic tissue levels can be achieved at the site of injury.

Mitomycin C (MMc) is an antibiotic agent with antiproliferative properties derived from the soil fungus Streptomyces caespiotosus.6 In addition to cancer therapy,7 MMc is a well-recognized antifibroblastic drug. In vitro studies using cultured human fibroblasts have shown that after exposure to a single dose of MMc, there is a profound inhibition of the proliferative phase of the wound-healing response, specifically through the inhibition of fibroblast replication.8 These findings have led to the successful clinical use of MMc as an adjuvant in procedures displaying excessive late-onset scar tissue formation,8–10 and suggest that MMc may be suitable as an agent to decrease neointima formation. Accordingly, we sought to evaluate the growth modulating potential of MMc on cultured rat arterial VSMCs and the impact of MMc on neointima formation in a rat model of arterial balloon injury.

Methods

Materials

MMc was purchased from Henan Kangtai Pharmaceutical Group (Zhengzhou, China). Streptomycin and serum were from Gibco BRL (Rockville, Md). Antibodies against cyclin D1, cyclin E, cyclin A, cyclin B1, p21, p27, cdk1, and nonimmune IgG were from Santa Cruz Biotechnology (Santa Cruz, Calif). A phosphorylation-specific antibody against retinoblastoma protein was from Cell Signaling Technologies (Beverley, Mass); [32P]UTP (400 Ci/mmoll) was from Amersham (Arlington Heights, Ill); γ[32P]ATP (3000 Ci mmoll) was from NEN-Dupont (Boston, Mass). All other reagents were from Sigma Chemical (St. Louis, Mo).
Cell Culture
VSMCs were isolated by elastase and collagenase digestion of rat thoracic aorta and characterized by morphological and immunologic criteria. Cells (passages 6 to 18) were serially cultured in minimum essential medium containing 10% serum; Earle’s salts, 5.5 mmol/L glucose, 2 mmol/L L-glutamine, 5 mmol/L L-Tes, 5 mmol/L Hepes and 100 U/mL streptomycin.

Proliferation and Cell Cycle Analysis
VSMCs were seeded at a density of $2.5 \times 10^4$ cells/well in 12-well plates in serum (10%)-containing media. After 24 hours, culture media were exchanged for serum-free media for an additional 48 hours. VSMCs were then treated with serum (5%) in the presence or absence of MMC. Media with appropriate additions were replenished every second day. Cell number determinations were performed after 7 days of treatment by dissociating cells with trypsin and counting cells in a Coulter Counter (model ZF; Coulter Electronics, Hileah, Fla.). For cell cycle analysis, quiescent VSMCs were treated with serum (5%) in the presence and absence of MMC for 24 hours. Cells were then stained with propidium iodide and DNA fluorescence measured in a Dickinson FACSscan flow cytometer (Franklin Lakes, NJ).

Apoptosis
Apoptosis was monitored by measuring DNA fragmentation and caspase-3 activation, as we have previously described.

Protein Analysis
VSMCs were lysed in electrophoresis buffer (125 mmol/L Tris [pH 6.8], 12.5% glycerol, 2% SDS, and trace bromophenol blue) and proteins resolved by SDS-polyacrylamide gel electrophoresis (PAGE). Blots were blocked in phosphate-buffered saline (PBS) and nonfat milk (5%) and then incubated with antibodies directed against cyclin D1 (1:500), cyclin E (1:500), cyclin A (1:500), cyclin B1 (1:500), cdki (1:100), p21 (1:500), p27 (1:300), or phosphorylated retinoblastoma protein (1:500). Membranes were then washed in PBS, incubated with horseradish peroxidase-conjugated goat anti-rabbit or rabbit anti-goat antibody, developed with commercial PBS, and bands quantified by scanning densitometry.

mRNA Analysis
Total RNA (30 μg) was loaded on 1.2% agarose gels and fractionated by electrophoresis. RNA was blot transferred to Gene Screen Plus membranes (Perkin Elmer Life Sciences) and prehybridized for 4 hours at 68°C in hybridization buffer (rapid hyb-buffer; Amer sham). Membranes were hybridized overnight at 68°C in hybridization buffer containing [32P]DNA probes (1×10^6 cpm) for p21 and 18 S ribosomal RNA. DNA probes were labeled with α-[32P]dCTP using a random priming kit (Amersham). After hybridization, membranes were washed, exposed to X-ray film, and p21 mRNA levels measured in a Dickinson FACSscan flow cytometer (Franklin Lakes, NJ).

