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# RELATION BETWEEN EXPRESSION OF TNF ALPHA, iNOS, VEGF mRNA AND DEVELOPMENT OF HEART FAILURE AFTER EXPERIMENTAL MYOCARDIAL INFARCTION IN RATS

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An injury to the heart due to myocardial infarction (MI) may progress to heart failure. Among factors, whose interactions promote remodeling of ischemic myocardium, the increased expression of tumor necrosis factor α (TNFα), inducible nitric oxide synthase (iNOS) and Vascular Endothelial Growth Factor (VEGF) was found. However, little is known about the temporal and spatial relation between expression of iNOS, cytokine TNFα, and growth factor VEGF during pathological process of development of heart failure after the myocardial infarction. Male Sprague-Dawley rats were used for experimental myocardial infarction. The procedure was performed by anterolateral thoracotomy and snearing LAD with the metal clip. The hemodynamic measurements were done with the Langendorff preparation converted into a working heart system. The hemodynamic parameters were recorded at day 6, 11, 28, 40 and the myocardium for gene expression was collected at day 1, 4, 11, 28, 40. Control group was sham operated rats. The VEGF, TNFα, iNOS, and GAPDH genes were detected by RT-PCR assay from samples taken at border zone of myocardial infarction. Expression of isoform $VEGF_{120}$ was found at day 1 and 4 after MI, whereas isoforms $VEGF_{164}$ and $VEGF_{188}$ along with expression of TNFα and iNOS was found at day 1, 4, 11, 28, 40. No expression of examined genes was detected in the myocardium of control rats. The expression of studied factors was parallel with development of heart failure after myocardial infarction assessed by hemodynamic measurements. These findings confirm the postulated involvement of TNFα, iNOS and growth factor VEGF in the remodeling of the myocardium and development of heart failure after experimental myocardial infarction.

Key words: *TNFα, VEGF, iNOS, myocardial infarction, remodeling, heart failure.*

## INTRODUCTION

A large transmural myocardial infarction (MI) initiate a cascade of progressive structural and geometrical changes in left ventricle that is commonly referred to as remodeling. The remodeling process is believed to serve as a compensatory process to maintain cardiac output (1). However, these

architectural changes may also contribute to the development of congestive symptoms from afterload mismatch and exacerbation of left ventricle (LV) dysfunction (1). The mechanisms responsible for the transition to the heart failure are still unknown, however the expression of several growht factors and cytokines is activated and a role of the insufficient angiogenesis and the endothelial dysfunction in the pathophysiology of heart failure is widely discussed (2, 3). Remodeling of the heart is characterised by hypertrophy of surviving myocytes and hyperplasia of nonmyocytes (4). This observations suggest that growth factors and cytokines may be important modulators in process of postmyocardial infarction remodeling, including infarction associated inflammation, cardiac hypertrophy, fibrosis of myocardium and cardiac dysfunction (5).

The clinical and experimental data have demonstrated that among the cytokines and growth factors, the elevated TNFα and enhanced expression of iNOS may be involved in progression to the heart failure (6, 7). Both factors were postulated to be responsible for cardiac deterioration causing a negative inotropic effect and apoptosis (8).

Vascular Endothelial Growth Factor (VEGF) is involved in embryonic vasculogenesis and pathological angiogenesis (9). It may also be involved in angiogenic response to myocardial ischemia and is now being tested for use in gene therapy of ischemic heart and peripheral vascular disease (10).

The aim of present study was to investigate the relation between expression of TNFα, iNOS and growth factor VEGF during development of heart failure after the experimental myocardial infarction.

## METHODS

### *The model of experimental myocardial infarction in rats*

All experiments were performed on Sprague-Dawley male rats weighing 250—350 g, kept under standardized housing conditions. The local Bioethic Committee for Animal, Silesian Academy of Medicine approved the experiments. All animals tested were carried out in accordance with NIH regulation of animals care, as described in "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985).

