

Macrophage colony stimulating factor expression in human cardiac cells is upregulated by tumor necrosis factor- α via an NF- κ B dependent mechanism

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Summary. *Introduction:* Macrophage colony stimulating factor (M-CSF) is a key factor for monocyte and macrophage survival and proliferation. M-CSF has been implicated in cardiac healing and repair after myocardial infarction. *Methods and results:* We show by immunohistochemistry and Western blotting analysis that M-CSF protein is present in human heart tissue. Cultured human adult cardiac myocytes (HACM) and human adult cardiac fibroblasts (HACF) isolated from human myocardial tissue constitutively express M-CSF. When HACM and HACF were treated with tumor necrosis factor- α (TNF- α) M-CSF protein production and M-CSF mRNA expression, determined by ELISA or by using RT-PCR, respectively, was significantly increased. To determine a possible role of nuclear factor κ B (NF- κ B) and activating protein 1 (AP-1) in M-CSF regulation, blockers to both pathways and an adenovirus overexpressing a dominant negative (dn) form of I κ B kinase 2 (IKK2) were used. Only the NF- κ B blocker dimethylfumarate and the dn IKK2, but not januskinase inhibitor-1 (JNK-I), were able to block the TNF- α -induced increase in M-CSF production in these cells, suggesting that the induction of M-CSF through TNF- α is mainly dependent on the activation of the NF- κ B pathway. The monocyte activation marker CD11b was significantly increased after incubating U937 cells with conditioned medium from HACM or HACF as determined by FACS analysis. *Conclusions:* Our *in vitro* data taken together with our immunohistochemistry data suggest that human cardiac cells

constitutively express M-CSF. This expression of M-CSF in the human heart and its upregulation by TNF- α might contribute to monocyte and macrophage survival and differentiation.

Keywords: cardiac cells, M-CSF, TNF- α .

Introduction

Macrophage colony stimulating factor (M-CSF), also known as colony stimulating factor-1 (CSF-1), is the most pleiotropic mononuclear growth factor, stimulating the survival, proliferation and differentiation of mononuclear cells [1]. Its receptor, CSF1-R, is expressed by primitive multipotent hematopoietic cells, mononuclear phagocyte progenitor cells, monocytes, tissue macrophages and smooth muscle cells [2]. Monocytes and macrophages have been reported to be a main source of M-CSF. Besides mononuclear cells, also endothelial cells, fibroblasts, smooth muscle cells, chondrocytes, epithelial cells, bone marrow stromal cells and tumor cells have been identified as a source for M-CSF [3,4]. Accumulating evidence suggests that besides its role in innate immunity, M-CSF is also critically involved in the pathophysiology of a variety of disease processes characterized by an inflammatory state, such as arthritis, atherosclerosis and obesity [5].

Various recent reports have implicated M-CSF also in the development of myocardial infarction and heart failure. Dewald *et al.* reported a marked and sustained upregulation of M-CSF in canine infarcts [6]. Frangiogiannis *et al.* speculated that local upregulation of M-CSF in the heart may have an important role in providing the inflammatory milieu during the development of heart failure characterized by infiltration of mononuclear cells into the infarct area [7]. Recently, macrophages in healing myocardial infarcts in dogs were shown to express M-CSF [8]. The authors suggested that M-CSF expression in the infarct area might play a role in promoting cardiac healing. This notion is supported by a recent report in

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which Yano *et al.* demonstrated that treatment of rats with M-CSF after myocardial infarction attenuated left ventricular dysfunction [9]. Furthermore, combined granulocyte colony stimulating factor and M-CSF treatment resulted in the suppression of border zone remodeling in rats [10]. On the other hand, other inflammatory mediators such as the proinflammatory cytokine tumor necrosis factor- α (TNF- α) have also been identified in clinical and experimental studies as important factors in the development and progression of cardiac disease [11]. With respect to cytokine expression in the human heart, we could show recently that the expression of monocyte chemoattractant protein 1 (MCP-1), a main chemoattractant for mononuclear cells, in human cardiac cells is significantly upregulated by TNF- α [12].

In this context we questioned whether human cardiac cells could serve as a source of M-CSF. Interestingly, no information is yet available on such a role of cardiac cells. Furthermore, we wanted to determine if TNF- α , a key inflammatory cytokine in the development of cardiac disease as outlined above, affects the expression of M-CSF in these cells.

Methods

Western blot

Human myocardial tissue was homogenized using a ball mill (Retsch, Haan, Germany). Samples were immediately frozen and stored at -80°C . Western blot under reducing conditions (5% beta-mercaptoethanol) was performed using a BioRad system (BioRad, Hercules, CA, USA). Conditioned media of HACM and HACF and purified recombinant human (rh) M-CSF (R&D Systems, Minneapolis, MN, USA) expressed in CHO-cells used as a standard were also subjected to Western blotting under these conditions. A 10% SDS-PAGE gel was used for protein separation. For the detection of M-CSF a monoclonal mouse antibody (R&D Systems) was used. Visualization with the ECL system (GE Health Care, Chalfont St Giles, UK) was performed. To guarantee equal protein loading total protein content was determined using a commercially available Bradford reagent (BioRad) according to the manufacturer.

