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REGULAR ARTICLE

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Induction of cytokine expression in rat post-ischemic sinoatrial node (SAN)

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Abstract The aim of this study was to determine the spatial and temporal expression of various pro-inflammatory cytokines in the peri-sinoatrial nodal area after atrial infarction. Rats were subjected to permanent atrial infarction, in particular, sinoatrial node (SAN) infarction and sacrificed at various time points up to 7 days. Real-time polymerase chain reaction analysis demonstrated that mRNA levels of tumor necrosis factor alpha (TNF-α), interleukin-1β, interleukin-6, and transforming growth factor beta 1 (TGF-β_1) were upregulated in the peri-sinoatrial nodal area after atrial infarction. Immunostaining for TNF-α and TGF-β_1 proteins revealed that both cytokines were expressed persistently up to 7 days after atrial infarction around the peri-sinoatrial nodal area. Furthermore, the infiltrating inflammatory cells immunoreactive for both cytokines were predominant within the infarct SAN. In situ hybridization analysis showed that TNF-α gene expression was enhanced in the inflammatory cells and myocardium within the peri-sinoatrial nodal area in response to the infarction. These results provide evidence for the local expression of cytokines in the post-ischemic peri-sinoatrial nodal area, suggesting that the upregulation of the cytokines might be associated with the atrial arrhythmia observed after acute myocardial infarction.

Keywords Sinoatrial node · Ischemia · Arrhythmia · Cytokine · Rat (Wistar)

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Introduction

During various forms of myocardial stress and injury, many cytokines are produced and released by either the myocardial cells and/or inflammatory cells in response to the injury and activated interconnected inflammatory cascade (Gulick et al. 1989; Chung Beutler and Cerami 1989; Finkel et al. 1992; Lane et al. 1993). Cytokines are highly potent, pleiotropic, endogenous peptides produced by a variety of cell types (Oppenheim et al. 1988). Tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), interleukin-1α (IL-1α), interleukin-10 (IL-10), and interleukin-6 (IL-6) have been classified as pro-inflammatory cytokines (Mann and Young 1994); these cytokines initiate both primary host responses and tissue repair. Recent evidence suggests that pro-inflammatory cytokines are capable of modulating cardiovascular function by a variety of mechanisms, such as promoting left ventricular remodeling (Pagani et al. 1992), inducing contractile dysfunction (Finkel et al. 1992), and uncoupling myocardial beta-adrenergic receptors (Fowler et al. 1986). Although the majority of studies have focused on the role of cytokines in heart failure during various forms of left ventricle injuries, it is unclear how the cytokines are expressed and regulated in the sinoatrial node (SAN) in response to stress and injury.

SAN, the primary pacemaker of the heart, is located at the junction of the superior vena cava and the right atrium (Bouman and Jongsman 1986; Opthof 1988) and is composed of specialized cardiomyocytes responsible for the generation and conduction of electrical impulses to the working myocardium. Clinical studies have shown that ischemia of SAN attributable to coronary occlusion proximal to the origin of the SAN artery is one of the probable causes of sinus arrythmia and sick sinus syndrome (SSS) in myocardial infarction (Kyriakidis et al. 1992; Alboni et al. 1991). Relatively little is known about the relationship between the inflammatory responses (in particular cytokine signaling molecules) and the dysfunction of SAN after myocardial infarction.

The purpose of the present study is to establish a rat model of atrial infarction, especially SAN infarction, to determine the pattern of pro-inflammatory cytokine gene and protein expression in the area of SAN. To differentiate the rat SAN clearly, we have used the general neural marker protein gene product 9.5 (PGP9.5) as a positive marker and connexin 43 as a negative marker of the rat SAN, as clearly established previously (Oosthoek et al. 1993).

Materials and methods

Induction of myocardial infarction

Early studies showed that the SAN of rat was supplied dominantly by the extracoronary artery originating from the right cardiocomediastinal artery. Therefore, in the present investigation, a right atrial infarcion (in particular SAN infarction) was established by ligating the right cardiocomediastinal artery and right coronary artery (Halpern 1955, 1957). Adult Wistar rats of either sex weighing 250–300 g and in generally good health were anesthetized with a dose of 0.5 ml/100 g 7% chloral hydrate. Under sterile conditions, the trachea was cannulated to facilitate mechanical ventilation. An incision was made in the right first intercostal space to expose the right cardiocomediastinal artery crossing from lateral to medial via the ventral aspect of the right superior vena cava according to previous studies (Halpern 1955, 1957). The right cardiocomediastinal artery was ligated at its point of origin with a 6-0 silk thread. To occlude the blood vessels supplying the peri-sinoatrial nodal area, the right thoracic cavity was opened, and the right part of the thymus was dissected to expose the right atrium and pulmonary artery. The right coronary artery was then ligated near its branch point from the aorta, between the pulmonary artery and the right atrio-ventricular junction. Following ligation, the chest was closed, and the animals were allowed to recover for various time points up to 7 days under medication with antibiotics. In sham-operated animals, the heart was exposed and returned to the thoracic cavity without further manipulation. Myocardial infarction was confirmed by histological examination (hematoxylin and eosin staining). In the experimental model, six animals from each group were sacrificed at 1 h, 6 h, 24 h, 3 days, and 7 days after occlusion. The sham-operated rats were sacrificed 3 days after operation; these were expected to have an intense inflammatory response to the injury.

