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TNF and manipulation of the tumor cell – stromal interface: "ways to make chemotherapy effective"

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1. ABSTRACT

Growth of solid tumors depends largely on the development of a functional vasculature, which has been the focus in anti-tumor therapy since Folkman in 1971 proposed that prohibiting the formation of new vessels could inhibit tumor growth. The recognition of the tumor vascular bed as an important target led to the development of 3 vascular-targeted strategies. I) The anti-angiogenesis strategy that prevents the formation of new blood vessels and normalizes the remaining vessels. II) Applying vasculo-destructive agents to induce apoptosis in the endothelium of the tumor-associated vasculature that results in vascular collapse and tumor necrosis. III) Promoting further abnormalization of the already abnormal features of the tumor-associated vasculature with vaso-active agents to enhance vessel permeability. Tumor necrosis factor alpha (TNF) is a very promising vaso-active agent because of its anti-tumor effects but its severe systemic toxicity is a major drawback. Therefore a new setting, in which the optimal therapeutic benefit of TNF could be exploited, needed to be found. Through an isolated perfusion high dose of TNF can be administered in the blood circulation of the tumor-bearing extremity or organ. Alternatively, systemically low doses can be safely administered for several times. Importantly, TNF has no anti-tumor effect by itself and the combination with a conventional chemotherapeutic drug that targets the tumor cell is a prerequisite for a good tumor response. In this dual approach, TNF enhances intratumoral accumulation of the chemotherapeutic drug resulting in an impressive tumor response.

2. INTRODUCTION

At the end of the 19th century Dr. William Coley, a surgeon from New York, observed spontaneous tumor regression in patients suffering from a bacterial infection. Later, he developed a vaccine from two dead bacteria stains Streptococcus pyogenes and Serratia marcescens, the so-called Coley's Toxin, to successfully treat sarcoma patients. This toxin was also used for carcinomas, melanomas, lymphomas and myelomas and inducing a fever seemed the essential requirement for a good tumor response (1). It was not until 1975 that Dr. Carswell isolated tumor necrosis factor (TNF) in serum form mice treated with bacterial endotoxin and found it to induce identical haemorrhagic necrosis of methylcholanthrene A fibrosarcoma (Meth A) tumors as the endotoxin itself (2). With the development of new recombinant DNA techniques, human TNF became available for pre-clinical and clinical application (3, 4). However, TNF appeared to exert cytotoxic activity only towards some animal and human tumor cell lines (5) while the majority of malignant cells and most normal cells, including fibroblasts and endothelial cells are relative insensitive to TNF *in vitro* (6-9). Also, systemic and intramuscular TNF therapy proved less successful than originally anticipated with ineffective tumor response and severe toxic effects including fever, fatigue, hypotension, shock and cachexia (10, 11).

The development of chemotherapeutic agents, that would kill cancer cells, has long been the focus for the treatment of solid tumors. Agents may seem promising in a culture disk but when administered as a single agent in

patients the results are often disappointing. Insufficient delivery at the target site, the tumor cell, results often in failure of the therapy and development of alternative strategies to increase chemotherapeutic agents at the tumor site proved to be a major success in treatment of solid tumors. TNF induces a better delivery of these agents at the tumor site by manipulating the tumor-associated vasculature. The models used and the mechanism behind this effect will be further discussed.

3. THE FORMATION OF NEW VESSELS

3.1. Vessel development

Angiogenesis, the process in which new blood vessels are formed form pre-existing blood vessels, is required for further growth and development of most tissues. Once the vasculature has been established, it remains mostly quiescent except in the ovary, uterus and the placenta during pregnancy. All other activation of the endothelium is found in processes during wound healing and inflammation and several other pathological conditions, like cancer, artherosclerosis, diabetic retinopathy and arthritis. There is a delicate balance between pro-angiogenic and anti-angiogenic molecules and in many pathological conditions the balance changes in favor of angiogenesis, the so-called angiogenic switch (12). Vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) are among the initial factors involved in the angiogenic vessel growth. Mature blood vessels consist of endothelial cells (ECs), that form the luminal lining of the vessel wall, and perivascular cells (pericytes in capillaries and smooth muscle cells in larger vessels), which remain in close association with the ECs. These perivascular cells are responsible for vasoconstriction and dilation and prevent rupture due to blood flow pressure (13). Blood vessels are embedded in the extracellular matrix (ECM), existing of several proteins including fibronectin, vitronectine, collagen and laminin (14).