Immunohistochemistry

Paraffin embedded healthy human heart tissue was stained using the MultiLink[®] horse radish peroxidase system (Biogenex, San Ramon, CA, USA) according to the manufacturer's instructions. For staining of M-CSF a monoclonal mouse antibody was used ($25\ \mu\text{g ml}^{-1}$; R&D Systems). Using the M-CSF antibody to stain mid-term placenta sections, a similar staining pattern as in the literature was observed (data not shown) [13]. To stain macrophages in the heart tissue we used a monoclonal mouse anti-CD68 antibody ($8.2\ \mu\text{g ml}^{-1}$; Dako, Glostrup, Denmark). Muscle cells were stained with a monoclonal mouse antibody recognizing all muscle actins (ready to use concentration provided by the manufacturer,

Biogenex, USA). Antigen retrieval for M-CSF was performed using Protease (Sigma, St Louis, MO, USA) for 10 min at 37°C . For CD68 and actin antigen retrieval, samples were boiled for 5 min at 90 W in citrate buffer (pH 6.0) in a microwave. In order to allow comparison of the sections stained with the different antibodies we performed serial tissue staining. Slides were counterstained with hematoxylin (Merck, Darmstadt, Germany).

Isolation and cultivation of human cells

Human adult cardiac myocytes (HACM) and human adult cardiac fibroblasts (HACF) were isolated and maintained in culture as previously described [14,15]. Both cell types were isolated from failing human hearts and from healthy donor hearts unsuitable for transplantation and stained positive for their respective markers [12]. Cells were cultured in cell culture flasks coated with 1% gelatine (Sigma) in M199 (Sigma) containing 20% fetal calf serum (Biochrom, Berlin, Germany), $100\ \text{U ml}^{-1}$ penicillin, $100\ \text{U ml}^{-1}$ streptomycin, $0.25\ \mu\text{g ml}^{-1}$ fungizone and $2\ \text{mM}$ L-glutamine (L-Glut-AB, all Cambrex, East Rutherford, NJ, USA) at 37°C in a humidified atmosphere of 5% CO_2 :95% air.

Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated using a Ficoll-Hypaque density gradient (Amersham Biosciences, Uppsala, Sweden) as described recently [16]. Positive isolation of peripheral blood monocytes from PBMC was performed by a CD14 + monocyte isolation kit (Stem Cell, Germany) according to the manufacturer's instructions. Cells were cultured in Ultra Culture medium (Cambrex) containing 10% human serum (Sigma) and L-Glut-AB. Monocyte derived macrophages (MDM) were generated as described [16].

All human material was obtained and processed according to the recommendations of the hospital's ethics committee, including informed consent.

Stimulation of human cells

Twenty-four hours prior to experiments confluent cells were starved with M199 containing 0.1% bovine serum albumin (BSA; Sigma). For stimulation experiments human recombinant TNF- α , interleukin-1 α (IL-1 α) (both Roche, Switzerland), IL-10, IL-8 (both R&D Systems), NF- κ B inhibitor dimethylfumarate [17] (Sigma, pre-treatment for 1 h) or the januskinase inhibitor JNK Inhibitor I (Merck, pre-treatment for 1 h) were added at various concentrations as indicated to the cells in fresh M199 containing 0.1% BSA. After the experiments supernatants were collected and stored at -80°C for further analysis. For adenoviral overexpression of a mutant dominant negative I κ B kinase 2 (dnIKK2) cells were transfected as described [18]. Briefly, cells were incubated with adenovirus containing the dnIKK2 or with a control vector containing green fluorescence protein (GFP) at a multiplicity of infection of 100 in PBS for 30 min. Cells were cultivated for 2 days before stimulation with TNF- α . MDM were

starved with Ultraculture (0.1% BSA) and stimulated with 2000 U ml⁻¹ TNF- α for 24 h.

Antigen determination

M-CSF antigen was determined in cell culture supernatants using a specific enzyme linked immunosorbent assay (ELISA) with a detection limit of 9 pg ml⁻¹ according to the manufacturer's instructions (R&D Systems).