The rats were anaesthetized with pentobarbital (60 mg/kg intraperitoneally, i.p.). Surgical procedure was performed after Seyle (11) *et al.* and others (12) with own improvements (13). In brief, the trachea was incised longitudinally and cannulated. The chest was opened under ventilation with room air (Rodent Ventilator-UB 7025, stroke volume 0.8 ml/100 g of body weight, rate 54 strokes/min with the positive end-respiratory pressure of 1 cm $H_2O$) by left thoracotomy. After opening of the pericardium the heart was exteriorised and a sling (6/0 Prolene 0.7 suture, EH 7406H, Ethicon GmbH, Norderstedt, Germany) was placed around the left anterior descending coronary artery (LAD) close to its origin. Then the ligature was passed through a plastic pad. The

coronary artery was occluded by applying tension to the ligature. Tension was maintained by clamping a climb clip (LT-100, Ethicon). The rat awaked in few hours after closing the thorax. The postoperative mortality rate of all rats was 15%.

Animals were scarified at day 1, 4, 11, 28, 40 after myocardial infarction and myocardium was isolated from myocardial infarction border zone. The sham operated rats (without the LAD closure) served as the control animals. Each experimental group consisted of six animals.

## *Hemodynamics*

Rats survived with myocardial infarction after 11, 21, 28, 40 days were heparinized (500 IU/100 g body weight, i.p.) and anaesthetized with pentobarbital (60 mg/kg, i.p.). Hearts were rapidly excised together with lungs and arrested by chilling in the beaker with ice-cold modified Krebs-Henselheit bicarbonate buffer and weighted. The heart was cannulated through the ascending aorta, and mounted with stainles-steel cannula, connected to a non-recirculating perfusion system according to the method of Langendorff (7).

The coronary arteries were retrograde perfused with a constant pressure at 60 mm Hg during preparation procedure. The perfusion medium was a modified Krebs-Henselheit buffer (pH 7.4—7.45 at 37°C) consisted of: NaCl 118 mM, KCl 4.7 mM, $NaHCO_3$ 24.88 mM, $CaCl_2$ 2.52 mM, $KH_2PO_4$ 1.18 mM, $MgSO_4$ 1.64 mM, glucose 11.1 mM, pyruvate 2.0 mM, saturated with 95% $O_2$ and 5% $CO_2$ ($pO_2$ in perfusate measured just above aorta was $>560$ mmHg).

The air temperature in the heart chamber was maintained at 37°C by a beaker with a jacket containing water. The lungs lobes were subsequently cut off. To convert the Langendorff preparation into a working heart, the veins were ligated close to the surface of the right atrium and the left atrium was cannulated through an incision into the left auricle with steel cannula. A plastic cannula was placed in the pulmonary artery to drain the coronary effluent perfusate for $pO_2$, $pCO_2$ and pH measurement in the volume of 0.5 ml every 30 s. Perfusion through the aorta was switched to perfusion through left atrium, which was regulated through adjustable resistance. The starting atrial filling pressure was adjusted at 12 mm Hg (preload). The left ventricle ejected the perfusion fluid through an aortic cannula into an overflow system in which the aortic pressure and thus the coronary perfusion pressure was held constant at 60 mm Hg (afterload). Inflow (preload) and outflow (afterload) elasticity chambers (air/fluid "windkessel") were attached to the left atrial and aortic cannula, respectively. At the end of the preparation, the suction electrodes were attached onto the heart surface for electrogram (EG) recording $pO_2$, $pCO_2$, pH in effluent perfusate were measred ($pO_2>530$ mm Hg just before left atrium). The hearts were allowed to beat spontaneously. Following parameters were measured, calculated and computerized min of the experiment every 30 s in all control and infarcted hearts: heart rate (HR), left atrial filling pressure (PP, preload pressure), Aortic systolic and diastolic pressures (AoS, AoD), $+dP/dt$, $-dP/dt$, Aortic flow (AF), Coronary flow (CF), $pO_2$, $pCO_2$ and pH in pulmonary effluent, Myocardial oxygen consumption ($MVO_2$) was calculated according to Zander *et al* (3) with formula: CF/g wwt × (affluent $pO_2$ — effluent $pO_2$) × c × 100, where: wwt — heart wet weight, c=0.0240 (Bunsen oxygen solubility for Krebs-Henselheit solution at 37°C).

## *RNA isolation*

Total RNA was isolated from the myocardial tissue samples homogenates by guanidine-thyocyanate-phenol-chloroform extraction method (14). After addition of 600 ml of

GTC-γ-merkaptoethanol solution, the tissue samples were homogenized and stored in freezer (−72° C). Then, 600 ml of acidic phenol-chloroform-isoamyl alcohol solution (pH = 4,3 Sigma) was added and followed by centrifugation at 14,000 × g for 30 min. The supernatant was collected and mixed with a double volume of cold absolute ethanol and stored overnight at −20°C. Sedimentation at 14,000 × g was performed again for 30 min and the resulting RNA pellet was washed with 70% ethanol, centrifuged again, air dried and dissolved in 25 ml of DEPC — treated water. Then the RNA concentration was measured with spectrophotometer and adjusted to 100 ng RNA per 1 μl.