Cyclin B1-Cdk1 Complex Activity
VSMCs were collected in lysis buffer (150 mmol/L Tris [pH 7.5], 200 mmol/L NaCl, 2.0 mol/L EDTA, 2.0 mmol/L EGTA, 10% glycerol, 0.1% Tween-20, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mmol/L PMSF, 1 mmol/L Na3VO4, 50 mmol/L NaF, and 1 mmol/L DTT) and sonicated on ice. Protein (300 to 500 μg) was immunoprecipitated using protein A agarose beads, anti-cyclin B (1 μg) or nonimmune IgG (1 μg), and lysate buffer overnight at 4°C. Agarose beads were washed sequentially with lysis buffer and kinase buffer (50 mmol/L Tris [pH 7.5], 10 mmol/L MgCl2, 1 mmol/L DTT, and 1 mmol/L Na3VO4), and then incubated with kinase buffer, histone H1 (2.0 μg; Roche, Indianapolis, Ind), ATP (50 μmol/L), and γ-[32P]ATP (6 μCi) for 20 minutes at 30°C. Reactions were terminated by adding Laemmli buffer and boiling samples for 5 minutes. Proteins were separated by SDS-PAGE, fixed and exposed to X-ray film. Lysates immunoprecipitated with nonimmune IgG did not express kinase activity, confirming the specificity of the assay.

Animal Experiments
Sprague-Dawley rats (400 to 450 grams; Charles River Laboratories, Wilmington, Mass) were anesthetized (ketamine, xylazine, acepromazine; 0.5 mL/kg, intramuscularly; VetMed Drugs, Houston, Tex) and the left common carotid artery injured with an embolome- tomy catheter, as we previously described. Immediately after balloon injury, a local perivascular polymer-based delivery system was used to administer MMC to the injured vessel wall in one group of animals (n=8). A separate cohort of animals received an empty gel (n=8). Previous studies from our laboratory and others confirmed that the empty gel has no effect on neointima forma-
tion. The delivery system consisted of 250 μg of a 25% copolymer gel (PLF127; BASF Corporation, Florham Park, NJ) containing MMC at a concentration of 1 μg/mL (maximal saturation load of the polymer) that was topically applied in a circumferential manner to the exposed adventitia of the carotid artery. PLF127 gels exhibit reverse thermal behavior remaining liquid at refrigerator temperature, but becoming soft gels at body temperature. In vitro release experiments using this gel have indicated that PLF127 acts as a rate-controlling barrier serving as a vehicle for sustained-release preparations of MMC; releasing an accumulative amount of drug over a 3-hour period of ~5.5% from a PLF127–mitomycin C gel containing 25% of PLF127.14 Based on this study, we estimated that the delivered dose at 3 hours was 13.75 μg.

Histology
Two weeks after arterial injury, all animals were euthanized and the vasculature perfusion-fixed with 10% buffered formalin. The com-
mmon carotid artery was excised, paraffin-embedded, and sections (5 μm) stained with Verhoffs–Van Gieson for measurement of vessel dimen-
sions. Microscopic quantitation of vessel dimensions was performed using Zeiss Image 3.0 (Media Cybernetics) and Adobe Photoshop 6.0 software systems linked through a charge-coupled device (CCD) color camera (Leaf Microlumina; Leaf Systems, USA) to a Zeiss Axioskop 50 light microscope (Carl Zeiss, Germany), as we have previously described. Apoptosis was also monitored by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining.

Statistics
Results are expressed as the mean±SEM. Statistical analysis was performed with the use of a Student 2-tailed t test, and an analysis of variance with the Bonferroni post-hoc test when >2 treatment regimens were compared. P<0.05 was considered statistically significant.