Polymerase chain reaction

Real-time-PCR (RT-PCR) was performed using LightCycler-RNA Master SYBR Green I (Roche, Basel, Switzerland) according to the manufacturer's instructions. Primers were designed using Primer3 Software (<http://frodo.wi.mit.edu/>; accessed 22 October 2007), GAPDH-forward primer: 5'-acagtcacatgcatcactgcc-3', GAPDH-reverse primer: 5'-gcctgcttcacacctctctg-3', M-CSF-forward primer: 5'-tagccacatgattgggagtg-3', M-CSF-reverse primer: 5'-tatctctgaagcgcgatgtg-3'. The amplification conditions consisted of an initial incubation at 61 °C for 20 min, followed by incubation at 95 °C for 30 s, 50 cycles of 95 °C for 1 s, the respective annealing temperature (65 °C for GAPDH, 68 °C for M-CSF) for 10 s and 72 °C for 10 s, a melting step from 45 °C to 95 °C increasing 0.1 °C per second and a final cooling to 40 °C. Data were analysed using LightCycler Software Version 3.5 (Roche). RNA was isolated using the High Pure RNA Isolation Kit (Roche).

Transcription factor activity determination

For preparation of the cell nuclear fraction, HACM or HACF were seeded in gelatine-coated six-well plates. For nuclear extraction, the NE-PER[®] Nuclear Extraction Reagents and Halt[™] Protease Inhibitor cocktail (both Pierce, Waltham, MA, USA) were used. Cells were manually scratched and isolation of the nuclear fraction was performed according to the manufacturer's instructions. Nuclear extracts were immediately snap frozen and stored at -80 °C until used. Total protein content of the nuclear extract was determined using Bradford Reagent. For determination of transcription factor activity for p50, p65 and c-jun the TransAM[™] ELISA system (Active Motif, Rixensart, Belgium) was used according to the manufacturer's instruction.

U937 differentiation

The monocytic cell line U937 was purchased from ATCC (lgcprochem, Wesel, Germany) and maintained in RPMI 1640 Medium (Sigma) supplemented with 10% FCS (Biochrome) and L-Glut-AB at 37 °C in a humidified atmosphere of 5% CO₂:95% air. U937 were stimulated with serum free conditioned medium (0.1% BSA, L-Glut-AB) from HACM or HACF for 4 days. For control cells serum free medium (0.1% BSA, L-Glut-AB) was used. The expression of CD11b was determined by FACS analysis using a FACSCanto

(BD, Franklin Lakes, NJ, USA). Data analysis was performed using FACSDiva software (BD). CD11b was labeled using an r-phycoerythrin (R-PE) conjugated mouse anti-human monoclonal antibody (BD) according to the manufacturer's instruction. Isotype control was performed using a R-PE conjugated mouse IgG₁ (BD).

Statistical analysis

Data were compared statistically by ANOVA. Values of $P < 0.05$ were considered significant.

Results

To determine if M-CSF is present in human cardiac tissue we performed Western blot analysis of homogenized human cardiac tissue (Fig. 1A). We used M-CSF expressed in CHO cells as a positive control. M-CSF is a multiply glycosylated protein with a molecular mass ranging from 25 to 100 kD under reduced conditions [19]. Our results showed that M-CSF protein species in this molecular range are present in human healthy and diseased heart tissue and also in conditioned media of HACM and HACF (Fig. 1A). As we did not use protease inhibitors, proteolytic cleavage could have occurred, resulting in multiple forms of M-CSF with various molecular masses as seen in the Western blot. It should be noted that proteolytic cleavage of M-CSF has been shown not to affect its biologic activity [20].

The presence of M-CSF in human cardiac tissue was confirmed by immunohistochemical analysis of paraffin embedded heart tissue, as can be seen by the brown staining pattern in Fig. 1B. As shown in Fig. 1C, CD68 positive cells were mainly found around vessels whereas heart muscle cells as well as smooth muscle cells in the vessel wall stained positive for actin (Fig. 1D). Stains of serial sections showed that myocardial cells positive for M-CSF protein stained also positive for muscle actin (Fig. 1B2,D1). As expected CD68 positive cells stained also positive for M-CSF (Fig. 1B1,C1). Note that no CD68 positive cells are present in insert 1B2 (Fig. 1C2 shows the CD68 staining of area 1B2).

Incubation of HACM with increasing concentrations of TNF- α (2–2000 U ml⁻¹) resulted in a dose-dependent protein accumulation of M-CSF (Fig. 2A). When the cells were incubated with 2000 U ml⁻¹ TNF- α for different time periods we observed a significant time-dependent protein accumulation in M-CSF by HACM after 12, 24, 36 and 48 h (Fig. 2B). Similar results were seen with 200 U ml⁻¹ TNF- α (Fig. 2A). To test if this increase in M-CSF protein production can also be seen at the level of mRNA expression, we determined specific mRNA levels for M-CSF in HACM treated with and without 2000 U ml⁻¹ TNF- α for 4, 8 and 24 h, respectively. As can be seen in Fig. 2C, mRNA was upregulated at all time points. No cytotoxic effect of TNF- α was observed at the concentrations used in this study as determined by LDH leakage (data not shown). As can be seen from Table 1, HACM isolated from four failing hearts and one non-failing heart express M-CSF

