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Free heme is a danger signal inducing expression of proinflammatory proteins in cultured cells derived from normal rat hearts

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ABSTRACT

Endogenous molecules from damaged tissue act as danger signals to trigger or amplify the immune/inflammatory response. In this study, we examined whether free heme induced pro-inflammatory proteins in cultured cells derived from normal hearts and investigated the cells targeted by heme, together with its mechanism of action in these cells. We cultured collagenase-isolated heart-derived cells from normal rats and examined whether free heme induced pro-inflammatory proteins, reactive oxygen species (ROS) production and NF-κB activation, by quantitative RT-PCR, ELISA and flow cytometry. Free heme increased mRNA of various pro-inflammatory proteins in cultured cardiac resident cells (CCRC) (at least 100-fold) and induced intracellular ROS formation. Approximately 85–90% of CCRC are fibroblast/smooth muscle cells and 10–15% are CD11bc-positive macrophages; therefore to examine individual target cells, macrophage-deleted (CD11bc-negative) CCRC, primary cultured cells (cardiac fibroblasts, arterial smooth muscle cells and cardiac microvascular endothelial cells) and macrophage cells lines (NR8383) were similarly treated. Free heme activated NF-κB and induced expression of some pro-inflammatory proteins, including IL-1 and TNF-α in NR8383. On the other hand, macrophage-deleted CCRC strongly increased expression of these proteins on treatment with IL-1 or TNF-α, but not free heme. Induction of expression of pro-inflammatory proteins by free heme was not inhibited by intracellular ROS reduction, but by protease and proteasome inhibitors capable of regulating NF-κB. These data suggest that free heme strongly induces various pro-inflammatory proteins in injured hearts through NF-κB activation in cardiac resident macrophages and through cross-talk between macrophages and fibroblast/smooth muscle cells mediated inter alia by IL-1, TNF-α.

1. Introduction

In response to cardiac injury, resident cells such as cardiomyocytes, endothelial cells, pericytes, smooth muscle cells, fibroblasts, mast cells and resident macrophages are thought to rapidly react to environmental changes and cross-talk mediated by various mediators (Frangogiannis, 2008; Yoshida et al., 2005). Recent reports have indicated that endogenous intracellular contents released by dying cells initiate an intense inflammatory response by activating the innate immune mechanism. Heat shock proteins (Chen et al., 1999), hyaluronan (Scheibner et al., 2006), fibronectin fragments (Okamura et al., 2001) and uric acid (Shi et al., 2003) serve as "danger signals" that trigger an inflammatory cascade. Even in the absence of microbial pathogens, "danger signals" can activate the innate immune system (Frantz et al., 2007; Shi et al., 2003; Trendelenburg, 2008). However, the identity of "danger signals" in myocardium remains largely unknown; nor is it known which resident cells react to these signals and produce pro-inflammatory proteins such as cytokines and chemokines.

On the other hand, our previous gene expression analysis in hearts of rats with myocarditis by DNA microarray and real time RT-PCR has demonstrated that gene expression of several iron metabolism-related proteins, such as lipocalin-2/NGAL/alpha-2μ globulin-related protein (Lcn2/NGAL), hepcidin and heme oxygenase-1 (HO-1), is markedly increased in such cardiomyocytes (Ding et al., 2010; Isoda et al., 2010; Watanabe et al., 2008). The role of these proteins in injured hearts still remains largely unknown; however, this finding led us to hypothesize that resident cells in injured hearts are rapidly and strongly influenced by a molecule

Abbreviations: CCRC, cultured cardiac resident cells; CRC, cardiac resident cells; EAM, experimental autoimmune myocarditis; HO-1, heme oxygenase-1; IL-1, interleukin-1; Lcn2, lipocalin-2; ROS, reactive oxygen species; RASMC, rat aortic smooth muscle cells; RCF, rat cardiac fibroblasts; RHMVEC, rat heart microvascular endothelial cells; TLCK, Tosyl-Lys-chloromethylketone; TPCK, Tosyl phenylalanyl chloromethyl ketone.