One of the initial steps in the formation of a new blood vessel is weakening of contact between neighboring ECs, between ECs and underlying basal membrane (BM), detachment of pericytes and production of proteases that degrade the components of the ECM. The molecular mechanism involved in capillary sprouting has been extensively studied (15, 16). Pericytes have been shown to restrict the proliferation and migration of endothelial cells so loss of pericytes from the vessels is required in the initiation of endothelial sprouting (17-20). In the presence of VEGF, angiopoietin-2 (Ang2) binds to the Tie receptor tyrosine kinase, Tie-2, which is upregulated in angiogenic vessels and results in the loss of interaction between endothelial cells, pericytes and ECM (21). Next, for an EC to migrate and subsequently form a new blood vessel, the BM and the ECM have to be demodulated. Additionally, the ECM is a reservoir of several growth factors like VEGF, bFGF and transforming growth factor-1 (TGF-1), which are released during angiogenesis contributing to further migration, proliferation and survival of the cells. The rearrangement of the ECM is facilitated by matrix metalloproteinases (MMPs), zinc-dependent endopeptidases capable of degrading and reorganizing the matrix proteins to suit better migration (22, 23).

The leading EC in the angiogenic sprout is a non-proliferating cell with long protrusions scanning the microenvironment and migrating towards the angiogenic stimulus. Second, the VEGF-A gradient also stimulates proliferation of the stalk cells forming a bipolar (basal-luminal) cord (24). After an endothelial sprout is formed, perivascular cells are needed to stabilize the tubular structure. Platelet-derived growth factor B (PDGFB) is secreted by endothelial cells in the presence of VEGF and signals though the PDGF-β receptor, which is expressed on perivascular cells. Also, angiopoetin-1 (Ang1) is produced by perivascular cells and binds to the Tie-2 receptor at the EC membrane. The Ang1/Tie-2 association promotes interaction between ECs and perivascular cells and is therefore important in stabilization of newly formed vessels (25, 26). Maturation of the sprout is initiated by transforming growth factor-ß (TGF-ß), secreted by ECs and perivascular cells. In a dose-dependent manner, TGF-ß can promote proliferation in ECs but also maintains EC quiescence to induce maturation of the vessel by stimulating the migration and recruitment of perivascular cells to the endothelial tube (19, 27). Furthermore, TGF-ß stimulates synthesis and deposition of ECM proteins and prevents their degradation by inducing plasminogen activator inhibitor 1 in ECs (28, 29).

3.2. The Tumor-associated vasculature

The presence of blood vessels is essential for growth and survival of a tumor. Tumors start as avascular masses that depend on diffusion of oxygen from pre-existing nearby vessels. When tumor cells starts to proliferate further growth will rely on the formation of blood vessels. In areas localized beyond the diffusion distance of 200 μm hypoxia will arise and will stimulate tumor cells to produce several pro-angiogenic factors, like VEGF-A and bFGF shifting the angiogenic balance in favor of angiogenesis (30). In contrast to the structurally precise organization of the vascular bed of the basic organs, the tumor-associated vasculature (TAV) is “abnormal”. They display a lack of hierarchical branching organization in which the recognizable features of arterioles, capillaries and venules is lost. They are tortuous and unevenly dilated. As a result, tumor blood flow is chaotic, might be stationary and can even change direction. This leads to hypoxia and acidosis in solid tumors (31).

