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Strategies to enhance the anticancer potential of TNF

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1. ABSTRACT

Although tumor necrosis factor (TNF) antitumor activity is evident in several preclinical models and in non-comparative clinical trials, no evidence exists that TNF-based treatments increase patient survival. Furthermore, due to systemic toxicity, TNF can only be administered via sophisticated drug-delivery systems in patients with solid tumors confined to one extremity or organ. The impossibility to administer TNF systemically does not allow to test the effectiveness of this cytokine in other clinical settings for the treatment of a broader spectrum of tumor types. Dissecting the cascade of molecular events underlying tumor sensitivity to TNF researchers will allow to further exploit the anticancer potential of this molecule. The rational for the development of strategies aimed at sensitizing malignant cells to TNF is to modulate tumor-specific molecular derangements in order to maximize the selectivity of TNF cytotoxicity towards cancer. This would enhance the anticancer activity of current TNF-based locoregional regimens and would pave the way to the systemic administration of this cytokine and thus to a much wider clinical experimentation of TNF in the oncology field.

2. INTRODUCTION

Although chronically sustained low levels of tumor necrosis factor (TNF) have been linked to tumor development and progression (1, 2), it is widely accepted that TNF pharmacological doses can have potent antitumor activity (2, 3). In fact, TNF possesses both direct and indirect (mainly by disrupting the tumor vasculature) cytotoxic effects towards different cancer types both *in vitro* and *in vivo*.

In humans, despite the antitumor activities just mentioned, TNF alone is only marginally active in inducing tumor regression, even when high doses are given through locoregional drug-delivery systems (4-6). Conversely, the combination of TNF with conventional antineoplastic agents (e.g. melphalan, doxorubicin, paclitaxel, actinomycin-D, cisplatin) significantly increases the tumor response rates, although this effect is more striking in some animal models rather than in humans (3, 7, 8).

The other key feature of TNF use in humans regards the cytokine toxicity. The dose-limiting side effect of TNF systemic administration is represented by dose-

dependent hypotension that can progress to a shock–like syndrome due to a depression of the cardiac function combined with a capillary leak syndrome resembling that of septic shock. Unfortunately, at the maximum tolerated dose (150-300 microg/m^2), TNF systemic administration is not associated with significant antitumor activity, as demonstrated by phase I-II trials (6). This is the reason why in the clinical setting the cytokine is currently administered only through locoregional drug-delivery systems (e.g. isolated limb and hepatic perfusion), which claim the advantage of yielding high drug concentrations within the tumor and minimizing the risk of systemic toxicity (4, 9).

The third fundamental observation regarding the administration of TNF as an anticancer agent is that no survival advantage has thus far reported in patients receiving this cytokine, even in combination with conventional anticancer drugs.

The impossibility to administer TNF through the systemic route likely prevents clinicians from assessing the effectiveness of this cytokine in terms of patient overall survival, which mainly depends upon the metastatic spread throughout the body and thus is not affected by locoregional treatments.

Overall, these considerations point at concluding that the future of TNF as an anticancer agent is linked to the identification of pharmacokinetics (e.g. development of novel delivery systems) and or pharmacodynamics (e.g. development of TNF sensitizers) solutions that allow to improve the tumor specificity of TNF, which would ultimately lead to increase the therapeutic index (i.e. the activity/toxicity ratio) of this cytokine to the extent that systemic administration becomes feasible (in terms of toxicity) and at the same time effective (in terms of anticancer activity).

In this review we summarize the available findings regarding some among the most promising and molecularly best characterized approaches to improve the clinical usefulness of TNF as an anticancer agent.

3. TNF MUTEINS

The development of TNF analogues (also known as muteins) has represented one of the first strategies to split the molecular domains of TNF responsible for the cytokine antitumor properties from those causing systemic toxicity (10). Moreover, TNF-induced cytotoxicity towards some malignant cells lines is mediated by interaction with TNF receptor-1 (TNFR1), whereas TNF-induced proliferation of these cells is mediated by the triggering of TNFR2: accordingly, muteins selectively binding to a specific TNF receptor have also been designed (11). Despite the reported therapeutic advantage of TNF muteins in some animal tumor models (12, 13), TNF analogues have not yet been implemented in the clinical setting.