Results
Dose Response Effect of MMC on VSMC Proliferation
Treatment of VSMCs with MMC inhibited serum-stimulated proliferation in a concentration-dependent manner (Figure 1). VSMC growth inhibition was evident at a dose of 3 nmol (22% inhibition) and doses >100 nmol resulted in near-total aboli-
tion of cell growth. VSMC proliferation was inhibited by ~50% at MMC concentration of 10 nmol. MMC concentra-
tions <1 μmol/L did not stimulate DNA laddering (Figure IA, available online at http://atvb.ahajournals.org). In contrast, doses of MMC of 1 μmol/L and higher (1 to 30 μmol/L) induced cell rounding and blebbing (data not shown) that was associated with pronounced DNA fragmentation and a marked increase in caspase-3 activity (Figure IA and IB).

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Effect of Nonapoptotic Doses of MMc on Cell Cycle Progression

The effect of lower, nonapoptotic concentrations of MMc on cell cycle progression was determined by flow cytometry. Subconfluent VSMCs accumulated in the G0/G1 phase of the cell cycle after serum deprivation for 48 hours. Administration of serum for 24 hours induced cell cycle progression and the population of cells in G0/G1 decreased, whereas the number of cells in S and G2/M increased (Figure 2). However, MMc (3 to 100 nmol) induced a concentration-dependent increase in the fraction of cells in G2/M and this was accompanied by a decrease in the percentage of cells in G0/G1 (Figure 2A). At a 100 nmol MMc concentration, ∼2-fold increase of VSMCs in G2/M was observed (Figure 2B). No apparent toxicity with these lower doses of MMc was noted as reflected by the lack of a sub-G0/G1 fraction (Figure 2A).

To elucidate the mechanism by which MMc disrupts cell cycle progression, we examined the effect of this drug on cell cycle regulatory proteins. MMc failed to inhibit the serum-stimulated expression of the G1 cyclins, cyclin D1, E, A, the phosphorylation/activation of retinoblastoma protein, or the serum-mediated downregulation of the cdk inhibitor, p27 (Figure 3). In fact, MMc enhanced the expression of cyclin E (Figure 3). Interestingly, MMc potentiated the serum-stimulated expression of p21 protein (Figure 3), and this was associated with a time-dependent increase in p21 mRNA (Figure 4A and 4B). MMc also induced p21 expression under serum-free conditions (Figure 3 and 4A and 4B). Because the progression of cells through G2 and entry into mitosis is under the control of the cyclin B1–cdk1 complex,20 the effect of MMc on the activity of this complex was also examined. We found that MMc potently inhibited the serum-stimulated activity of the cyclin B1–cdk1 complex while having no effect on the expression of cyclin B1 or cdk1 protein (Figure 4C and 4D).

Impact of MMc on Neointima Formation

The effect of a single-use delivery of MMc on neointimal proliferation was investigated in an in vivo rat model of carotid artery injury. Figure 5A depicts representative cross-sections of perfusion-fixed, Verhoffs–Van Gieson-stained tissues 2 weeks after injury. Two weeks after initial injury, the maximal neointimal thickness was 0.046±0.009 mm in the MMc group versus 0.100±0.021 mm in the control group (P<0.05) (Figure 5B). Morphometric analysis further re-
revealed that intimal area was 0.079±0.017 mm² in the MMc group versus 0.169±0.030 mm² in the control group (P<0.05) that represents a 50% reduction in neointima formation in vessels treated with the MMc-containing gel (Figure 5B). In contrast, despite the fact the intima/media area ratio was also reduced by 50% (0.393±0.30 in the MMc group versus 0.780±0.138 in the control group, P<0.05), the medial area was not changed in MMc-treated segments (0.127±0.007 mm² in the MMc group versus 0.126±0.008 mm² in the control group) (Figure 5B). In the MMc-treated vessels, there was no evidence of vascular toxicity including fibrin deposition, excessive infiltration of inflammatory cells, or tissue necrosis. TUNEL staining evaluation for evidence of apoptosis revealed no difference in the rate of apoptosis in empty gel-treated vessels (2.1±0.4%) compared with MMc-treated vessels (2.5±0.3%). Finally, perivascular application of MMc stimulated the expression of p21 protein in balloon-injured arteries (Figure 5C).

