Tumor necrosis factor-α and cardiovascular diseases

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Tumor necrosis factor (TNF)-α is a potent inducible cytokine with pleiotropic biological effects, now implicated as a mediator of various physiologic and pathophysiologic events including inflammation, cell survival, growth, differentiation and apoptosis. TNF-α functions within a complex and tightly regulated cytokine network, activating multiple signal transduction pathways and inducing or suppressing a wide variety of genes, including those encoding for other cytokines, adhesion molecules and the inducible nitric oxide synthase. TNF-α has recently been implicated as a transducer of cardiovascular diseases, namely coronary artery disease and congestive heart failure. This review will summarize established and newer findings on this molecule.

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History

Tumor necrosis factor (TNF)-α is a molecule with a dual history. A "tumor necrosis factor" was first indirectly discovered around the turn of the century by a surgeon named William Coley[1]. Coley noticed that tumors of cancer patients who developed bacterial infections became necrotic. In the hope of curing cancer, Coley started injecting cancer patients with bacterial broths containing cultures of Streptococcus and Serratia. These injections, which were called "Coley's toxins", were partially successful in producing necrosis of human tumors, but had negative side effects. In 1936, Shear and Andervont[2] isolated the bacterial product responsible for the induction of hemorrhagic necrosis in murine transplantable tumors, and termed this material "bacterial polysaccharide". Now known as "lipopolysaccharide" (LPS), the agent responsible for the induction of tumor necrosis is also the most toxic factor of Coley's broth. In 1962 O'Malley and Shear[3] observed that serum derived from LPS-treated mice contained an endogenous factor capable of inducing hemorrhagic necrosis of a tumor grown in another animal. A similar observation was reported in 1975 by Carswell et al.[4]. While studying the tumor hemorrhagic necrosis produced by endotoxin, these authors found that the serum of mice infected with the Calmette-Guerin bacillus contained a substance which mimics the tumor necrotic action of endotoxin. They named such activity "tumor necrosis factor". A variety of tests indicated that TNF activity was not due to the residual endotoxin, but rather to a factor released by endotoxin from host cells, probably macrophages. Therefore, they proposed that TNF mediates endotoxin-induced tumor necrosis, and that it might be responsible for the suppression of transformed cells by activated macrophages[4]. In 1985, Parrillo et al.[5] discovered that subjects with septic shock have, among other cytokines, a myocardial-depressant substance that later proved to be TNF.

Independently of this research line, in the mid '70s, Cerami and coworkers began to investigate cachexia in chronic diseases. Trypanosome-induced cachexia was used as a model system, since in rabbits and some other species trypanosome induces, during the terminal phases of infection, a marked wasting syndrome with loss of more than half of the initial body mass. It was observed at that time that, paradoxically, experimental animals infected with trypanosome also developed an impressive hypertriglyceridemia[6]. Rouzer and Cerami[6] determined that the elevation in triglycerides was due to a systemic suppression of the enzyme lipoprotein lipase. Kawakami and Cerami[7] subsequently showed that lipoprotein lipase suppression also occurred in endotoxin-treated mice, and that suppression was conferred by a transferable serum factor. This factor, termed "cachectin", was shown to be principally produced by macrophages.

TNF-α, as such, was isolated by Aggarwal et al.[8], and within a short time its cDNA sequence was reported by several groups, showing that this molecule was identical to cachectin.

TNF-α needs to be distinguished from TNF-β, also described as "lymphotoxin-α". TNF-β is a larger molecule, less potent, not as abundant, and is mainly produced by T-cells. This lymphokine shows an inflammatory activity similar to that of TNF-α and binds to the same receptors[9].

Structure of tumor necrosis factor-α

TNF-α is a trimeric polypeptide consisting of 157 amino acids and with a molecular weight of 17 kDa. It is first produced as a 26 kDa integral transmembrane precursor protein consisting of 233 amino acids, from which the 17 kDa subunit is released after proteolytic cleavage of a 76 residue signal peptide. This cleavage is catalyzed by a metalloproteinase named "TNF-α converting enzyme" (TACE)[10]. TNF-α exists both in a secreted (type I) and a transmembrane (type II) form, both of which are biologically active[11]. Membrane as well as secreted forms aggregate *in vitro* producing a protein of variable size: dimers, trimers, pentamers, and perhaps higher-order multimers have been reported. The active form of the protein that interacts with the receptors is trimeric. Its amino acid sequence shows a 30% homology with that of TNF-β. Both the mature peptide and the precursor sequences of TNF-α show high sequence similarity across mammalian species. The transmembrane form is characterized by an extra-cytoplasmic C-terminus. A conserved region of 150 amino acids within the C-terminus characterizes the TNF family of proteins. This region is used by all members of the TNF family to recognize their corresponding receptors[12].

