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Review

Tumor necrosis factor as an antineoplastic agent: pitfalls and promises

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Abstract. The discovery and cloning of the cytokine tumor necrosis factor α (TNF) gave rise to new hopes for a significant victory in the war against cancer. Preclinical in vitro studies in cell cultures and in vivo studies in animal models demonstrated the antitumor capacities of TNF. Although clinical studies were largely made possible by the availability of recombinant TNF, phase I and II clinical trials showed very quickly that the systemic administration of TNF induced severe side effects mainly due to its pleiotropic action on immunocompetent cells. The clinical manifestations of the side effects were similar to those observed during a severe infection and inflammation. Very recently, lessons from these clinical studies yielded refined approaches whereby the toxicity of TNF is limited through local administration, a combination with other therapeutic regimens and targeted gene therapy. These new approaches are slated for larger clinical trials and in the near future might demonstrate the limited but powerful usefulness of TNF as an antineoplastic agent for different types of cancer.

Key words. Tumor necrosis factor; cytokines; endotoxin; cancer therapy; gene therapy.

The biology of tumor necrosis factor α

Tumor necrosis factor α (TNF) or cachectin is a pleiotropic cytokine initially identified as a protein released by endotoxin-stimulated macrophages [1], although several other types of cells are able to synthesize small amounts [2, 3] as well. The term *tumor necrosis factor* originates from the observation that this protein plays a crucial role in the killing of tumor cells by activated macrophages and cytotoxic T-lymphocyte subsets involved in the early stages of anticancer surveillance. Besides its effect on cancer cells, TNF exerts a wide range of biological activities related to such diverse functions as inflammation, mitogenesis and differentiation [4]. TNF can induce both necrotic and apoptotic (programmed) forms of cell death [5]. Necrosis is characterized by cell swelling, destruction of cell organelles and lysis. In contrast, the cells shrink as a consequence of apoptosis, where in most cases specific DNA fragmentation occurs in the nucleus. Apoptotic bodies are formed without lysis and therefore without immune reaction by the organism. Although TNF was originally described as a necrosis factor, in many cases the initiation of apoptosis seems to play a more important role in the death of cancer cells.

The molecular facts

The membrane-bound precursor form of TNF is composed of 233 amino acids resulting in a molecular weight of 26 kDa [6]. Processing of this precursor by

metalloproteinases releases a soluble 17,350-Da cytokine consisting of 157 amino acids [7–9]. The monomer forms an elongated, antiparallel β-pleated sheet sandwich. Three monomers associate closely around a threefold axis of symmetry to form a compact bell-shaped trimer [10]. Its homotrimer with a molecular mass of 52 kDa is the biologically active form that can bind to surface receptors with high affinity [11]. These specific receptor molecules are expressed on the cell surface of virtually all somatic cell types except erythrocytes, highlighting the multipotent nature of TNF. The number of TNF receptors per cell can vary from 100 to more than 10,000 [12, 13].

