MAST CELL DEFICIENT W/W^v MICE HAVE LOWER SERUM IL-6 AND LESS CARDIAC TISSUE NECROSIS THAN THEIR NORMAL LITTERMATES FOLLOWING MYOCARDIAL ISCHEMIA-REPERFUSION

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Myocardial ischemia-reperfusion (IR) injury complicates all forms of coronary artery revascularization. Circulating interleukin-6 (IL-6) has been implicated in cell death following a variety of stimuli. Macrophages, platelets, neutrophils and the endothelium have been shown to release IL-6 after IR injury. Cardiac mast cells have been implicated in IR; however, their involvement has never been quantified. In this randomized, prospective study, we compared cardiac tissue susceptibility and serum IL-6 changes between mast cell deficient (W/W^v) mice and their normal littermates (+/+). Twenty-eight male W/W^v mice (n=14) and their +/+ littermates (n=14) were anaesthetized with 2.5% isofluorane. The left coronary artery (LCA) was ligated for 30 minutes or a sham procedure was performed. After 6 hours of reperfusion, the animals were sacrificed. The muscle viability was assessed on fresh whole-mount slices by nitroblue tetrazolium (NBT) histochemical assay and serum IL-6 concentrations measured by ELISA. Cardiac muscle viability was significantly higher in W/W^v mice than the +/+ mice. Serum IL-6 levels were higher in the +/+ sham mice (465 ± 32 pg/ml, n=6) than the W/W^v mice (185 ± 31 pg/ml, n=6), $p < 0.001$. The IL-6 levels increased significantly after reperfusion only in the +/+ mice (698 ± 41 pg/ml, n=8, $p = 0.001$), while it remained similar in the W/W^v mice (202 ± 48 pg/ml, n=8, $p = 0.783$). These results show that the absence of mast cells reduces the myocardial damage associated with IR injury. Furthermore, there is an attenuation in the inflammatory response, as measured by serum IL-6 levels, following this local insult. This finding entertains the prospect of developing prophylactic therapy - targeting selective inhibition of cardiac mast cell activation, in clinical situations involving medical or surgical myocardial revascularization.

Coronary Artery Disease (CAD) is the leading cause of morbidity and mortality in the United States; it accounts for 1.5 million of myocardial infarctions (MI) and 500,000 deaths each year. Unfortunately, in spite of earlier predictions, CAD continues to be the leading cause of death globally. CAD is a clinical manifestation of atherosclerosis, which is now considered to be a chronic vascular inflammatory disorder (1). Angioplasty removes cholesterol plaques in CAD and allows reperfusion,

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which unfortunately is associated with a high rate of ischemia-reperfusion (IR) and restenosis (1). IR injury also occurs following thrombolysis, percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABG), and is associated with further tissue damage from the release of inflammatory mediators. These could be released from macrophages, platelets and neutrophils, as well as from the endothelium and resident mast cells. *In vivo* studies of animal models show evidence of the role of mast cell activation in IR injury. For instance, mast cell numbers increase in cardiac tissue during IR, while pretreatment with a mast cell stabilizer reduces the size of infarct (2). Mast cells are well known for their involvement in allergic reactions (3) and neuroinflammatory conditions that are precipitated or exacerbated by stress (4). Mast cells are not only a rich source of vasoactive and pro-arrhythmogenic molecules, such as histamine, but also many cytokines, including IL-6 (5). Increased numbers of activated cardiac mast cells are found in ventricles, the sinoatrial node and the shoulder region of the fibrous plaque in association with atherosclerosis (6-7). It has also been suggested that coronary inflammation may depend on mast cell-derived mediators (8).

IL-6 was originally identified in monocytes/ macrophages, fibroblasts and endothelial cells (9). It is a pleiotropic cytokine that has multiple functions including: (a) stimulatory effects on the proliferation and differentiation of lymphocytes (9); (b) activation of the hypothalamic-pituitary adrenal (HPA) axis; (c) induction of the hepatic acute phase response proteins, particularly CRP; (d) increase of fatty lesions and increase of atherosclerosis in ApoE knockout mice. The importance of IL-6 in the pathophysiology of CAD has been documented in several studies (10-11) and it is probably the link between inflammation, stress and CAD (12). Both IL-6 and C reactive protein (CRP), are strong independent predictors of cardiovascular mortality. IL-6 can also be released from cardiomyocytes in response to hypoxia, IL-1 from mast cells (13) in acute stress.