Activated macrophages are the main source of TNF-α, both for the cell-associated (cytoplasmic) form which is then released, and the membrane-bound form[13]. Analysis of the kinetics of TNF secretion has demonstrated that bioactivity appears after 2 hours of macrophage culture stimulation, peaks at 4-8 hours and disappears within 12 hours. The cytokine is rapidly synthesized and released on demand, and is not stored in the cytoplasm.

Other cells releasing TNF include lymphocytes, fibroblasts, neutrophils, smooth muscle cells, mast cells and adipocytes. It has also been shown that adult mammalian myocardial cells are able to release TNF-α after endotoxin stimulation[14].

Molecular regulation of tumor necrosis factor-α production

Because macrophages are the main source of TNF-α, mechanisms of TNF-α production have been studied prevalently in these cells after LPS stimulation. The interaction of LPS with its main ligand, CD14 (through the LPS-binding protein), leads to a rapid intracellular tyrosine phosphorylation of *ras*, functioning as a guanosine triphosphatase, a process that initiates the protein kinase cascade (Fig. 1)[15].

The activation of *ras* activates the system *Raf-1*/MEK (MAP/ERK kinase), which in turn activates members of the mitogen-activated protein kinases (MAPK, in particular P-38 MAPK), the stress-activated protein kinase (SAPK), the extracellular signal-related kinase (ERK) and the *jun* nuclear kinase (JNK). These signals ultimately determine the phosphorylation of inhibitory-kappa B (I-κB), an inhibitory factor normally sequestering the active transcription factor nuclear factor-κB (NF-κB) in the cytoplasm. The phosphorylation of I-κB causes I-κB degradation. Once liberated from I-κB, NF-κB translocates from the cytoplasm into the nucleus where it docks to DNA at one of the four NF-κB binding sites in the TNF promoter sequence. Thus, TNF transcription is initiated. Once TNF gene transcription has occurred, TNF mRNA is translated into the 26 kDa TNF precursor (pro-TNF) in the cytoplasm. Myristoylation in the cytoplasm facilitates membrane insertion/association, where pro-TNF is cleaved by TACE. The mature 17 kDa TNF is then released in the extracellular space[15,16].

Production of TNF-α is tightly regulated, both at the transcriptional and – chiefly – at the post-transcriptional level. While TNF gene transcription increases almost 3-fold in response to macrophage stimulation by LPS, intracellular mRNA levels may increase 100-fold, and TNF protein production may increase 1000-fold or more. In part, this post-transcriptional control may depend upon the variable instability of the TNF mRNA, which, in turn, may be attributable to the presence of an

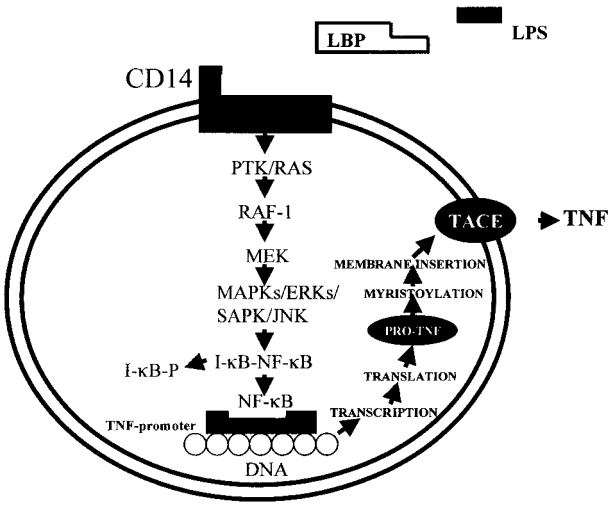

Figure 1. *A scheme of the lipopolysaccharide (LPS) signal transduction pathway and of tumor necrosis factor (TNF)-α gene expression. See text for details. ERK = extracellular signal-related kinase; I-κB = inhibitory-kappa B; JNK = jun nuclear kinase; LBP = LPS-binding protein; MAPK = mitogen-activated protein kinase; NF-κB = nuclear factor-kappa B; PTK = protein kinase; SAPK = stress-activated protein kinase; TACE = TNF-α converting enzyme.*

AU-rich -3' untranslated sequence that constitutes a recognition site for the action of a specific ribonuclease[17] (Fig. 1).

Tumor necrosis factor-α receptors

The wide range of TNF activities is explained by the presence of TNF receptors (TNFR) on almost all nucleated cell types (Fig. 2)[18]. Both in humans and in mice, two distinct types of TNFR have been identified and molecularly cloned: TNF-R1 (also referred to as TNF-R55, p55) and TNF-R2 (also called TNF-R75, p75), with a molecular mass of 55 and 75 kDa respectively[19]. Both these receptors belong to the TNFR superfamily, also including Fas, CD40, CD27 and RANK[20].