**Discussion**

The present study demonstrates that MMc is a potent inhibitor of VSMC proliferation and neointima formation. These inhibitory actions of MMc are associated with the induction of the cyclin-dependent kinase inhibitor, p21, and the arrest of VSMCs in the G2M phase of the cell cycle. These findings suggest a potentially important therapeutic role for MMc in treating occlusive vascular disease.

Although MMc exerts a powerful antiproliferative effect in tumor cells and fibroblasts, its action on VSMC growth has not been fully characterized. In the current study, we found that MMc is a robust inhibitor of VSMC proliferation. Analysis of cell cycle distribution indicates that MMc inhibits cell cycle progression by specifically arresting VSMC in the G2M phase. Previous work established that the cyclin B1–cdk1 complex functions as a master mitotic regulator and is the ultimate target of most agents that prevent mitotic entry. Consistent with such a hypothesis, we found that MMc-

**Figure 3.** Effect of mitomycin C (MMc) on the expression of cell cycle regulatory proteins. A, Effect of MMc on the expression of cyclin D1, E, and A, p21, and p27 protein, and the phosphorylation of retinoblastoma protein (pRb-P). VSMCs were treated with serum (5%) for 24 hours in the presence or absence of MMc (100 nmol). B, Quantification of protein expression by scanning densitometry relative to serum-free controls. Results are means±SEM of 4 separate experiments. *Statistically significant effect of serum. †Statistically significant effect of MMc.

**Figure 4.** Effect of mitomycin C (MMc) on p21 mRNA expression and cyclin B1–cdk1 complex activity. A, VSMCs were treated with MMc (100 nmol) for various times (0 to 24 hours) and mRNA levels determined by Northern blotting. B, Quantification of p21 mRNA levels by scanning densitometry relative to serum-free controls. VSMCs were incubated with or without serum (5%) in the presence and absence of MMc (100 nmol). C, VSMCs were treated with MMc (100 nmol) for 24 hours and cyclin B1–cdk1 complex kinase activity determined as described in Methods section, whereas cyclin B1 and cdk1 protein levels were monitored by Western blotting. D, Quantification of cyclin B1–cdk1 complex kinase activity and protein expression by scanning densitometry. VSMCs were incubated with or without serum (5%) for 24 hours in the presence and absence of MMc (100 nmol). Results are means±SEM of 4 separate experiments. *Statistically significant effect of serum. †Statistically significant effect of MMc.
induced G2-M arrest is associated with a marked decrease in cyclin B1-cdk1 complex kinase activity that occurs independent of any change in the level of either cyclin B1 or cdk1. However, MMc selectively increases the expression of p21, which has been shown to promote G2-M arrest and block the activation of the cyclin B1-cdk1 complex.24,25 Thus, it is likely that the elevation in p21 expression contributes to the inhibition of cell cycle progression and VSMC growth by MMc. Although p21 can also mediate G1 arrest by inhibiting the activity of the G1 cdk's,26 flow cytometry experiments did not detect G1 arrest by MMc. Moreover, MMc had no effect on the phosphorylation of retinoblastoma protein, a key event required for S phase entry. Failure of p21 to inhibit G1 to S phase transition following MMc treatment may be due to the delayed induction of p21 expression or, alternatively, the inhibitory activity of p21 on G1 cdk's may be counteracted by increases in the expression of G1 cyclins, such as cyclin E. Interestingly, induction of p21 by MMc has also been reported in other cell types and may provide a general mechanism by which this agent inhibits cell growth.27,28 The mechanism by which MMc stimulates p21 expression is not known but it may involve the induction of p53, which is a known inducer of p21.28

