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Tumor necrosis factor-α (TNF-α): Biological activities and mechanisms of action

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Tumor necrosis factor-α/cachectin (in further text TNF-α) was originally defined for its ability to cause hemorrhagic necrosis of different types of tumors. In the meantime, it has become clear that TNF-α is a multifunctional immunoregulatory cytokine with a broad spectrum of activities upon hematopoetic and nonhematopoetic cells. Some of the pleiotropic activities of TNF-α are growth inhibition of some tumor cells; stimulation of human fibroblast, B cell and thymocyte proliferation; activation of phagocytic and endothelial cells; induction of prostaglandin synthesis as well as regulation of oncogenes, transcription factors and major histocompatibility complex antigen expression. The effect of TNF-α (antiproliferative or stimulative) depends upon the type of target cells, presence of TNF receptors, TNF-α concentration in tissues or upon presence of other mediators capable of affecting the activities of this cytokine. In this paper we are reviewing biological as well as physico-chemical properties of the cytokine, its production and some mechanisms of TNF-α action.

Key words: tumor necrosis factor

TNF history

More than 200 years ago some physicians noticed tumor reduction in patients with bacterial inflammations; a logical conclusion was that bacteria or their products somehow retard the growth of tumors.[1] This finding encouraged the physicians of the 19th century to treat patients with solid tumors by means of a direct introduction of microorganisms into the tumors. Such a therapy gave different outcomes ranging from complete disappearance of tumors or partial reduction of tumor burden to complete failure of the therapy.[2] The high infectiveness of microorganisms (*Streptococcus pyogenes*) and consequently a serious risk for bacterial infection in patients was the motive that forced dr. William B. Coley to treat his patients with the toxins from bacterial cultures instead of using microorganisms themselves. For the preparation of bacterial toxins he chose *Streptococcus pyogenes* and *Bacillus prodigious* (now called *Serratia marcescens*). With the above stated therapy Coley achieved noteworthy results (disappearance or partial reduction of tumors) that are described in his articles from 1894, 1896 and 1898.[3, 4, 5] On the account of higher efficacy and safety of Coley's toxins (as they were called

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later) for the treatment of cancer patients this method found acceptance and became commercially attractive. Coley's toxins were produced and used for treatment until the end of 1920 when they gave up their place to radiotherapy.

However, the Coley's toxins were not forgotten. In the years 1931,[6] 1932,[7] 1935[8, 9] and 1936[10] various researchers executed quite a few experiments on tumors in mice using filtrates of Gram negative bacterial cultures as antitumor agents. All these experiments share a common fact: tumors with good vascular supply became centrally necrotic at the beginning of therapy and afterwards, necrosis spread out to the external parts of tumors. Bacterial toxins were injected intratumorally - i.e. locally in all cited experiments. In the year 1943 Shear[11] reported about experiments with tumor model Sarcoma-37 (Sa-37) where he injected tumor-bearing mice intraperitoneally (i.p.) with polysaccharide isolated from *S. marcescens*. Such kind of systemic treatment caused necrosis in the centre of tumors and even more, systemically treated tumors, in which necrosis spread out to periphery, later on completely disappeared (like in the case when they were treated locally).

All the above stated experiments were a good starting point for Carswell and co-workers. In the year 1975 they published an article in which they, for the first time, used the appellation "tumor necrosis factor" and speculated about its production and its effects.[12] Namely, the very same researchers established that animals, infected with *Bacillus Calmette Guerin* (BCG) and afterwards treated intravenously with endotoxin, produce "with endotoxin induced serum factor" which causes hemorrhagical necrosis of tumors. This serum factor responsible for necrosis of tumors was named simply "tumor necrosis factor" (TNF). TNF displayed cytotoxic activity against numerous tumor cells, yet not against normal mouse fibroblasts (later it turned out to be growth factor for normal fibroblasts). On the basis of these findings Carswell and co-workers described TNF as an agent with selective antiproliferative effect only on tumor cells. In the same article authors also presumed that macrophages are the main producers and bacterial endotoxins the main inducers of TNF. Both presumptions later turned out to be correct. It is well known today, that TNF-α (i.e. the agent which Carswell and co-workers named TNF) can be produced not only by macrophages but also by other types of cells, and that there are other factors beside endotoxins which can serve as inducers of TNF-α production.

TNF-α is not the only tumor necrosis factor known nowadays, since another substance with similar activities, was discovered already in 1968. Ruddle and Waksman[13] and also Granger and Wiliams,[14] independently one from another, described a substance, produced by lymphocytes, with powerful cytotoxic effect on syngenic embrional fibroblasts or on L929 cells. Ruddle and Waksman named this substance "cytotoxic factor", while Granger and Wiliams called it "lymphotoxin". Because of the similarity in the amino acid sequence (35% homology)[15] and in their activities (ranging from the cytotoxicity against L929 cells to the ability to produce necrosis of some sorts of sarcomas *in vivo),*[16] lymphotoxin was classified among tumor necrosis factors and called TNF-β, while Carswell's TNF was renamed into TNF-α.

Today, it is well known that TNF-α can be synthesised not only by macrophages but also by other cells while TNF-β is produced exclusively by T lymphocytes. Yet, the mechanisms of stimulation of TNF-α production in macrophages are completely different from the mechanisms of stimulation in other cells.[17]

Production of TNF-α

TNF-α production is a multistep process which includes the induction of gene transcription and the amplification of TNF-α mRNA, the synthesis of prohormone (233 aminoacids-AA), the activation of prohormone with cleavage of the molecule to 157 AA (molecular weight approximately about 17 kDa), and the secretion of TNF-α.[18]

Inducers of TNF-α production

Substances that trigger TNF-α production can be divided according to their source into extracellular (exogenous) and intracellular (endogenous) inducers. One of well known and most often used extracellular inducers of TNF-α production is bacterial lipopolysaccharide (LPS), with its active part - lipid A, which is responsible for most of the biological properties of LPS.[19, 20] The second (by frequence of its use) extracellular inducer is muramyldipeptide - MDP (or its structural analogues) that can be employed, either alone or in combination with other agents (LPS, IFN-γ), to stimulate the production of TNF-α in macrophages. Besides, a higher degree of transcription of TNF-α gene *in vitro* was ascertained in the cells exposed to radiation, viruses, bacteria, parasites, as well as to their products.[21-26] Some tumor cells[27] and plant polysaccharides[28] are also known to act as extracellular inducers.