Tumor ECs are disorganized with irregular shape, sometime even overlapping each other or displaying fenestrae (32). These intracellular openings make the vessel highly permeable and allow passage of molecules across the vasculature. Dvorak *et al.* demonstrated that the leaky vessels are predominantly mature veins and venules lined by a continuous endothelium and that immature interface vessels and tumor penetrating vessels do not leak macromolecules (33). This leakage subsequently results in an increased interstitial pressure that is maintained by the absence of functional lymphatics. Tumor-associated pericytes also display phenotypic differences not found in normal conditions. They are loosely associated with the

ECs, display long extensions into the tumor stroma and are irregularly scattered (34). Also, ECs and pericytes are loosely associated with the basal membrane, which is irregular in thickness, matrix composition, assembly and structure (35). The TAV varies greatly among tumor types. Eberhard *et al.* quantified maturation in glioblastoma and 5 different carcinoma's and found that microvessel pericyte coverage ranges form 10 to 20 % in glioblastoma and renal cell carcinoma to approximately 65 % in mammary and colon carcinomas (36). Therefore, it seems logically that tumors that are less dependable on their vessel structure are less likely to react to tumor vessel therapy. In the K1735 murine melanoma tumors angiogenic sprouts lacking pericytes are evolving into functional endothelial tubes. Coverage of pericytes comes later and these vessels are larger in size and lack proliferating ECs (37). In contradiction, in Lewis lung carcinoma and MCa-IV pericytes are present in practically all vessels undependably of size. Moreover, the endothelial sprouts were closely associated with pericytes and these even extended the endothelial tip cell (34).

4. TREATMENT OF SOLID TUMORS BY USING THE TUMOR-ASSOCIATED VASCULATURE

In 1971 Folkman proposed the hypothesis that prohibiting the development of newly formed tumor blood vessels would be an attractive new approach in cancer treatment (38). Starving the tumor to death through withdrawal of the inflow of nutrients using vascular targeting agents was in theory a strait forward approach. However, putting the theory in practice proved not so simple, but this idea led to several strategies focusing on the TAV. First, the anti-angiogenesis strategy interferes with the formation of new tumor blood vessel and deprives the tumor of oxygen and nutrients required for tumor development. Several growth factors are important in this process and VEGF is believed to be the most important. VEGF is an important mitogen for vascular endothelial cells, mediates secretion and activation of MMPs and increases vascular permeability (39-42). All these actions promote tumor vessel formation and it is not surprising that development of VEGF antagonists became the major focus in anti-angiogenic therapy. Besides preventing the development of new blood vessels, the anti-angiogenic therapy also normalizes the existing TAV. These normalized tumor vessels are believed to be more susceptible to conventional chemotherapy (43, 44).

A second approach is the direct damage of the established tumor vasculature by vascular disrupting agents (VDAs) that will initiate vascular collapse, shutdown of tumor blood flow depriving the tumor of oxygen and nutrients leading to tumor necrosis (45). VDAs exploit the difference in tumor ECs, because these cells have a the higher proliferating status and dependence on a tubulin cytoskeleton to maintain their shape then ECs in normal vessel (46, 47).

A third approach consists of further enhancing the abnormal features of the TAV to improve a more homogenous drug delivery. A chemotherapeutic agent may show promising anti-tumor effects *in vitro*, but *in vivo* the anti-tumor effect will be limited if insufficient amounts of the agents reach its target, the tumor cell. This inadequate drug delivery has always been a major problem in the treatment of solid tumors. After systemic injection the drug dilutes massively in the blood stream and is rapidly cleared by liver and kidney. Because of the severe side effects of most chemotherapeutic drugs simply increasing the injected dose is not an option. When reaching the tumor site, a homogeneous drug distribution is difficult to accomplice due to the irregular blood flow and inhomogeneous perfusion of tumors. Also, the drug has to leave the blood circulation across the endothelial lining into the tumor interstitial space. Although ECs in tumor vessels display fenestrae and mature veins appear to be permeable for macromolecules with a cut off around 400 nm (32, 33, 48, 49), extravasation of drug is often limited to the rim of the tumor. The dense tumor microenvironment, rich in a variety of matrix components (i.e. collagen, laminins, trombospondin, fibronectin, hyaluronate) and multiple types of stromal cells (fibroblasts, myofibroblasts, inflammatory cells) accompanied with a high interstitial fluid pressure, provide a solid barrier. Therefore, a dual approach is required combining an agent with vaso-active properties with a conventional chemotherapeutic agent that target the tumor cell.