### *The Reverse transcription — PCR reaction*

mRNA level was studied using a coamplification of the target gene with a control gene (internal standard — glyceraldehyde phosphate dehydrogenase (GAPDH)) by means of RT-PCR assay (reverse transcription-polymerase chain reaction) (15). RT-PCR was carried out on 100ng total RNA using Tth DNA plymerase (Promega) following the manufacturer's recommended conditions at a concentration 1,5 U/tube. Briefly, the reverse transcription step was carried out in 5 μl volume (1U Tth DNA Polymerase, 1 mM $MnCl_2$, 10 mM Tris — HCl (pH = 8,9), 50 mM KCl, 200 μM dNTPs and 200 nM downstream (3') primer (iNOS 5' — tgg ctt gcc ctt gga agt ttc tc, VEGF 5' — cac cgc ctt ggc ttg gtc aca t, TNFα 5' — acc agg gct tga gct cag ctc cc, GAPDH 5' — tcc acc acc ctg ttg ctg ta) at 62.5°C for 20 minutes. Afterwards, 20 μl of chelating buffer (750 μM EGTA, 2.5 mM $MgCl_2$, 200 nM upstream (5') primer (iNOS 5' — tgt ctc tgg gtc ctc tgg tca aa, VEGF 5' — ctg ctc tct tgg gtg cac tgg, TNFα 5' — ctc gag tga caa gcc cgt agc cc, GAPDH 5' — acc aca gtc cat gcc atc ac) and 0,5 U per tube of Prime Zyme Polymerase (Th. brockianus, Biometra, Germany) were added. The 30-cycle PCR was performed (at the temperature profile 94°C/20 s, 62.5°C/20 s. 72°C/20 s), followed by final 10 min extension in 70°C and cooling to 4 °C. All PCR tests were completed in Biometra UNO Thermoblock themocycler.

PCR products were analysed with electrophoresis in TEA (pH = 8,0) buffer with ethydium bromide (0,5 μg/ml) at 7 V/cm, in 2% agarose gel (3:1 agarose, PCR grade, Sigma, USA).

## RESULTS

### *The time-related changes of TNFα, iNOS, VEGF gene expression after myocardial infarction*

The sequence of TNFα, iNOS mRNA expression in the border zone of myocardial infarction is depicted in *Fig. 1* and *2*, respectively. Analysis demonstrated the permanent expression of TNFα and iNOS mRNA starting from the first day after the experimental myocardial infarction contrary to the sham operated control rats where no expression was found.

No expression of VEGF mRNA was found in myocardium from the sham operated rats. The expression of VEGF in infarcted myocardium was time dependent and the different VEGF isoforms were found in different time points. Expression of isoform $VEGF_{120}$ was deected only on day 1 and 4 after myocardial infarction, whereas the mRNA of $VEGF_{164}$ and $VEGF_{188}$ isoforms was present during the whole experimental period (*Fig. 3*).

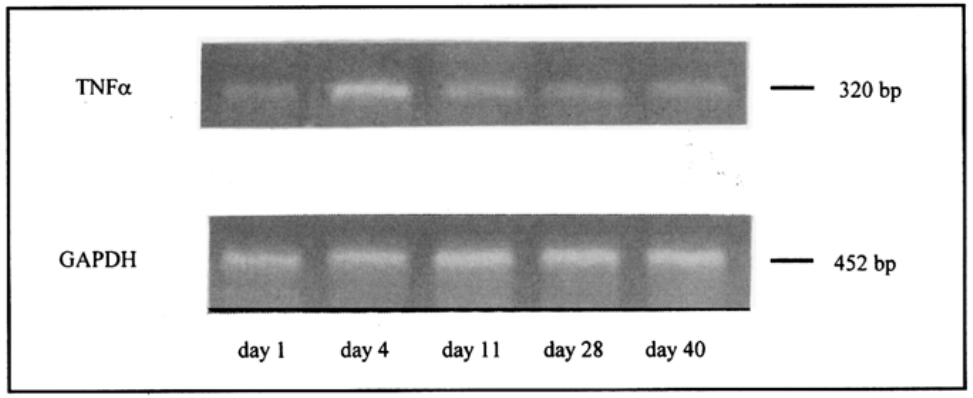


*Fig. 1.* Reverse transcriptase — PCR. Expression of TNFα mRNA in border zone of myocardial infarction at day 1, 4, 11, 28, 40 after LAD ligation. bp = base pair.