On the other hand, interferons (IFN-α and IFN-γ), growth factors (GM-CSF), interleukin-2 (IL-2) and TNF-α itself are the most frequently applied intracellular inducers.[29-35]

Inhibitors of TNF-α

Excessive production of TNF-α can be very harmful for the organism. As a consequence - the production and activities of TNF-α as well as of other cytokines in the organism are strictly controlled. The control is realised mostly through the supervision of gene transcription and translation, or through the production of substances that block cytokine's effects (by binding either to the cytokine molecule or to cytokine's specific membrane receptors).

Mechanisms of action of the inhibitors, which directly influence transcription and translation of TNF-α, are still quite unclear. However, the researchers are already acquainted with the fact that individual cytokines, as for example interleukins (IL-4, IL-6), transforming growth factor β (TGF-β), prostaglandins and corticosteroids, can operate as inhibitors in these processes.[36-40]

The second group of TNF-α inhibitors, which block the activities of the very cytokine, are proteins that were discovered in urine samples of febrile patients. One of these proteins is called uromodulin; i.e. a glycoprotein with molecular weight of 85 kDa. It operates by binding to TNF-α molecules and thus preventing the attachment of these molecules to receptors.[41, 42] The second specific protein, isolated from urine samples, has molecular weight of 40-60 kDa; this one also inhibits activities of TNF-α by means of binding to its molecule and blocking of its attachment to receptors.[43, 44]

Beside the inhibitors that were isolated from urine another group of proteins with similar effects was described by Scuderi and co-workers[45] in serum. The authors assumed that these proteins were plasma α-globulins. They supposed that the blockade of activities of secreted TNF-α was carried out through the binding of α-globulins to TNF-α molecule which prevented the binding of TNF-α to cell receptors.

A more thorough biochemical analysis of TNF receptors demonstrated that proteins (isolated from urine and serum) which inhibit activities of TNF are nothing but the soluble form of TNF receptors. In this light, uromodulin was connected with TNF R75 (TNF receptor with molecular weight of 75 kDa), while the other inhibitory protein isolated from urine was identified (according to its molecular weight) as a substance related to TNF R55 (TNF receptor with molecular weight of 55 kDa).

Moreover, there is some evidence of existence of substances capable of blocking the activities of TNF cytotoxic products. Among the most thoroughly examined substances of this character belong manganese superoxide dismutase (MnSOD) and metaloproteins (e.g. metalothionein).[46, 47] MnSOD prevents the production of oxygen free radicals in mitochondria while metaloproteins bind these free radicals and thus neutralise their effect on cell structures.

The best known synthetic TNF inhibitors originate from the group of serine protease inhibitors. Serine proteases play an important

role in cleavage of prohormone molecules and production of biologically active form of TNF-α. The inhibitors of these proteases interfere with serine protease activity and consequently block the secretion of biologically active form of TNF-α.[48]

The control of TNF-α production and its activities is a complex process that most likely includes (beside above cited mechanisms of regulation of transcription, translation and secretion, as well as beside the direct effect on the very cytokine) other manners of supervision. All these mechanisms and factors interweave and work together as an entirety.

TNF-α producers

Quite a few years ago Beutler and co-workers[40] established that monocytes and macrophages are the basic producers of TNF-α. Today, it is known that also other cells are capable of TNF-α production. Among the most important producers we classify NK cells,[49] T lymphocytes,[50] some non-hematogenous cells like for example muscle cells, endothelial cells and microglia,[21, 51-53] as well as some tumor cells: cells of colorectal adenocarcinoma, Hodgkin's lymphoma, ovarian carcinoma, breast carcinoma.[54-56]

Production of TNF-α in monocytes/macrophages

As it was already mentioned before LPS, i.e. the endotoxin isolated from cell walls of Gram negative bacteria, is the best known extracellular inducer of transcription of TNF-α gene and of TNF-α synthesis in leukocytes.[40] That explains why the LPS's effect on monocyte/macrophage population and its stimulation of lymphocytes to produce TNF-α are most thoroughly examined. Despite of that, exact molecular mechanisms of signal transduction and of initiation of TNF-α gene transcription remain uncleared. The researchers presume, that the process of lymphocyte stimulation (to produce TNF-α) is somehow connected to the activity of phospholipase A_2 and afterwards to the metabolism of arachidonic acid and cAMP activity.[22, 31] Namely, Motrri and co-workers[31] ascertained that LPS statistically significantly increases the activity of membrane-bound phospholipase A_2. On the other hand, the inhibitors of phospholipase A_2 statistically significantly reduce TNF-α mRNA production in monocytes stimulated with LPS. Unfortunately, all these findings are not sufficient for exact conclusions about molecular mechanisms involved in regulation of transcription, translation and secretion of TNF-α. Not until these mechanism are understood, the possibility to influence effectively the quantity of cytokine produced and its cooperation in different activities in organism will be given.

Physical and chemical properties of TNF-α

TNF-α is a glycoprotein whose sequence consists of 156-157 amino acid residues (156 AA mouse TNF-α, 157 human and rabbit TNF-α); its molecular weight is 16-18 kDa (16-18 kDa mouse, 17 kDa human, and 18 kDa rabbit TNF-α). These molecular weights are the ones of TNF-α after electrophoresis in sodium-dodecylsulfate-polyacryl-amide gel (SDS-PAGE), when the active form decomposes into monomers. Molecular weight of unaltered protein (as for example after gel filtration) ranges from 34 kDa (rabbit) to 45 kDa (human).[57] The above stated facts lead us toward conclusion that the active form of TNF-α is its trimeric form, which has also been proved by means of ultracentrifugation and crystallography.[58, 59]

Trimeric form of TNF-α is susceptible to temperature changes, since the increase of temperature causes decomposition of trimmer form. The isoelectric point (pI) of human TNF-α is somewhere between 5 and 6, of mouse TNF-α between 3 and 5 and of rabbit TNF-α about 5.[57] Besides, TNF-α is susceptible to trypsin and chimotrypsin action, as well as to the action of some other proteases (V8),[57] for it looses its biological activities when being exposed to these proteases. On the other hand, TNF-α retains its biological activities when exposed to low pH or organic solvents.

