Marimastat Inhibits Neointimal Thickening in a Model of Human Arterial Intimal Hyperplasia


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Objective: matrix metalloproteases (MMPs) produced by vascular smooth-muscle cells (VSMCs) degrade extracellular matrix and facilitate the migration of these cells. This is a fundamental process in arterial intimal hyperplasia. This study investigated whether Marimastat (a selective but non-specific MMP inhibitor) can prevent intimal hyperplasia in cultured human internal mammary artery (IMA).

Materials and methods: segments of IMA from 8 patients were prepared and cultured for 14 days in serum-supplemented medium (control) or in medium supplemented with Marimastat at 2 concentrations (treatment groups). The tissue was fixed, sectioned, stained and neointimal thicknesses measured by computer-aided image analysis. Further sections were cultured in the same manner and prepared for gel enzymography to quantify the production of MMPs.

Results: neointimal thickness was significantly reduced by Marimastat in a dose-dependent manner when compared to controls (p = 0.008 Wilcoxon). Gel enzymography demonstrated a reduction in levels of MMP2 and MMP9. This was most significant for the active forms of the enzymes (p = 0.03).

Conclusions: our results suggest that there is a potential therapeutic role for specific inhibition of the gelatinases in the prevention of human arterial restenosis.

Key Words: Intimal hyperplasia; Marimastat; Metalloproteases; Restenosis.

Introduction

Restenosis caused by intimal hyperplasia is the most common cause of failure of vascular intervention within the first postoperative year. Intimal hyperplasia (IH) is characterised by the migration of medial vascular smooth-muscle cells (VSMCs) through the internal elastic lamina into the arterial intima and their subsequent proliferation to produce the intimal hyperplastic lesion. The lesion also consists of extracellular matrix (ECM) which must be degraded to allow for VSMC migration. Vascular smooth-muscle cells are able to produce and secrete the necessary proteases to degrade all components of the ECM.

Matrix metalloproteases (MMPs) are a family of zinc-dependent enzymes, which are the principal regulators of the ECM. They are produced in latent form by a range of cell types including inflammatory cells, fibroblasts and VSMCs. They are all secreted as proenzymes, except the membrane type metalloproteases (MT-MMPs), which have a transmembrane domain and are an integral part of the plasma membrane. Metalloprotease latency is ensured by the presence of a prodomain, with activation occurring by limited proteolysis. In vivo, plasmin acts as the principal activator of MMPs promoting cleavage of the latent propeptides. Metalloprotease activity is regulated at several levels. A number of growth factors and cytokines have been shown to either stimulate or inhibit the synthesis of MMPs by regulating transcription.

Specific tissue inhibitors of metalloproteases (TIMPs) are secreted by the same cells and are important in establishing a balance between matrix synthesis and degradation. Matrix metalloproteases are classified according to the specific substrate that they degrade. The arterial basement membrane consists predominantly of type IV collagen. This is degraded by the MMPs, gelatinase A (MMP-2) and gelatinase B (MMP-9). These gelatinases are required by the VSMCs to facilitate their progress from media to intima in the progression of IH. Southgate et al. demonstrated that migration and proliferation of VSMC explants from rabbit artery was inhibited by MMP inhibitors. Other studies have suggested a role for...
MMPs in the remodelling which occurs after arterial angioplasty in the rat and therefore in the restenotic process. Both increased gelatinase secretion and mRNA production have been demonstrated in post-angioplasty tissue in animal models. Although there is evidence for the role of MMPs in IH in animal models, there is a paucity of data regarding human arteries. The aim of this study, therefore, was to investigate the role of MMPs in the production of IH in an in vitro model of human arterial intimal hyperplasia.

Methods

Culture methods

Sections of internal mammary artery (IMA) were obtained from eight consecutive patients undergoing coronary artery bypass grafting. They were prepared for organ culture by a method previously described. Briefly, segments were cleaned of their fat and adventitial pedicle and divided into 3 segments of approximately 5-mm length. Each of these was then opened longitudinally and laid, endothelial surface uppermost, in a culture dish containing pre-cast Sylgard resin (Dow Corning, Seneffe, Belgium) to which the sample was pinned. To each dish was added 5 ml of standard culture medium (RPMI 1640, 30% foetal calf serum (v/v), penicillin 50 U/ml, streptomycin 50 μg/ml, and L-glutamine 2 mmol/l). One segment served as a control specimen, the other two being the treatment groups. In these groups the culture medium was supplemented with Marimastat at 10-6 M and 10-5 M concentrations. The samples were maintained in a humidified cell culture incubator at 37°C with 5% CO2 in air for 14 days. The medium and drug were renewed every 2-3 days. At the end of the 14-day culture period each sample was fixed in 4% paraformaldehyde for 18 hours, processed, paraffin-embedded and sectioned to 4-μm thickness.