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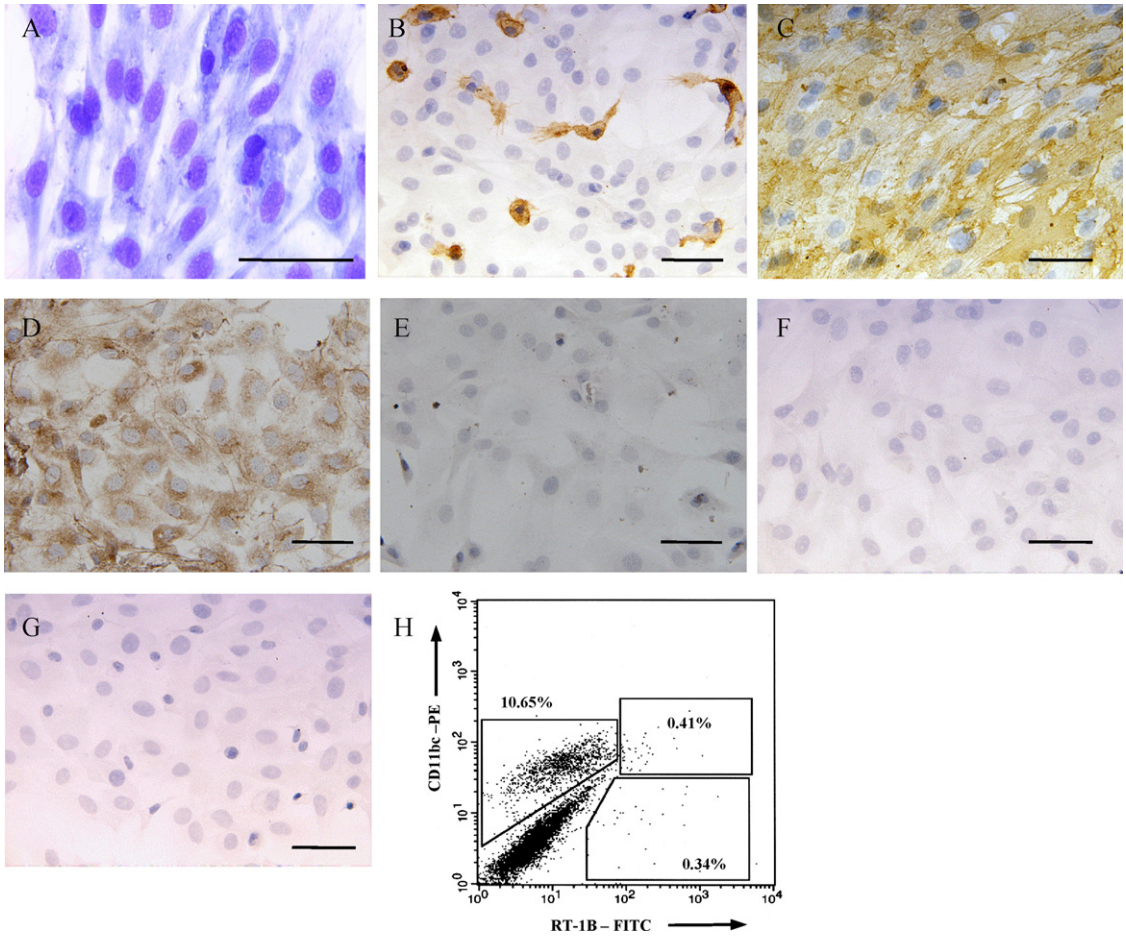

Fig. 1. Immunohistochemical staining and flow cytometry of CCRC from normal hearts. CCRC were isolated from the hearts of 8-week old normal rats and cultured for 2 weeks on chamber slides. (A) May-Giemsa stain, (B) CD11bc, (C) α-smooth muscle actin, (D) collagen type III, (E) factor VIII-related Ag, (F) rabbit serum as a negative control, (G) mouse IgG2a as a negative control, and (H) flow cytometry of CCRC. Bar represents 50 μm.

containing iron, which is critically involved in inflammation. In other words, it has been speculated that this may be one of the "danger signals" released from damaged cardiac tissue. In fact, cardiomyocytes are a cell type containing a large amount of iron (Brown et al., 2007). Cardiomyocytes must maintain high intracellular oxygen levels to generate sufficient energy for contraction and relaxation (Wittenberg and Wittenberg, 1987); therefore, cardiomyocytes possess high levels of myoglobin containing heme (iron protoporphyrin IX), which is involved in oxygen storage and facilitation of oxygen diffusion to the mitochondria (Wittenberg and Wittenberg, 2003). On the other hand, serum myoglobin is also a biomarker for the early detection of myocardial infarction (Stone et al., 1975). This means that, in case of cardiac injury, myoglobin is released into the extracellular space and leaks into the circulation. Moreover, it suggests that dispersed myoglobin is partially degraded in cardiac lesions, thereby increasing the concentration of free heme at the site of damage (Nakahira et al., 2003). Because free heme is highly lipophilic, it will rapidly intercalate into the lipid membranes of adjacent cells (Beri and Chandra, 1993). Recent studies have revealed that free heme is a potent pro-oxidant by virtue of its ability to promote reactive oxygen species (ROS) formation (Hasan and Schafer, 2008); furthermore, ROS are involved in interleukin-1 (IL-1)β production by triggering inflammasome signaling (Allen et al., 2009; Cruz et al., 2007). Moreover, free heme has also been reported to induce the expression of pro-inflammatory adhesion molecules both on endothelium and blood cells (Wagener et al., 1997, 2001), monocyte chemoattractant protein (MCP)-1 in immortalized proximal tubular cells (Kanakiriya et al., 2003) and tumor necrosis factor (TNF)-α in peritoneal macrophages (Figueiredo et al., 2007). Free heme is a major regulator of gene expression (Tsiftsoglou et al., 2006).

For these reasons, we here investigated whether ferriprotoporphyrin IX (hemin), into which heme released from globin is converted by iron oxidation in the presence of chloride ions (Tsiftsoglou et al., 2006), induced pro-inflammatory proteins in heart. Approximately 75% of the total cell number in normal heart is considered to be non-cardiomyocytes in the interstitium (Zak, 1973) and the majority are fibroblasts (Eghbali et al., 1988). On the other hand, recent studies have suggested that tissue resident macrophages react to endogenous products of damaged cells and tissues (Medzhitov, 2008; Zitvogel et al., 2010). In this study, we have investigated the influence of hemin on cultured cardiac resident cells (CCRC) with cardiac resident macrophages and fibroblasts.

2. Materials and methods

2.1. Conditions for culture of cardiac resident cells

Cardiac resident cells (CRC) were isolated by forcing aseptically well-washed hearts of 8-week-old normal male Lewis rats through a 200-gauge stainless steel mesh after trypsin and collagenase digestion using 0.25% trypsin–EDTA solution (Invitrogen Life Technologies, Tokyo, Japan) plus 0.0075% collagenase Type