TNF-α exists in two different forms, i.e. as a free (soluble) cytokine or bound to cellular membranes (of monocytes for example). Membrane-bound TNF-α was at first supposed to be the excreted cytokine that reversely attached itself to its own membrane receptors.[60] Hafsli and co-workers[61] proved that this statement is incorrect. Namely, they ascertained by means of immunofluorescent microscopy that the number of membrane-bound TNF-α molecules remains unaltered regardless of the presence of added soluble TNF-β. As a matter of fact, if membrane-bound TNF-α was the soluble form reversely attached to its own receptors, then, in the presence of TNF-β, there would be less TNF-α molecules bound to membrane, because of the competition for free receptors. Membrane-bound TNF-α acts cytotoxically as a transmembrane protein; the exact mechanism of action is still unclear, yet it is known to kill target cells in direct cell-to-cell contact.

Biological properties and actions of TNF-α

TNF-α is classified together with TNF-β, interferons, interleukins and growth factors into the group of hormone-like substances called cytokines. Its name "tumor necrosis factor" originates from its first known biological property, i.e. from its ability to cause necrosis of tumors.[12]

In the year 1984 Pennica and co-workers[62] succeeded in cloning of TNF-α cDNA, thus enabling the synthesis of recombinant TNF-α (rTNF-α). Since physical and chemical properties, as well as biological activities of rTNF-α are identical to the ones of native TNF-α and because of low cost production of rTNF-α this accelerated experimental work with the cytokine. Today it is known that biological activities of this very cytokine are not confined to cytostatic/cytotoxic effect upon tumor cells only, but that TNF-α co-operates in many other processes (Table 1).

TNF-α has (beside the above cited activities) also quite a few side-effects (just like the other cytokines do). The most frequent side-effects are fever, anorexia, diarrhea, nausea and hypotension.[63-68]

The cited activities rank among the most important ones of this cytokine, yet the list of TNF-α effects is by no means at the end. A complete register of processes, in which TNF-α takes part directly or indirectly, is very difficult to elaborate, since this pluripotent cytokine is secreted by different cells (blood, liver, spleen, kidney, muscle cells and cells of central nervous system) and participates in a large number of processes in the organism. Also, the pathogenesis of cerebral malaria and the destruction of tissues in inflammation are connected to hyperproduction of TNF-α.[69, 70]

Table 1. Short review of biological activities of TNF-α.

Antitumor activities
• modulates MHC I and II antigenic expression (thus it operates as an immunomodulator);[71, 72]
• induces transcription of enzyme inhibitors;[73]
• induces differentiation of tumor cells;[74]
• participates in cytotoxic activities of monocytes and macrophages (membrane-bound or soluble TNF-α);[75, 76]
• stimulates IL-1, IL-6 and IL-8 production in macrophages/monocytes;[77-79]
• stimulates prostaglandine E_2 (PGE2) production in macrophages/monocytes;[77]
• induces synthesis of IL-2 receptors on T lymphocytes;[80]
• activates NK cells;[81]
• induces synthesis and expression of transforming growth factor (TGF-α) and of receptors for epidermal growth factor (EGFR) on human pancreatic cancer cell line.[82]
Other activities
• operates as a mediator of inflammation and cellular immune response;[83-85]
• participates in regulation of cellular and physiological processes: in differentiation of cells, in regulation of sleep (i.e. inducer of sleep);[67, 68, 86-88]
• influences cellular metabolism;[89]
• operates as a growth factor (stimulates fibroblasts);[90-92]
• has antiviral, antibacterial and antiparasitic effects;[93]
• operates as a modulator for neurons in hypothalamus (regulates growth and their functions);[94]
• has radioprotective effect (most probably by means of stimulating the stromal cells in bone marrow to produce growth factors CSF, that are known to stimulate hematopoiesis).[95, 96]

Antitumor activities of TNF-α

Carswell discovered TNF-α as a factor capable of causing hemorrhagical necrosis of tumors in mice.[12] Later, Carswell and different researchers found out that TNF-α acted cytostatically and cytotoxically on various tumor cells, while having no effect on growth of normal human cell lines.[97, 98] On the basis of these facts they concluded that TNF-α acts directly cytostatically/cytotoxically on tumor cells, and that this very cytokine possesses a distinctively selective antiproliferative potential only against tumors cells. Further studies disproved the above conclusion, since TNF-α acts either antiproliferatively on tumor and normal cells or stimulates the growth of both types of cells. The mode of its action depends upon growth conditions in the cell culture and upon the quantity of cytokine added.[82, 99, 100] As an example we would like to mention the study of Palombella and Vilček,[100] where rTNF-α had a cytotoxic effect on 3T3 fibroblasts in non-confluent layers, while promoting growth of the very same fibroblasts in confluent layers. In the same study researchers noticed, that the higher the dose of TNF-α the more intensive was the DNA synthesis.

The above mentioned *in vitro* studies[82,99,100] thus do not support the presumption about selective antiproliferative function of TNF-α (only against tumor cells), but prove that the mode of its activities *in vitro* depends upon external conditions, as for example confluency of cell layer, composition of growth medium or presence of other cytokines.

