Ischaemia–reperfusion injury activates matrix metalloproteinases in the human heart

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Aims Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) regulate matrix remodelling in the heart and play a pivotal role in myocardial dysfunction immediately following ischaemia–reperfusion injury ex vivo in rats. We investigated the changes in MMPs and TIMPs in acute myocardial ischaemia–reperfusion injury in humans.

Methods and results Fifteen patients with stable angina undergoing coronary artery bypass graft surgery with cardiopulmonary bypass were enrolled. Left ventricular stroke work index was monitored prior to bypass and for 24 h following reperfusion. Left atrial biopsy samples were obtained at the start of bypass before cardioplegia and within 10 min after removal of the aortic cross-clamp. Plasma samples were collected from the radial artery and coronary sinus 1, 5, and 10 min following removal of the cross-clamp. In cardiac biopsies there was a marked increase in 72 kDa MMP-2 and 92 kDa MMP-9 activities, and a decrease in TIMP-1 upon reperfusion. Increased MMP activity correlated positively with cross-clamp duration and inversely with cardiac mechanical function 3 h following reperfusion. TIMP-1 correlated inversely with cross-clamp time and positively with cardiac mechanical function. Plasma samples revealed a significant increase in both 92 kDa MMP-9 and 64 kDa MMP-2 activities 1 min following removal of cross-clamp.

Conclusion Reperfusion following cardioplegia activates MMPs in the myocardium and plasma of patients undergoing coronary artery bypass grafting. This is the first correlation of MMP myocardial activity with cardiac function in humans. The early increase in MMP activity produces a proteolytic environment that may contribute to myocardial stunning injury in humans.

Introduction

Coronary artery bypass graft (CABG) surgery with cardiopulmonary bypass (CPB) is associated with stunning injury following reperfusion of the ischaemic myocardium.
Cellular mechanisms proposed to explain this reversible impairment of cardiac mechanical function include alterations in cardiac metabolism, production of reactive oxygen species, and activation of myocardial proteinases.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases which are synthesized as zymogens. Of particular interest in the heart are the gelatinases MMP-2 (72 and 62 kDa) and MMP-9 (92 and 84 kDa) as they are found in cardiac myocytes, cardiac fibroblasts, and endocardial cells. Their activity is regulated by the tissue inhibitors of metalloproteinases (TIMPs), four of which have been identified thus far in humans. They all inhibit a broad variety of MMPs.

MMP-2 and -9 are well recognized for their proteolytic action on extracellular matrix proteins and their involvement in long-term remodelling processes. These occur in several physiological as well as pathological events such as heart failure and atherosclerosis. However, recent data implicate MMP-2 in a variety of acute physiologically relevant processes involving proteolysis of novel substrates unrelated to the extracellular matrix. These include platelet aggregation, regulation of vascular tone, and modulation of the inflammatory response.

Stunning injury following reperfusion of the ischaemic myocardium is another acute process in which MMPs have been implicated. We have shown that MMP-2 is directly involved in acute stunning injury seen during reperfusion following ischaemia in isolated rat hearts. During reperfusion, MMP-2 is activated intracellularly and cleaves the contractile protein regulatory element troponin I. MMP-2 is also released from the rat heart during acute reperfusion, probably to abrogate the extent of injury, and inhibition of MMP activity significantly improves cardiac function during reperfusion. These actions occur on a rapid time scale (seconds to minutes) where MMPs are thought to act as signalling proteinases to impair cardiac mechanical function.

A previous study demonstrated increased MMP-2 and -9 activities in the human heart following CPB, although the time of atrial biopsy sampling and the ischaemia time were not considered. Therefore our aim was to determine, in the setting of CABG with CPB, the changes in myocardial and plasma MMPs and TIMPs within 10 min of reperfusion and to determine whether these correlate with changes in acute post-ischaemic myocardial function.