Immunohistochemistry and immunofluorescence

To localize cytokine proteins within SAN, three rats at each time point from the ischemic model and control groups were used. The hearts were fixed for 24 h with 4% paraformaldehyde and cryoprotected by immersion in 20% sucrose in phosphate buffer. Transverse sections of the peri-sinoatrial nodal area were cut at a thickness of 20 µm on a cryostat and mounted on 3-aminopropyltriethoxy-silane-coated slides. The primary antisera used were goat anti-TNF-α (R and D systems), and goat anti-TGF-β_1 (Santa Cruz Biotechnology). The secondary reagents used for cytokine localizations were biotinylated rabbit anti-goat IgG and ABC kit (Vector Laboratories). The peroxidase reaction was visualized by using 3,3'-diaminobenzidine tetrahydrochloride (Sigma) as a peroxidase substrate.

Double-immunofluorescence labeling was carried out for the identification of rat SAN. Two different primary antibodies, polyclonal rabbit anti-PGP9.5 (Incstar, USA) and mouse anti-connexin 43 (Transduction Lab) were applied to the same sections simultaneously. After incubation, the sections were washed in PBS and incubated for 1 h at room temperature with fluorescence-isothiocyanate-labeled anti-rabbit or anti-mouse IgG (Biolab Technological Company). After further washes, the preparations were mounted in Vectashield mounting medium (Vector Laboratories). Negative controls were performed routinely by incubating the sections in a buffer or normal buffered serum instead of the primary antibody.

Table 1 PCR primers for the reverse transcription/PCR assay

Marker	Sense (5'–3')	Antisense (3'–5')	Size (bp)
GAPDH	accaccatggagaaggctgg	ctcagtgtagcccaggatgc	528
IL-1β	caccttcttttccttcatctttg	gtcgttgcttgtctctccttgta	241
IL-6	gggactgatgttgttgacag	tgttcttcacaaactccagg	304
TNF-α	cccagaccctcacactcagat	ttgtcccttgaagagaacctg	215
TGF-β_1	gagagccctggataccaactactg	gtgtgtccaggctccaaatgtag	173

RNA extraction and real-time polymerase chain reaction assay

At each time point, the rats were killed, and the peri-sinoatrial nodal areas were removed rapidly and frozen in liquid nitrogen (each peri-sinoatrial nodal area is supposed to be at the anterior and lateral part of the junction between the superior vena cava and right atrium and continues along the crista terminalis in the smooth-walled part of the right atrium; Oosthoek et al. 1993). Total RNA was isolated from the tissue by using TRIzol reagent. For reverse transcription, the reaction mixture containing 2 µg RNA, 2.5 µM oligo(dT) primer, and 200 U Molony Murine Leukemia Virus Reverse Transcriptase (M-MLV, Promega, USA) in a total volume of 25 µl was incubated for 1 h at 42°C and stopped by heating for 5 min at 99°C. Aliquots (0.5 µl) of the reverse transcriptase products were amplified in the reaction mixture (20 µl) containing LightCycler-FastStart DNA Master SYBR Green I, 0.5 µM of each primer, and 4 mM $MgCl_2$ in a LightCycler (Roche Molecular Biochemicals). The primers corresponding to the rat TNF-α, IL-1β, IL-6, and TGF-β_1 are listed in Table 1. After pre-incubation at 95°C for 10 min, the polymerase chain reaction (PCR) was performed as follows: 50 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 10 s, and elongation at 72°C for PCR product size/25 s. The level of specific mRNA was quantified, expressed as C_t, the cycle number at which the LightCycler System detected the upstroke of the exponential phase of PCR product accumulation, and normalized by the level of expression of D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each individual sample.

Localization of TNF-α gene expression by in situ hybridization

For in situ hybridization, 20-µm-thick sections from sham and experimental rats were processed. A cocktail of two 28-oligonucleotide probes complementary to bases 162–189 and 427–454 of rat TNF-α (GenBank accession no. NMO12675) was used for in situ hybridization. The antisense oligonucleotide sequence was as follows: probe I, 5'-cttctcctccttgttgggaccaatcacc-3'; probe II, 5'-cgtagtcggggcagccttgtcccttgaa-3'. The probes were labeled at the 3' end with DIG-dUTP/dATP by using the DIG Oligonucleotide Tailing Kit (Roche, Boehringer Mannheim).