Activities of TNF-α in *in vivo* systems are even more complicated. Carswell and co-workers[12] observed a pronounced *in vivo* antitumor effect of TNF-α (then only TNF) on transplanted sarcomas in mice. But they were surprised to see that TNF-α was completely inefficacious as an cytostatic/cytotoxic agent on Meth-A sarcoma cell line *in vitro*. This fact led towards conclusion that TNF-α in vivo performs its antitumor activities also indirectly and that this indirect action depends upon the treated organism to a great extent. Experiments with tumor models in nude mice confirmed that the antitumor effect of TNF-α virtually depended upon the treated organism (i.e. upon host), actually upon its immune system. Namely, the treatment of experimental tumors in nude mice with TNF-α was a complete failure and even application of high doses of the cytokine did not result in hemorrhagical necrosis of tumors.[101] Indirect antitumor action of TNF-α is brought about mostly by activation of host immune system. The cytokine activates macrophages so that they become tumoricidal (in less than 20 minutes triggers the synthesis of mRNA for TNF and IL-1 in these cells). At the very same time TNF-α activates T lymphocytes, NK cells and neutrophils,[81, 102-104] exerts an effect upon stromal cells of the host organism and triggers the synthesis of growth factors,[95, 96] as well as the production of other cytokines that participate in antitumor activities (IL-1, IL-6 and TGF)[77-79] (see Table 1).

Besides, TNF-α exerts an effect upon endothelial cells where it induces the synthesis of several adhesion molecules (which are located on the cell surface) and modulates the coagulation properties of cell surface, thereby increasing vascular permeability. The effect upon endothelial cells of tumor blood vessels is potentiated if the vessels are newly formed. Most probably these changes result from the direct and indirect actions of TNF-α, which lead to intravascular thrombosis and complete destruction of tumor blood vessels.[105-107]

Obstruction of blood vessels represents an important mode of TNF-α antitumor activities, especially against non-immunogenic tumors. Poorly immunogenic tumors are quite resistant to the action of TNF-α and destruction of tumor tissue is brought about mostly by means of destroying of blood vessels, which leads to reduced tumor blood supply and afterwards to the phenomenon of central necrosis.[101, 108]

Immunogenic tumors (e.g. with methylcholanthrene induced sarcomas) are more susceptible to TNF-α activities, since such tumors can be completely destroyed with very low doses of this cytokine (injected intratumorally). High susceptibility of immunogenic tumors to TNF-α activities suggests that the immunogenicity of a

tumor is an important factor in antitumor action of this cytokine and that there are also other mechanisms (above stated), beside the effect on tumor blood vessels, which participate in affecting of immunogenic tumors.

Mechanisms of cytotoxic activity of TNF-α

For most of the cytokines it is true that their effect upon target cells is conditioned with the presence of specific receptors on target cell membranes, with binding of the cytokine to these receptors and with transduction of the signal into the interior of the cells.

Certain authors assume that TNF-α - receptor complex is transferred into the interior of the cell (by endocytosis), where it disintegrates in lysosomes, excessive material is then excreted, while TNF-α participates in different cellular processes.[109, 110]

Besides, Hasegawa and Bonavida[111] noticed that the cytocidal activity of TNF-α depended upon the presence of substances capable of inducing formation of pores in cell membrane, i.e. perforins. This led towards conclusion that TNF-α enters cells directly through membrane pores without binding to receptors (nonspecifically). The mechanisms of action of TNF-α after its direct entrance into the cytoplasm differ from the mechanisms of action after its specific entrance (which is brought about by binding of TNF-α to receptors). Direct entrance of TNF-α into the cell generally results in a strong cytotoxic effect in contrast to a broader spectrum of activities of TNF-α after specific binding to cell receptors. Also, Smith and co-workers[112] proved that the activities of this cytokine in the cell interior depend upon the mode of cytokine's entrance and that the cell membrane represents some kind of selective barrier of TNF-α activities. When TNF-α was introduced into the cytoplasm of normal macrophages directly, the researchers expected these cells to become stimulated (as in the feedback loop of TNF-α effect upon macrophages), yet the only outcome was a strong cytotoxic activity.

On the other hand, the latest studies report that internalisation of TNF is not obligatory for the activation of intracellular processes. Namely, binding of TNF to receptors causes segregation of receptors and trimerization of intracellular parts of receptors, which is sufficient for the activation of signaling pathways.[113-118]

The cytotoxic action of TNF-α results in "programmed cell death" - apoptosis or/and necrosis of target cells. The "nucleus dependent" mechanism of action is supposed to be brought about by means of stimulation of gene transcription and of synthesis of different proteins. It is well known that TNF-α activates protooncogenes *c-fos* and *c-jun* and that the products of these protooncogenes operate as activation factors for promotors of numerous other genes: e.g. for synthesis of various endonucleases, MHC I antigenes, TGF-α, and EGFR.[119]

Apoptosis is a consequence of TNF-α action upon microfilaments (TNF-α causes the disintegration of microfilaments) and/or upon the activation of endonucleases which "cut" (cleave) the cell chromatin (DNA) to short fragments of approximately 200 base pairs.[75, 98] This chromatin cleavage proceeds nonspecifically at internucleosomal loci. Cells, damaged this way, are no longer capable of repairing the damage and instead of entering the S phase of cell cycle they pass over to apoptosis (Figure 1).

Necrosis is the result of production of free radicals, which have an effect on mitochondria and on cell structures different form mitochondria (i.e. cytoskeleton), as well as a result of inhibition of mitochondrial functions.[101, 120] This theory is relatively old and it is supported by the fact that antioxidants, as for example mitochondrial enzyme MnSOD, are capable of protecting the cells from TNF-α effects.[121, 122] Another evidence that speaks for the above mentioned theory are experiments of Yamauchi and co-workers.[123] Namely, they quantified the cellular production of OH- and found out that the amount of this powerful oxidant depends upon the duration of cell exposure to TNF-α. Such kind of dependence was observed only with cells that were susceptible to TNF-α, while

there were no signs of increased production of OH- in cells resistant to TNF-α action.

On the other hand, Wong and co-workers[121] and later on Okamoto with co-workers[122] proved that the group of proteins induced by TNF-α includes also MnSOD. They observed a statistically significant increase of MnSOD concentrations following the transplantation of TNF-α gene into tumorigenic mouse fibroblasts which are originally susceptible to TNF-α. Cells, changed genetically in such a way, were now able to produce both endogenous TNF-α, as well as MnSOD and thus became resistant to TNF-α. This means that TNF-α operates autoregulatively and that the mechanisms of its action interrelate and integrate with each other.