5. MANIPULATING THE TUMOR-ASSOCIATED VASCULATURE

5.1. The isolated limb perfusion

The technique of the isolated limb perfusion (ILP) was first described in 1958 by Creech *et al.* and was used for the treatment of a patient with multiple in transit melanoma who refused amputation. With this technique, isolation of the blood circulation is achieved by clamping the major vein and artery, ligating the collateral vessels and application of a tourniquet around the basis of the limb to compress the remaining minor vessels. The main artery and vein is cannulated and connected to an oxygenated extracorporeal circuit. After isolation, chemotherapeutic agents can be injected into the circuit and regional concentrations 15 to 25 times higher can be reached compared to systemic administration (50). After the procedure a washout is performed to ensure minimal systemic exposure to the drug. Also, ILP can be performed with mild hyperthermia (38.5 – 40°C) that improves local drug uptake whereas true hyperthermia (>41°C) is associated with increased toxicity. The main advantage of this method is that locally very high concentrations of cytotoxic drugs can be accomplished with minimal systemic leakage and side effects. To use the anti-tumor properties of TNF, while limiting the side effects, Lejeune introduced TNF in the ILP (51, 52). The combination of TNF with the chemotherapeutic drug melphalan is now primarily used for the treatment of soft tissue sarcoma (STS) and melanoma in transit metastasis (IT-mets) of the extremities. STS are fast growing tumors and IT-mets exist of several large ulcerating lesions providing much painful discomfort for the patients and amputation was often the only option available. However, with the development of the ILP limb salvage and tumor control has replaced

amputation in managing STS and IT-mets (53-55). The excellent response rates and limb salvage of the TNF-based ILP with melphalan led to the approval of TNF by the European Medicine Evaluation Agency (56, 57). Angiograms taken from patients before perfusion show a well-developed tumor vasculature, which is selectively destroyed after TNF-based ILP while leaving the normal vessels intact (58-60).

For pre-clinical studies a rat ILP is developed that resembles the clinical setting to further investigate the role of TNF in this model (61). Two different sarcomas are used: the rapidly growing and metastasizing high grade soft-tissue sarcoma BN175 and the rapidly growing and metastasizing intermediated grade osteosarcoma ROS-1. A perfusion of BN175-bearing rats with TNF alone has no effect on the tumor growth and treatment with only melphalan resulted in a stable disease. However, complete remission in 75% of the animals is found with the combination of TNF and melphalan (62). Also, in the ROS-1 tumor, the combination TNF and melphalan results in an significant increased tumor response (63). *In vitro*, TNF has no direct cytotoxic effects in these cell lines and, more importantly, no synergistic effects between TNF and melphalan could be observed (63, 64). These observations indicate that the activity of TNF is most likely not directed towards the tumor cells but to the stromal compartment of the tumor. Histopathology of the tumors revealed that TNF induces edema, extravasation of erythrocytes and haemorrhagic necrosis suggestive of radical alteration in permeability and integrity of the tumor vasculature (65). Indeed, directly after perfusion with TNF plus melphalan, taking 30 minutes, vascular destruction was detected and erythrocyte extravasated into the surrounding tumor tissue from these damaged vessels (66). This endothelial damage, however, is not directly responsible for the tumor response since these effects are also observed with TNF alone and the combination with melphalan is necessary to obtain an efficient tumor response. Further studies revealed that immediately after TNF-based perfusion a six-fold increase of intratumoral melphalan concentration has been demonstrated compared to a perfusion with melphalan alone. This event is specific for the tumor environment as no increase was found in the skin and muscle (66, 67). Bauer *et al.* performed ILP on nude rats bearing a human melanoma xenograph and also reported high response rates with TNF and melphalan. Although they found no TNF-induced increase in intratumoral melphalan concentration, they observed extensive erythrostasis in the tumor vasculature (68).