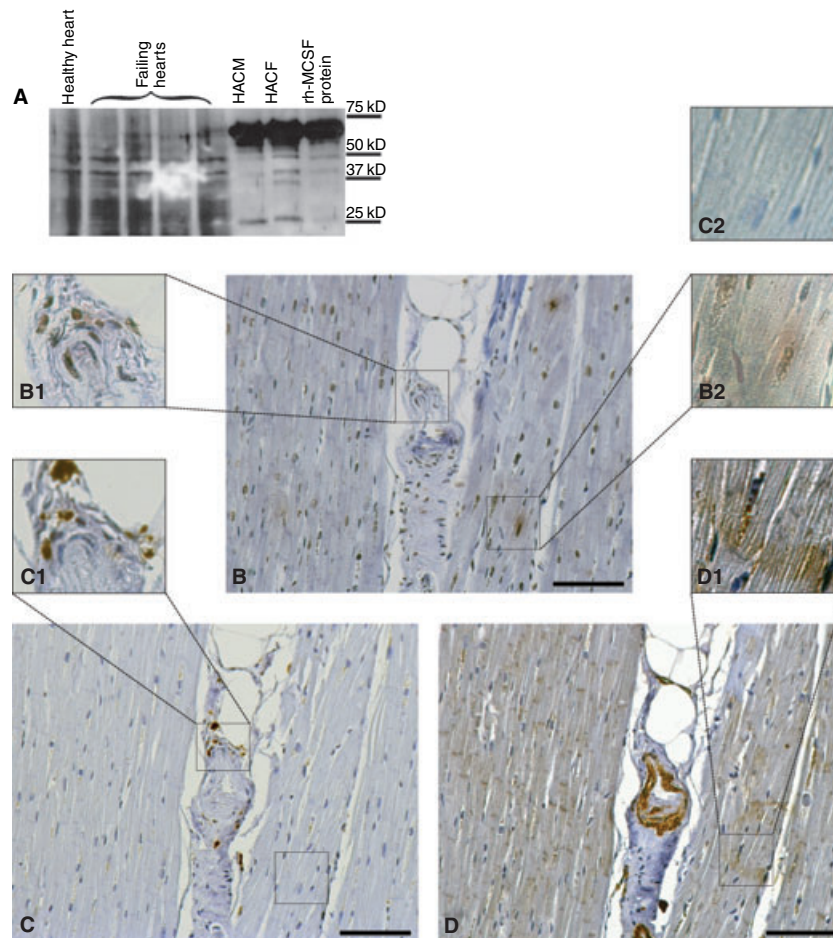


Fig. 1. M-CSF protein is present in human myocardial tissue. Myocardial tissue from one healthy heart (lane 1) and four failing hearts (lanes 2 to 5) was homogenized and Western blotting for M-CSF protein was performed. Conditioned media from HACM (lane 6) and HACF (lane 7) and purified rh M-CSF (lane 8) were also subjected to Western blotting as described in the Methods section (panel A). M-CSF (panel B), CD68 (panel C) and muscle actin (panel D) in human healthy myocardial tissue were visualized in serial sections by immunohistochemistry using specific antibodies as described in the Methods section. Inserts show comparable areas from serial sections at higher magnification. Black bars in panel B–D represent 100 μm .

in vitro. When these cells were treated with 2000 U ml^{-1} TNF- α for 24 h M-CSF protein production increased from 2- to 3-fold, respectively.

Similar results were obtained when HACF were treated with TNF- α (Fig. 2D–F). Stimulating HACF from four failing hearts and one non-failing heart with TNF- α resulted in a 2- to 3.5-fold increase in M-CSF protein, respectively, as compared with untreated cells (Table 2).

Notably, MDM secreted M-CSF at levels comparable with HACM and HACF (215.3 $\text{pg ml}^{-1} \pm 157.2$, mean value \pm SD of three independent healthy donors). The increase of M-CSF protein secretion in response to TNF- α was 1.45- to 1.88-fold compared with untreated MDM ($P < 0.05$). When comparing these values with values seen in HACM and HACF, possible differences in cell size between these different cell types should be considered.

Treatment of HACM and HACF with 200 U ml^{-1} IL-1 α for 24 h resulted in an up to 1.9-fold increase of M-CSF in HACM and an up to 1.4-fold increase in HACF, whereas IL-10 and IL-8 had no effect on M-CSF protein secretion (data not shown).

The nuclear transcription factors AP-1 and NF- κB are involved in regulating the expression of M-CSF in various cells on the one hand, and both transcription factors have been shown to be induced by TNF- α in cardiac myocytes on the other hand [21]. To determine a possible involvement of AP-1 and NF- κB in the TNF- α -induced M-CSF upregulation in HACM and HACF we used the NF- κB blocker dimethylfumarate (DMF) and the januskinase JNK Inhibitor I. Only dimethylfumarate blocked the induction of M-CSF production by TNF- α (Fig. 3A,B). No cytotoxic effect of DMF or a combination of DMF plus TNF- α was found using a commercially available assay for photometric determination of lactate dehydrogenase leakage (Sigma) as described (data not shown) [22].