4. NOVEL DRUG DELIVERY SYSTEMS

Different strategies have been adopted to selectively deliver TNF into the tumor microenvironment. For instance, selective intratumoral TNF expression can be achieved by means of radiation-induced activation of replication-deficient viral vectors (14). Using this gene delivery method (called TNFerade), phase I-II trials in patients with prior treatment-refractory solid tumors have been carried out (15-17). In the largest of these studies no dose-limiting toxicities have been observed and 21 of 30 patients (70%) experienced objective tumor regression (15). Other clinical trials - including a phase III randomized controlled study - are ongoing that use TNFerade coupled with radiation therapy alone or in combination with other anticancer agents (Table 1). Interestingly, it has been recently demonstrated in a preclinical model that TNFerade therapeutic effect might not be limited to the irradiated area but might be spread to the entire body thanks to the host reaction elicited within the irradiated tumor mass (18); clearly, if this phenomenon held true in humans as well, the treatment with TNFerade might result effective not only towards the clinically detectable local disease but also for the clinically occult distant micrometastatic tumor deposits.

Other authors, using a replication-deficient adenovirus coding for the constitutively active version of the TNFR1 under the control of a melanoma-specific promoter/enhancer element, have shown that melanoma cells (but not other cells) undergo programmed cell death *in vitro* (19). This drug delivery system might allow investigators to selectively target tumor masses without administering TNF, thus bypassing the issue of systemic toxicity.

Using another bioengineering "trick", researchers have modified the TNF molecule so to render it "sticky" towards the tumor vasculature (20). This synthetic molecule, called NGR-TNF is prepared by coupling TNF with the tumor-homing peptide Cys-Asn-Gly-Arg-Cys (CNGRC), a ligand of a CD13 (aminopeptidase N) isoform expressed by endothelial cells in tumor vessels. Systemic administration of picogram doses of NGR-TNF can enhance the antitumor activity of melphalan and doxorubicin in mouse models, with no evidence of increased toxicity (21). Clinical trials testing this therapeutic solution are ongoing (Table 1).

A similar approach to improve the therapeutic index of TNF consists of fusing the cytokine to monoclonal antibodies capable of a selective localization at the tumor site. Some investigators have constructed fusion proteins of TNF with L19 (TNF-L19), an antibody fragment specific to the extradomain B of fibronectin, which has been shown to target tumors in animal models and in patients with cancer (22). These fusion proteins display a potent antitumor activity in several immunocompetent murine models of cancer but do not lead to complete remissions of established aggressive tumors. Only the combination of the fusion protein interleukin-12(IL12)-L19 with TNF-L19 has displayed potent synergistic anticancer activity and led to the eradication of F9 teratocarcinomas grafted in immunocompetent mice (23). Clinical trials with these fusion proteins are being planned (Prof. L. Zardi, National Cancer Institute of Genova, Italy, personal communication).

Table 1. Ongoing clinical trials using TNF as an anticancer agent

Regimen	Phase	Tumor	Trial ID[1]
TNFerade + 5-fluorouracil + radiotherapy	III	Unresectable pancreatic cancer	NCT00051467
Capecitabine + radiotherapy with versus without TNFerade	II (randomized)	Rectal cancer (before surgery)	NCT00072241
NGR-TNF	II	Metastatic SCLC	NCT00483509
NGR-TNF	II	Advanced HCC	NCT00484211
NGR-TNF + doxorubicin	II	Advanced ovarian cancer	NCT00484432
TNFerade + cetuximab + radiotherapy	I-II	Locally advanced H&N cancer	NCT00496236
TNFerade + 5-fluorouracil + hydroxyurea + radiotherapy	I-II	Recurrent H&N cancer	NCT00496535
TNF-bound colloidal gold	I	Advanced solid tumors	NCT00356980

[1] IDs are from the National Cancer Institute clinical trial database (http://www.cancer.gov/clinicaltrials/search/) H&N: head&neck; HCC: hepato-cellular carcinoma; SCLC: small cell lung carcinoma

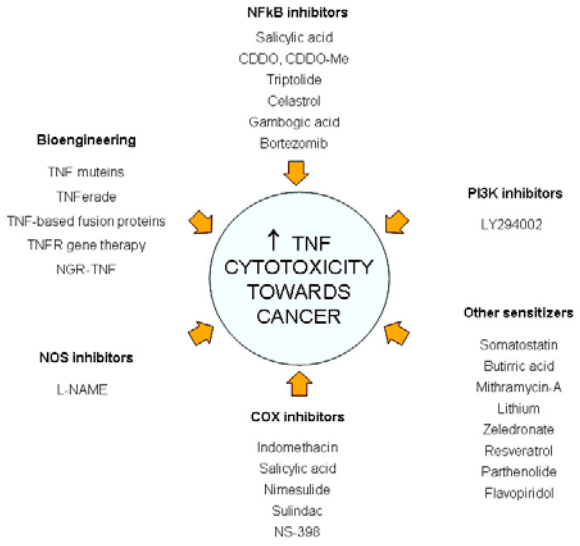

Figure 1. Synopsis of strategies developed to increase the therapeutic index of tumor necrosis factor (TNF) in oncology (see text for more details). COX: cyclooxygenase; NFkB: nuclear factor kappa B; NOS: nitric oxide synthase; NSAID: non steroidal anti-inflammatory drugs; PI3K: phosphatidyl-inositol 3-kinase; L-NAME: N-nitro-L-arginine-methyl-ester; CDDO: 2-cyano-3,12-dioxooleana-1,9,-dien-28-oic acid; CDDO-Me: C-28 methyl ester derivative of CDDO.