The receptors consist of an N-terminal extracellular ligand-binding domain, which recognizes TNF, of a transmembrane helix and of a cytoplasmic domain[21]. The extracellular portion (182 residues long for TNF-R1 and 235 residues long for TNF-R2) contains four characteristic domains with regularly spaced cysteine residues, with a significant sequence homology. Contrary to the conserved extracellular domains, the cytoplasmic domains of the two receptors lack homology. Thus, investigators have proposed that these receptors may be coupled to activation of different downstream transduction pathways.

Signaling occurs when a TNF trimer induces cross-linking of two or three receptors in an extracellular complex, which permits the aggregation and activation of the cytoplasmic domains. In most cells, following receptor triggering, the ligand TNF-R1 complex is rapidly internalized by coated pits and degraded in the lysosomes. Unlike TNF-R1, TNF-R2 contains no tyrosine residues in its intracellular domain, and therefore lacks a consensus sequence for the rapid cellular internalization through coated pits[19].

The precise role of each receptor in different cell types has not yet been completely unraveled. Antibodies against TNF-R55 as well as those against TNF-R75 can inhibit TNF-induced cytotoxicity of tumor cells and TNF-enhanced expression of adhesion molecules on endothelial cells. This suggests that both receptor types contribute to these bioactivities. However, the use of agonistic antibodies or of high concentrations of TNF suggests that, in most cases, only triggering by means of TNF-R1 is responsible for the cytotoxic activity or gene induction in endothelial cells. Therefore it is difficult to explain the inhibition of TNF-dependent signaling by antibodies antagonistic to TNF-R2[19].

The ligand-passing model reconciles these paradoxical findings. TNF-R2, having a higher affinity and dissociation rate than TNF-R1, preferentially binds TNF at low ligand concentrations[22]. The ligand is then passed to the neighboring TNF-R1, which monopolizes all TNF-mediated signaling. Thus TNF-R2 enhances and synergizes the effects mediated by TNF-R1.

Recent data indicate that there are soluble circulating TNFR which can bind and inactivate TNF. These are membrane-bound TNFR that have been proteolytically cleaved from the cell membrane. Two TNFR are found in the circulation: the 55 kDa and the 75 kDa soluble TNFR. The soluble receptors are held responsible not only for the inactivation of TNF, but also for its clearance[23]. According to a few reports, soluble TNFR en-

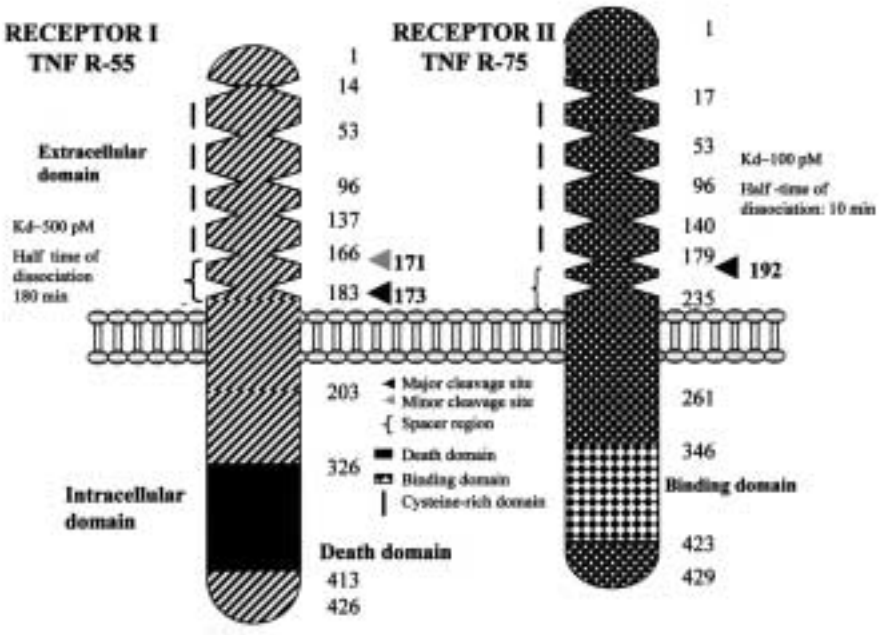

Figure 2. *A schematic representation of the structural organization of TNF-α receptor type 1 (TNF-R1, also referred to as TNF-R55, p55) and of TNF-α receptor type 2 (TNF-R2, also called TNF-R75, p75) and of the different domains involved in the activation of specific signal transduction pathways. See text for details.*

hance, rather than attenuate, the biological actions of TNF. By binding to trimeric TNF, soluble TNFR prevent its monomerization and subsequent inactivation, thus increasing its half-life and biological function. According to this school of thought, soluble TNFR act as a circulating "slow-release reservoir", allowing a more prolonged persistence of the cytokine[24].