To date, there is no published report evaluating the effects of IR in mast cell deficient W/W^v mice. Here, we report that IR-induced serum IL-6 increase, and significantly less myocardial damage in these mice. These findings point to mast cell-derived IL-6 as a key player in the inflammation and damage during IR.

MATERIALS AND METHODS

Ischemia-reperfusion

Fifteen male (30-35 g) W/W^v mast cell deficient mice (WBB6F1/J-W/W^v) and 15 normal +/+ littermates (Jackson Laboratories, Bar Harbor, ME) were allowed food and water *ad libitum* and were maintained in a 14: 10 hr dark-light cycle. Animals were kept in the animal facility for at least one week before use. Each mouse was anesthetized with 2.5% isoflurane. The mice either had their left coronary artery (LCA) ligated 4 mm from the aorta for 30 minutes followed by a 6 hr reperfusion period, or were sham-operated. The animals were then sacrificed; blood was collected, allowed to clot at 37ºC, centrifuged and the serum was removed and stored at –80ºC for future IL-6 analysis. The heart was removed rapidly, fixed and stored. This investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

IL-6 measurements

Mouse serum samples were assayed for IL-6 with the quantitative sandwich enzyme immunoassay kit technique (Quantikine M Murine, Mouse IL-6, R&D Systems Inc., Minneapolis, MN).

Light microscopy

The heart was rapidly removed and fresh whole mount slices were cut using a Vibratome (Micro-cut H1200, E.M. Core, Chestnut Hill, MA). Cardiac sections were stained with nitroblue tetrazolium (NBT) to assess cardiac muscle viability as previously described (14). Two random sections from each heart were evaluated by two researchers, blinded to the experimental conditions, using a Diaphot inverted Nikon microscope (Don Santo, Natick, MA). Results are presented as percent cardiac tissue not stained.

Statistical analysis

The IL-6 values were subjected to the Levine's Test for the equality of variances (SPSS version 10.1, Chicago, IL). As the variances were not significantly different, each group data is presented as the mean±S.E.M. The cardiac tissue viability results were compared using non-parametric *U* test. One way ANOVA run by SigmaStat was used to compare the differences between the groups. A student's *t*-test was used to compare the treatments between each group of animals. A p value of less than

0.05 was considered statistically significant.

RESULTS

Serum IL-6 levels in wild-type sham-operated mice without IR were 465±32 pg/ml and were significantly higher than the corresponding sham-operated W/W^v mice (185±33 pg/ml, $p< 0.05$, Fig. 1). After reperfusion, the wild type mice showed a significant increase in IL-6 levels (698±41 pg/ml, $p<0.01$). However, the serum IL-6 concentration for the W/W^v mice did not increase (Fig. 1) following reperfusion (202±48 pg/ml, $p<0.783$). The difference in the serum IL-6 levels between wild type IR and W/W^v IR was also very significant ($p<0.001$). There was no apparent difference in cardiac tissue viability between sham-operated +/+ and W/W^v mice (Table I); however, cardiac tissue viability was strikingly higher in W/W^v mice than the +/+ mice after IR (Table I). In +/+ mice following IR, there was extensive cardiac necrosis, as seen with absence of nitroblue tetrazolium staining (Fig. 2).