All members of the large TNF/nerve growth factor receptor family act through a common set of signaling molecules and contain a characteristic repeating extracellular cystein-rich motif [14, 15]. TNF homotrimers are capable of binding specifically to two members of this family, the 55- and 75-kDa TNF receptors [16–23]. The two molecules differ in glycosylation and their affinity constants for TNF. Calculation of the dissociation constant (K_d) from the association and dissociation rate constants determined at 37 °C revealed that the 75-kDa myeolid cell type receptor has an affinity constant of 0.42 nM, whereas the 55-kDa receptor of epithelial origin has a higher affinity of 0.019 nM [24]. The high affinity determined for the p55 TNF receptor is mainly due to the marked stability of ligand-receptor complexes in contrast to the transient interaction of soluble TNF with the p75 TNF receptor. These data may in part explain why the p55 TNF receptor is predominant in the induction of cellular responses by soluble TNF; they suggest the stability of the TNF-TNF receptor complexes as a rationale for the differential signaling [25, 26]. When TNF binds to the TNF receptors, phospholipase C is activated and diacylglycerol is produced from membrane phospholipids, leading to the activation of protein kinase C. Upon binding of the ligand the TNF receptors also employ the sphingomyelin pathway, initiating the hydrolysis of plasma membrane sphingomyelin by sphingomyelinase [27]. Ceramide serves as a second messenger stimulating a cascade of protein kinases including mitogen-activated protein (MAP) kinase and, via Raf, the nuclear translocation of nuclear factor kappa B (NFκB) [28]. Some other protein kinases such as β casein kinase and hsp27 kinase are also activated, but their roles in mediating TNF-dependent cell responses are not yet entirely clear. The TNF-TNF receptor complex is rapidly internalized and degraded in lysosomes [29, 30]. This process seems to be mediated by protein kinase C [31–33]. Thus, the TNF receptors are not recycled to the cell surface, but new synthesis is required to maintain receptor density on the plasma membrane [34]. However, some reports indicate that in some cells the TNF receptor may be recycled to the cell surface [35, 36]. On the other hand, an internalization or shedding of both the p55 and the p75 TNF receptors was shown to occur, indicating differential receptor inactivation upon ligand binding [37–39]. In most cells, inactivation of the p55 receptors seems to occur mainly by internalization, whereas the p75 receptor is removed from the cell surface by shedding. The soluble receptor forms may act as inhibitors for TNF-mediated cellular responses by binding and, hence, neutralizing circulating TNF [40, 41].

The p55 receptor mediates the cytotoxic and cytostatic effects of TNF in malignant cells [42–48]. The p75 receptor does not seem to be essential for the cytotoxic activity of TNF, but may have its own biological properties. It has been suggested that the p75 receptor might have a helper function for enhanced cytotoxicity triggered by the p55 receptor. Human HeLa cells containing mainly the p55 receptor are not killed by TNF alone, but overexpression of the p75 receptor induces cell death [49, 50]. In addition, NIH3T3 mouse fibroblasts overexpressing human p75 receptors became sensitive to human TNF, which causes cell death. This series of experiments demonstrates that the p75 receptor can induce cytotoxicity. However, it is not clear yet whether the cytotoxicity is directly induced by the p75 receptor or via an interaction with the p55 receptor. Recent findings indicate that a cooperation of both TNF receptors via TNF-receptor-associated factors 1 and 2 (TRAF1/TRAF2) binding domains may induce apoptosis in rat and mouse T-cell hybridoma PC60 cells [51–53].

The antitumoral activity of TNF: a brief history

The story of TNF as an antitumor agent begins some centuries ago with the accidental observation that advanced cancer patients suffering concomitant bacterial infections showed tumor regression and, in a few cases, a complete cure. In 1891, a New York surgeon, W. E. Coley, discovered that a filtered medium from bacterial cultures showed anticancer activity in terminally ill patients. He went on to treat over 1200 cancer patients with 'Coley's mixed toxin', a crude filtrate of *Erysipelas* and *Serratia marcescens*, having but 'mixed success'. Repeated inoculation of the toxin caused severe side effects such as high fever and chills – side effects reminiscent of infection and inflammation. Despite an initial success rate of 22% this treatment was discontinued mainly because standard bacterial preparations could not be obtained and the causes of the severe side effects were not yet understood. Half a century later, namely in 1943, Shear and colleagues isolated endotoxin from *S. marcescens* culture filtrates, a fraction of which was able

to induce necrosis in tumors [54]. Although the clinical use of endotoxin is very limited because of toxic side effects, over the last decades a number of attempts to revive Coley's toxin in different forms were made. Highly purified endotoxin was intravenously administered by a bolus injection in phase I and II trials [55–58]. In virtually all trials using tolerated doses of either endotoxin or the lipid-A portion of the molecule no direct or only marginal objective antitumor activity or necrosis were observed. Only a few case histories of colorectal cancer patients show partial remission, and in a single case a complete remission occurred [58].