Tumor necrosis factor-α signaling pathways

Both TNF-R1 and TNF-R2 lack any intrinsic kinase activity and therefore associated molecules are required to mediate signal transduction and amplification.

In the intracellular region of TNF-R1, a domain of 80 amino acids responsible for its cytotoxicity has been identified near the C-terminus. This region is called the "death domain" (Fig. 2). It has also been found that this domain is necessary for other functions of TNF, such as its antiviral activity and the induction of the inducible nitric oxide synthase (iNOS). By engaging TNF-R1, TNF determines its own trimerization, thus resulting in the association of three receptor death domains. Subsequently, an adapter protein termed TNFR-associated death domain (TRADD) binds through its own death domain to the clustered receptor death domains. TRADD functions as a platform for the recruitment and adaptation, in a proper steric arrangement, of several signaling molecules close to the activated receptor. These include TNFR-associated factor 2 (TRAF-2) and receptor-interacting protein (RIP). These stimulate a transduction pathway including the MAPK, which, in turn, leads to the final activation of the transcription factors NF-κB and JNK/activator protein-1 (AP-1) implicated in the induction of cytoprotective genes. The latter are important for cell survival and for a number of inflammatory responses (Fig. 3)[25].

In some cell types, TNF-α also induces apoptosis through TNF-R1 signaling. In fact, TRADD can also bind Fas-associated death domain (FADD), another adapter protein, leading to apoptosis through the activation of caspase-8.

TNF-R1 activation is not usually associated with apoptosis, perhaps owing to the preexistence of cellular factors that can suppress the apoptotic stimulus generated by TNF. Expression of these suppressor proteins is probably controlled through NF-κB and JNK/AP-1[26] (Fig. 3).

Following membrane receptor triggering, TNF-α also activates a cell membrane neutral sphingomyelinase. Neutral sphingomyelinase hydrolyses sphingomyelin, a phospholipid preferentially found in the plasma membrane of mammalian cells, to produce ceramide and sphingosin. In turn, ceramide acts as a lipid second messenger that induces a variety of cell regulatory phenomena such as programmed cell death, cell differentiation, cell proliferation and sterol homeostasis[27].

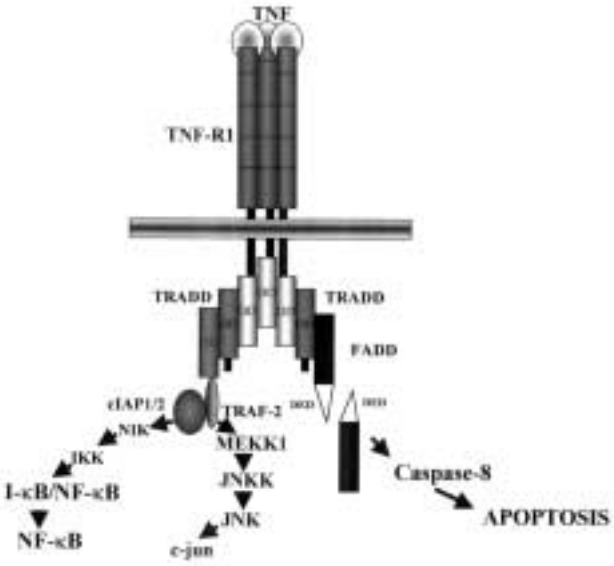

Figure 3. *Main signal transduction pathways activated by the binding of TNF-R1 by TNF-α in mammalian cells. See text for details. AP-1 = activator protein-1; cAP1/2 = cellular inhibitor of apoptosis-1 and -2; DD = death domains; DED = death effector domain; FADD = Fas-associated death domain; IKK = inhibitor of kappa B kinase complex; JNKK = JNK kinase; MEKK = MAP/ERK kinase kinase-1; NIK = nuclear factor-kappa B-inducing kinase; N-Smase = neutral sphingomyelinase; RIP = receptor-interacting protein; TRADD = TNFR-associated death domain; TRAF-2 = TNFR-associated factor-2. Other abbreviations as in figures 1 and 2. From Ashkenazi and Dixit[25], modified.*

Pleiotropic effects of tumor necrosis factor-α

The cellular effects of TNF-α are highly pleiotropic. At low concentrations, TNF-α exerts paracrine and autocrine effects on leukocytes and endothelial cells, and thus serves as an important regulator of the inflammatory response. TNF-α enhances chemotaxis of macrophages and neutrophils, increases their phagocytic and cytotoxic activity, and promotes leukostasis by inducing increased expression of adhesion molecules at sites of inflammation[28].

At higher concentrations, TNF-α production exceeds the binding capacity of TNFR located on the cell surface and may thus exert endocrine or exocrine effects, including the initiation of metabolic wasting, thrombosis, hypotension and fever.