To verify a selective blocking of NF- κB by dimethylfumarate without involvement of AP-1 we determined the nuclear levels of NF- κB subunits p50 and p65 as well as the AP-1 component c-jun in cells stimulated with TNF- α in the absence and presence of dimethylfumarate. TransAM ELISA revealed that TNF- α induced nuclear translocation of p50, p65 and c-jun but dimethylfumarate only blocked active p50 and p65 protein in

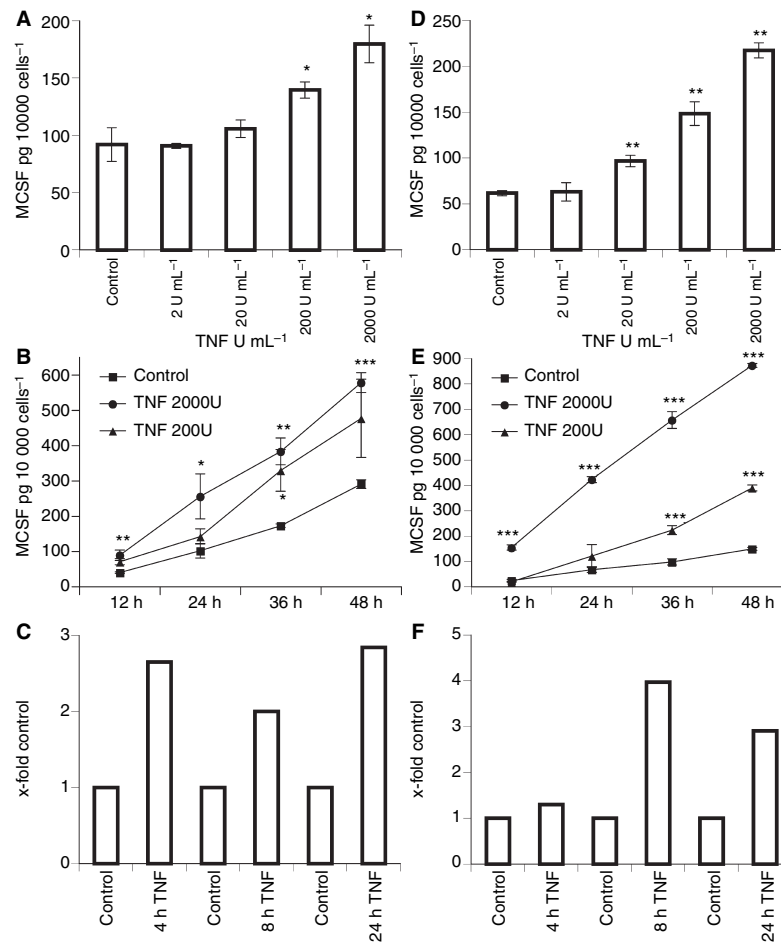


Fig. 2. TNF- α upregulates M-CSF in cultured human adult cardiac myocytes (HACM) and in cultured human adult cardiac fibroblasts (HACF). Confluent monolayers of HACM (panels A and B) or HACF (panels D and E) were incubated for 24 h in the absence or presence of 2, 20, 200 or 2000 U mL⁻¹ TNF- α (panels A and D) or in the absence or presence of 2000 U mL⁻¹ or 200 U mL⁻¹ TNF- α for 12, 24, 36 and 48 h (panels B and E). M-CSF in the supernatants was determined by a specific ELISA as described in the Methods section. Values are given in pg/10 000 cells and represent mean values \pm SD of three independent determinations. Experiments were performed three times with HACM and HACF isolated from three different donors, respectively. A representative experiment with cells from the heart of a patient suffering from ischemic cardiomyopathy is shown. Data were compared by ANOVA. *** P < 0.00005; ** P < 0.005; * P < 0.05. Confluent monolayers of HACM (Panel C) and HACF (Panel F) were incubated for 4, 8 and 24 h with or without 2000 U mL⁻¹ TNF- α . RNA was prepared and RT-PCR with specific primers for GAPDH and M-CSF was performed as described in the Methods section. M-CSF values were normalized according to the GAPDH levels. Values are given as x-fold control. Experiments were performed two times with HACM and HACF isolated from two different donors, respectively. A representative experiment with cells from the heart of a patient suffering from ischemic cardiomyopathy is shown.