5. TNF SENSITIZERS

Many investigators have instead focused on a different strategy to maximize TNF therapeutic index. In particular, several attempts have been made to exploit the anticancer synergism between TNF and different compounds, called TNF sensitizers (24) characterized by the ability of reducing cytokine-related toxicity or making malignant cells more sensitive to the lethal effect of the cytokine, although - to the best of our knowledge - none of them has entered the clinical phase of experimentation thus far.

Some authors have tested the ability of certain molecules (e.g. BB-94, a broad-spectrum matrix metalloproteinase inhibitor; pentoxifylline, a methylxanthine derivative with vascular protective activity) to reduce TNF toxicity both in animal and human models (25, 26).

However, most researchers have described TNF sensitizers that interact with the metabolic/survival/apoptotic machinery of malignant cells so to favor the cytotoxicity of the cytokine (Figure 1). This ultimately might allow clinicians to administer therapeutically effective TNF doses through the systemic route, without patients incurring in the toxicity typical of the cytokine. The following paragraphs are an overview on some of the most promising and best characterized of such TNF sensitizers.

5.1. NFkB pathway modulators

TNF is a potent activator of the transcription factor nuclear factor kappa B (NFkB) pathway, a survival pathway often upregulated in tumors that leads to upregulation of anti-apoptotic proteins (27, 28). Hence, TNF induces apoptosis in the presence of inhibitors of protein synthesis, a phenomenon routinely exploited for experimental purposes when TNF-related apoptosis is evoked by co-administering cycloheximide, a protein synthesis inhibitor. It has been demonstrated that TNF-mediated NFkB activation leads to the overexpression of caspase inhibitor FLIP (Fas-associated protein with death domain-like IL-1converting enzyme), and that FLIP inhibition restores the sensitivity of human ovarian epithelial cancer cells to the TNF pro-apoptotic stimulus (29). According to these findings, sodium salicylate selectively enhances the apoptotic effects of TNF in human erythroleukemia cells but does not affect primary human lymphocytes or monocytes (30). In fact, these leukemia cells possess high basal NFkB responses and elevated FLIP levels; therefore, sodium salicylate exerts its synergistic effect with TNF by reducing the elevated NFkB responsiveness and FLIP levels and restoring the apoptotic response of TNF rather than the proliferative/proinflammatory effects of the cytokine in these cancer cells.

NFkB anti-apoptotic effect in cancer cells appears to depend not only upon the activation of anti-apoptotic genes but also the downregulation of pro-apoptotic factors such as PTEN (31), a tumor suppressor gene that functions as a negative regulator of the phosphatidyl-inositol 3-kinase (PI3K) - protein kinase B (PKB)/Akt cell survival pathway. Given the current possibility of mimicking the effect of PTEN by the use of small molecule inhibitors of PI3K (e.g. LY294002), the spectrum of potential NFkB silencers (and thus TNF

sensitizers) is constantly growing, as already demonstrated in an esophageal carcinoma model (32).

Investigators have reported that a novel triterpenoid, 2-cyano-3,12-dioxooleana-1,9,-dien-28-oic acid (CDDO) inhibits NFkB-mediated gene expression at a step after translocation of activated NFkB to the nucleus (33). Of note, CDDO in combination with TNF causes a dramatic increase in apoptosis in ML-1 leukemia cells, which is associated with activation of caspase-8 and caspase-3, cleavage of Bid, translocation of Bax, and cytochrome-C release. In this model, apoptosis is acutely induced by CDDO/TNF in every leukemia cell line tested including those overexpressing Bcl-xL, which suggests that the mitochondrial pathway is not required for apoptosis by this combination. Similar data have been more recently reported on CDDO-Me, the C-28methyl ester derivative of CDDO (34).

Many other *in vitro* studies have demonstrated that the resistance of both solid and hematological malignancies to TNF can be reversed by inactivation of NFkB (35, 36). For instance, stable transfection and adenoviral-mediated expression of NFkB inhibitor IkB can be used to confer TNF sensitivity to colorectal carcinoma cells previously refractory (37). Also pharmacological inhibition of NFkB (e.g. triptolide, celastrol) increases malignant cell sensitivity to TNF cytotoxicity and might limit TNF-related pro-inflammatory effects (38, 39).