Methods

Patient population

Fifteen patients with stable angina undergoing elective CABG surgery were recruited at the San Rocco Hospital between September 2000 and January 2001 (nine patients) and between July 2002 and September 2002 (six patients). The second cohort of patients was recruited so that the total sample size reflected other studies that have analysed MMPs in human cardiac tissue. This study was approved by the local institutional ethics committees. Exclusion criteria were: (i) reduced ejection fraction; (ii) previous myocardial infarct; (iii) enlargement or hypertrophy of cardiac chambers; (iv) concomitant valvular diseases; (v) arrhythmia; (vi) metabolic disorders; and (vii) concomitant liver, pulmonary, or kidney diseases. All patients received calcium channel blockers and ß-blockers and all oral medication was discontinued 2 days prior to surgery. All patients underwent multiple isolated aorto-coronary bypass grafts with internal mammary arteries and saphenous veins (n = 2–4).

Surgical procedure

In all patients, anaesthesia and muscle relaxation were induced with propofol (2.0–2.5 mg/kg) and atracurium (0.08 mg/kg), respectively. Propofol was continuously infused (4–6 mg/kg/h) in order to maintain anaesthesia. Ventilation was controlled with 50% O2 in air. Before sternotomy, an 18 gauge cannula was placed in the radial artery for arterial sampling. A Swan-Ganz catheter was introduced through the left jugular vein for haemodynamic measurements. After median sternotomy, the aorta, inferior vena cava, and coronary sinus were cannulated and heparin (3 mg/kg) was administered. CPB was conducted with non-pulsatile flow at a rate of 2.4 L/min/m². The aorta was cross-clamped and intermittent antegrade and retrograde warm blood cardioplegia was used in all patients as previously described. Cardioplegia was initiated through the infusion of whole blood (flow rate 300 mL/min) plus 20 mEq/L of K⁺ (flow rate 2 mL/h). The cardioplegic solution was injected at 37°C for 2 min into the aortic root and then into the coronary sinus. This dose of cardioplegic solution was injected every 15–20 min. CPB was maintained with moderate haemodilution (haematocrit 24–33%).

After completion of distal anastomoses, the aortic cross-clamp was removed and the construction of the proximal anastomoses was begun. At the end of the grafting procedure, protamine (3 mg/kg) was injected to reverse the effect of heparin. Inotropic drugs were not used.

Haemodynamic measurements

Left ventricular stroke work index (LVSWI) was used as a measure of global left ventricular function. Haemodynamic measurements were performed in the operating theatre before starting CPB, and 1, 3, 6, 12, and 24 h after cross-clamp release.

Tissue and blood samples

Tissue biopsy samples were sampled by one surgeon only. Immediately after the start of CPB, but before cardioplegia, a biopsy sample (~15 mg wet weight) was obtained from the right atrium. Ten minutes after aortic cross-clamp release, another biopsy was taken as close as possible to the previous one. No clinical complications resulted from sample collection. Biopsies were immediately frozen (liquid N2) in the operating theatre and stored at −80°C. Myocardial extracts were prepared as previously described.

Before surgery, 4 mL fresh venous blood was collected from the antecubital vein; during surgery, 4 mL radial artery (arterial) and coronary sinus (venous) blood were collected simultaneously before CPB and 1, 5, and 10 min after cross-clamp release. Blood samples were immediately centrifuged (3000 g, 10 min, 4°C). The plasma fraction was then removed, frozen in liquid N2, and stored at −80°C. Protein concentrations were assessed by the bicinchoninic acid assay (Sigma) with bovine serum albumin as the standard.
Measurement of MMP-2 and -9 activities by zymography

To determine MMP-2 and -9 activities, 20 µg of either myocardial extract or plasma were analysed by gelatin zymography as described.17,24 Intensities were normalized to an internal standard (supernatant from HT-1080 human fibrosarcoma cell culture) loaded in each gel.

Collagenase activity

Collagenase activity (MMP-1, -8, and -13) was determined in 50 µg of myocardial homogenates using a collagenase activity kit (ECM 710, Chemicon). The samples, however, were not chemically treated to activate latent collagenases.

Western blot analysis

An aliquot of 20 µg of myocardial extract or plasma was loaded onto 8% acrylamide gels, electrophoresed under reducing conditions, and then electroblotted onto polyvinylidene difluoride membranes (BioRad). Samples were probed with either mouse anti-human MMP-2 that has higher affinity for the 72 kDa form (1:200, MAB13405, Chemicon), mouse anti-human MMP-2 that has higher affinity for the 64 kDa form (1:1000, MAB3308, Chemicon), rabbit anti-mouse MMP-9 (1:1000, AB19047, Chemicon), mouse anti-human TIMP-1 (1:133, NS608, Neomarkers), rabbit anti-human TIMP-2 (1:133, RB1489, Neomarkers), or rabbit anti-human TIMP-4 (1:4000, AB816, Chemicon). Appropriate horseradish peroxidase conjugated antibodies were used (either anti-mouse or anti-rabbit, Transduction Laboratories), and a chemiluminescence reaction kit was used to visualize the protein bands (Amersham Pharmacia Biotech).