Thus, the basic effect of TNF-α in process of necrosis is the stimulation of production of free oxygen radicals that later on affect cellular structures. Additional proofs speaking for this theory came from experimental results which indicated that the inhibition of cell respiration is a consequence of generation and activities of free radicals (after TNF-α treatment of cells). Further confirmations are results of studies where previous treatment with antioxidants protected cells from cytotoxic activity of TNF-α.[120, 121, 123, 124]

TNF-α as a growth factor

With regard to all biological activities of TNF-α it is difficult to define which one is primary: its antitumor activity, its role in inflammation processes or its activity as a growth factor. Sugarman and co-workers[97] were among the first to describe TNF-α effects upon different normal and malignant cell lines. They established that TNF-α statistically significantly reduced the cell number of only seven out of twenty-two malignant cell lines (after exposure time of 72 h) while having a stimulative effect on growth of some normal cell lines (different human fibroblast cell lines).

On the other hand, Piacibello and co-workers[125] ascertained that TNF-α not only has a stimulative effect on growth of normal cell lines, but also promotes growth of some malignant cell lines. Namely, they found out that low doses of TNF-α in the presence of GM-CSF (granulocyte-macrophage colony stimulating factor) stimulate the growth of human normal, as well as leukemic stem cells whereas higher doses of TNF-α in the presence of G-CSF (granulocyte colony stimulating factor) inhibit the growth of only normal stem cells. These observations were confirmed by other authors who demonstrated that low doses of TNF-α stimulate growth of, while high doses have an antiproliferative effect on, certain tumor cell lines.[101] In normal human fibroblast cell lines TNF-α operates as growth factor and in most of the cases there is a dose dependence: the higher concentrations the better the cell growth.[97] It is important to stress again that beside the dose there are different external factors like confluence of cell cultures, composition of growth mediums, phases of cell cycle and presence of growth factors or cytokines, which influence the mode of TNF-α action.[100, 126]

The TNF-α activity as a growth factor is, just like its antitumor activity, direct and indirect. The direct action comprises activation of genes responsible for synthesis of proteins that direct the cell from G0 to G1 phase of cell cycle thus increasing the number of cell divisions and accelerating the proliferation of cells.[127] The indirect action includes stimulation of cells to produce other growth factors and specific receptors for growth factors. Direct and indirect mode of TNF-α action intertwine and it is impossible to fix their limits.

TNF-α as an immunomodulatory agent

TNF-α in its immunomodulatory role has an effect upon T and B lymphocytes, affects the expression of MHC class I and II antigens, and stimulates macrophages as well as other cells to produce cytokines.[71, 128]

Effect on T and B lymphocytes

TNF-α affects T and B lymphocytes predominantly as a mitogenic factor. Vine and co-wor-

kers[127] established that this cytokine accelerates transition of T lymphocytes from G0 to G1 phase of cell cycle and thus stimulates multiplification of these cells while not stimulating the production of IL-2 (i.e. one of the elementary products of T lymphocytes) at the same time. The effect upon immune system executed by stimulation of B lymphocytes is even more indirect, since TNF-α is capable of promoting the proliferation of these cells, yet only in the presence of IL-2. Because it is well known that TNF-α affects predominantly the division of T and B lymphocytes and co-operates only indirectly in the induction of cytokine synthesis in lymphocytes, there still remains a question about its immunomodulatory role in activation of T and B lymphocytes. It is quite interesting that all lymphocytes after treatment with TNF-α demonstrate a statistically significant increase of receptors for TNF-α.

Effect on NK cells

The effect of TNF-α on NK cells represents a special pattern of autocrine stimulation. Bancroft and co-workers[129] treated immunodeficient mice (suppressed B and T lymphocytes) with dead bacteria of *Listeria monocytogenes species,* isolated spleen cells of these animals and measured their production of IFN-γ. They ascertained that IFN-γ production depended upon the dose of injected bacteria or upon the number of activated macrophages, respectively. Since the macrophages do not produce IFN-γ (or at least this has not been demonstrated yet), the researchers concluded that NK cells are being stimulated with macrophages or their products, respectively. Because IFN-γ is known to be a powerful stimulator of TNF-α production in macrophages Bancroft and co-workers assumed that it is TNF-α that stimulates NK cells to produce IFN-γ: 1. bacteria activate macrophages and trigger synthesis of TNF-α in these cells; 2. TNF-α acts upon NK cells and IFN-γ production; 3. IFN-γ in a feedback loop triggers additional synthesis of TNF-α and the cycle repeats.

Effect on macrophages

The effect of TNF-α on macrophages represents a classical example of autocrine activity. TNF-α, which is itself a product of macrophages, binds in a feedback loop to specific cell receptors and stimulates these cells.[130] It is well known that activated macrophages act cytostatically/cytotoxically on tumor cells that are susceptible to TNF-α. This fact leads towards conclusion that the antitumor activity of macrophages is brought about by producing TNF-α which then affects tumor cells. However, this is not the only form of cytotoxic activity of activated macrophages. These cells can act cytotoxically also by means of other products as for example IL-1, hydrogen peroxide (H_2O_2) and nitric oxide (NO). The combined treatment with TNF-α and NO has a synergistic cytotoxic effect on tumor cells.[131, 132] Especially effective production of NO and TNF-α in macrophages is achieved after stimulation with IFN-γ and IL-2, whereas the stimulation of macrophages with IFN-γ and MDP or its structural analogues primarily increases the TNF-α production.[132, 133]

Receptors for TNF-α

In the year 1990 different researchers cloned the cDNA for two types of cell surface receptors for TNF (TNF-α and TNF-β).[70, 134] Both types of receptors are present on the cell surface of most of the cell lines, yet in a different mutual percent relation.[135,136] These two types of receptors, named TNF R-1 (55 kDa) and TNF R-2 (75 kDa), consist of an extracellular and an intracellular part. The extracellular part of TNF R-1 comprises 182 amino acids and the extracellular part of R-2 235 amino acids. The intracellular parts of receptors are larger - the one of R-1 includes 221 amino acids and the one of R-2 439 amino acids.