The mechanism of action of some TNF sensitizers has been studied in detail. For instance, gambogic acid, a xanthone derived from the resin of the *Garcinia hanburyi*, has been recently demonstrated to bind transferrin receptor and exhibit potential anticancer effects (40). In human leukemia cancer cells, treatment of cells with gambogic acid enhances apoptosis induced by TNF and inhibits the expression of gene products involved in anti-apoptosis (inhibitor of apoptosis [IAP] -1 and -2, Bcl-2, Bcl-xL, and TNF receptor associated factor 1 [TRAF1]), proliferation (cyclin D1 and c-Myc), invasion (cyclo-oxygenase-2 [COX-2] and matrix-metalloproteinase-9 [MMP-9]) and angiogenesis (vascular endothelial growth factor [VEGF]) (41), all of which are known to be regulated by NFkB. Gambogic acid suppresses NFkB activation induced by various inflammatory agents and carcinogens and this is accompanied by the inhibition of TAK1/TAB1-mediated IkB kinase (IKK) activation, thus inhibiting inhibitor of NFkB (IkB) phosphorylation and degradation, suppressing p65 phosphorylation and nuclear translocation, and finally abrogating NFkB-dependent reporter gene expression; the effects of this TNF sensitizer appear to be mediated through transferrin receptor as down-regulation of the receptor by RNA interference reverses its effects on NFkB and apoptosis (41).

Another class of NFkB pathway inhibitors - proteasome inhibitors (e.g. bortezomib) - has already entered the clinical phase in combination with conventional antineoplastic drugs (42, 43). Preclinical results with the combination bortezomib-TNF are encouraging (44), but no clinical trial is ongoing yet. Recently, some investigators have demonstrated that combining TNF with bortezomib in an experimental model of colon carcinoma not only inhibits tumor growth but also significantly prolongs animal survival (45). Intriguingly, in this model the synergism was biunivocal, since bortezomib inhibits the NFkB activated by TNF, and the cytokine prevents the upregulation of heat shock protein-27 (HSP27, a chaperon protein that protects NFkB from proteasome mediated degradation) induced by bortezomib (45).

Finally, the growing knowledge of the molecular disruptions underlying cancer development and progression offers the opportunity to exploit tumor specific derangements to enhance malignant cell sensitivity to antineoplastic agents (such as TNF) while sparing normal cells the drug related toxicity (46, 47). For instance, in breast carcinoma cells the involvement of mitogen-activated protein kinase (MAPK) p38 in the activation of the transcription factor NFkB suggests a potential role and mechanism for regulation not only of cell survival but also drug resistance (48). Some investigators have dissected the role of the p38-MAPK pathway in the regulation of drug resistance by using p38-MAPK inhibitor SB-203580 (49). As compared to the sensitive MCF-7N parent cell line, the MCF-7TN-R cell line displays significant resistance to TNF-induced cell death coupled with an increased basal activation of p38-MAPK. The p38-mediated phosphorylation and transcriptional activity are suppressed by pharmacologic inhibition with SB-230580 and treatment of MCF-7TN-R cells with SB-230580 restores sensitivity to TNF-induced cell death, while no further toxicity is observed in normal cells. The ability of p38 inhibition to resume apoptotic sensitivity is correlated with suppression of the TNF-induced cell survival pathway NFkB. The increased activation of p38-MAPK in MCF-7TN-R cells demonstrates that this signaling pathway through activation of NFkB is an important route for control of resistance to cell death in breast carcinoma and suggests that pharmacological inhibition of p38-MAPK signaling may represent a mechanism for sensitizing cancer cells to TNF-based therapeutic regimens. These findings represent a proof-of-concept evidence that molecular characterization of cancer can lead to the identification of the targets suitable for the development of tumor specific (and thus highly effective) drugs with direct cytotoxic activity or with sensitizing properties that make "conventional" antineoplastic agents (such as TNF) more effective.

5.2. Nitric oxide scavengers and donors

The interaction between TNF and nitric oxide (NO) pathways is complex and still incompletely elucidated (50, 51). Following the above mentioned principle, the disruption of the NO pathway frequently observed in malignant cells has led many researchers to explore the opportunity to interfere with this pathway in order to increase cancer sensitivity to TNF.

Incubation of normal (e.g. endothelial) and malignant cell lines with TNF leads to the expression of inducible NO synthase (NOS) (52) through the activation of NFkB (53). NO can modulate TNF signaling, but its effects on TNF-driven programmed cell death are not

univocal. When generated after cytokine-stimulated expression of inducible NOS, NO may contribute to later stages of apoptosis (54). Accordingly, NOS inhibition prevents TNF-induced apoptosis in some tumor cell lines, and TNF-resistant tumor cell lines are NOS-negative (52). By contrast, administration of NO prior to, or together with TNF has been shown to inhibit cytokine-driven programmed cell death both in normal and malignant cells (55).