When chronically administered, TNF-α/cachectin leads to a state of anorexia. This occurs over a period of months. This state resembles the cachexia associated with invasive neoplastic diseases and a variety of infectious disorders. In this situation, hypertriglyceridemia characteristically occurs and results from systemic suppression of the enzyme lipoprotein lipase[29]. Paradoxically, TNF also plays a role in obesity[30]. TNF expression is increased in obese human adipose tissue, and contributes to the insulin resistance related to obesity[31]. TNF-α is indeed a key component of the obesity-linked serum elevation of plasminogen activator inhibitor-1 (PAI-1)[32].

TNF has also been shown to elicit the expression of tissue factor on the surface of cultured endothelial cells, and leads to a down-regulation of the protein C antico-

agulant pathway due to a loss of thrombomodulin activity[33]. High levels of TNF in patients with disseminated intravascular coagulation support the hypothesis of the existence of a relationship between the production of this cytokine and hypercoagulability. This phenomenon may also be related to the pathogenesis of hemorrhagic necrosis of tumors and of the migratory thrombosis accompanying certain neoplastic diseases[28].

TNF also exerts effects on the central nervous system since it is a potent endogenous pyrogen, capable of causing fever, both through a direct effect on hypothalamic neurons, and indirectly by triggering the peripheral production of interleukin (IL)-1[34].

Paradoxically, TNF-α also appears to modulate both tissue proliferation (and rebuilding) and tissue destruction. On the one hand TNF-α directly stimulates fibroblast and mesenchymal cell proliferation whereas, on the other, it simultaneously induces the biosynthesis of collagenases, proteases, reactive oxygen intermediates and arachidonic acid metabolites implicated in tissue remodeling[35].

TNF-α has also been shown to have an important role in cell death. A variety of mechanisms, including stimulation of the synthesis of arachidonate metabolites, activation of protein kinases and the production of oxygen free radicals, nitric oxide, and regulation of nuclear regulatory (transcription) factors are implied. Under normal conditions, these cytotoxic effects are important for the host defense since they initiate or increase antitumor activity and modulate cell growth and differentiation. However the role of TNF in the pathogenesis of malignancies remains controversial: while therapy with TNF improved survival in tumor-bearing mice, in other animal models it also promoted tumor cell adhesion, nodule development and metastasis[36,37]. Excessive activation of TNF-α is clearly linked to tissue necrosis and apoptosis.

Recent studies have demonstrated a causal role for TNF-α in a group of diseases including septic shock, rheumatoid arthritis, preeclampsia, the hemolytic uremic syndrome, allograft rejection and regional enteritis[38,39]. In particular, the role of TNF-α in the pathogenesis of septic shock has received great attention. Septic shock typically occurs through a sequence of events beginning with the establishment of a nidus of infection. This is followed by the release of active antigenic structural components (including LPS and exotoxins) from the infecting organisms, with subsequent generation and release of proinflammatory cytokines such as TNF-α and IL-1β by macrophages and other cells[40]. These endogenous products, particularly TNF-α, determine a strong induction of iNOS in endothelial and myocardial cells, thus resulting in the pathophysiologic manifestations of septic shock, which include severe hypotension, myocardial dysfunction, organ hypoperfusion and lactic acidosis.

Genetic variation of tumor necrosis factor-α levels

Different forms of a given gene that are contained in a stable manner in the same chromosomal site within a species are termed alleles. In a single individual, only two alleles are possible, one from each parent. Within a species, however, more than two alleles may exist with varying frequency. The term "genetic polymorphism" is related to this situation, and indicates a stable gene mutation that is sustained within a species. These mutations generate the different alleles that result in phenotypic variants in the populations. Genetic polymorphism can partly explain the considerable variation existing within the normal population in the amount of TNF-α[41].

The gene for human TNF-α is located on the short arm of chromosome 6 (chromosome 6p21.1-6p21.3), in tandem with TNF-β (lymphotoxin) and in close proximity to the HLA-DR locus within class III of the human major histocompatibility complex (MHC)[42]. This is a highly polymorphic region, and the TNF-α gene itself contains a large number of genetic variants[43]. The location of the TNF-α gene within the HLA-locus on chromosome 6 prompted speculation about the existence of an association between certain HLA-DR alleles and variations in the production of TNF-α. HLA-DR3 and HLA-DR4 correlate with the "high-production" phenotype for TNF, whereas HLA-DR2 co-segretates with the "low-production" phenotype[44]. Genetic polymorphism in the TNF-α locus has been associated with higher levels of TNF-α production and is known to be related to several autoimmune, infectious and neoplastic diseases[45]. Almost all known TNF-α polymorphisms are located within a region of the gene that is crucial for the transcriptional regulation of TNF-α expression (promoter region). Single-base changes in such a region may have significant effects on gene expression.