After postfixation in 4% paraformaldehyde, sections were incubated for 2×15 min in phosphate-buffered saline (PBS) and equilibrated for 15 min in 5×SSC (0.75 mM NaCl, 0.075 M Na-citrate). The sections were then prehybridized for 2 h at 55°C with hybridization solution (DAKO, Denmark). Hybridization was carried out with labeled probe (500 ng/ml hybridization solution) in a humified chamber at 55°C overnight. After hybridization, the sections were washed for 30 min in 2×SSC (room temperature), 1 h in 2×SSC (65°C), and 1 h in 0.1×SSC (65°C) and equilibrated for 5 min in TBS buffer (100 mM TRIS-HCl, 150 mM NaCl, pH 7.5). The hybridization signal was detected by anti-digoxigenin conjugated with alkaline phosphatase followed by incubation in the NBT/BCIP color substrate solution (Roche, Boehringer Mannheim). The sections were fixed, mounted with glycerol, and coverslipped for analysis on

a Zeiss microscope. As a control, adjacent sections were hybridized with the corresponding sense oligonucleotide probe. Moreover, some sections were digested with Rnase before hybridization.

Statistical analysis

The comparisons of cytokine mRNA level values at various time points in the experimental groups versus sham-operated heart samples were performed by using the $2^{-[\Delta\Delta Ct]}$ method based on early studies (Winer et al. 1999; Livak and Schmittgen 2001). Statistical significance was estimated as the fold induction among the various groups in different cytokine production by a 1-way ANOVA. Results were considered to be significant at $P<0.05$.

Results

Immunohistochemistry of TNF-α and TGF-β_1 protein expressions

Numerous PGP9.5-immunoreactive neuronal cell bodies, nerve trunks, and varicose nerve fibres were observed in

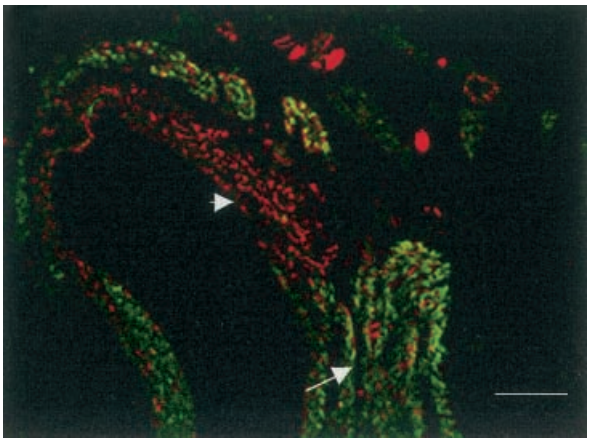

Fig. 1 Double labeling for PGP 9.5 (*red*) and connexin 43 (*green*). Numerous PGP9.5-immunoreactive nerve trunks, fibers, and neuronal cells are distributed in nodal tissue (*arrowhead*), whereas sparse cells and fibers immunoreactive for PGP9.5 lie in the atrial myocardium. Staining for connexin 43 reveals a homogeneous distribution in the atrial myocardium (*arrow*) and none in SAN. *Bar* 100 µm