Statistical analyses

Data are expressed as mean ± SEM. Three blocks of variables were collected from each patient. The first block consists of data on MMP activity from tissue biopsy samples of heart before and after ischaemia–reperfusion. These data were analysed using repeated measures ANOVA with one ‘within subject’ factor (site) having two levels (arterial and venous). A value of $P < 0.05$ was taken to be statistically significant for the overall test. A significant $F$ value does not allow us to conclude that any particular mean is significantly larger or smaller than any other particular mean, thus comparisons between means were done with the Bonferroni critical value procedure. Multiple comparisons were performed (each time point vs. ‘Pre’) and $P < 0.01$ was considered to be statistically significant according to the Bonferroni adjustment for multiple comparisons (two-sided).

The second block of data represents cardiac function measured at six time points (‘Pre’, 1, 3, 6, 12, and 24 h) for each patient. These data were analysed using repeated measures ANOVA. A value of $P < 0.05$ was taken to be statistically significant for the overall test. A significant $F$ value does not allow us to conclude that any particular mean is significantly larger or smaller than any other particular mean, thus comparisons between means were done with the Bonferroni critical value procedure. Multiple comparisons were performed (each time point vs. ‘Pre’) and $P < 0.01$ was considered to be statistically significant according to the Bonferroni adjustment for multiple comparisons (two-sided).

The third block of data represents the measurements of MMP activity in blood samples collected from two sites (arterial and venous) at four time points (‘Pre’, 1, 5, and 10 min). For MMP activity there were a total of eight measurements for each subject. These data were analysed using repeated measures ANOVA with one ‘within subject’ factor (site) having two levels (arterial and venous). A value of $P < 0.05$ was taken to be statistically significant for the overall test. Multiple comparisons were performed and $P < 0.002$ was considered to be statistically significant according to the Bonferroni adjustment for multiple comparisons (two-sided).

Results

CABG surgery with CPB produces reversible cardiac mechanical dysfunction

Table 1 provides descriptions of the patients and the operative procedures. All patients tolerated the surgery and survived without complications. CABG with CPB produced an acute depression in post-operative cardiac mechanical function that was maximal 3 h post-reperfusion and returned to baseline levels by 24 h (Figure 1).

MMP-2 and -9 activities are increased in reperfused myocardium

Gelatinolytic activities were detected at 92 and 72 kDa in the myocardium prior to aortic cross-clamping (Figure 2A). Weak activities were also detectable at 135 and 64 kDa when gels were incubated for a longer time. Activities at 92 and 135 kDa were identified as MMP-9 (the latter probably represents a lipocalin-associated MMP-9),25 and 72 and 64 kDa activities were identified as MMP-2. The rank order of gelatinolytic activities prior to ischaemia was 92 > 72 > 135 > 64 kDa. There was no significant linear correlation between 92 and 72 kDa activities in the pre-ischaemic biopsies and pre-ischaemic LVSWI (data not shown).

Within 10 min of aortic cross-clamp release and reperfusion, myocardial 135 and 92 kDa MMP-9 and 72 kDa MMP-2 activities were markedly elevated without any change in 64 kDa MMP-2 (Figure 2B). Post-ischaemia there was a significant inverse correlation between

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<thead>
<tr>
<th>Table 1 Patient demographics</th>
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<tr>
<td><strong>Surgical variables</strong></td>
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<td>Bypass time, min</td>
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<td>Cross-clamp time, min</td>
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<td>Number of grafts (range)</td>
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Mean ± SEM values are given; ICU, intensive care unit.
92 kDa MMP-9 or 72 kDa MMP-2 activities in the reperfused myocardium and LVSWI at 3 h when cardiac function was most severely depressed (Figure 3A). A significant positive correlation was found between 92 or 72 kDa activities and aortic cross-clamp duration (Figure 3B). No correlation existed between any of the MMP activities and the duration of CPB (data not shown). Thus, elevated MMP-2 and -9 activities in the biopsies taken within 10 min of reperfusion correlated with the nadir of left ventricular function 3 h into reperfusion. Moreover, the longer the ischaemic time the higher the MMP activities upon reperfusion.