Eight polymorphisms have been so far identified: six are located upstream to the coding region of the gene at positions -857(CA), -851(CT), -376(GA), -308(GA), -238(GA) and -163(GA) from the first transcribed nucleotide; two have been found in a non-translated region at position +691 (G deletion) and +70 (C-insertion, resulting in an 8C rather than 7C repeated sequence).

The most extensively studied polymorphism is a point mutation at position -308, involving the substitution of guanine by adenosine. The two allelic forms of the gene are referred to as TNF-A1 (G) and TNF-A2 (A), respectively. The presence of the TNF-A2 allele in the promoter region increases the expression of TNF-α protein *in vitro* and *in vivo*. Less than 5% of normal controls are homozygous for this allele, but its frequency is increased in patients with rheumatoid arthritis and systemic lupus erythematosus. Indeed, it has been shown that the TNF-A2 allele is strongly associated with the autoimmune MHC haplotype HLA-A1-B-8-DR-3 which is associated with high TNF-α levels[46]. Mortality due to

mucocutaneous leishmaniasis, to meningococcal *purpura fulminans* and to cerebral malaria (possibly due to microvascular thrombosis of the cerebral vessels) is also increased in subjects who are homozygous for the TNF-A2 allele.

Tumor necrosis factor-α production in the heart

The heart is a TNF-α-producing organ. Both myocardial macrophages and cardiac myocytes themselves synthesize TNF-α. Indeed, in response to endotoxin, the myocardium produces as much TNF-α per gram tissue as either the liver or the spleen, both of which possess a large macrophage population and are known to be major sources of TNF-α[14]. However, contrary to the spleen, the liver and the kidneys, TNF-α mRNA expression in the adult myocardium is not constitutive, but only induced.

To confirm the production of TNF-α by myocardial cells, suspensions of highly purified adult myocytes have been exposed to LPS in an *in vitro* experimental setting. TNF-α was found to be secreted into the culture medium[14]. These observations have been extended to the intact heart: adult feline hearts, maintained in Langendorff preparations and stimulated with LPS, produced TNF-α, whereas this factor was not synthesized in hearts perfused with vehicle alone[14].

Although infection and endotoxemia are potent stimulants of myocardial TNF-α production, studies have also documented that the heart has an endogenous capacity to produce TNF-α (as well as other cytokines) in response to diverse pathophysiological stimuli such as ischemia-reperfusion, pressure or volume overload and trauma. Clinical situations in which an increase in myocardial TNF-α production has been detected include myocardial infarction, cardiopulmonary bypass, congestive heart failure, dilated cardiomyopathy, allograft rejection and acute viral myocarditis.

Role of tumor necrosis factor-α in coronary artery disease

There are many reasons to consider TNF-α as a candidate transducer of the risk of coronary artery disease. These are briefly summarized in table I.

TNF-α affects lipid metabolism and may lead to hypertriglyceridemia by decreasing lipoprotein lipase activity in adipose tissue and by increasing *de novo* hepatic synthesis of fatty acids[47]. Moreover, in hyperlipidemic patients, TNF-α levels correlate significantly with the concentrations of very low-density lipoproteins, triglycerides and cholesterol, and negatively with high-density lipoprotein cholesterol[48].

TNF-α probably also plays a pivotal role in obesity-related insulin resistance. It attenuates insulin receptor signaling by decreasing both insulin-stimulated autophosphorylation and the tyrosine kinase activity of the insulin receptor in cultured adipocytes as well as in muscle and fat tissues of obese rats[49].

Table I. Main biological effects of tumor necrosis factor-α with potential impact on the cardiovascular system.

Adhesion molecule expression	↑
MHC molecule expression	↑
Vascular permeability	↑
Activation of inflammatory cells and cytokine release	↑
Turnover of extracellular matrix	↑
Production of reactive oxygen intermediates	↑
Disruption of calcium handling	↑
Uncoupling of β-adrenergic receptors	↑
Inotropy	↓
Left ventricular ejection fraction	↑
Apoptosis	↑
Synthesis and plasma levels of triglycerides	↑
Hepatic fatty acid synthesis	↑
Lipoprotein lipase activity	↑
Procoagulant activity	↑

MHC = major histocompatibility complex; ↑ = increase; ↓ = decrease.

TNF-α may contribute to atheroma development through its direct actions on endothelial function (a decrease in the constitutive form of endothelial nitric oxide synthase and an increase in iNOS), by stimulating the synthesis of growth factors, chemoattractants and adhesion molecules as well by direct stimulation of adhesion molecules (including intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and E-selectin)[33].

Finally, TNF-α may contribute to the risk of coronary artery disease by interfering with thrombosis. Indeed, TNF-α enhances coagulation (by increasing tissue factor activity and the expression of PAI-1 and by suppressing the antithrombotic protein C pathway in endothelial cells)[33].