Two observations support the protective effect of NO. First, NO inhibits key pro-apoptotic signal transduction events triggered by TNF, including ceramide accumulation, TNF receptor associated death domain (TRADD) recruitment to the death inducing signaling complex (DISC), cytochrome-C release, and activation of initiator (caspase-8, -9, -10) and effector (caspase-3, -6,-7) caspases (56, 57). Second, inhibition of endogenous NOS activity during stimulation with TNF increases apoptosis induction by the cytokine (58, 59). In accordance with these data, some investigators have reported that coadministration of TNF and N-nitro-L-arginine-methyl-ester (L-NAME, an inhibitor of NOS) significantly increases the tumor regression rate in an animal model of soft tissue sarcoma (60).

The complexity of the interactions between NO and TNF pathways has been further underscored by recent studies and opens new avenues of therapeutic intervention. Ceramide generation ensuing TNFR1 engagement by TNF acts as an amplifying factor for programmed cell death by increasing TRADD recruitment (57) and triggering other apoptosis-related signaling events (61). Although NO downregulates the accumulation of the lipid messenger (57), ceramide has been found to stimulate NOS activity (58), which implies that NO production is regulated by, and itself regulates, the apoptotic process triggered by TNF in a built-in feed-back circuit.

Other investigators have observed that NO donating drugs sensitize human carcinoma cell lines to TNF-driven apoptosis and inhibits NFkB activation and DNA-binding property (62): therefore, NO-mediated TNF sensitization of cancer cells might involve the disruption of other TNF-related pathways (e.g. NFkB), which in turn release the break to cell apoptosis.

In line with these findings, we have observed that endothelial NOS mRNA levels are higher in human melanoma metastases responding rather than those non-responding to TNF-based isolated limb perfusion (63). Moreover, depletion of NO by L-NAME before TNF addition to human endothelial cells conditioned with melanoma supernatant inhibits TNF-induced apoptosis *in vitro*, suggesting that high intratumoral levels of NO might favor the induction of programmed cell death triggered by TNF; remarkably, addition of L-NAME together with TNF does not inhibit apoptosis of endothelial cells, but still decreases their proliferation rate (according to the pro-angiogenic effect of NO) (63). These findings, coupled with those from animal models (60), lead to hypothesize that coadministration of TNF and L-NAME to patients with high-NOS expressing tumors might represent an effective strategy to maximize the anti-angiogenic attack on cancer.

5.3. Non steroidal anti-inflammatory drugs (NSAID)

In preclinical models, tumor susceptibility to TNF-mediated apoptosis directly correlates with TNF ability to generate reactive oxygen species (ROS) and inversely with malignant cell ability to produce ROS scavengers via the upregulation of redox enzymes, such as glutathione reductase/transferase (64) or superoxide dismutase (65). Interestingly, activation of the NFkB pathway can induce the expression of enzymes (e.g. superoxide dismutase) that neutralize free radicals (66). Traditional NSAID such as indomethacin and salicylic acid, which increase the production of ROS by cancer cells, can sensitize TNF resistant tumor cell lines to the apoptotic activity of the cytokine (67).

Currently, several investigators are focusing their attention on a novel class of NSAID - i.e. COX-2 inhibitors - and believe that their sensitization activity towards TNF-induced programmed cell death mainly relies on the disruption of the NFkB pathway (68, 69). Both COX-2 and NFkB are often overexpressed in cancer cells and this phenomenon has been repeatedly reported to favor tumor cell survival and resistance to antineoplastic agents (27, 70). Moreover, TNF interaction with TNFR1 is followed by increased levels of both COX-2 and NFkB, which would counteract cytokine-driven apoptosis. Not surprisingly, several investigators have reported on the ability of selective COX-2 inhibitors (e.g. nimesulide, sulindac, NS-398) to significantly augment the antitumor activity of TNF both *in vitro* and in animal models (68, 69, 71, 72).

Despite these encouraging results and the availability of clinical grade COX-2 inhibitors, to the best of our knowledge no clinical trial testing the synergism of these drugs with TNF is currently ongoing.