Table 1 TNF- α upregulates M-CSF in human cardiac myocytes (HACM) from five different donors. Confluent monolayers of HACM, isolated from five different donors (donors 1 to 4, explanted hearts from patients suffering from ischemic cardiomyopathy; donor 5, healthy donor heart, unsuitable for transplantation) were incubated for 24 h in the absence or presence of TNF- α (2000 U mL⁻¹). Values are given in pg M-CSF/10000 cells and represent mean values \pm SD of three independent determinations

	HACM no. 1	HACM no. 2	HACM no.3	HACM no.4	HACM no.5
Control	54.2 \pm 5.5	118.5 \pm 8.4	58.2 \pm 14.9	102.3 \pm 20.5	80.9 \pm 8.7
TNF- α	162.3 \pm 22.5	257.7 \pm 3.8	155.3 \pm 23.1	256.3 \pm 63.5	193.2 \pm 2.9
Fold increase	2.99	2.17	2.67	2.51	2.39

the nucleus, whereas c-jun was not affected (Fig. 4). Nuclear translocation of p65 was also induced with 200U TNF- α in HACM and HACF and increased 2.4-fold and 2-fold compared with control after 2 h, respectively.

In addition, we transfected HACM and HACF with a dominant negative mutant of IkappaB kinase 2 using a

recombinant adenovirus. IKK2 acts as an upstream activator of NF- κ B. Expression of dnIKK2 results in a strongly reduced nuclear translocation of NF- κ B [18]. We analyzed mRNA expression after 8 h of stimulation with TNF- α and found a dramatically reduced upregulation of M-CSF mRNA in dnIKK2 HACM compared with GFP transfected cells and

Table 2 TNF- α upregulates M-CSF in human cardiac fibroblasts (HACF) from five different donors. Confluent monolayers of HACF, isolated from five different donors (donors 1 to 4, explanted hearts from patients suffering from ischemic cardiomyopathy; donor 5, healthy donor heart, unsuitable for transplantation) were incubated for 24 h in the absence or presence of TNF- α (2000 U ml⁻¹). Values are given in pg M-CSF/10 000 cells and represent mean values \pm SD of three independent determinations

	HACF no.1	HACF no. 2	HACF no. 3	HACF no. 4	HACF no. 5
Control	59.7 \pm 4.5	79.3 \pm 7.4	79.1 \pm 9.1	68.6 \pm 1.1	102.8 \pm 18.6
TNF- α	127.1 \pm 3.0	275.4 \pm 19.4	168.3 \pm 3.8	156.9 \pm 2.8	261.7 \pm 31.2
Fold increase	2.13	3.47	2.13	2.28	2.55

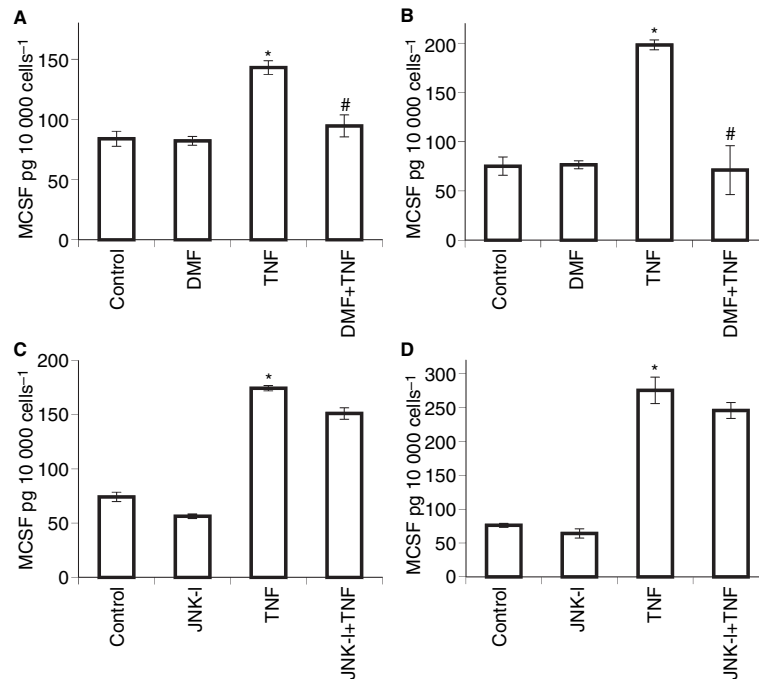


Fig. 3. Effect of dimethylfumarate (DMF) or januskinase inhibitor-1 (JNK-I) on TNF- α -induced M-CSF expression in human adult cardiac myocytes (HACM) and human adult cardiac fibroblasts (HACF). Confluent monolayers of HACM (panels A and C) and HACF (panels B and D) were incubated for 24 h with or without 2000 U ml⁻¹ TNF- α , 100 μ M DMF (HACM), 50 μ M DMF (HACF) or a combination of TNF- α and DMF at the respective concentrations (panels A and B) or with or without 2000 U ml⁻¹ TNF- α , 10 μ M JNK-I or a combination of TNF- α and JNK-I at the respective concentrations (panels C and D). M-CSF in the supernatants was determined by a specific ELISA as described in the Methods section. Values are given in pg/10 000 cells/24 h and represent mean values \pm SD of three independent determinations. Experiments were performed three times with cells isolated from three different donors. A representative experiment with cells obtained from the heart of a patient suffering from ischemic cardiomyopathy is shown. Data were compared by ANOVA. Values of $P < 0.05$ were considered significant. * $P < 0.0005$ as compared with control; # $P < 0.005$ as compared with TNF- α .

no upregulation in HACF treated with dnIKK2 compared with cells transfected with the control vector (Table 3).