While absent from normal tissues, TNF-α has been demonstrated in 88% of atherosclerotic lesions. In these sites its expression increases with the severity of the lesion. This observation suggests that TNF-α plays a role in the evolution of the disease[50].

Myocardial TNF-α production has been well documented during acute ischemia. Such myocardial TNF-α production and release may theoretically reduce cardiac contractility, thus providing a cardioprotective effect by attenuating the myocardial oxygen demand. Mice with genetic deletion of TNF-R1 and TNF-R2 developed larger myocardial infarcts compared to wild-type littermate controls when subjected to an experimental acute infarction *in vivo*[51]. This suggests that myocardial production of TNF-α in response to ischemia-reperfusion may in fact be an endogenous pathway activated by the heart to induce short-term intrinsic cardioprotection against subsequent ischemia-reperfusion injury.

Given the possible implications of TNF-α in cardiovascular pathophysiology, it has been hypothesized that polymorphisms of the TNF-α gene might be associated with a genetic predisposition to or protection against coronary artery disease. Herrmann et al.[52] have studied the TNF-α/-308 polymorphism in relation to coronary artery disease. The TNF-α/-308 polymorphism is functional, being associated with increased constitutive and inducible levels of expression of the TNF-α gene. Homozygotes or heterozygotes for the TNF-α/-308 polymorphism were significantly more frequent among subjects with a family history of myocardial infarction than among those with no family history, but globally the allele frequency was similar in patients with myocardial infarction and control subjects. Therefore, in this study, there was no overall case-control difference with regard to the -308 polymorphism, whereas the TNF-α-308 allele was associated with a family history of myocardial infarction. However, the results of this study may have been flawed as a consequence of the strong selection bias in favor of survivors of coronary artery disease, estimated to constitute as much as 40% of patients developing an acute myocardial infarction. Family history, on the other hand, includes both fatal and non-fatal myocardial infarction. The possibility that the TNF-α/-308 polymorphism be preferentially associated with fatal myocardial infarction should therefore be taken into consideration[52].

The same results have been found in a study where the frequency of the alleles of the TNF-α/-308 gene were measured in healthy control subjects, in patients with angiographically normal coronary arteries, in patients with single-vessel coronary disease and in patients with multivessel coronary disease. In fact, no significant associations were seen between this polymorphism and any coronary artery disease phenotype[53].

Another study that explored the relevance of the TNF-α/-308 polymorphism in the evaluation of the risk of coronary artery disease has reported that the TNF-α-308 allele was positively associated with the levels of extracellular superoxide dismutase, an antioxidant abundant in the extracellular space, but also with the levels of total homocysteine, which is considered to be an oxidant. The mechanism behind this association is not clear. It is established, however, that TNF-α plays a significant role in oxidative stress. It can either directly or indirectly cause oxidative stress or potentiate lipid-related oxidation, both of which are highly relevant in atherosclerosis[54].

Role of tumor necrosis factor-α in heart failure

Patients with end-stage congestive heart failure show some clinical features and profound metabolic abnormalities, similar to those observed in patients with chronic inflammatory or neoplastic disorders. In 1990, Levine et al.[55] demonstrated that 30-40% of patients with advanced heart failure had increased circulating TNF-α levels. In parallel, it has long been recognized that patients with advanced sepsis, a condition characterized by increased TNF-α production, may develop reversible cardiac dysfunction[56]. Secondary to these observations, the hypothesis was advanced that TNF-α may contribute to the development of contractile abnormalities within the failing myocardium. However, although causing the clinical features of cardiac cachexia, the concept that TNF-α pathogenetically contributes to the syndrome of congestive heart failure is still at present little more than a hypothesis.

The possibility that the heart itself is a target for TNF-α is supported by the evidence that circulating levels of TNF-α are high in patients with congestive heart failure, whereas such levels are low or undetectable in subjects with a non-failing myocardium[57]. Further evidence on this is the fact that the expression of myocardial TNFR in the failing myocardium, similarly to what occurs with β-adrenergic receptors, is significantly decreased in comparison with that observed in the non-failing myocardium. Interestingly, the soluble (circulating) forms of TNF-R1 and TNF-R2 are elevated in patients with moderate-to-severe heart failure[23]. These observations suggest that the "shedding" of myocardial membrane-bound TNFR may contribute to the increased soluble TNFR levels in spite of decreased membrane expression of functional TNFR. The role of soluble TNFR is not known, but clearly they can neutralize the biological effects of circulating TNF-α. Based on these observations, it is reasonable to postulate that the myocardium responds to pathologic concentrations of TNF-α by decreasing cardiac TNFR levels and by increasing those of soluble TNFR, possibly in an attempt to decrease TNF-α toxicity[24].