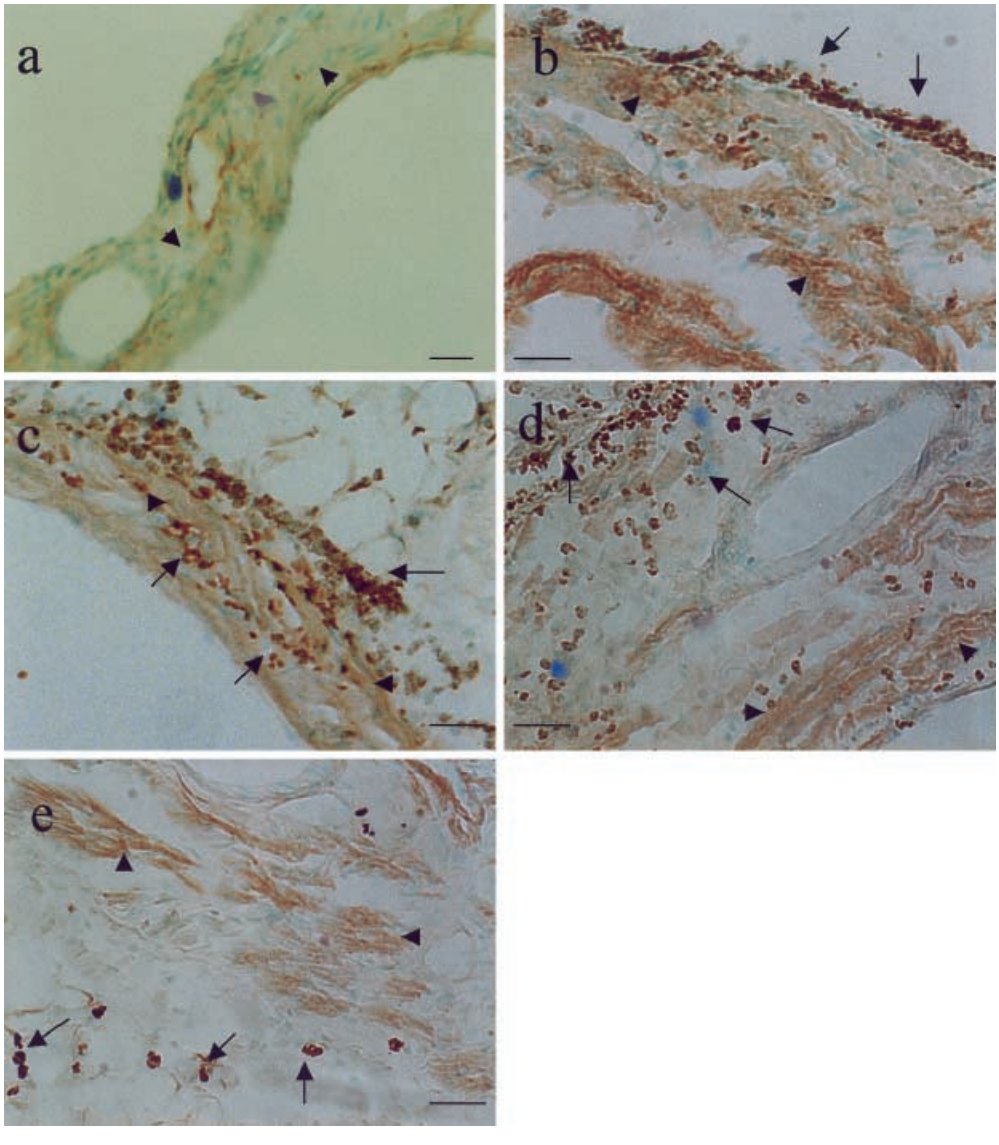

Fig. 2a–e Photomicrographs of TNF-α protein expression in peri-sinoatrial nodes in controls and at 1 h, 6 h, 1 day, and 7 days after atrial infarction. TNF-α-staining cells can be seen in the venous endothelium (**b** *arrows*) in 1 h post-ischemic SAN; the stained cells are mainly infiltrating neutrophils and macrophages. Abundant inflammatory cells infiltrate into the area of SAN at 6 h (**c**) and 1 day (**d**) after atrial infarction. After 7 days of SAN ischemia, TNF-α immunoreactive macrophages (*arrows*) are found throughout the peri-sinoatrial node (**e**). After infarction, the myocytes also weakly express the TNF-α protein in the SAN (**b–e** *arrowheads*). No immunostaining of TNF-α protein can be detected in the control (**a,** *arrows*). *Bar* 50 µm (**a**), 25 µm (**b–e**)