We tested a possible effect of conditioned media (CM) of HACM or HACF on the differentiation of U937 cells using the monocyte differentiation marker CD11b [23]. Cultivation of U937 cells in CM of HACM and HACF increased the expression of CD11b significantly (Fig. 5).

Discussion

Accumulating evidence suggests that the hematopoietic growth factor M-CSF, which stimulates the survival, proliferation and differentiation of cells of the mononuclear phagocytes lineages, plays also an important role in inflammatory processes. It is produced by a variety of cells,

including monocytes, macrophages, endothelial cells, smooth muscle cells and fibroblasts [3]. We were able to show that M-CSF is also present in the human heart, where M-CSF was not only detected in macrophages but also in cardiac muscle cells, as revealed by immunohistochemistry. In addition, we support these findings by showing here for the first time that cultured human cardiac cells, namely primary human cardiac myocytes and primary human cardiac fibroblasts, constitutively express M-CSF.

In this context it should be emphasized that recent studies have implicated M-CSF in cardiac healing after myocardial infarction. In a mouse hindlimb ischemia model M-CSF was shown to stimulate differentiation of bone marrow cells into endothelial cells and increase blood flow [24]. Whereas Woldbaek *et al.* [25] reported a decrease of M-CSF in the

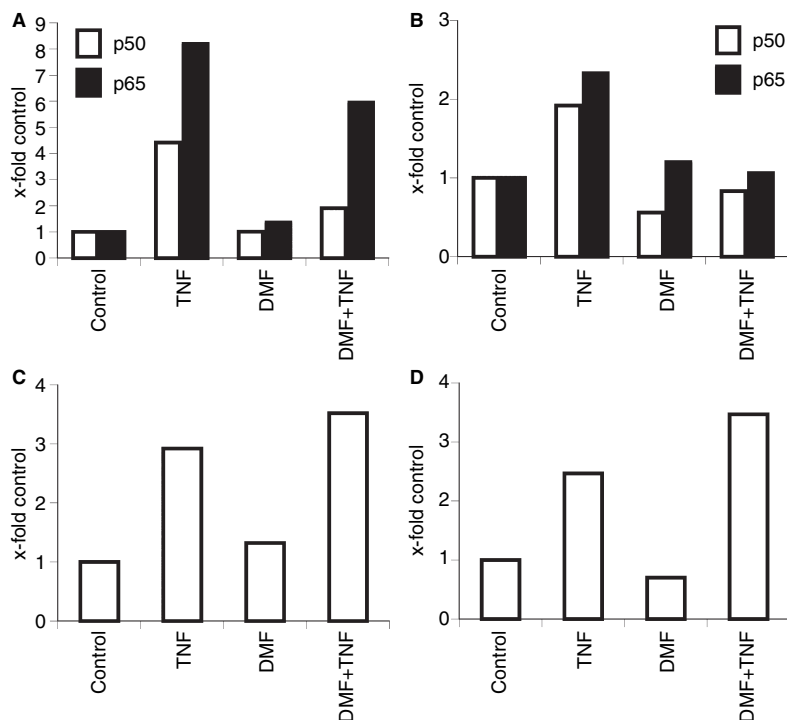


Fig. 4. Effect of TNF- α and/or dimethylfumarate (DMF) on NF- κ B activation and AP-1 activation in human adult cardiac myocytes (HACM) and human adult cardiac fibroblasts (HACF). HACM (panels A and C) and HACF (panels B and D) were treated for 60 min with or without 2000 U ml⁻¹ TNF- α , 100 μ M DMF or a combination of TNF- α and DMF at the respective concentrations. Nuclear extracts were prepared from these cells and levels of p50 and p65 (panels A and B) or c-jun (panels C and D) in these extracts were determined as described in the Methods section. The combined data of two experiments with cells obtained from hearts of patients suffering from ischemic cardiomyopathy is shown.

Table 3 Overexpression of dominant negative IKK2 blocks TNF- α -induced M-CSF mRNA upregulation. Confluent monolayers of HACM and HACF were transfected with an adenovirus containing construct for dominant negative IKK2 or a construct for GFP as described in the Methods section. Forty-eight hours later cells were incubated for 8 h with TNF- α (2000 U ml⁻¹). RNA was prepared and RT-PCR with specific primers for GAPDH and M-CSF was performed as described in the Methods section. M-CSF values were normalized according to the GAPDH levels. Values are given as x-fold control. Experiments were performed two times with HACM and HACF isolated from two different donors. A representative experiment with cells from the heart of a patient suffering from ischemic cardiomyopathy is shown.