In 1975 it was discovered that the tumor necrosis effect is not directly caused by endotoxin but is due to an endotoxin-induced serum factor, concomitantly called tumor necrosis factor (TNF) [1]. It has been clear for some time that all the bacterial extracts and endotoxins used to treat patients trigger the release of endogenous cytokines such as TNF, interleukin-6, interleukin-8 and granulocyte-macrophage colony-stimulating factor by activated macrophages. TNF has been shown to be cytotoxic for some, but not all, tumor cells. Some cells need to be activated by a second or third cytokine in order to become sensitive to TNF. The mechanism leading to this sensitization is not clear, although it seems certain that the number of surface TNF receptors is not enhanced by the sensitizing cytokine. For other tumor cells TNF is not cytotoxic but only cytostatic, inhibiting growth while not affecting maintenance of cells. Lastly, a number of tumor cell lines do not respond at all to TNF. In fact, the majority of tumor cell lines are not growth-inhibited by TNF in vitro. The cytotoxic effect on some tumor cells is largely mediated by a complex machinery leading to apoptosis of the target cells. Of interest for a therapeutic approach is the observation that induction by TNF of cell death can occur in cells whose protein synthesis has been completely blocked. This implies that death-triggering TNF receptors can activate this latent machinery through a preexisting signaling cascade. The mechanism initiated by the binding of TNF to its receptors can also be activated by the binding of a ligand to CD95/Fas/Apo-1, resulting in the highly controlled activation of killer proteases, the caspases or ICE (interleukin-1β converting enzyme). The activation of the same suicide machinery in tumor cells is also induced by drugs such as chemotherapeutics, which either damage the DNA irreparably or interfere with the cell cycle or the cellular metabolism.

Although the cellular pathways to death were characterized in detail during the last few years, it is still not clear why some tumor cells are resistant to TNF action. One explanation has been offered by recent observations that mutations in proteins with central functions in the apoptotic mechanisms might protect cells from dying. In particular, cells with mutations in the p53 tumor suppressor can survive hypoxic conditions, although the lack of oxygen induces cell death via apoptosis in cells containing wild-type p53. Thus, the obvious approach is to concentrate on developing a new generation of drugs which manipulate the apoptotic machinery selectively in target cells either by inducing apoptosis or by overcoming drug resistance in combination with conventional chemotherapeutics.

The pluripotent effect of TNF is also reflected in its action on vascular endothelial cells affecting vascularization of growing tumors. Neovascularization is an early requirement for both tumor growth and dissemination. This angiogenesis is a result of a balance between angiogenic and antiangiogenic factors, with TNF exhibiting both pro- and antiangiogenic activities [59–64]. On the one hand, some reports indicate that TNF exerts an antiangiogenic effect in part by modulating the vascular endothelial growth factor (VEGF)-specific angiogenic pathway through downregulation of vascular endothelial growth factor receptors (KDR) in cultured human vascular endothelial cells [65] or through its local tissue concentration [66]. On the other hand, the stimulation of endothelial cell growth by TNF seems to depend on other cells, in particular TNF-activated monocytes and macrophages [60, 67, 68]. Thus TNF, a potent inhibitor of endothelial cell growth in vitro, under certain circumstances is indirectly angiogenic in vivo. Whether therapeutically administered TNF will have an angiogenic or antiangiogenic effect in vivo is therefore not entirly predictable, since the complex mechanisms mediating the effects on angiogenesis are poorly understood. However, if TNF is able to eradicate cancer cells in situ, the proangiogenic effect of this cytokine might not play a role at all in the efficacy of tumor therapy.

Another potential pitfall for the use of TNF as an antineoplastic agent is the observation that this cytokine can stimulate proliferation of certain tumor cells in vitro [69–72]. The growth-promoting effect can be mediated via enhanced expression of growth factors [73] and direct or indiret MAP kinase activation [74, 75] and might require the c-jun/AP-1 function [76]. These experimental data led to the conclusion that TNF needs to be blocked, rather than stimulated or administered, in cancer treatment [77]. In view of the numerous reports demonstrating the antiproliferative potency of TNF, the in vitro proliferatory effects of TNF on certain types of cancer cells will need to be further defined and extended to in vivo studies.