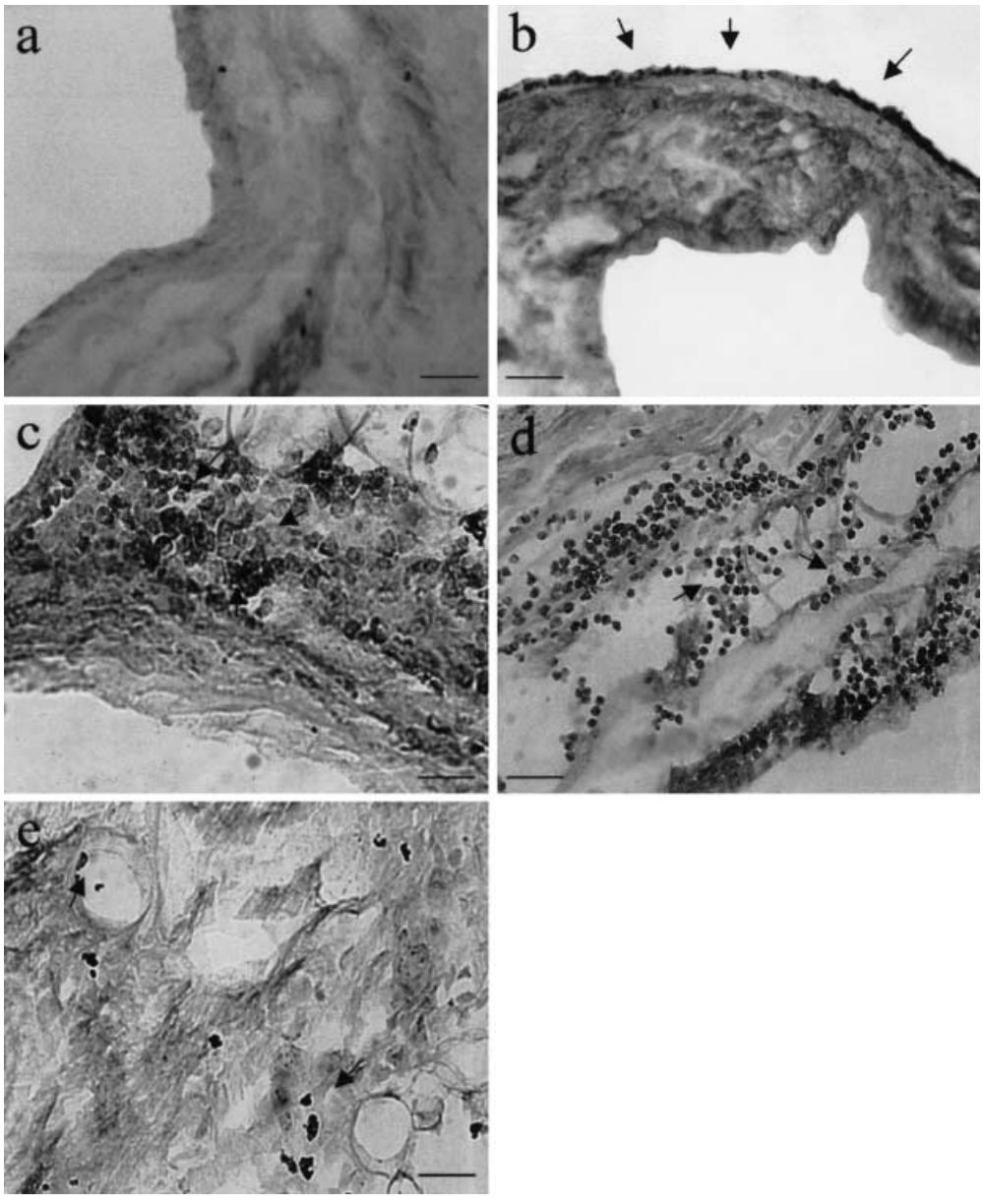

Fig. 3a–e Immunohistochemical staining for TGF-β_1 in peri-sinoatrial nodes after infarction. In the sham-operated rats, no TGF-β_1 is observed around the area of SAN (**a**). Numerous infiltrating cells (*arrows*) immunoreactive for TGF-β_1 within SAN are seen 1 h after atrial infarction (**b**). There are abundant lymphocytes and macrophages (*arrows*) with TGF-β_1 immunoreactivity infiltrating SAN in 6 h (**c**) and 1 day (**d**) post-ischemic rats. After 7 days of right atrial infarction, TGF-β_1 immunoreactivity (*arrows*) is found in the inflammatory cells within SAN (**e**). *Bar* 25 μm

the nodal tissues compared with a significantly lower staining intensity in the surrounding right atrium (Fig. 1). In addition, an absence of staining with anti-connexin 43 was observed in SAN of the rat heart, whereas abundant staining was present in the atrial myocardium. The tissues staining for the two markers were mostly exclusive, and a boundary between the non-stained nodal area and the stained atrial myocardium was evident.

The results of the immunohistochemistry demonstrated that the expression of TNF-α was predominately in the infarct zone. Within the infarct zone, TNF-α protein was localized mainly in the infiltrating cells and weakly in the cardiomyocyte (Fig. 2b–e). In experimental animals during early post-ischemia time points, immunoreactivity of TNF-α protein was limited in the endothelial and infiltrating cells in the superior vena cava (Fig. 2b). At later times after the operation, TNF-α immunostaining cells were localized predominantly in the infarct site. e.g., at day 1 after ligation (Fig. 2c). The staining was more intense on day 3 (Fig. 2d) and persisted up to day 7 (Fig. 2e). No obvious immunostaining of TNF-α was detected in the SAN of the control rats.

Similar to the spatial and temporal expression of TNF-α protein in the SAN, TGF-β_1 was observed mainly in the endothelial and infiltrating cells in the superior vena cava 1 h after SAN infarction (Fig. 3b). By 6 hour and 1 day after the occurrence of ischemia, abundant macrophages and lymphocytes immunoreactive for TGF-β_1 were observed to be infiltrating the infarcted sinoatrial nodal area (Fig. 3c, d). At day 7 after infarction, numerous mononuclear cells expressing TGF-β_1 infiltrated predominantly around the microvascular bed within SAN (Fig. 3e).