Since the cloning of two distinct receptors for TNF (each of which binds TNF-α and TNF-β), the past few years have witnessed the rapid emergence of two superfamilies, of which TNFs and their receptors are only representatives. To

date 12 receptors have been identified with which we can associate eight TNF-related proteins.[137]

According to the amino acid sequence the two types of receptors differ from each other to a great extent. Human TNF receptors demonstrate an amino acid homology of only 27% and most of the homologous amino acids (70%) are placed in the extracellular parts of receptors. Thus the structure of intracellular parts differs considerably between the two types of receptors, which indicates that TNF is connected to different functions or different processes, respectively (according to the type of receptor to which TNF binds). It is interesting that there is a higher resemblance in the structure of human TNF R-1 and mouse TNF R-1 (homology of 64%), which is also true for human TNF R-2 and mouse TNF R-2 (homology of 62%). The resemblance between human and mouse receptors appears mostly in the intracellular parts of receptors (homology of 73% between human and mouse TNF R-2).[136, 138] On the basis of cited facts we can conclude that the activity of TNF is relatively species unspecific and that the intracellular processes in which TNF co-operates (after binding to receptors) are quite similar in different animal species.

Human TNF R-1 and TNF R-2 thus demonstrate only a slight resemblance in the amino acid sequence of their extracellular parts and differ almost completely in the structure of their intracellular parts. Besides, the intracellular parts (according to their amino acid sequence) are not even similar to any of the known proteins.[138] The recapitulation of the above statements is that TNF binds to the two types of receptors, induces different processes in the cell (in dependence upon the type of receptor to which it binds) and that the details of TNF activities inside the cell remain to be explained.

Thoma and co-workers[139] blocked TNF R-1 with antagonistic monoclonal antibodies and in this way prevented the cytostatic/cytotoxic effect of TNF-α on different cell lines. From these results they made an inference that TNF acts cytotoxically trought binding to TNF R-1 receptors.

Brouckaert and co-workers[140] demonstrated a good antitumor effect of recombinant mouse TNF-α (rMuTNF-α) on B16 BL6 melanoma in C57Bl/6 mice, whereas the therapy with recombinant human TNF-α (rHuTNF-α) turned out to be unsuccessful. They also ascertained that 50% more experimental animals died owing to toxic side effects of rMuTNF-α when compared to the number of lethal outcomes after rHuTNF-α therapy. On the other hand, when the mice were pretreated with galactosamine, also rHuTNF-α demonstrated some degree of antitumor activity, but also the toxic side effects were more pronounced. These results lead towards conclusion that the species specific activity of TNF-α (if it is present) can be neutralised and that such activity does not depend only upon the binding to receptors but also upon other factors.

In January 1993 Nature published an article about TNF-α activities after its binding to different receptors.[141] Authors of this article cited some results which are identical to the ones mentioned before. Namely, they confirmed that the cytotoxic effect on tumor cells results from binding of TNF-α to TNF R-1 and described also an interesting example of species specific activity of human and mouse TNF-α; human TNF-α demonstrated an antitumor effect in experimental mice but caused no toxic side effects. The presence of antitumor effect and the absence of toxic side effects of human TNF-α in mice were explained with its ability to bind only to TNF R-1 but not to TNF R-2. Mouse TNF-α, on the other hand, bound to both types of receptors on human cell lines and did not act species specifically.[141]

Contrary to the above stated results, from which could be concluded that the antitumor activity of TNF-α is a consequence of its binding to and operating by means of TNF R-1, other authors established that mouse fibroblasts (TA 1 cells) are insensitive to human TNF-α but very sensitive to mouse TNF-α. Since human TNF-α binds only to TNF R-1 on mouse cells, while mouse TNF-α binds to both types of receptors, there is a logical inference that the cytotoxic effect upon these cells was mediated

by TNF R-2 receptors. The second confirmation, that the cytotoxic effect of TNF-α can also be mediated by TNF R-2, is the fact that HeLa cells, which are otherwise completely insensitive to the antiproliferative activity of human TNF-α and have only TNF R-1, become very sensitive after insertion of a gene coding for TNF R-2 into their DNA.[119] The existence of two types of receptors for TNF-α is supposed to be important also for its activity as a growth factor. In the year 1991 Tartaglia and co-workers[142] ascertained that mouse TNF-α accelerated cell division of mouse thymus cell line and of mouse T lymphocyte cell line (CT-6) while human TNF-α had no effect on the proliferation of cells employed in the experiment. Since human TNF-α binds only to TNF R-1 on mouse cells, the authors substituted TNF-α with agonistic polyclonal antibodies against TNF R-2 and TNF R-1. Agonistic antibodies against TNF R-2 stimulated cell division of both cell lines whereas agonistic antibodies against TNF R-1 were inefficacious. A logical conclusion is that TNF R-2 most probably mediate the stimulation of cell division.

The researchers of Hoffman-La Roche[135] incubated human monocytes from peripheral blood with TNF-α and various inhibitors of TNF-α binding to receptors: i.e. with antibodies against TNF-α, or with recombinant receptor proteins, or with specific neutralising antibodies against TNF R-1 and TNF R-2. In contrast to the above cited data they established that the stimulation of cell division by TNF-α is mediated through both types of receptors, yet the mechanisms of stimulation differ and depend upon the type of receptor.

The antiviral effect of TNF-α is supposed to be mediated by TNF R-1. Namely, Wong, Tartaglia and co-workers[143] stimulated the antiviral activity by means of using agonistic antibodies against TNF R-1. The addition of antibodies against TNF R-2 had no antiviral effect.