A further six samples from consecutive patients were prepared in exactly the same manner as above, and after completion of a 14-day culture period each specimen was immediately snap-frozen in liquid nitrogen and stored at −80°C for subsequent processing and metalloprotease extraction.

Immunohistochemistry

Sections of 4-μm thickness were stained using a combined monoclonal anti-smooth-muscle actin/Miller’s elastin stain in order to identify the layers of the arterial wall. An average of 30 measurements of neointimal thickness were made on each of 2 consecutive sections of each sample using a computer-aided image analysis system (Improvision, Coventry, U.K.) as previously described.

Gelatin enzymography

Samples from six patients were prepared for metalloprotease extraction in a manner previously described. Frozen tissue samples were thawed over ice, weighed, diced into pieces of approximately 1 mm2 and then homogenised in 1 ml of buffer per 1 mg of tissue (urea 2 mM, Tris-HCl 50 mM pH 7.6, NaCl 137 mM, EDTA 1 g/l, Brij-35 1 ml/l and PMSF (a serine protease inhibitor) 0.1 mM – all supplied by Sigma, Poole, U.K.).

The homogenate was then centrifuged at 11,000 g for 1 hour at 4°C and the resultant supernatant dialysed against a dialysing buffer (Tris-HCl 25 mM pH 8.5, CaCl2 10 mM, Brij-35 1 ml/l, PMSF 0.1 mM) for 18 hours at 4°C using 10 kDa Visking tubing (Merck, U.K.). The protein content of each sample was determined using spectrophotometry and the samples standardised. Equivalent protein loads were resolved on a non-reducing SDS–polyacrylamide gel impregnated with 1 mg/ml gelatin. Conditioned medium from the HT 1080 human fibrosarcoma cell line was loaded onto one lane of each gel to act as a positive control, and protein standards (BioRad, Hemel Hempstead, U.K.) were loaded onto another lane for estimation of the molecular weights of each band. After completion of the separation, SDS was removed by incubation in three consecutive washes of 2.5% Triton X-100 (3 x 15 min) and the gels were then incubated at 37°C for 18 h in incubation buffer (50 mM Tris-HCl, 10 mM CaCl2 and 0.05% Brij-35, pH 7.4). Inhibition of protease activity was used to confirm the nature of the protease being detected on the zymograms. EDTA (20 mM), 1,10 phenanthroline (5 mM) or PMSF (1 mM) was included in the incubation buffer to verify the presence of specific MMPs. Following incubation, the gels were fixed and stained in 0.1% Coomassie blue R250 (in 50% methanol/20% acetic acid/30% double-distilled water) for 2-3 hours. Proteinases were visualised as clear bands of lysis against a dark background of substrate. The molecular weight of each band was estimated by comparison against positions of known molecular weight standards. The relative density of each band was determined from negative photographic images of the gels with a Pharmacia LKB
Imagemaster scanning densitometer (Pharmacia LKB, St Albans, Herts., U.K.) and was expressed as a product of the optical density and area of the band. Previous experiments (data not shown) demonstrated that the protein concentration used in this analysis was within the range for densitometric quantification. Paired treatment samples and their controls were run on the same gels. The product of the optical density and area of the band was compared to the HT 1080 control (same sample for each gel) to obtain a ratio that could be compared between gels. No direct comparison was made between different gels. Western blotting experiments (data not shown) confirmed that the bands corresponded to MMP-2 and MMP-9.

**Statistics**

Measurements of neointimal thickness are expressed as median and 95% CI. Differences between treatment groups were analysed using the Wilcoxon paired-rank test, and significance was assumed at the 95% confidence level. For the zymographic analysis, the relative density was quantified and compared between samples run on the same gel. Values are expressed as median and 95% CI as a percentage of HT 1080 control value. Differences between groups were analysed using the Wilcoxon paired-rank test. All statistical analyses in this study were performed using the computer software package SPSS 8.0 for Windows.

**Results**

**Neointimal thickness**

During the 14-day culture period, all control specimens developed a significant neointimal layer with a median thickness of 5.5 (95% CI 4.2–7.1) μm. Median neointimal thickness in artery treated with Marimastat at $10^{-5}$ M was 0.75 (95% CI 0.5–1.2) μm, ($p=0.008$). At $10^{-4}$ M concentration, the median neointimal thickness was 1.7 (95% CI 1.3–2.7) μm ($p=0.008$). These results are summarised in Figure 1. A representative histological section of internal mammary artery cultured in the absence and presence of Marimastat at each concentration is shown in Fig. 2(a and b). CD 31 staining (Fig. 2c) confirmed the retention of an intact endothelium on the arterial segments after the culture period.