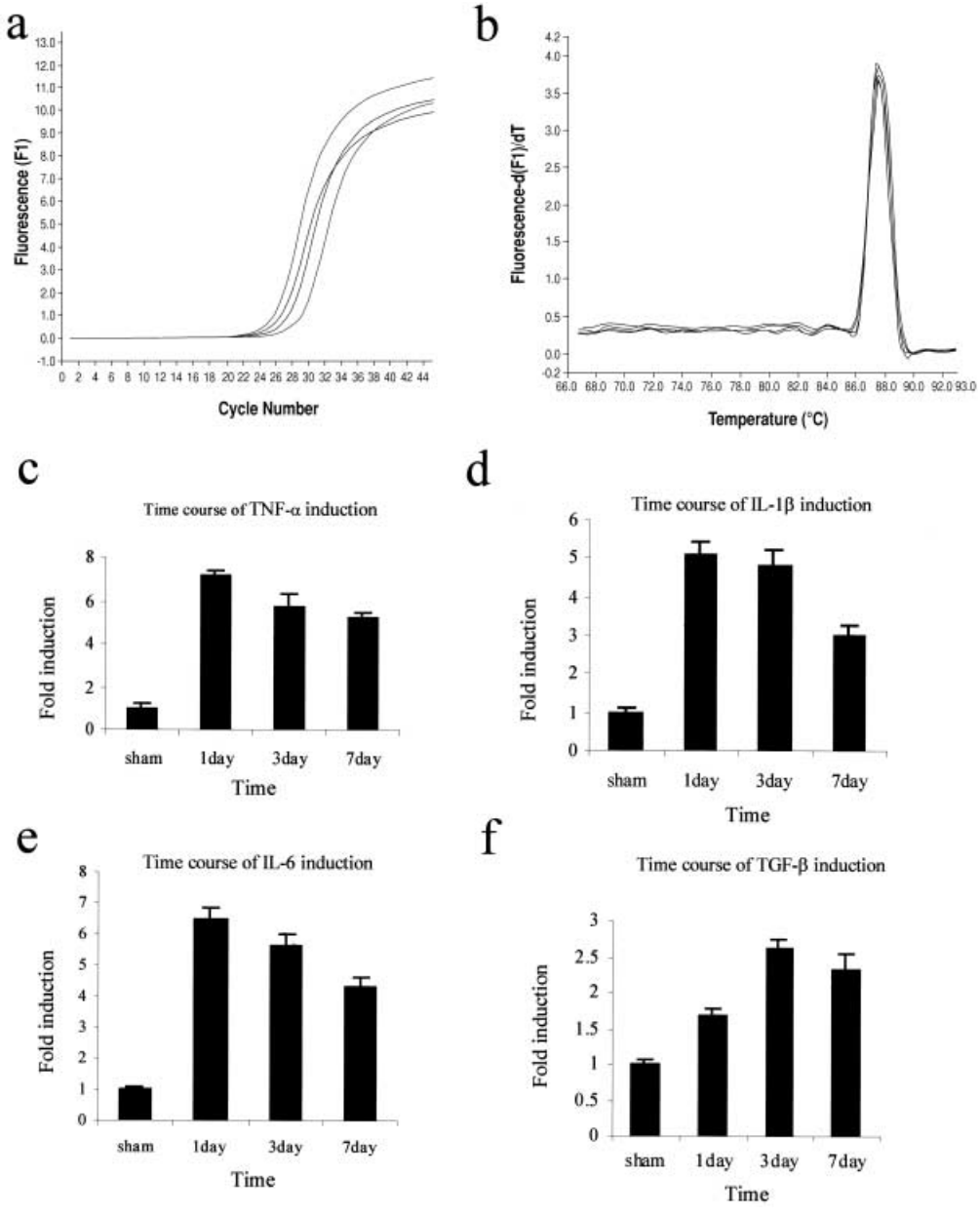

Fig. 4a–f Graph and data analysis of LightCycler Real-Time PCR. Typical examples of amplification plots (**a**) and melting curve (**b**) of mRNA for TGF-β_1 from sham, 1 day, 3 day, and 7 day ischemic SAN are shown. Relative quantification of TNF-α (**c**), IL-6 (**d**), IL-1β (**e**), and TGF-β_1 (**f**) mRNA levels at various times in SAN; the different cytokines are dramatically upregulated after infarction compared with the control. Relative fold induction was calculated by using the $2^{-[\Delta\Delta Ct]}$ method ($P<0.001$ in all groups compared with the control)

Cytokine mRNA level in the heart

Real-time PCR technology, which is a sensitive and rapid method that needs no post-PCR handling (Wittwer et al. 1997), was used to study the mRNA expression levels of various cytokines. Relative quantitation was performed to analyze the changes in the gene expression levels of the various cytokines. The results were presented as the fold induction in mRNA expression relative to the amount present in the sham operated group and were normalized to an internal control gene (Fig. 4). The analysis of real-time PCR showed that all the test cytokine mRNA levels were increased greatly after ligation and persisted to day 7. The mRNA of TNF-α reached a peak level at day 1 after the operation (about 6.3-fold that of the mRNA level of the sham-operated group). At days 3 and 7, the amount of mRNA of TNF-α was still very high (5.6-fold and 5.2-fold of the mRNA level of the sham group). Similarly, IL-6 and IL-1β mRNA levels were also remarkably increased after SAN infarction compared with those of the control. After SAN infarction, the mRNA level of TGF-β_1 was dramatically increased and reached peak levels at day 3 after ligation (2.6-fold compared with the control).