Kalthoff and co-workers[144] demonstrated that TNF-α actions, mediated through binding to different receptors, are not completely explained yet. They ascertained that binding of TNF-α or agonistic antibodies to TNF R-1 of human malignant pancreatic cell lines (HPAF, Capan 2) causes a rapid transcription of TNF R-2 gene and that TNF R-2 operates as a specific receptor through which TNF-α mediates transcription of TGF-α gene. In the very same study the authors state that binding of agonistic antibodies to TNF R-1 triggered transcription of EGF receptor gene.

On the basis of cited data it is quite difficult to draw universal conclusions about activities of TNF-α mediated either by one or by the other type of receptors. Common to all known reports are the facts that the species specific activity (if such activity exists) is expressed by means of both types of receptors and that the effect of TNF-α can be substituted by binding of specific agonistic antibodies to receptors. We can also conclude that TNF R-1 and TNF R-2 (after binding of TNF-α to either of the receptors) mediate or stimulate, respectively, different processes in cells. However, quite often the transduction of signals for intracellular processes is realised through simultaneous binding of TNF-α to both types of receptors.

Biochemical mechanisms of TNF-α actions

Biochemical processes that follow the entrance of TNF-α into the cell or the segregation of receptors are still not known: neither the proteins which bind complementarily to the complex TNF-α - TNF-R after internalization, nor the exact procedure of its further action on cellular organelles or cell processes, respectively.[138] Variety of its activities inside the cell (Table 1) indicates that TNF-α is invovlved in different chemical processes which represent additional complications at creating a general sheme of biochemical mechanisms of TNF-α action.

The cytotoxic effect of TNF-α is one of its most thoroughly examined activities. Since such an effect reflects either as apoptosis or as necrosis of cells there are at least two different biochemical mechanisms of cytotoxic activity. The first one is orientated directly to cell nucleus, whereas the other mechanism affects

cellular organelles or cytoskeleton, respectively. However, regardless of the fact that there are different mechanisms by which TNF-α acts upon the cells, it is known that during its intracellular action this cytokine activates protooncogenes *c-fos* and *c-jun* (i.e. immediate early genes) and stimulates the synthesis of various proteins which direct the cell towards either apoptosis or necrosis.

Most probably also the growth factor-like activity of TNF-α is realised through activation of protooncogenes and stimulation of synthesis of proteins responsible for transition of cells into S phase of cell cycle. The activation of protooncogenes *c-fos* and *c-jun* is mediated through nuclear factor kappa B (NF ϰB), which is itself supposed to be activated by protein kinase C.

Biochemical mechanisms of TNF-α action can also be triggered through activation of phospholipase A_2 (Figure 1). Namely, the cells following the addition of TNF-α produce arachidonic acid and prostaglandins,[145-147] which indicates that activation of phospholipase A_2 is also involved in signal transduction. Besides, Palombella and Vilček[100] suceeded in blocking both the cytotoxic and the mitogenic activity of TNF-α by means of inhibiting phospholipase A_2 with dexamethasone.

Undoubtedly TNF-α is a cytokine with a very broad spectrum of effects, owing to which we could hardly expect a simple explanation for its biochemical mechanisms of intracellular activities. Figure 1 represents the authors' global idea of TNF-α activities based on more or less known data from the literature.

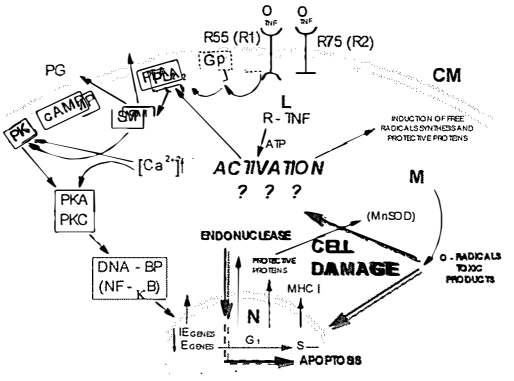

Figure 1. Biochemical mechanisms of TNF-α (TNF) activities at the cellular level. Binding of TNF to receptors [R55 (R1), R75 (R2)]; putative passage of TNF through the cell membrane (CM) into the interior of the cell (endocytosis); degradation of TNF-receptor - TNF complex (R-TNF) with lysosomal enzymes (L); generation of adenosine-triphosphate (ATP); activation of G proteins (Gp); activation of phospholipase A_2 (PLA_2); production of second messengers (SM) as for example cyclic adenosine-monophosphate (cAMP), arachidonic acid, diacylglycerol and inositolphosphate; activation of protein kinases (PK); arachidonic acid is either excreted from the cell or represents a substrate for PLA_2 - products of which are prostaglandins (PG); activation of protein kinases A and C, which results with generation of "DNA binding proteins" (DNA-Bp) like for example "nuclear factor ϰB" (NF-ϰB); NF-ϰB operates in the nucleus (N) and activates "immediate early genes" (IE genes) as *c-myc, c-fos* and *c-jun,* which are further responsible for activation of early genes (E genes); the next step is transcription of genes that are normally being transcribed in G1 phase of cell cycle; products of these genes determine whether the cell continues to S phase or is directed into apoptosis (which is supposed to be a consequence of endonucleases' activities); in the synthesis of endonucleases and "DNA binding proteins" as well as in most of energy-dependent cell processes participate Ca^{2+} ions; TNF affects cell organelles as mitochondria (M) where it stimulates synthesis of oxygen free radicals and other toxic products; TNF also triggers production of protective proteins (which protect the cell from its activities) e.g. manganese superoxide dismutase (MnS●D).

TNF-α and other cytokines

The role of TNF-α in the defence of organism against foreign or own antigens and the mode of TNF-α action quite often depend upon the presence and activity of other cytokines. When interacting in such a way the cytokines either stimulate or inhibit mutually their activities. The stimulative activity includes stimulation of cytokine as well as of cytokine receptor synthesis. On the other hand, the inhibitory action (of cytokines upon other cytokines) is realised through inhibition of transcription, translation and/or secretion of cytokines or their specific receptors. Besides, the cytokines can compete for the very same receptors on the cellular

membrane (e.g. TNF-α and TNF-β) and in respect to the efficiency of their binding to receptors direct cellular processes. Cytokines thus represent some sort of polypeptide hormones at the cellular level which through interreactions transduce different signals to the cells.