**Gelatin zymography**

Densitometric values are expressed as a percentage of control (HT 1080 cell line). For latent MMP-9, the median value for the control group was 100% (95% CI 89–111). For Marimastat at $10^{-5}$ M, the median value was 70% (95% CI 58–91) ($p=0.05$). At $10^{-4}$ M, the median value was 69% (95% CI 45–90) ($p=0.03$). For active MMP-9, the median value for the control group was 100% (95% CI 73–151). At $10^{-5}$ M Marimastat the median value was 42% (95% CI 21–64) ($p=0.03$) and at $10^{-6}$ M the median was 48% (95% CI 30–64) ($p=0.03$).

For latent MMP-2, the median densitometric value for the control group was 109% (95% CI 83–126). For Marimastat at $10^{-5}$ M, the median value was 89% (95% CI 54–117) ($p=0.03$) and at $10^{-6}$ M the median value was 85.7% (95% CI 61.9–101.2) ($p=0.03$). For active MMP-2, the median value for the control group was 139% (95% CI 127–161). At $10^{-5}$ M Marimastat, the median value was 68% (95% CI 41–85) ($p=0.03$) and at $10^{-6}$ M the median value was 74% (95% CI 64–84) ($p=0.03$). These results are summarised in Figure 3.

A representative gelatin zymogram for segments of internal mammary artery cultured both with and without Marimastat at each concentration is shown in Figure 4 within the molecular weight range 60–100 kDa. No other bands outside this range were detected (up to 250 kDa).
Fig. 2. Histological sections of cultured internal mammary artery stained with a combined smooth-muscle actin and Miller’s elastin stain, showing the development of a neointima (arrowed) in representative segments from (a) control, and (b) Marimastat-treated artery. IEL = internal elastic lamina, M = media, A = adventitia. CD 31 staining (c) demonstrates the presence of intact endothelium at the end of the culture period (arrow).
Fig. 3. Scatter plots showing the protease activity of cultured IMA homogenates for (a) MMP-9 and (b) MMP-2. Horizontal bars represent the median and 95% confidence intervals.

Fig. 4. Representative gelatin zymogram for paired control and Marimastat-treated arterial segments. Fibrosarcoma-derived HT-1080 cells served as positive controls.

**Discussion**

The study clearly demonstrates that Marimastat, in therapeutic doses, is able to significantly reduce the formation of neointima in this laboratory model of human arterial IH. This attenuation in the thickness of the neointima is accompanied by a reduction in the tissue levels of MMP-2 and MMP-9. Previous work in this laboratory in cultured human saphenous vein has demonstrated that Marimastat reduces both neointima formation and the tissue levels of both latent MMP-2 and MMP-9 detected by gel zymography. Production of MMPs was detected by immunohistochemistry and found to be predominantly localised to the endothelium and neointimal smooth-muscle cells, and in the smooth-muscle cells adjacent to the internal elastic lamina.27

Southgate et al.28 demonstrated increased levels of MMP-2 and MMP-9 in arterial tissue damaged by balloon angioplasty in pigs compared with untreated control segments. Similar findings have also been demonstrated in a rat model of angioplasty.19 However, although the use of peptide MMP inhibitors reduced MMP activity in the rat model, neointima formation...
after a 2-week period was not significantly attenuated. This catch-up phenomenon was not observed in our study, perhaps because of the time period involved, but possibly because the human tissue culture lesion is more akin to the human arterial restenosis lesion in being less cellular and containing more ECM than the lesion produced in rat arterial injury models. Several studies support this view. Post-mortem studies of human restenosis specimens after PTCA suggest that the ECM plays a major role in restenotic lesions and Schwartz et al. have demonstrated that cellular elements account for only 11% of the neointimal volume of human restenotic lesions. Re-injury models provide a more valid model, Strauss et al. showed that ECM accumulation after injury. I. Smooth muscle cell growth in the absence of extracellular matrix active and generally well tolerated. Side-effects include muscle and joint pain affecting 30% of patients after 5 months of taking a dose of 10 mg twice daily. This has been shown to be the maximally effective dose. These side-effects are reversible on withdrawal of the drug. Marimastat inhibits MMPs by binding to their active site, thus rendering them inactive. This does not explain why a reduction in MMP levels was observed in both latent and active forms. Previous studies have demonstrated an altered balance between latent and active forms of MMPs after arterial injury and that reduced levels of MMPs can be found in human atherectomy specimens post-PTCA compared with control tissue. This was associated with increased levels of TIMPs. Due to the small sample size involved in our study, measurement of TIMPs was not possible.

Although Marimastat is an inhibitor of MMPs, suggesting that its principal mode of action is to inhibit SMC migration, this study as it stands is unable to differentiate between the events of migration and proliferation. Experiments are currently in progress using migration assays of isolated arterial SMC in response to a chemotactic stimulus, to further elucidate the mode of action of Marimastat. In conclusion, this study suggests that Marimastat may be an attractive agent for clinical trials investigating the effect of MMP inhibition on arterial restenosis in the clinical setting.

Acknowledgements

The authors would like to thank British Biotechnology Pharmaceutical Ltd (Oxford, U.K.) for the gift of Marimastat.

References


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Accepted 22 November 1999