Table I. *Effect of ischemia-reperfusion on cardiac viability.*

Condition	Wild-type mice	W/W^v mice
Sham-operated	+/-	None
IR	+++	None

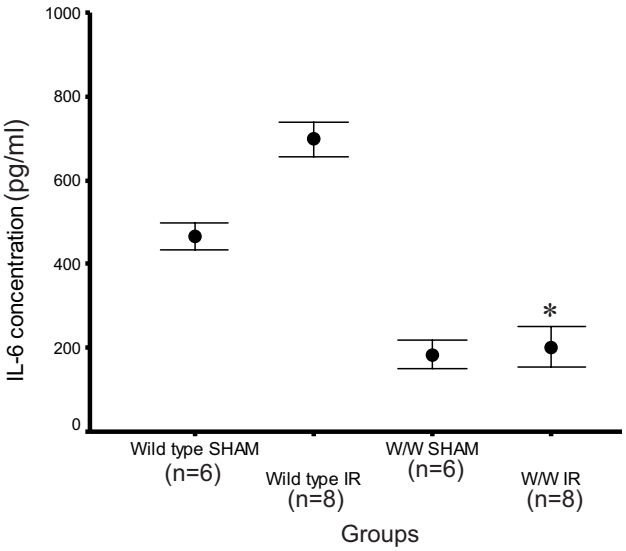

Fig. 1. *Comparison of serum IL-6 levels in wild type controls (n=15) and W/W^v mast cell deficient (n=15) mice. Results are presented as mean ± S.E.M. (± means least and +++ most tissue necrosis).*

DISCUSSION

Our present data show that IR induces serum IL-6 release and myocardial damage absent in W/W^v mice, indicating that this process is mast cell-dependent. In other studies using W/W^v mast cell deficient mice, their immune system was otherwise found to be normal, thus excluding the possibility of defective monocyte/macrophage function that may otherwise explain our results (15). Our findings are important because mast cells have been considered as the link between inflammation and atherosclerosis (16).

The importance of cardiac mast cell activation to the onset and progression of CAD is increasingly recognized (17). Activated cardiac mast cells are found in the coronary arteries and the heart in association with atherosclerosis (6-7). We previously showed that acute stress induces cardiac mast cell activation (18), as well as release of histamine (19) and IL-6 (13), both of which were higher in ApoE k/o mice.

IL-6 is elevated in patients with ACS and is expressed in atherosclerotic lesions of both mice (20-21) and humans (22-24); intracoronary IL-6 release in patients with acute CAD is also documented (25). In fact, IL-6 is considered a possible link between inflammation, stress and CAD (12). IL-6 was shown to be a potent independent marker of increased mortality in unstable CAD (26). Mast cells can release many cytokines, including IL-6 (5), especially in response to the inflammatory cytokine IL-1 (27). Mast cell-derived IL-6 and TNF-α induce the expression of vascular adhesion molecules that participate in the pathophysiology of CAD (28).

Increasing evidence implicates focal inflammation in the pathophysiology of CAD (25, 29-30), and acute stress is well known to worsen (31-36) or precipitate cardiac ischemia especially in patients with CAD (31-35, 37). Neurogenic stimulation of cardiac mast cells could occur since adventitial mast cells were in contact with sensory nerve fibers