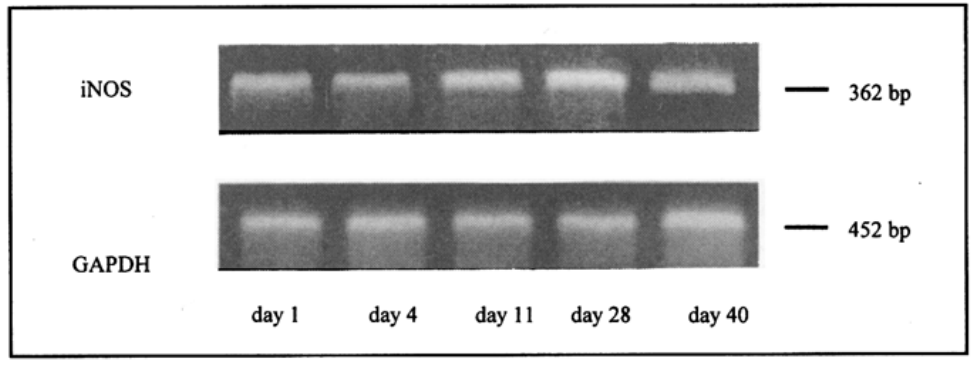


*Fig. 2.* Reverse transcriptase — PCR. Expression of iNOS mRNA in border zone of myocardial infarction at day 1, 4, 11, 28, 40 after LAD ligation. bp = base par.

### *Hemodynamics*

Results showed that there was decrease in aortic systolic blood pressure both at day 6 and 40 after myocardial infarction, whereas on day 11 and 28 after myocardial infarction the systolic blood pressure did not differ from control animals (*Fig. 4A, B*). Decrease in diastolic pressure was only found on day 40 after myocardial infarction. No changes in diastolic pressure was found in other time points.

On day 28 and 40 after myocardial infarction, progressive, statistically significant depression of isovolumetric contraction parameters was present. +dP/dt was also depressed on day 6 after myocardial infarction, whereas on day 11 it returned to control values (*Fig. 5A, B*). −dP/dt was depressed on day 6 after myocardial infarction, on day 11, 28, 40 it was not different from control values (*Fig. 5C, D*).

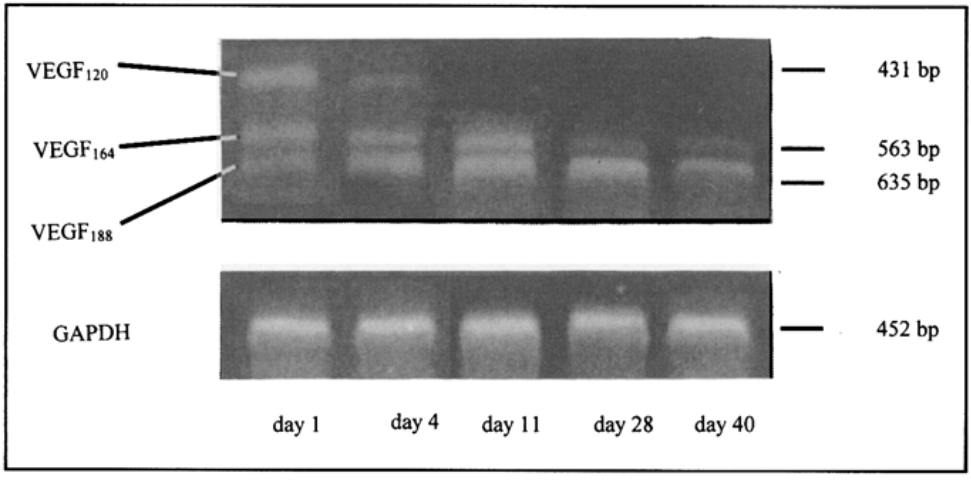


*Fig. 3.* Reverse transcription — PCR. Expression of $VEGF_{120}$, $VEGF_{164}$ and $VEGF_{188}$ isoform mRNA in border zone of myocardial infarction at day 1, 4, 11, 28, 40 after LAD ligation. bp = base pair.