5.4. Other TNF sensitizers

Several other agents have been tested as TNF sensitizers potentially exploitable to increase the therapeutic index of TNF. As an example, some investigators have explored the possibility of therapeutically exploiting the fact that somatostatin receptor subtype 2 (SST2) gene expression is lost in 90% of human pancreatic adenocarcinomas (73). They found that stable SST2 transfection of human pancreatic BxPC-3 cells, which do not endogenously express SST2, inhibits cell proliferation, tumorigenicity, and metastasis. Interestingly, these effects occur as a consequence of an autocrine SST2-dependent loop, whereby SST2 induces expression of its own ligand, somatostatin. Since SST2 transfection also sensitizes these cells to apoptosis induced by TNF (by up-regulating expression of TNFR1 and by downregulating the expression of the anti-apoptotic mitochondrial Bcl-2 protein), it can be hypothesized that somatostatin administration together with TNF might lead to a synergistic antineoplastic effect.

Findings obtained in preclinical models suggest that TNF anticancer effect might be enhanced by the

concomitant administration of interferon-gamma (IFNγ). This hypothesis has been recently supported by the observation that IFNγ upregulates the expression of key elements of TNF-driven apoptosis, such as caspase-8 (74). However, when TNF-based isolated limb perfusion is combined with subcutaneous administration of IFNγ for the treatment of patients with locally advanced melanoma or soft tissue sarcomas (75, 76), tumor response rates are not higher than those achieved without IFNγ.

Nevertheless, other cytokines might serve as TNF sensitizers. Endothelial-monocyte-activating-polypeptide II (EMAP-II) can sensitize initially resistant human tumors to TNF-induced regression (77), likely increasing TNFR1 expression on endothelial cells (78).

Inhibitors of histone deacetylase represent a novel class of anticancer agents (79). One such inhibitor, sodium butyrate, presents a synergistic antineoplastic effect when used along with TNF (80). In particular, using breast cancer cell lines some authors have demonstrated that this synergism is associated with increased expression of death receptors, Bid and cytosolic cytochrome-C, and that synergistic induction of apoptosis is strongly inhibited by dominant-negative Fas associated death domain (FADD) as well as inhibitors of caspases-8 and caspase-9, indicating that enhancement of programmed cell death involves key elements of the TNF receptor signaling machinery (80).

Also the conventional chemotherapeutic agent mithramycin-A considerably increases the direct cytotoxic effect of TNF on tumor cells by acting on the balance of apoptotic factors (81). *In vitro*, TNF-induced activation of NFkB-dependent gene expression is not modulated by mithramycin-A treatment, while FLIP protein levels are downregulated, indicating that mithramycin enhances TNF-induced apoptosis in an NFkB-independent manner by releasing the apoptotic brake FLIP.

For long time lithium salt has been known to considerably increase the direct cytotoxic effect of TNF on tumor cells, both *in vitro* and *in vivo* (82). Recently, some light has been shed on the mechanism underlying TNF-lithium synergism, which might renew the interest for the clinical implementation of such anticancer association. *In vitro* lithium sensitizes soft tissue sarcoma cells to both types of TNF-driven programmed cell death in a NFkB-independent manner, which is supported by the fact that apoptosis is associated with an increased processing of pro-caspase-8 and an early release of cytochrome-C from mitochondria (83). This indicates that lithium acts upstream of the point of bifurcation of signaling pathways leading to apoptotic or necrosis-like programmed cell death, supposedly at the level of TNF receptor itself or TRADD.

The PKB/Akt pathway has emerged to be of pivotal importance in promoting cell survival and conferring protection against death induced by antineoplastic drugs, including TNF (84-86). Constitutionally sustained activity of PKB/Akt might also explain the characteristic resistance of endothelial cells to TNF-driven programmed cell death, as demonstrated by the fact that pharmacological inhibition of PI3K (an essential activator of PKB/Akt) sensitizes endothelial cells to TNF cytotoxicity (87). However this effect is not universal. For instance, TNF resistant B16 murine melanoma cells are not sensitized by selective inhibition of PKB/Akt, while specific blockade of protein kinase-C (PKC) - another cancer survival related kinase - does elicit apoptosis following exposure to TNF (88, 89).

Bisphosphonates (e.g. zeledronate) are potent inhibitors of osteoclast function but can also exert anticancer properties, which seems mainly due to their ability to suppress tumor related angiogenesis (90). Some investigators have reported that, *in vitro*, zoledronate strongly sensitizes human endothelial cells to TNF-induced caspase-independent (necrosis-like) programmed cell death (91). This phenomenon has been associated with the inhibition of the PKB/Akt pathway, while NFkB activation is not affected. Further experiments are warranted to address the *in vivo* efficacy of bisphosphonates as sensitizers of TNF anti-angiogenic activity.