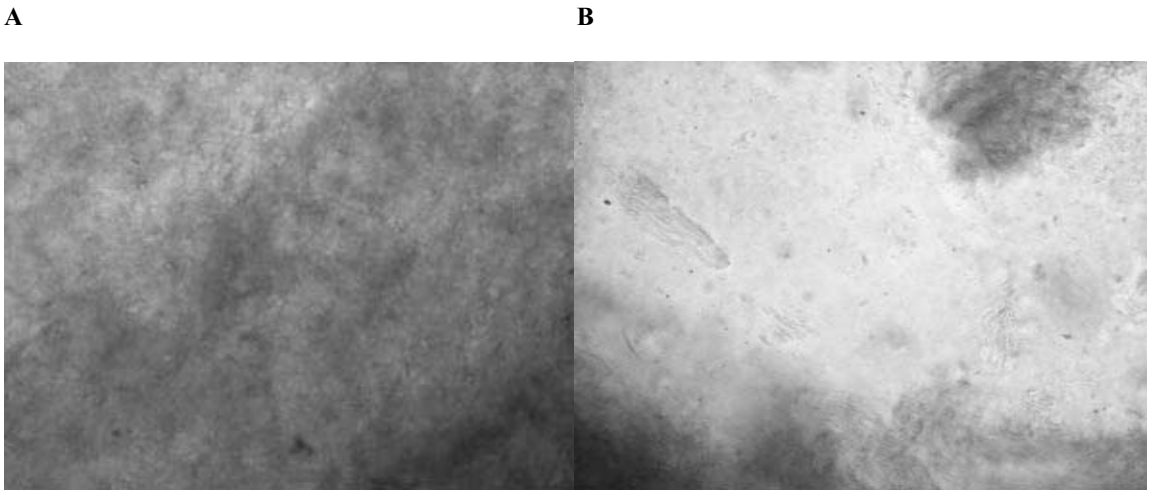

Fig. 2. *Representative photographs of cardiac sections (n=5 in each case) after IR from (A) W/W^v and (B) +/+ wild-type hearts stained with tetrazolium blue chloride, which generates red/purple color when acted upon by dehydrogenases present in live cells. Note the extensive cardiac tissue necrosis apparent in the +/+ mouse.*

in human atherosclerotic coronary arteries (38). A number of studies also suggested that mast cells may be involved in IR. An increased number of activated mast cells was reported in IR and they were noted to be degranulated with corresponding release of IL-6 and TNF-α. (2). Pretreatment with a mast cell stabilizer lodoxomide was reported to decrease the infarct size following IR in isolated rabbit hearts (39). Finally, *skeletal* muscle viability was reported to be higher in W/W^v mice (40).

It was recently shown that certain naturally occurring flavonoids, especially quercetin, could inhibit IL-6 release from human cultured mast cells (41). These findings entertain the prospect of developing prophylactic therapy by targeting selective inhibition of cardiac mast cell activation, in clinical situations involving medical or surgical myocardial revascularization.

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REFERENCES

1. **Hansson G.K.** 2005. Inflammation, atherosclerosis, and coronary artery disease. *N. Engl. J. Med. 352: 1685.*
2. **Frangogiannis N.G., M.L. Lindsey, L.H. Michael, K.A. Youker, R.B. Bressler, L.H. Mendoza, R.N. Spengler, C.W. Smith and M.L. Entman.** 1998. Resident cardiac mast cells degranulate and release preformed TNF-α, initiating the cytokine cascade in experimental canine myocardial ischemia/ reperfusion. *Circulation 98:699.*
3. **Galli S.J., J. Kalesnikoff, M.A. Grimbaldeston, A.M. Piliponsky, C.M. Williams and M. Tsai.** 2005. Mast cells as "tunable" effector and immunoregulatory cells: recent advances. *Annu. Rev. Immunol. 23:749.*
4. **Theoharides T.C. and D.E. Cochrane.** 2004. Critical role of mast cells in inflammatory diseases and the effect of acute stress. *J. Neuroimmunol. 146:1.*
5. **Krüger-Krasagakes S., A.M. Möller, G. Kolde, U. Lippert, M. Weber and B.M. Henz.** 1996. Production of inteuleukin-6 by human mast cells and basophilic cells. *J. Invest. Dermatol. 106:75.*
6. **Constantinides P.** 1995. Infiltrates of activated mast cells at the site of coronary atheromatous erosion or rupture in myocardial infarction. *Circulation 92:1083.*
7. **Kaartinen M., A. Penttilä and P.T. Kovanen.** 1994. Accumulation of activated mast cells in the shoulder region of human coronary atheroma, the predilection site of atheromatous rupture. *Circulation 90:1669.*
8. **Laine P., M. Kaartinen, A. Penttilä, P. Panula,**

T. Paavonen and P.T. Kovanen. 1999. Association between myocardial infarction and the mast cells in the adventitia of the infarct-related coronary artery. *Circulation 99:361.*