Net myocardial collagenase activity was measured in atrial biopsies from six patients. Prior to ischaemia, collagenase activity was detectable and after reperfusion this was significantly increased (1.8 ± 0.8 vs. 3.7 ± 1.2 arbitrary activity units, P < 0.05). Post-reperfusion collagenase activity, however, did not correlate with any clinical parameter.

Western blots for MMPs and TIMPs

Western blots for MMP-2 and -9 in the biopsies showed that there was no change in either 92 kDa MMP-9 or 72 kDa MMP-2 levels, although there was a ~30% decrease in the level of 64 kDa MMP-2, between the samples taken prior to ischaemia and those obtained within 10 min of reperfusion (n = 9, data not shown).

TIMP-1 was significantly decreased following reperfusion (pre-ischaemia 4.4 ± 1.2 vs. 1.2 ± 0.3 arbitrary units post-ischaemia, n = 6). Post-ischaemic levels of TIMP-1 correlated positively with 3 h LVSWI (r = 0.931, P = 0.007) and inversely with ischaemic duration (r = −0.934, P = 0.006). Levels of both TIMP-2 (n = 6) and TIMP-4 (n = 15) did not change (data not shown).

MMP-2 and -9 activities increase in the plasma following CPB

To determine whether the human heart releases MMPs, as observed in isolated rat hearts following ischaemia–reperfusion injury,17,18 plasma samples were collected from the coronary sinus and radial artery. Prior to ischaemia, but after the onset of CPB, weak 135 and 92 kDa MMP-9 activities were detected, along with 72 kDa MMP-2 (Figure 4A). The predominant activity was 72 kDa. When the gels were incubated for a longer time in order to resolve weaker activities, 84 kDa MMP-9 and 64 kDa MMP-2 were also detected (data not shown).

Within 1 min of reperfusion, 92 and 135 kDa MMP-9 activities were significantly increased (Figure 4B and D) but not 84 kDa MMP-9 (data not shown). Of MMP-2 activities, 72 kDa did not change whereas 64 kDa was significantly elevated (Figure 4C and E). Increased activities were accompanied by significant increases in 64 kDa MMP-2 and 92 kDa MMP-9 as measured by immunoblot (data not shown).

The increased activities did not correlate with changes in cardiac function, cross-clamp time, or cardiopulmonary bypass time (data not shown). As the surgical procedure itself could possibly alter plasma MMP activities, we compared samples taken during surgery (pre-ischaemia) with those taken from the antecubital vein before the patient went into surgery. Of the MMP activities, only 92 kDa MMP-9 was significantly increased (pre-surgery antecubital vein 13 ± 9 vs. 49 ± 23 pre-ischaemia coronary sinus and 36 ± 17 arbitrary units

Figure 1 Cardiac mechanical function in patients prior to cardioplegia (Pre), and post-reperfusion. LVSWI was monitored for 24 h post-reperfusion, *P < 0.01 vs. pre, n = 15.

Figure 2 MMP activities in myocardial biopsies prior to (Pre) and following reperfusion injury (Post). Pre samples were obtained after the initiation of CPB but prior to cardioplegic arrest, and Post samples were taken within 10 min of reperfusion. (A) Representativezymogram of myocardial MMP activities in biopsies from three patients. STD (standard): HT-1080 cell culture medium. (B) Densitometric analysis of gelatinolytic activities from all patients (*P < 0.05 vs. Pre value, n = 15).
pre-ischaemia radial artery, $P < 0.05, n = 6$). Following reperfusion there were no detectable transcardiac differences (i.e. between arterial and venous samples) in any plasma MMP activity following reperfusion.