The natural phytoalexin resveratrol (3,5,4'-trihydroxystilbene), which is under investigation for cancer chemoprevention (92), induces a redistribution of death receptors into lipid rafts (93). This effect sensitizes human colon cancer cell lines to death receptor-mediated apoptosis; in particular, in resveratrol-treated cells TNF activates a caspase-dependent death pathway that escapes Bcl-2-mediated inhibition (93). Resveratrol does not enhance the number of death receptors at the surface of tumor cells, but induces their redistribution into lipid rafts and facilitates the caspase cascade activation in response to death receptor stimulation: in fact, the cholesterol sequestering agent nystatin prevents resveratrol-induced death receptor redistribution and cell sensitization to death receptor stimulation (93).

Parthenolide, one of the principal active components in medicinal plants, is a sesquiterpene lactone with potent anti-inflammatory and antitumor activities (94). Pre-treatment with parthenolide greatly sensitizes various human cancer cells to TNF-alpha-induced apoptosis. Such sensitization is closely associated with the inhibitory effect of parthenolide on TNF-mediated NFkB activation by disrupting the recruitment of the IKK complex to TNF receptor, which then blocks the subsequent signaling events including IKK kinase activation, IkB-alpha degradation, p65 nuclear translocation, DNA binding and transactivation (95). In addition, parthenolide also markedly enhances and sustains TNF-mediated c-Jun N-terminal kinase (JNK) activation, as demonstrated by the fact that a specific JNK inhibitor (SP-600125) abolishes the sensitization effect of parthenolide on TNF-induced apoptosis of malignant cells.

Another TNF sensitizer that deserves consideration is flavopiridol, one of the first cyclin-dependent kinase inhibitors undergoing clinical tests (96). In apoptosis-refractory A549 and PC3M cancer cells, flavopiridol inhibits cell proliferation but does not cause programmed cell death; however, as a reversible inhibitor of transcription, flavopiridol sensitizes these cells to

apoptotic stimuli, allowing TNF to cause rapid and massive apoptosis (97). Other investigators (98) have independently confirmed these findings. The molecular mechanisms underlying this synergism are being elucidated (97, 98). Flavopiridol inhibits gene expression and causes apoptosis, and these effects cannot be explained by inhibition of cyclin-dependent kinases that govern cell cycle. By inhibiting Mdm-2 (the chaperon protein that targets p53 and delivers it to the proteasome machinery for degradation), flavopiridol dramatically induces p53 and causes tumor cell apoptosis. However, induction of p53 has been proved to be a marker, not a cause, of flavopiridol-related cytotoxicity, as it causes rapid apoptosis in p53-null leukemia cells. In A549 cells, the cytotoxic synergy by the combination treatment involves the activation of caspase-1, caspase-3, and caspase-8 and generates significant chromosomal degradation. Remarkably, the timing of TNF/flavopiridol administration is crucial, as only the treatments of flavopiridol concomitantly with or followed by TNF induces pronounced apoptosis in A549 cells; by contrast, prior treatment of TNF inhibited the apoptosis by the following combination treatment, leading to scarce cell death. Yet, such inhibition is reversed when 5,6-dichloro-1-beta-D-ribofuranosyl-benzimidazole (a transcription inhibitor) is present during TNF pre-treatment, suggesting that the inhibitory pre-treatment of TNF might involve anti-apoptotic gene expression at the transcriptional level. While TNF treatment results in NFkB activation, flavopiridol has been found to inhibit the NFkB-dependent gene transcription, which might explain the synergistic effect of flavopiridol with TNF.

Finally, it is worth mentioning that, besides "conventional" TNF sensitizing drugs, gene expression modulation by means of RNA interference has been experimented in this field. RNA interference is not only an innovative tool to dissect the molecular mechanisms underlying tumor development/progression but also a potential anticancer weapon itself (99).

For instance, it has been demonstrated that concurrent blockade of both NFkB and PKB/Akt gene expression by small interfering RNA synergistically enhances the cytotoxicity of TNF towards lung carcinoma cells (85). Other authors (100) have used this approach to silence the expression of phosphatidylethanolamine-binding protein-4 (hPEBP4), a gene encoding a protein for which no inhibitory drugs are known. hPEBP4 belongs to an evolutionarily conserved family of proteins with pivotal biological functions and is potentially involved in tumor cell sensitivity to TNF. MCF-7 breast cancer cells overexpress hPEBP4 and are resistant to TNF-mediated apoptosis: however, downregulation of hPEBP4 expression by RNA intereference sensitizes these cells to TNF-induced apoptosis, seemingly by inhibiting activation of the Raf-1/MEK/ERK cell survival pathway (100).

6. PERSPECTIVE

Although TNF antitumor activity appears quite evident in some non-comparative clinical studies, the failure to demonstrate a significant tumor response advantage in the only phase III randomized controlled trial (8) coupled with the fact that no TNF-based treatment has thus far proved to increase patient survival does not allow oncologists to use TNF as a routine antineoplastic agent.