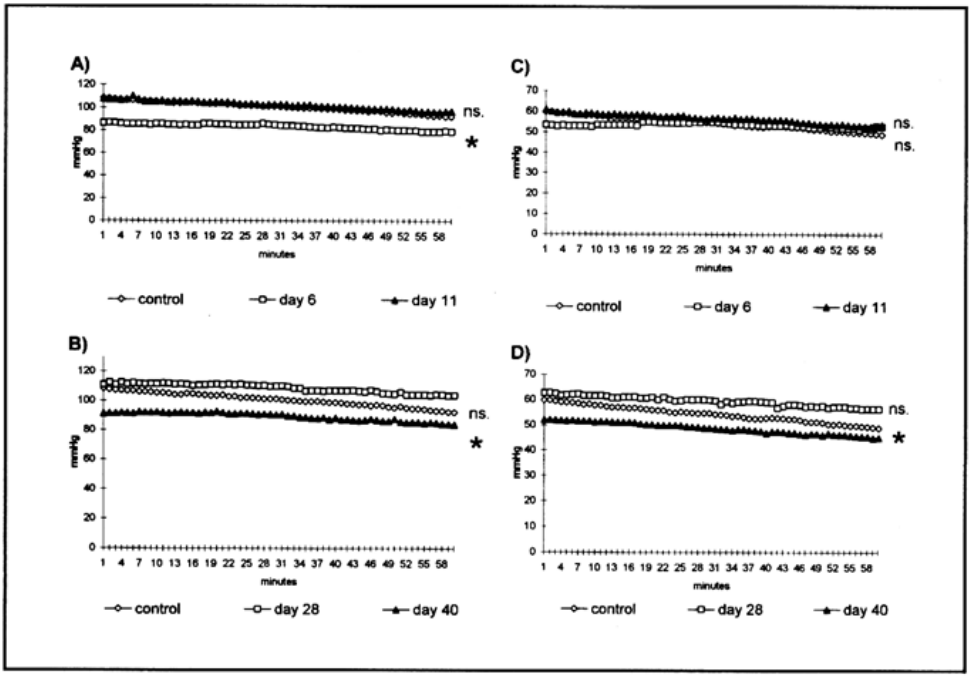


*Fig. 4.* Aortic systolic (A, B) and diastolic (C, D) blood pressure. +dP/dt and −dP/dt. Differences statistically significant for (A) day 6, $p<0{,}005$ and (B) day 28, $p<0{,}01$, (C) day 6, $p<0{,}001$ vs. control. All results expressed as mean ± SD, n = 7, 7, 10, 7, 8, for day 6, 11, 28, 40 respectively, t-Student test. Recording during 60 min of isolated working heart.