**Discussion**

We studied the expression and activity of MMPs and TIMPs in the myocardium and plasma MMPs of patients undergoing CABG with CPB. During reperfusion following warm blood cardioplegia there was a rapid (within 10 min) increase in MMP-2, -9, and collagenase activities in the myocardium that was accompanied by a decrease in TIMP-1. The enhanced MMP-2 and -9 activities and the loss of TIMP-1 correlated with the post-bypass impairment of global left ventricular function and the duration of ischaemia. MMP activity in the plasma was also increased following CABG with CPB. Although this investigation was limited to sampling atrial biopsies, this is the first study of either acute or long-term cardiac pathologies that clearly correlates both MMP and TIMP in the human heart with cardiac dysfunction.

**MMP-2 and -9 in cardiovascular pathology**

We focused on the gelatinases since MMP-2 is ubiquitously expressed throughout the body and MMP-9 is a cytokine inducible MMP. MMP-2 activity is increased in the myocardium of spontaneously hypertensive heart failure rats and an inhibitor of MMPs ameliorated both the ventricular remodelling and the dysfunction. Targeted deletion of MMP-9 attenuated left ventricular remodelling after myocardial infarction. As well, both MMP-2 and -9 activities increased in the myocardium of patients with dilated cardiomyopathy. Clearly, these studies provide evidence that MMP-2 and -9 are involved in the development of long-term heart failure.

Other investigations have established MMPs as mediators of acute (minutes to hours time scale) stunning injury following ischaemia–reperfusion. MMP-2 is activated and released during the first minutes of reperfusion from isolated rat hearts following ischaemia. The activation of MMP-2 correlated directly with the duration of ischaemia and inversely with the recovery of mechanical function following reperfusion. Moreover, infusion of neutralizing MMP-2 antibody or MMP inhibitors attenuated...
In vivo animal models showed that myocardial MMP-1 and MMP-9 activities are upregulated some hours into reperfusion following ischaemia. Also, targeted deletion of MMP-9 decreased infarct size following ischaemia–reperfusion injury. Here we demonstrate activation of MMP-2 and -9 in the human myocardium, in accordance with a previous study. In this previous work, atrial biopsies were taken at the end of CPB, thus the time of the biopsy sampling in relation to reperfusion is unknown, nor was cardiac function measured in the acute post-operative period. In our present study we obtained atrial biopsies within 10 min of aorta cross-clamp release and correlated changes in MMP activities with the acute mechanical dysfunction in the first 24 h post-operative period. Both MMP-2 and -9 activities correlate with the severity of the ischaemic insult and the resulting mechanical dysfunction. This raises the possibility that MMP inhibition may be a novel therapeutic strategy to prevent stunning injury in humans.

Patients without pre-existing left ventricular dysfunction were selected for this study; thus, the resulting post-reperfusion cardiac dysfunction was mild and reversible. Studies have demonstrated that patients with
Acute regulation of MMP activity in the human heart

MMP activity in cells can be regulated at the transcriptional and post-translational levels, and through inhibition by endogenous inhibitors (TIMPs). Pro-inflammatory cytokines, which are elevated following bypass surgery, increase MMP transcription in vitro. This mechanism, however, cannot account for the rapid increase in myocardial MMP activity seen here since MMP protein levels were not elevated. The absence of increased protein levels also excludes neutrophil infiltration as a possible source for increased 92 and 72 kDa MMP activities. However, the 135 kDa MMP-9 may be derived from human neutrophils since it has only been characterized to date in these cells.

Since MMPs are synthesized as zymogens they must be activated post-translationally through either proteolytic removal of the pro-peptide domain, or modification by oxidant stress. In vitro, both mechanisms can activate MMPs by reacting with a critical cysteine residue without loss of the pro-peptide. This provides a possible explanation for the discordance between MMP activity and protein levels noted in this study. Thus, increased myocardial peroxynitrite biosynthesis, which occurs immediately upon reperfusion in human hearts during bypass surgery, may increase MMP activity without loss of the pro-peptide or increased expression. In isolated rat hearts, peroxynitrite infusion activates MMP-2 prior to the loss of mechanical function, and a MMP inhibitor prevented the acute loss of mechanical function. MMPs may be effectors of the increased oxidative stress which occurs post-bypass.