9. **Papanicolaou D., R.L. Wilder, S.C. Manolagas and G. Chrousos.** 1998. The pathophysiologic roles of interleukin-6 in human disease. *Ann. Intern. Med. 128:127.*
10. **Baraille R. and B. Klein.** 1992. C-reactive protein levels as a direct indicator of interleukin-6 levels in humans *in vivo*. *Arthritis Rheum. 35:982.*
11. **Harris T.B., L. Ferrucci, R.P. Tracy, M.C. Corti, S. Wacholder, W.H. Ettinger Jr, H. Heimovitz, H.J. Cohen and R. Wallace.** 1999. Associations of elevated interleukin-6 and C-reactive protein levels with mortality in the elderly. *Am. J. Med. 106:506.*
12. **Yudkin J.S., M. Kumari, S.E. Humphries and V. Mohamed-Ali.** 2000. Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? *Atherosclerosis 148:209.*
13. **Huang M., X. Pang, K. Karalis and T.C. Theoharides.** 2003. Stress-induced interleukin-6 release in mice is mast cell-dependent and more pronounced in Apolipoprotein E knockout mice. *Cardiovasc. Res. 59:241.*
14. **Cooper M. and K. Ytrehus.** 2004. Cell survival signalling in heart derived myofibroblasts induced by preconditioning and bradykinin: the role of p38 MAP kinase. *Mol. Cell. Biochem. 259:83.*
15. **Ha T.Y., N.D. Reed and P.K. Crowle.** 1986. Immune response potential of mast cell-deficient W/W^v mice. *Int. Arch. Allergy Appl. Immunol. 80:85.*
6. **Kovanen P.T.** 1993. The mast cell - potential link between inflammation and cellular cholesterol deposition in atherogenesis. *Eur. Heart J. 14:105.*
17. **Chester A.H.** 2002. Mast cells feel the strain. *Cardiovasc. Res. 55:13.*
18. **Pang X., N. Alexacos, R. Letourneau, D. Seretakis, W. Gao, D.E. Cochrane and T.C. Theoharides.** 1998. A neurotensin receptor antagonist inhibits acute immobilization stress-induced cardiac mast cell degranulation, a corticotropin-releasing hormone-dependent process. *J. Pharm. & Exp. Therap. 287:307.*
19. **Huang M., X. Pang, L. Letourneau, W. Boucher and T.C. Theoharides.** 2002. Acute stress induces cardiac mast cell activation and histamine release, effects that are increased in apolipoprotein E knockout mice. *Cardiovasc. Res. 55:150.*
20. **Sukovich D.A., K. Kauser, F.D. Shirley, V. DelVecchio, M. Halks-Miller and G.M. Rubanyi.** 1998. Expression of interleukin-6 in atherosclerotic lesions of male ApoE-knockout mice. *Arterioscler. Thromb. 18:1498.*
21. **Huber S.A., P. Sakkinen, D. Conze, N. Hardin and R. Tracy.** 1999. Interleukin-6 exacerbates early atherosclerosis in mice. *Arterioscler. Thromb. 19: 2364.*
22. **Kanda T., M. Inoue, N. Kotajima, S. Fujimaki, Y. Hoshino, M. Kurabayashi, I. Kobayashi and J. Tamura.** 2000. Circulating interleukin-6 and interleukin-6 receptors in patients with acute and recent myocardial infarction. *Cardiology 93:191.*
23. **Hojo Y., U. Ikeda, T. Katsuki, O. Mizuno, H. Fukazawa, K. Kurosaki, H. Fujikawa and K. Shimada.** 2000. Interleukin 6 expression in coronary circulation after coronary angioplasty as a risk factor for restenosis. *Heart 84:9.*
24. **Miyao Y., H. Yasue, H. Ogawa, I. Misumi, T. Masuda, T. Sakamoto and E. Morita.** 1993. Elevated plasma interleukin-6 levels in patients with acute myocardial infarction. *Am. Heart J. 126:1304.*
25. **Deliargyris E.N., R.J. Raymond, T.C. Theoharides, W.S. Boucher, D.A. Tate and G.J. Dehmer.** 2000. Sites of interleukin-6 release in patients with acute coronary syndromes and in patients with congestive heart failure. *Am. J. Cardiol. 86:913.*
26. **Lindmark E., E. Diderholm, L. Wallentin and A. Siegbahn.** 2001. Relationship between interleukin 6 and mortality in patients with unstable coronary artery disease: effects of an early invasive or noninvasive strategy. *JAMA 286:2107.*
27. **Kandere-Grzybowska K., R. Letourneau, D. Kempuraj, J. Donelan, S. Poplawski, W. Boucher, A. Athanassiou and T.C. Theoharides.** 2003. IL-1 induces vesicular secretion of IL-6 without degranulation from human mast cells. *J. Immunol. 171:4830.*
28. **Klein L.M., R.M. Lavker, W.L. Matis and G.F. Murphy.** 1989. Degranulation of human mast cells induces an endothelial antigen central to leukocyte adhesion. *Proc. Natl. Acad. Sci. USA 86:8972.*
29. **Alexander R.W.** 1994. Inflammation and coronary