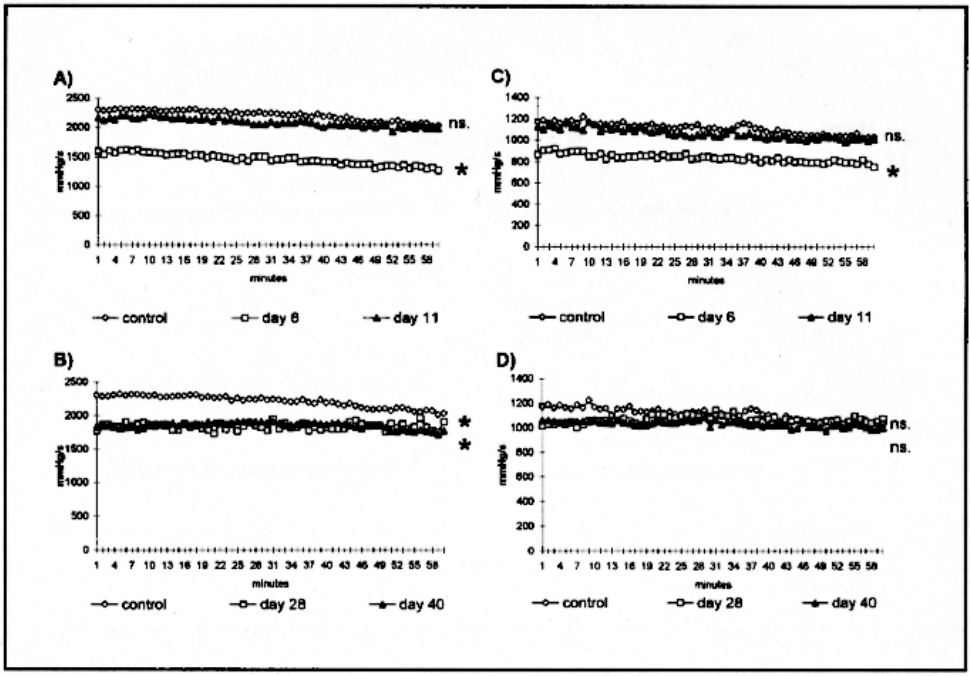


*Fig. 5.* +dP/dt (A, B) and −dP/dt (C, D). Differences statistically significant for t-Student test. * $p<0{,}005$ mean ± SD, $n=7$ for day 6 (A), mean ± SD, $n=7$ for day 28 and 40 (B), $p<0{,}001$, mean ± SD, $n=7$ for day 6 (C). All results expressed as mean ± SD, $n=7$, 7, 10, 7, 8 for day 6, 11, 28, 40 respectively, t-Student test. Recording during 60 min of isolated working heart.

There were no differences in aortic blood flow in all time points. However we observed changes in coronary blood flow (CBF): marked decrease on day 6 after MI, and later, CBF returned to control values. On day 28 and 40 statistically significant increase in CBF was observed (*Fig. 6A, B*).

Myocardial oxygen consumption was depressed only on day 6. On day 11 it did not differ from control values. Myocardial oxygen concentration increased on day 28, but it was significantly decreased on day 40 (*Fig. 4C, D*).

We did not find any differences in $pCO_2$ in pulmonary perfusion fluid. The significant increase in $pO_2$ in pulmonary perfusion fluid was found on day 6, 11, 40. On day 28 decrease in $pO_2$ was found. pH of pulmonary perfusion fluid was unchanged in any time point.

There were no changes in heart rate and left atrial filling pressure during observation, the former showed only tendency to decrease on day 40 after MI.

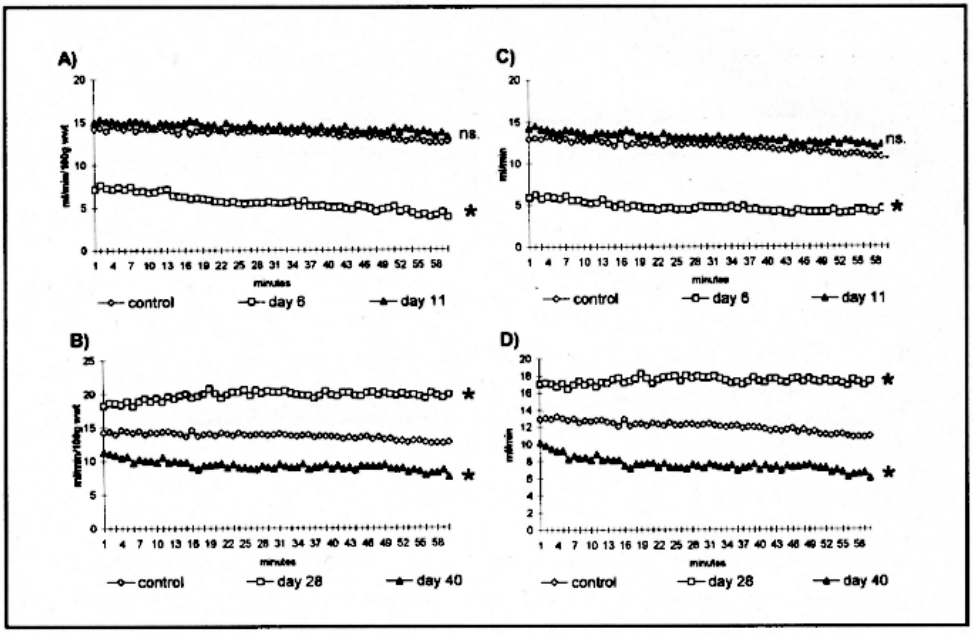


*Fig. 6.* Myocardial oxygen consumption ($MVO_2$) (A, B) and coronary blood flow (CBF) (C, D). Differences statistically significant for (A) day 6, $p < 0{,}001$ and (B) day 28, $p < 0{,}05$, day 40 (C), day 6, $p < 0{,}001$, (D) day 28 $p < 0{,}05$ and day 40 vs. control. All results expressed as mean $\pm$ SD, n = 7, 7, 10, 7, 8 for day 6, 11, 28, 40 respectively, t-Student test. Recording during 60 min of isolated working heart.