Cellular localization of TNF-α mRNA expression

TNF-α mRNA expression was undetectable in the sham-operated rats by in situ hybridization, 3 days after the operation (Fig. 5a). By 6 h after infarction, expression of TNF-α was detected in the infiltrating macrophages and

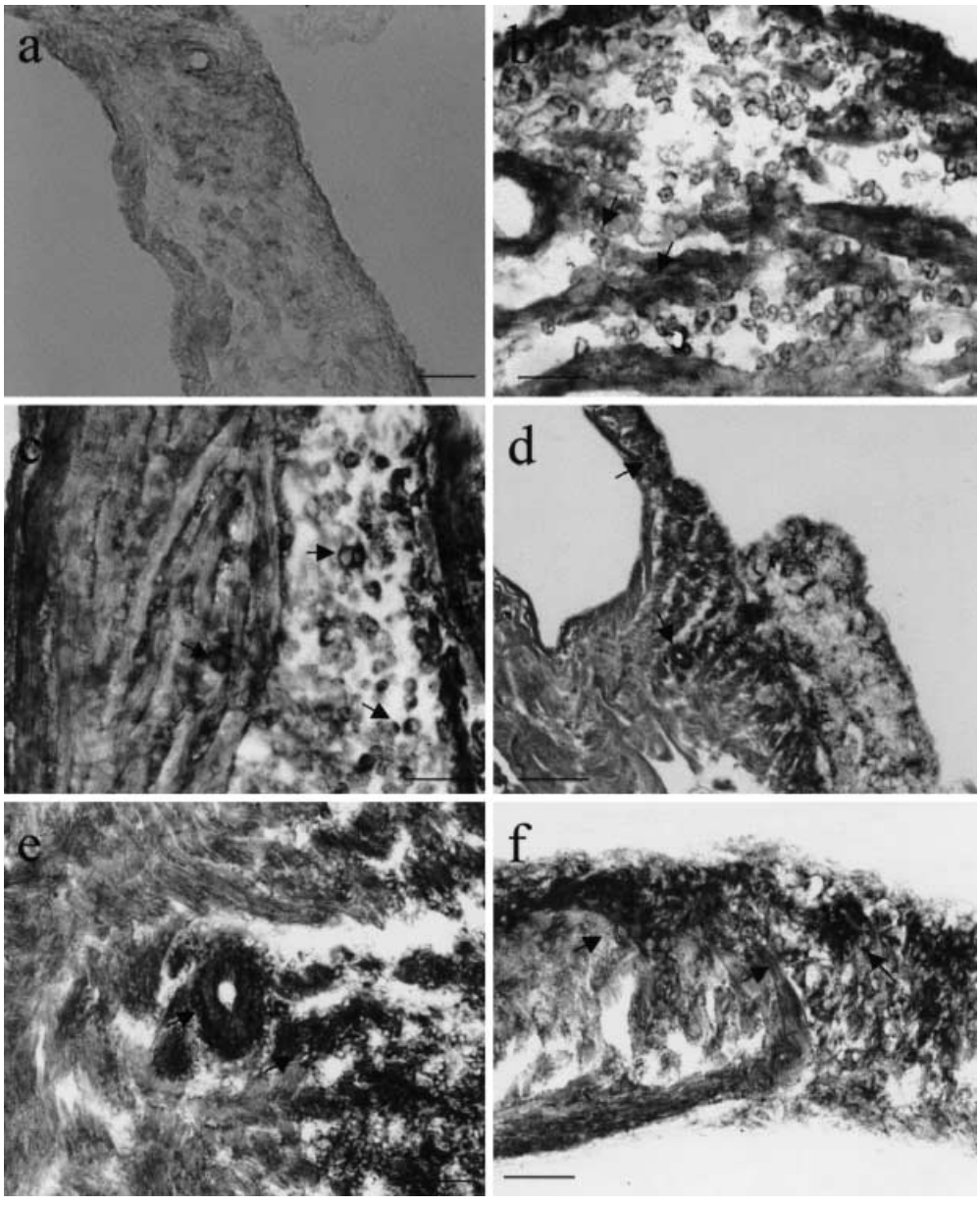

Fig. 5a–f In situ hybridization analysis of TNF-α mRNA expression in the peri-sinoatrial node after right atrial infarction. No obvious staining for TNF-α is detected in the control SAN (**a**). Evident TNF-α mRNA is observed in the infiltrating neutrophils and the endothelial cells around the microvascular bed (*arrows*) in 6 h post-ischemic SAN (**b**). In 1 day post-ischemic SAN, strong TNF-α mRNA (*arrows*) is expressed within SAN and adjacent areas where the main sources of TNF-α are the neutrophils and macrophages (**c**). After 3 days' infarction, a high level of TNF-α mRNA (*arrows*) is predominantly expressed in the endothelial cells and smooth muscle cells in the microvascular bed and infiltrating cells in SAN and adjacent atrial myocardium (**d, e**). Significant TNF-α mRNA is also expressed in the area of the SAN and the adjacent atrial myocardium (**f** *arrows*). *Bar* 100 μm (**a**), 200 μm (**d**), 40 μm (**b, c, e, f**)