TIMPs provide another level of regulation of MMP activity by complexing with these enzymes and inhibiting their activity. We found a significant decrease in myocardial TIMP-1 levels that correlated positively with ischaemic time and inversely with cardiac function. This finding is supported by previous studies which demonstrated decreased myocardial TIMP-1 mRNA expression following acute ischaemia-reperfusion injury in isolated rabbit hearts, and decreased serum levels of TIMP-1 following acute myocardial infarction in humans. We found no significant changes in TIMP-2 or -4 levels following bypass. TIMP-2 and -4 have a higher sequence homology to one another than to TIMP-1. Thus it is not surprising to note a difference between the effects of ischaemia-reperfusion on TIMP-1 compared with TIMP-2/4. Moreover, TIMP-1 is acutely regulated by a number of cytokines and growth factors whereas TIMP-2 and -4 are not as highly regulated.

Potential targets for MMP activity

Most studies ascribe the deleterious effects of MMPs to their ability to degrade extracellular matrix proteins. The brief time frame of ischaemia-reperfusion injury here makes it unlikely that sufficient collagen degradation occurred to contribute to stunning injury. MMP-2 has been shown to cleave a number of non-extracellular matrix substrates. MMP-2, -9, and MT1-MMP are closely associated with the sarcomeres in human cardiomyocytes. In rat hearts, MMP-2 co-localizes with the contractile protein regulatory element troponin I and causes stunning injury by degrading it. Thus, MMP activation seen in the present study may contribute to troponin I proteolysis or the deranged actin cross striation pattern following stunning injury in humans. In order to address this possibility, future studies will need to determine the cellular 'source and sink' of increased MMP activity following stunning injury.

MMP activity in the plasma following CABG with CPB

A previous report demonstrated that many MMPs are elevated in the plasma by CPB; however, only protein levels were examined. We have found here that reperfusion increased 92 and 135 kDa MMP-9 and 64 kDa MMP-2 activities. The increases in plasma 92 kDa MMP-9 and 64 kDa MMP-2 activities were accompanied by increased protein levels. Neutrophils or platelets may have been a potential source of this increased plasma activity, since white blood cells contain large stores of MMPs. As these cells came into contact with the foreign surfaces of the extracorporeal circuit they probably became activated and degranulated, liberating MMPs from intracellular stores into the plasma. Although the increase in plasma MMP activities did not correlate with any clinical parameters, it probably contributes to an elevated systemic proteolytic state that may alter cardiovascular homeostasis in the post-operative period. MMPs may cause coronary artery vasoconstriction by activating pro-endothelin, stimulating platelet aggregation, and contributing to coagulation disorders following bypass surgery.

Limitations

The limitations of this study should be recognized. First, MMP activities from atrial biopsy samples were correlated with left ventricular function. Atrial tissue was sampled pre-existing ventricular dysfunction who are subjected to cardiac revascularization represent a high mortality risk group. It is possible that MMP activation would be enhanced in these patients and ultimately contribute to adverse clinical outcomes (post-operative myocardial infarction, ventricular failure, cardiac death). Overall, following ischaemia-reperfusion a picture emerges of increased proteolysis within the heart. At the same time that MMP activities are increased the level of their primary inhibitors are either decreased or unchanged in the heart, resulting in an imbalance between MMPs and TIMPs. Moreover, the inhibitory activity of TIMPs following ischaemia-reperfusion may also be acutely diminished by peroxynitrite production within the heart.
to provide an appropriate amount of tissue without compromising the left ventricle. Secondly, a limited number of patients were enrolled in this study. This sample size was adequate to identify major relationships between ischaemia–reperfusion and MMPs. Future studies, however, will involve more patients and further refine this relationship. Third, the observational nature of this study allows only correlative conclusions to be drawn from the data. Future studies will determine whether MMP activation is a direct cause of ischaemia–reperfusion injury. Finally, the effects of ischaemia–reperfusion may be enhanced by the effects of CPB; however, in the present study myocardial MMP activity correlated with ischaemic time but not CPB time.

**Clinical implications**

Our findings in patients undergoing CABG with CPB indicate that MMPs are activated when the heart is subjected to ischaemia–reperfusion. The increased activity of these enzymes may produce a proteolytic environment that contributes to post-surgical complications. Our observational data, however, does not provide conclusive evidence that MMPs are directly involved in cardiac stunning injury in humans. Future studies will address this issue by either inhibiting MMPs or enhancing TIMP activity. MMP inhibition may prove to be a novel therapeutic strategy to prevent acute post-operative cardiac dysfunction.

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