## DISCUSSION

Remodeling process leading to post-MI heart failure continues to progress over time. Myocardium adaptive hypertrophy undergoes transition, leading to further cardiac dilatation with a strong tendency toward a decrease in the thickness of the non-infarcted myocardium in relation to LV diameter. Than decrease in relative wall thickness ensues which implies the fact that the limits of compensation have been reached and that there is lack of appropriate hypertrophy of surviving myocardium (13).

Our results revealed non-linear development of hemodynamic changes after experimental myocardial infarction in rats. After acute ischemia (experimental myocardial infarction) marked hemodynamic dysfunction developed that was clearly measurable on day 6 (the earliest assessed time point). Main determinants of that process were manifested as decrease in $+dP/dt$, $-dP/dt$, systolic blood pressure, coronary blood flow, and the decrease in myocardial oxygen consumption. Hemodynamic function was partially compensated between day 11 ad 28 after MI, thereafter compensatory mechanisms were wasted between

28 and 40 day. Despite the apparent stabilization of hemodynamic function it was lower than in control animals on day 40.

In the early period after MI hemodynamic deterioration was most prominent and it was parallel to the expression of iNOS and TNFα mRNA (day 4 and 11). Considering these data we may suggest that the greatest decrease in hemodynamic function was accompanied by the expression of above factors. Expression of these cytokines was continuously present at the later time points. It might be postulated that during this time the continuous and progressive injury to the myocardium proceeded leading to development of the heart failure symptoms.

The suggested mechanism for participation of TNFα in myocardial insufficiency is the depression of cardiomyocyte contractility by both NO-dependent and NO-independent mechanism (16, 17). The NO-mediated blunting of beta-adrenergic signalling for TNFα induced myocardial depression was also considered (18, 19). TNFα has also been demonstrated to elicit apoptosis of cardiomyocytes, the process which has been recently proposed as an important mechanism for the pathogenesis of chronic heart failure (20).

TNFα has been found to contribute to the remodeling of infarcted rat myocardium and to development of heart failure after myocardial infarction. (21) TNFα has ability to activate metalloproteoinases that are capable to degrade the extracellular matrix proteins. The activation of latent collagenase (MMP-1) plays a predominant role in remodeling of myocardium at the site of infarction (22, 23) and TNFα is known to activate stromyelisin-1, gelatinases A and B (24, 25).

The local availability of TNFα could be attributed to the induction of iNOS and consequently high production of NO (26). We observed that expression of iNOS after myocardial infarction coincides with local production of TNFα. TNFα downregulates the eNOS in the endothelium (27), increases oxidative stress (28), induces apoptosis and endothelial dysfunction (29). The excess of NO produced by iNOS can be cytotoxic to endothelial and cardiomyocytes (30).

TNFα was found to demonstrate both anti and proangiogenic properties (31, 32). The equilibrium might be modified by changes in the local TNFα biosynthesis differently affecting angiogenesis. At lower concentrations TNFα promoted, when at the higher concentrations inhibited angiogenesis (33) (and our unpublished *in vitro* observations). This dual effects of TNFα may be related to the balance between angiogenic and antiangiogenic factors induced by this cytokine such as: VEGF (11), IL-8 (21), basic fibroblast growth factor (bFGF) (21), collagenase (34), prostaglandins (23), platelet activator factor (PAF) (35). On the other hand, TNFα exerts its antiangiogenic activity by the induction of antiangiogenic plasminogen activator inhibitor — 1 (PAI — 1) (36), downregulation of angiogenic integrin $\alpha_V\beta 3$ (37), activation of protein matrix biosynthesis, induction of transforming growth factor $\beta$ (TGF$\beta$) (38) or

tissue type — plasminogen activator (t-PA) (39) may add to understanding of the TNFα activity. A direct antiangiogenic influence of TNFα on endothelial cells may be also associated with the downregulation of the VEGF receptors (40). TNFα have been also demonstrated to promote endothelial cell apoptosis especially in presence of IL-1β and interferon gamma (IFN-γ) (41). Moreover the newly identified member of TNFα family named Vascular Endothelial Growth Inhibitor (VEGI) is a potent inhibitor of endothelial cells proliferation (42).