Clinical studies

The failures

The cloning of TNF in 1985 and its expression in *Escherichia coli* allowed study of its effects on tumor

cells and cancer in more detail [78–82]. Initially, the discovery of 'the magic bullet' was celebrated, and an important victory in the war against cancer was declared. During the following years a huge interest by the scientific community and the pharmaceutical industry led to numerous animal studies, demonstrating the efficacy of recombinant TNF against various types of cancer. Mouse tumor models and human tumor xenografts in nude mice have shown that intratumoral applied TNF can be very effective in inducing regression of tumors in recipient mice. However, only systemic administration was able to kill tumor cells metastasized to remote areas of the organism or to help eradicate subclinical metastases. Unfortunately, systemically administered TNF was barely effective in mice. This might be due to the pluripotent effects of TNF and, hence, its rapid clearance from the bloodstream by binding to sites other than the targeted tumors.

Once data from the animal studies were available, phase I clinical trials with patients exhibiting advanced malignancies were initiated. The primary objectives of a phase I trial are (i) to determine the maximum tolerance and recommended phase II doses, (ii) to describe all drug-related toxicities in terms of severity and duration and (iii) to determine the clinical pharmacology of the drug. These trials indicated that the maximum tolerated dose of TNF is about 150–300 $\mu g/m^2$ per day for bolus administration [55, 83, 84] and up to 500 $\mu g/m^2$ per day for continuous infusion. For example, in one phase I study TNF was administered intravenously for 5 consecutive days every 2 weeks for a total duration of 8 weeks. Twelve of 34 patients had no change in their evaluable disease for a median duration of 18 weeks (range, 8–30 weeks), and 22 patients showed progressive disease [85]. Although phase I studies are not designed to show efficacy of the drug, these results clearly demonstrated that continuous administration of TNF seems to be as ineffective in humans as in mice. TNF was therefore administered in other trials either intravenously or intramuscularly as a single bolus or as a bolus several times weekly. Dose-limiting toxicity was manifested in hypotension, fatigue and nausea. The most common clinical toxicities of TNF consisted of rigors, fever, chills, anorexia, vomiting, headache and fatigue – side effects similar to those observed during a severe infection and inflammation. Pharmacokinetic studies indicated a rapid plasma clearance and a short plasma half-life, generally less than 0.5 h [86, 87]. In fact, the clearance in the serum of the recombinant TNF had a calculated half-life of 14–18 min [88]. Peak concentrations were observed within 2 h after bolus intravenous administration and TNF concentrations were virtually undetectable 24 h post injection.

The biological effects of intravenous or intramuscular TNF were shown to enhance serum β_2-microglobulin, serum neopterin and serum interleukin-2 receptor (Tac antigen) levels [89]. TNF enhanced granulocyte bactericidal activity [55] in some studies but not in others [89]. Neither did the administration of TNF significantly increase the expression of cell surface proteins on monocytes, including HLA (human lymphocyte antigen)-DR, HLA-DQ, β_2-microglobulin, the Fc receptor and serum interleukin-1 activity. Thus, in humans TNF caused biological response modulation with evidence of HLA class I (β_2-microglobulin) increase and T-cell (Tac antigen) and monocyte (neopterin) activation [89]. Furthermore, serum C-reactive protein as an indicator for inflammation increased significantly [90]. Other phase I trials combined TNF with other cytokines such as interferon-γ [91] or interferon-α and interleukin-2 [92]. The addition of interferon-γ to TNF resulted in a greater than threefold increase in toxicity compared with TNF administered as a single agent, supporting the hypothesis that the combination of these cytokines may induce in vivo the synergistic effects observed in vitro [12, 93, 94]. The doses of TNF evaluated in the phase I trials were used in phase II clinical trials, which are by definition designed to evaluate efficacy (and safety) in selected populations of patients suffering from the disease to be treated. Thus, these trials should yield a success rate for the administered drug. Of the evaluable patients, some responded with no change, and the majority showed progressive disease [95–101]. In conclusion, since recombinant TNF has no demonstrable antitumor efficacy, it is inactive as a single agent in patients with different neoplastic diseases. Thus, it proved necessary to initiate novel means of delivery or combination therapy studies designed to exploit the biological activities of TNF.