The best known functional dependence of TNF-α upon other cytokines is its cytostatic/cytotoxic activity against tumor cells in combination with interleukins and interferons. Namely, in the year 1985 Sugarman and co-workers[97] ascertained that the antiproliferative effect of TNF-α on tumor cells in vitro can be potentiated with IFN-γ. Similar results were obtained by Serša and co-workers[148, 149] on human adenocarcinoma and human malignant melanoma cell lines where IFN-α enhanced the antitumor effect of TNF-α. Enhanced in vitro antitumor effect was also observed by other authors after treatment of tumor cells with TNF-α and IL-1.[150]

In vivo experiments demonstrated that combined treatment with TNF-α and IFN-γ not only has a direct antitumor effect but also the indirect one which is carried out through affecting of the immune system (synthesis and activities of other cytokines, potentiation of cytokine's own antiproliferative activity). Young and Wright[151] established that low doses of IFN-γ and TNF-α reduce the number of suppressor T lymphocytes and decrease the concentration of growth factors. Thus these two cytokines are capable of affecting the growth of primary or residual tumors and the development of metastases. The antiproliferative effect of TNF-α and IFN-α on leukemic cells in patients with chronic myelogenous leukemia (CML) represents a special pattern of co-operation between the two cytokines. Namely, Moritz and co-workers[152] ascertained that IFN-α treatment of leukemic patients gives very promising results but only until the development of resistance to IFN-α. However, when the patiens were pretreated with TNF-α, the resistance to IFN-α did not appear and further successful therapy with IFN-α was enabled.

A complete stop of tumor growth in mice (5 different types of subcutaneous tumors) was described by Winkelhake and co-workers[153] following combined treatment with TNF-α and IL-2. It is quite interesting that in the very same experiment monotherapy with a single cytokine was inefficacious and that the efficacy of combined treatment depended predominantly upon the concentration of TNF-α. Maximal effect of the combination was achieved when maximal sublethal doses of TNF-α were used, whereas the concentration of IL-2 even 90% lower than maximal sublethal dose did not affect the efficacy of treatment. Beside the fact that combined therapy inhibited the growth of subcutaneous tumors, the very same therapy prevented completely the development of lung metastases if only it was started early enough.

The fact that efficacy of combined therapy with TNF-α and IL-2 depends also upon other factors, like for example immunogenicity of tumors, general condition of immune system and scheduling of cytokine application was demonstrated by Agah and co-workers.[154] Additive effect of combined therapy (TNF-α and IL-2) on the development of lung metastases (induced by methylcholanthrene) was observed when the researchers treated animals first with IL-2 and later with TNF-α. Reverse order of cytokine application was less effective. On the other hand, the antitumor activity of combined therapy with TNF-α and IL-2 was extremely low when the very same experiment was repeated using mice with suppressed immune system.

Beside the antiproliferative effect also the growth factor-like activity of different combinations with TNF-α and other cytokines represented an interesting challenge for the researchers. Some of them established that TNF-α together with growth factors *in vitro* effectivelly affects bone marrow cells where on one hand it stimulates the growth of stem cells by means of inducing synthesis of growth factors and, on the other hand inhibits the growth of some leukemic cell lines.[155, 156] TNF-α acts synergistically together with TGF-β on differentiation of human leukemic cells (i.e. stimulates the differentiation process) which results in a reduction of their malignant potential.[157]

When speaking about combined activity of TNF-α with other cytokines we must also em-

phasise its pluripotent role in affecting the cells. Mechanisms of common action of TNF-α with other cytokines are complicated and quite often unclear. In general, combination of TNF-α with interleukins or interferons synergistically inhibits tumor growth; with TGF-β it acts synergistically on the process of cell differentiation and has in lower doses, combined with growth factors a synergistic effect on cell proliferation.

Future perspectives

Cloning of human TNF-α gene was accepted by many researchers as a great step towards the discovery of universal medication for different malignant diseases. Such expectations were a logical consequence of numerous reports confirming the fact that TNF-α demonstrates a distinctive antitumor activity *in vitro* and in some cases an even more pronounced antitumor effect in tumor models *in vivo*. Unfortunately, the results of experiments with tumor models never gained an approval in clinical praxis. The problems which accompanied TNF-α applications in clinical conditions were not a result of its insufficient antitumor activity but derived from severe dose-limiting toxicity (elevated body temperature, anorexia, diarrhoea, nausea and hypotension). Nevertheless, ten years of experiments with TNF-α in clinics are far from a complete failure. The researchers are seeking after such a mode of TNF-α application that would retain its antitumor activity while minimizing the toxic side effects. One of the possibilities is a synthesis of new analogues of TNF-α, which according to incomplete knowledge of the role of certain molecular domains represents quite a difficult task. Namely, the cytokine molecule should be changed in the domain responsible for dose-limiting toxicity, while preserving its structure responsible for cytostatic/cytotoxic effect on tumor cells. Besides, the efforts are being made to create analogues capable of a longer retention at the tumor site, which would limit the effects of cytokine to tumor cells only. In this prospect, there is an idea of synthesising TNF-α chimeric proteins with specific affinity for certain antigens on the surface of tumor cells. The second possibility of local treatment with TNF-α is isolation perfusion with high doses of TNF-α. Problems arising from such kind of therapy derive from incompetence to control and retain completely the cytokine within the treated organ. Quite often TNF-α "escapes" from the artificial circulation loop which results in serious adverse effects. The third but most prospective point of view of clinical uses of TNF-α and its analogues is a gene therapy. Gene therapy represents a kind of systemic treatment where genetically engineered cells (i.e. with TNF-α gene transfected cells) produce a controllable amount of endogenous cytokine. However, the genetic engineering techniques to date do not allow major interventions at the level of human genome without a serious risk. They remain time consuming and expensive, and further developments will be needed before it can become a commonplace treatment.

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