TNFα mediated response may be partially explained by TNFα receptors (TNF-R1 and R2) signaling pathways. Namely, TNFα induces apoptosis via TNF-R1, The "death domaine" of the cytosolic component of TNF-R1 has been linked to apoptosis in any cell types and likely mediates TNFα-induced cardiac myocyte apoptosis. TNFα binding either TNF-R1 or Fas activates pathway favouring apoptosis, whereas the type 2 TNF receptors (TNF-R2) activates a pathway leading to NF-κB induction. The TNF-R2 is linked to the TNFα receptor-associated factors (TRAFs). Although their biological function remains unknown, most TRAFs contain two protein motifs called "zinc" and "ring" fingers that likely convey proliferative signals by activation transcription factors such as NF-κB. These pathway are not absolute, however, and cross-activation occurs.

New and important finding of recent paper (43) was persistent expression of both TNF-R1 and TNF-R2 receptors after acute ligation of the coronary artery, implying that the signal transduction pathways necessary for TNFα signaling in the heart remain intact. This results showed that rat cardiac tissue expresses both TNF-R1 and TNF-R2, in agreement with previous studies (44). The presence of the TNF-R1/R2 throughout the period after infarction suggests that the physiological effects of increased TNFα production in the myocardium will be transmitted through these receptors to the cells. Many clinical studies have shown that the lvels of circulating sTNF-R1 and sTNF-R2 are significantly increased in advanced heart failure (45, 46). The levels of circulating sTNF-R1 and sTNF-R2 may reflect a generalized shedding of TNF receptors from a variety of different cell types, including the inflammatory cells. As we know, many cells infiltrating into the area of tissue injury, such as polymorphonuclear leukocytes, express high levels of TNF-R1 (47).

Induction of VEGF may serve as a signal for the necessary compensatory angiogenesis which may ameliorate the effects of ischemia and hypertrophy. Alternatively, the rapid induction of VEGF expression after a rapid increase in left ventricular end-diastolic pressure may serve to increase permeability of myocardial capillaries, thereby increasing its turgor by raising water content. This increase in myocardial stiffness may in turn serve to counteract the rising of intracavitary pressure (48).

In the present study we have observed a specific time-dependent pattern of the angiogenic VEGF isoforms expression. The $VEGF_{120}$ isoform is expressed

in the early period of experimental myocardial infarction, whereas isoforms $VEGF_{164}$ and $VEGF_{188}$ were permanently expressed in ischemic tissue. Recently Carmeliet *et al.* (49) have demonstrated the impairment of angiogenesis and development of ischemic cardiomyopathy in mice lacking the $VEGF_{164}$ and $VEGF_{188}$ isoforms. The data indicated that $VEGF_{120}$ by itself is insufficient for induction of angiogenesis in the ischemic heart. The authors suggest that various VEGF isoforms exert different biological functions, by binding to different receptors such as neuropilin-1 (50) and flk-1 (51) which are essential for endothelial cytoprotection and angiogenesis. The severe angiogenic defect in VEGF gene lacking mice demonstrate the functional complementary of the VEGF isoforms in induction of angiogenesis and indicate that specific VEGF isoform may be important for an optimal angiogenic response in vivo.

The selective endothelial mitogen — VEGF was found to protect endothelial cells against TNFα — induced apoptosis (12). The VEGF — induced up regulation of β3 integrin and fibronectin (12) as well as increased generation of NO in endothelial cells (52) is suggested for mechanisms of VEGF — induced angiogenesis and inhibition of endothelial cell apoptosis. In present study VEGF mRNA was found to be permanently expressed in the border zone of infarcted myocardium, but the generated protein might be not sufficient to overcome antiangiogenic effect of the high concentration of TNFα. A possibility exists that insufficient increase in capillarity to match the needs of persistent ischemia at the border zone of myocardial infarction may induce hypertrophy of cardiomyocytes (8).

We conclude that locally expressed TNFα, iNOS and VEGF in inflammed border zone of infarcted myocardium may play an important role in myocardial dysfunction and remodeling after MI and this process is related to progressive development of heart failure. It is reasonable also to assume that impaired angiogenesis takes place during remodeling and development of heart failure after MI.

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