There is therefore substantial evidence that TNF-α is increased in severe congestive heart failure. It should be borne in mind, however, that serum TNF-α levels do not correlate with those of myocardial TNF-α. Transgenic mice overexpressing myocardial TNF-α indeed develop a cardiomyopathy, but have undetectable levels of peripheral TNF-α[58], whereas congestive heart failure patients, with a high myocardial TNF-α content, may have low peripheral levels of TNF-α[57]. All these observations can be explained by the fact that only locally produced TNF-α can induce myocardial injury. Mann[59] has argued that the net effect of TNF-α on cardiac function will depend on the amount and duration of TNF-α expression. Short-term expression of TNF-α within the heart may be an adaptive response to stress, whereas long-term expression may be a contributory factor for cardiac decompensation.

The mechanisms through which excessive TNF-α levels produce left ventricular dysfunction are not yet completely understood. TNF-α could contribute to heart failure in several ways. These include the stimulation of myocyte hypertrophy, the generation of reactive oxygen intermediates and the induction of ventricular remod-

eling, the stimulation of extracellular matrix protein production and an increased matrix turnover. TNF-α may also cause cardiomyocyte loss by inducing cardiomyocyte necrosis or apoptosis. The latter may be induced directly, through TNFR, or indirectly, via the stimulation of nitric oxide production. TNF-α can however induce contractile dysfunction independent of cardiomyocyte loss. This is linked to a disruption of calcium handling with an uncoupling of the excitation-contraction mechanisms, causing both systolic and diastolic dysfunction. This depression of myocardial function occurs in a biphasic manner and includes an immediate phase and a delayed phase. This has suggested that TNF-α induces negative inotropic effects by at least two different mechanisms involving the sphingomyelinase pathway and the nitric oxide pathway in the short- and in the long-term phases, respectively. Activation of the sphingomyelinase pathway occurs within minutes of TNF-α administration. It results in the breakdown of the phospholipid sphingomyelin to its metabolites ceramide and sphingosine. Sphingosine inhibits Ca^{2+} release by blocking the sarcoplasmic ryanodine receptor, with a subsequent reduction in the peak systolic Ca^{2+} concentrations. Activation of the nitric oxide-dependent pathway, conversely, requires hours of TNF-α exposure. Through the induction of iNOS expression and the resultant increase in nitric oxide, an increase in intracellular cGMP occurs. This can act as an important intracellular signaling molecule desensitizing the contractile myofilaments to Ca^{2+} and consequently inducing negative inotropic effects on cardiac myocytes.

Finally, TNF-α, at low, non-toxic concentrations, depresses cultured myocyte contractile performance independent of nitric oxide. This is achieved through an uncoupling of the β-adrenoceptors from adenilyl-cyclase via an effect on G inhibitory proteins[60].

Independent of the mechanisms involved, a remaining step to prove a causal role of TNF-α in heart failure is the demonstration that treatment with agents inhibiting the production or activity of TNF-α can prevent or reverse myocardial dysfunction and remodeling in the failing human heart. This has recently been reported in a randomized, multidose study with etanercept (ENBREL™), a dimer of two molecules of the extracellular portion of the p75 TNFR (or TNF-R2), that binds TNF-α and prevents its interaction with membrane bound TNFR. This drug, given for 3 months to 47 patients in NYHA classes III-IV, was well tolerated and led to a dose-related improvement trend in the NYHA classification and in quality of life[61]. The long-term efficacy of etanercept, however, needs to be demonstrated in large-scale clinical trials that are currently underway in the United States, Europe and Australia.

Conclusions

There is currently a good deal of experimental and clinical data highlighting some of the roles of TNF-α in the development of coronary artery disease and congestive heart failure. These data may be however interpreted in diametrically opposite ways, and in any case they do not clarify whether TNF-α exerts a net beneficial or detrimental effect on the cardiovascular system. It has been shown how TNF-α increases endothelial and monocyte iNOS expression. This can induce vasodilation, but also begets further endothelial dysfunction by inducing oxidative stress and promoting apoptosis. Similar effects have been more recently found in myocytes where TNF-α can simultaneously induce resistance to hypoxia and improve contractile dysfunction.

Thus, it may be said that we are dealing with a double-natured cytokine which, according to the setting, reveals this Janus-faced behavior: in the short term, TNF-α may exert protective and adaptive effects in cases of hemodynamic overload and ischemia-reperfusion. In the long term, conversely, its effects on myocardial contractility are likely to be harmful.

Obviously we have to consider that TNF-α is one component of a complex system which consists of many positive and negative regulators, and which predominantly functions in a tightly controlled equilibrium. In such a complex scenario, the role of TNF-α may actually change from beneficial to harmful owing to dysregulation of other factors.

Thus, it is difficult to detach one single tile from a complex system and attribute the origin of all harms to it. In such a complex panorama, the simple hypothesis that TNF-α reflects simply the severity of the clinical syndrome and that variations in its levels and function are merely a consequence and not the cause of cardiac disease still has to be convincingly ruled out.

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