artery disease. *N. Engl. J. Med. 31:468.*

30. **Maseri A.** 1997. Inflammation, atherosclerosis and ischemic events - exploring the hidden side of the moon. *N. Engl. J. Med. 336:1014.*
31. **Deedwania P.C.** 1995. Mental stress, pain perception and risk of silent ischemia. *JACC 25:1504.*
32. **Freeman L.J., P.G.F. Nixon, P. Sallabank and D. Reaveley.** 1987. Psychological stress and silent myocardial ischemia. *Am. Heart J. 114:477.*
33. **Rozanski A., C.N. Bairey, D.S. Krantz, J. Friedman, K.J. Resser, M. Morell, S. Hilton-Chalfen, L. Hestrin, J. Bietendorf and D.S. Berman.** 1988. Mental stress and the induction of silent myocardial ischemia in patients with coronary artery disease. *N. Engl. J. Med. 318:1005.*
34. **Jiang W., M. Babyak, D.S. Krantz, R.A. Waugh, E. Coleman, M.M. Hanson, D.J. Frid, S. McNulty, J.J. Morris, C.M. O'Connor and J.A. Blumenthal.** 1996. Mental stress-induced myocardial ischemia and cardiac events. *JAMA 275:1651.*
35. **Deanfield J.E., M. Shea, M. Kensett, P. Horlock, R.A. Wilson, C.M. deLandsheere and A.P. Selwyn.** 1984. Silent myocardial ischaemia due to mental stress. *Lancet 2:1001.*
36. **Gottdiener J.S., D.S. Krantz, R.H. Howell, G.M. Hecht, J. Klein, J.J. Falconer and A. Rozanski.** 1994. Induction of silent myocardial ischemia with mental stress testing: relation to triggers of ischemia during daily life activities and to ischemic functional severity. *J. Am. Coll. Cardiol. 24:1645.*
37. **Specchia G., C. Falcone, E. Traversi, M.T. La Rovere, L. Guasti, G. De Micheli, D. Ardissino and S. De Servi.** 1991. Mental stress as a provocative test in patients with various clinical syndromes of coronary heart disease. *Circulation 83:II.*
38. **Laine P., A. Naukkarinen, L. Heikkila, A. Penttilä and P.T. Kovanen.** 2000. Adventitial mast cells connect with sensory nerve fibers in atherosclerotic coronary arteries. *Circulation 101:1665.*
39. **Wang P., J.M. Downey and M.V. Cohen.** 1996. Mast cell degranulation does not contribute to ischemic preconditioning in isolated rabbit hearts. *Basic Res. Cardiol. 91:458.*
40. **Lazarus B., A. Messina, J.E. Barker, J.V. Hurley, R. Romeo, W.A. Morrison and K.R. Knight.** 2000. The role of mast cells in ischaemia-reperfusion injury in murine skeletal muscle. *J. Pathol. 191:443.*
41. **Kempuraj D., B. Madhappan, S. Christodoulou, W. Boucher, J. Cao, N. Papadopoulou, C.L. Cetrulo and T.C. Theoharides.** 2005. Flavonols inhibit proinflammatory mediator release, intracellular calcium ion levels and protein kinase C theta phosphorylation in human mast cells. *Br. J. Pharmacol. 145:934.*