The promises

To restrict the toxic side effects of systemically administered TNF, direct delivery into the tumor or isolated limb perfusion was performed. Isolated limb perfusion is an established method for the treatment of regionally advanced melanoma and sarcoma [102]. It allows the delivery of high doses of the drug in a closed system with acceptable toxicity and minimal systemic side effects. Local therapy, particularly isolated limb perfusion, has resulted in complete and long-lasting tumor regression with necrotic activity in most cases confined to the tumor vascular bed [103–105]. Combination therapies were applied to reduce the toxicity of a single agent and to increase the efficacy of TNF. In one study, a protocol with a triple-drug regimen was based on the reported synergism of TNF with chemotherapy, interferon-γ and hyperthermia. In patients with melanoma-in-transit metastases (stage IIIA or AB), 91% complete

response was obtained. In unresectable soft tissue sarcomas, this protocol was found to produce a 50% complete response with 87.5% limb salvage, since most tumors became removable [106]. These successes also triggered other perfusion studies such as the isolated perfusion of the kidney with TNF for localized renal-cell carcinoma [107] and isolated hepatic perfusion in the pig with TNF with and without melphalan [108–110]. The clinical success of the application of TNF in the setting of isolated limb perfusions in patients with advanced sarcomas, melanomas and other tumors has sparked renewed interest in TNF as an anticancer drug [111, 112]. Currently a number of renowned cancer centers in Europe and the United States are exploiting this method for tumor treatment.

An even more locally confined action of TNF can be achieved by specific gene transfer into cells in the tumor or adjacent tissue. Implantation of tumor cells modified by in vitro cytokine gene transfer has been shown by many investigators to result in potent in vivo antitumor activities in mice. These methods are still in the developing stage, and to date only experiments in cell cultures and mice have been conducted. Transduction of the TNF gene to primary culture tumor cells by the use of a retrovirus vector resulted in up to 12 copies of the gene per cell. The cytotoxic activity of killer cells to nontransduced autologous tumor cells incubated with these TNF gene transfectants was augmented. These and other findings indicate the potential for using TNF gene-transduced tumor cells as a vaccine [113]. Others transfected fibroblasts with the TNF gene and showed that these cells started to produce TNF and had antitumor activities [114]. Intratumoral injection of the TNF gene in a suitable vector and concomitant irradiation resulted in a complete tumor regression in over 70% of xenografted malignant human gliomas in athymic nude mice [115]. Furthermore, the lack of the 55-kDa TNF receptor on some tumor cells may be responsible for an incomplete or missing response to TNF. Gene transfer of the 55-kDa TNF receptor to these cells restored TNF susceptibility and resulted in suppression of in vivo tumor formation in nude mice [116]. In contrast, human TNF expressed from adenovirus vector infected tumor cells is quite toxic to mice while inducing only a moderate antitumor response [117]. Although a number of problems need to be resolved before TNF gene transfer or therapy can be tested in humans, the use of the powerful anticancer activity of TNF by locally confining its toxicity appears to be a promising step in the direction of tumor eradication.

The strategy: suicide of cancer cells

The prerequisites for a modern antineoplastic agent are specificity, deliverability, nontoxicity and avoidance of side effects. TNF can meet some of these requirements if administered appropriately. In particular, triggering the self-destruction of cancer cells through the apoptotic machinery is one feature of TNF which might in the near future revive the use of this cytokine as an anticancer drug. Characteristic features of apoptosis are the active participation of the affected cell in its demise, the removal of the cell debris (apoptotic bodies) by cells in neighboring tissue through phagocytosis and the lack of inflammatory responses by the organism during this process. Employing this naturally occurring cell death mechanism will facilitate elimination of cancer cells in a specific manner without triggering side effects such as severe inflammation. However, in vitro experiments and animal studies showed that TNF alone is not able to eliminate all cancer cells. Thus, combination therapy with other cytokines, new therapies and/or conventional adjuvant therapies will be necessary to control and eventually cure a devastating disease like cancer. Combination therapies might also include agents to reduce or avoid side effects of the toxic substances used to treat tumors.

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