	GFP transfected		IKK2 transfected	
	Control	TNF- α	Control	TNF- α
HACM	1.00	4.47	1.00	1.46
HACF	1.00	3.54	1.00	1.02

heart after myocardial infarction in a mouse model, an increase in M-CSF was detected in atrioventricular valves after surgery in rabbits [26] and in healing myocardial infarctions in a dog model [8].

Proinflammatory cytokines such as TNF- α have been identified as important mediators in the development and progression of cardiac disease [11]. To mimic the inflammatory conditions associated with the pathology of myocardial infarction we treated cardiac myocytes and cardiac fibroblasts

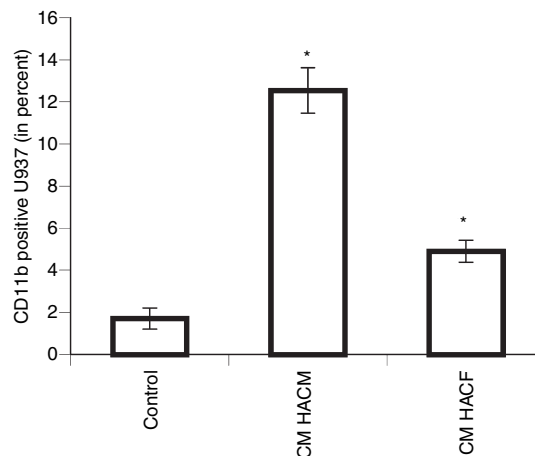


Fig. 5. Effect of conditioned media of HACM and HACF on the differentiation of U937 cells. U937 cells were incubated with serum-free medium (Control) or conditioned medium from HACM (CM HACM) or HACF (CM HACF) for 4 days. Expression of CD11b was determined by FACS analysis. Experiments were performed eight times. The combined data are shown. * $P < 0.005$ as compared with control.

with the potent inflammatory mediator TNF- α , which has been reported to be critically involved in this process. We show here that TNF- α increased the production of M-CSF protein in cardiac cells at concentrations of 200 and 2000 U ml⁻¹. However, caution should be used when extrapolating these

results to the *in vivo* setting because it is difficult to relate the concentrations of TNF- α used in our *in vitro* experiments to concentrations of this cytokine reached *in vivo*, as plasma concentrations do not necessarily reflect local tissue levels and data on tissue levels are, at least to our knowledge, not available for humans. Furthermore, TNF- α upregulated the expression of M-CSF specific mRNA in these cells. Similar effects of TNF- α on M-CSF production in other cells have been described [27–31]. TNF- α has been shown to induce activation of AP-1 and NF- κ B in rat neonatal cardiac myocytes [21]. The promoter region of M-CSF was reported to contain putative cis-acting sites for transcription factors AP-1, NF- κ B, EGR-1 and NF-IL6 [32]. In a human pancreatic cell line M-CSF gene expression was induced via NF- κ B activation by IL-1 and lipopolysaccharide and via AP-1 activation by phorbol ester [33]. TNF- α is known to activate the NF- κ B pathway mainly via phosphorylation of I κ B α [34,35] and the AP-1 pathway via JNK [36]. Based on our observation that TNF- α -induced upregulation of M-CSF in cardiac myocytes and fibroblasts was blocked by the NF- κ B inhibitor dimethylfumarate and by viral transfection of a dominant negative mutant of IKK2 but not by the januskinase inhibitor JNK inhibitor-1, we conclude that in human cardiac cells the TNF- α -induced upregulation of M-CSF is mainly mediated via NF- κ B activation. This hypothesis is further supported by our data, that TNF- α treatment of cardiac myocytes and fibroblasts results in an increase of the NF- κ B subunits p50 and p65 and of the AP-1 component c-jun in nuclear extracts of these cells and that the increase of p50 and p65 was specifically blocked by dimethylfumarate under these conditions.

In conclusion, we provide evidence that human cardiac cells could serve as a source for M-CSF and that its expression in these cells is upregulated by inflammatory mediators. Therefore we speculate that human cardiac cells, by generating a microenvironment suitable for survival of monocytes and their maturation and differentiation into macrophages, allow these cells to actively participate in the repair process, as suggested by recent work. In support of this notion we could show an increase of the differentiation marker CD11b in U937 cells after incubation with conditioned medium from HACM or HACF. Taken together with our previous data showing that human cardiac cells express MCP-1 [12], a key homing factor for monocytes, under inflammatory conditions, our results presented here suggest that, if similar mechanisms are also operative in the *in vivo* setting, human cardiac cells could not only initiate monocyte attraction to inflammatory sites but could also be actively involved in regulating monocyte and macrophage survival and differentiation.

Addendum

P. J. Hohensinner, C. Kaun, K. Rychli, G. Rega, K. Huber and J. Wojta made substantial contributions to the concept and design of this article; P. J. Hohensinner, S. Pfaffenberger, A. Niessner and R. de Martin were involved in the analysis and/or interpretation of the data; P. J. Hohensinner, G. Maurer, R.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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