the endothelial cells around the microvascular bed in the peri-sinoatrial nodal area (Fig. 5b). This expression extended to the SAN and adjacent areas in 1 day post-ischemic rats; high levels of TNF-α transcripts were observed in the infarct sinoatrial nodal area and peri-infarct atrial myocytes, and within the infarct sinoatrial nodal area, the TNF-α mRNA was identified mainly in the infiltrating cells (Fig. 5c). At day 3 after the occurrence of right atrial infarction, strong expression of TNF-α mRNA was seen in SAN and the adjacent atrial myocardium (Fig. 5d, f), together with a small cluster of inflammatory cells and smooth muscle cells in the microvascular bed (Fig. 5e).

Discussion

In the present study, we have shown that an inflammatory reaction occurs in response to myocardial injury after right atrial infarction; this reaction involves complex interactions between inflammatory cells and vascular cells. Various cytokines are induced at the sites of infarction, and each of these can potentially influence the nature of the inflammatory response. Several lines of evidence show that pro-inflammatory cytokines TNF-α and IL-1β, are involved in myocardial dysfunction in various experimental conditions (Yokoyama et al. 1993; Eichenholz et al. 1992; Dinarello et al. 1989; Dinarello 1996). TNF-α activates negative inotropic effects, apoptosis, and hypertrophic growth responses in myocytes (Torre-Aminoe et al. 1995; Yokoyama et al. 1997; Krown et al. 1996). On the other

hand, IL-1β induces myocardial depression with exogenous infusion (Cain et al. 1999) and suppresses the spontaneous rhythmic beating of cultured neonatal rat myocytes (Roberts et al. 1992). In addition, the exposure of excised papillary muscle to IL-1 prolongs the duration of the action potential and refractory period (Li and Rozanski 1993). Furthermore, a combination of TNF-α plus IL-1β has been shown to increase significantly the depression of myocardial cell contractility with synergistic effects (Kumar et al. 1996). In the present study, the upregulation of TNF-α and IL-1β observed in the perinodal area after the infarction might depress the contractile function of the right atrium and impair the rhythm of SAN; this might eventually lead to myocardial dysfunction.

TNF-α and IL-1β induce IL-6 expression through the cytokine cascade (Pang et al. 1994). There is also evidence that IL-6, in turn, regulates the release and activity of TNF-α and IL-1 by inhibiting the release of TNF-α directly and inducing the release of IL-1 receptor antagonist, respectively. Unlike the pro-inflammatory nature of TNF-α and IL-1β, IL-6 is reported to confer a variety of anti-inflammatory effects and to control the extent of the tissue inflammatory response. In the present study, the induction of IL-6 may contribute to the anti-inflammatory response in myocardial injury. An earlier study has shown that TGF-β reduces the amount of superoxide anions in the coronary circulation, maintains endothelial-dependent coronary relaxation, and reduces injury mediated by exogenous TNF-α (Lefer at el. 1990). Moreover, TGF-β_1 ablates the respiratory burst of macrophages induced by TNF-α (Tsunawaki et al. 1988). The upregulation of TGF-β_1 observed in the present study suggests that TGF-β_1 acts as a negative regulator in the cytokine network in response to injury.

The induction of different inflammatory cells expressing different cytokines within SAN and adjacent areas in the atrial myocardium in response to right atrium ischemia might be associated with atrial arrhythmia, which constitutes about 20% of cases of acute myocardial infarction in clinics (Jewitt et al. 1967; Lofmark and Orinius 1978; Pantridge et al. 1981). Although the underlying mechanism of atrial arrhythmia is not clear, the onset of the atrial arrhythmia may be attributable to the ischemia of SAN (Alboni et al. 1991; Kyriakidis et al. 1992; James 1961). Atrial arrhythmia has been reported as a side effect of the treatment of metastatic cancer patients with TNF-α (Eskander et al. 1997). In addition, arrhythmogenic effects have been observed in myocytes cultured in medium containing IL-1 and TNF-α (Weisensee 1993). All this information clearly indicates that the pro-inflammatory cytokines, TNF-α and IL-1β, are arrhythmogenic. Moreover, the localization of TNF-α mRNA expression around the area of SAN after infarction in our study suggests that this cytokine is closely related to the weakness of the SAN dominance of the heart and may impair the normal transmission of the impulse from SAN to the rest of the heart, thus leading to atrial arrhythmia.

In conclusion, various inflammatory cytokines are upregulated and persistently expressed in the peri-sinoatrial nodal area after atrial infarction, in particular SAN infarction. The expression of these cytokines is the result of the inflammatory response of SAN. This might represent one of underlying causes of atrial arrhythmia after acute myocardial infarction in clinics.

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