Due to its systemic toxicity, TNF administration through sophisticated locoregional drug-delivery systems is currently mandatory. The impossibility to administer TNF through the systemic route prevents from assessing the effectiveness of this cytokine in terms of patient overall survival, which mainly depends upon the metastatic spread throughout the body and thus is unlikely to be affected by locoregional treatments.

Despite these considerations, the interest in TNF as an antineoplastic agent is not fading, as it is witnessed by the continuous flow of scientific production on this subject (3, 101, 102). A challenge many researchers are tackling is to dissect the cascade of molecular events underlying tumor sensitivity to TNF so to fully explore the anticancer potential of this cytokine.

The rational for the development of strategies aimed at sensitizing malignant cells to TNF is to exploit tumor-specific molecular derangements to modulate TNF biological activities and ultimately maximize its tumor-selective cytotoxicity (or minimize its toxicity towards normal cells/tissues). This would not only enhance the anticancer activity of current TNF-based locoregional regimens, but would also open the avenue to the systemic administration of this cytokine and thus to a much wider clinical experimentation of TNF in the oncology field.

The task is particularly difficult due to the pleiotropic nature of the cytokine, which can stimulate multiple, complexly interconnected pathways often involved in opposite phenomena (e.g. apoptosis vs cell proliferation/survival). This potentially insuperable drawback might instead turn out to be the strength of TNF sensitizers development, provided that the derangement of the apoptotic/survival machinery of malignant cells could be exploited to selectively spare normal cells/tissues. For instance, most findings above reported support the hypothesis that the pharmacological inhibition of the anti-apoptotic pathways (which are generally upregulated in malignant, but not normal cells) induced by TNF engagement with its receptor might be a winning strategy in the attempt to selectively sensitize tumors to TNF cytotoxicity.

The interest in this “double hit” approach against cancer goes well beyond the development of TNF sensitizers and has been recently brought to the attention of the scientific community following the encouraging results obtained in the clinical setting with anticancer regimens combining conventional antineoplastic agents with molecularly targeted drugs (103-105). This strategy is based upon common notions of cancer biology that, surprisingly, appear to have been thus far substantially neglected while designing anticancer regimens. The aberrant behavior of cancer reflects the upregulation of certain oncogenic signaling pathways that promote

proliferation, inhibit apoptosis, and enable cancer to invade tissues and evoke angiogenesis. Since multiple pathways are dysfunctional in most cancers and tumors accumulate new oncogenic mutations as they progress, the greatest and most durable therapeutic benefit will likely be achieved with combination regimens that target multiple tumor-specific molecular derangements: ultimately, this should maximize the cytotoxicity of therapeutic regimens selectively towards malignant cells.

On the other hand, this cancer-tailored approach necessarily requires the molecular characterization of each single tumor to be successful, as different types of molecular dysfunctions can affect the metabolic/apoptotic/survival machinery of malignant cells, which may account for the wide range of TNF sensitivity observed in different tumor models. Besides intrinsic features of malignant cells, tumor microenvironment can also have a profound influence on the response to TNF-based therapy and thus should be taken into consideration to optimize this personalized therapeutic approach. For example, in a model of TNF-transfected murine carcinoma, tumor growth is inhibited in the lungs but not in the skin because of the different expression levels of TACE (TNF alpha converting enzyme, the enzyme responsible for the cleavage of membrane-bound TNF into its soluble form) in different normal tissues (106). Yet, the degree of tumor vascularization as well as the levels of nitric oxide produced by tumor endothelial cells have also been demonstrated to condition the sensitivity of malignant cells to TNF therapy (63, 107).

Overall, clinical trials testing the effectiveness of the above reported therapeutic strategies are urgently needed. Some studies are already ongoing (Table 1), and the results are eagerly awaited. Of note, most of these ongoing trials regard the use of sophisticated bioengineered TNF derivatives, whereas no TNF sensitizer is currently under clinical investigation despite the strong preclinical rational supporting their implementation. Since most TNF sensitizers are compounds already approved for the use in humans (e.g. lithium, NSAID, bortezomib, somatostatin, flavopiridol, zeledronate, mithramycin-A), the design and conduction of such trials should be facilitated and oncologists should be encouraged to explore this field.

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Abbreviations: IL12: interleukin-12, NFkB: nuclear factor kappa B, PI3K: phosphatidyl-inositol 3-kinase, PKB: protein kinase B, COX-2: cyclo-oxygenase-2, MMP-9: matrix-metalloproteinase-9, VEGF: vascular endothelial growth factor, IkB: inhibitor of NFkB, IKK: IkB kinase, MAPK: mitogen-activated protein kinase, NOS: nitric oxide synthase, NSAID: non steroidal anti-inflammatory drugs

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