The inhibitory effect of MMc on VSMCs is dose-dependent. A threshold effect on cell growth by MMc is observed at 3 nmol (22% inhibition) and is almost complete at 100 nmol (97.5% inhibition) without causing cell death. Only MMc concentrations of 1 μmol/L or higher induce apoptosis, as reflected by DNA fragmentation and caspase-3 activation. The almost 10-fold difference between cytostatic and cytotoxic dose provides a desirable therapeutic window needed to prevent local tissue toxicity if the drug is to be delivered locally. In our studies, the ID50 for MMc was ~10 nmol, which is comparable to the previously published data on paclitaxel and sirolimus.29,30

Several features make MMc a suitable candidate as a therapeutic agent to decrease restenosis after stent implantation: (1) MMc requires biological activation that is more favorably under anoxic conditions, this is an important feature considering that stent implantation occurs in an oxygen-deprived milieu;31 (2) the biological effect of a cytostatic dose of MMc is maintained for >3 weeks in cultured human fibroblasts after the initial dose is given to the cells;32 and (3) with the broad effective dose window suggested by the present study, it is plausible that the undesirable cytotoxic effects of the drug could be avoided if the right cytostatic dose is delivered to the tissue.

We further examined the effect of MMc on vascular remodeling using the well-established rat carotid artery balloon injury model. This model is characterized by its high degree of reproducibility and is associated with the development of a VSMC-rich intimal lesion. The dose of MMc chosen in the study was based on the range of doses (100 to 400 μg/mL) successfully used in clinical studies to ameliorate the wound-healing response in various pathological settings.33-36 In our series of experiments, a single-dose approach applying MMc topically to the adventitia of the blood vessel was used with a specific local delivery copolymer. Our laboratory and others have successfully used this approach to delivery drugs and oligonucleotides to the vessel wall.14-17,19,37-38 This local delivery system allows for the sustained release of MMc over the course of several days and avoids possible nonspecific effects associated with the systemic administration of the drug. We found that local application of MMc markedly attenuates neointima formation after arterial injury which is associated with the induction of p21 expression in the vessel wall. The inhibition of intimal thickening by MMc is independent of any overt signs of toxicity or with an increase in apoptosis, suggesting that vessel wall MMc levels were below the cytotoxic range. Of note, in one early study,39 MMc failed to block neointima formation in balloon-injured rabbit iliac arteries. However, this study used only a single acute intraluminal infusion of MMc into the arterial wall, and the rate and extent of MMc penetration into the vessel wall was not determined. A sustained delivery of MMc may be necessary to inhibit the vascular remodeling response following balloon injury.

In conclusion, the present study demonstrates that MMc is a potent inhibitor of VSMC proliferation with a broad range of cytostatic doses, and that it markedly attenuates neointima formation after arterial balloon injury in rat carotid arteries. In addition, it shows that MMc arrests VSMCs in the G1-M phase of the cell cycle and that this is associated with an increase in p21 expression and a decrease in cyclin B1-cdk1 complex activity. Thus, MMc represents an attractive therapeutic agent for attenuating the vascular response to injury, and may also

Figure 5. Effect of mitomycin C (MMc) on neointima formation and p21 expression after rat carotid artery injury. A, Representative cross-sections (magnification ×100) of injured carotid arteries treated with an empty gel (E) or a gel containing MMc (250 μg) 2 weeks after injury. B, Morphometric analysis of neointima formation 2 weeks after arterial injury. Results are means ± SEM of 8 experiments. *Statistically significant effect of MMc. C, Western blot of p21 protein expression in control, uninjured carotid arteries, or in vessels 1 day after balloon injury in the presence or absence of a gel containing MMc (250 μg/mL).
prove efficacious in preventing restenosis when eluted from the surface of a stent.

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References
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Figure I. Effect of mitomycin C (MMc) on VSMC apoptosis.  A. Effect of MMc (0.1 – 30 µM) on DNA laddering.  Data are representative of three separate experiments. B. Effect of mitomycin C (1-30 µM) on caspase-3 activity.  Results are means ± SEM of three separate experiments. *Statistically significant effect of MMc.