After the success with the TNF-based ILP other chemotherapeutic drugs, like actinomycin D and doxorubicin were investigated. Actinomycin D is an anticancer antibiotic that has been used in patients with osteogenic sarcoma in combination with bleomycin and cyclophosphamide but this treatment is associated with severe nausea and anorexia (69). Martijn *et al.* showed that melphalan-based ILP with actinomycin D in patients with IT-mets offer no improved response rates compared to melphalan alone (70). However, actinomycin D increase the TNF sensitivity of tumor cells *in vitro* (71) and local administration of TNF in combination with actinomycin D in mice delays the growth of several tumors (72, 73). In the rat ILP model synergy between TNF and actinomycin D is observed but is accompanied with severe idiosyncratic locoregional toxicity to the normal tissue and therefore abandoned for further studies in this setting (74). However, the TNF-based ILP with doxorubicin is more promising. Doxorubicin is the agent of choice for the treatment of sarcoma but is also accompanied with rigorous toxicity (75-77) and could therefore be a suitable agent for ILP. TNF-based ILP with doxorubicin results in an impressive tumor response compared to ILP with doxorubicin alone in both tumor models (54 % versus 0% for the BN175 and 100% versus 0% in the ROS-1) without any side effects. In accordance with the melphalan treatment this tumor effect was also accompanied with enhanced intratumoral doxorubicin concentrations (78).

5.2. Systemic liposomal treatment

Another strategy to increase the amount of chemotherapy at the site of the tumor is the systemic use of liposomal-encapsulated drug. Liposomes are vesicular structures that exist of one or more phospholipid bilayers and have been developed to improve drug delivery at the tumor site and decrease toxicity normally associated with the conventional drug. However, development of liposomal chemotherapeutic agents has been hindered primarily by their rapid uptake by the mononuclear phagocyte system. Coating the liposomes with polyethylene glycol (PEG) increases the hydrophilic properties of the liposomal surface, thereby avoiding phagocytosis and prolonging blood circulation with a reserved or even improved tumor response (79-83). As a result several of these sterically stabilized liposomes are developed for clinical use and two pegylated liposomal antracyclines are commercially available: pegylated liposomal doxorubicin (Doxil in the US, Caelyx in Europe) and liposomal daunorubicin (DaunoXome). Doxil, encapsulated doxorubicin in small (100 nm) unilamellar liposomes, shows a decrease in toxic effect, as alopecia, nausea, vomiting and cardiotoxicity, associated with the use of free doxorubicin (84-86). Systemic treatment with low dose TNF in combination with Doxil of BN175-bearing rats and B16BL6 or Meth A-bearing mice results in improved tumor response compared to treatment with Doxil alone (87-89). In agreement with the ILP, TNF-induced enhanced accumulation of the liposomes in the tumor appears to be crucial for the observed response. Also *in vitro*, no direct cytotoxic effect of TNF, or synergism with Doxil was observed indicating a TNF host-mediated effect.

The use of intravital microscopy allows a better insight in the intratumoral location of the liposomes and enables longitudinal studies on the kinetics of intratumoral events. We used this technique to understand the effect of low dose TNF on intratumoral fate of liposomes. Mice, implanted with the B16BL6 melanoma tumor in a dorsal skin-fold chamber were injected i.v. with liposomes together with a low well-tolerated dose of TNF. Liposomes, without the addition of TNF, remain predominantly in the vessels and hardly any accumulation in the tumor tissue could be observed. In contradiction, co-administration of

TNF and liposomes resulted in abundant extravasation of liposomes from the blood stream into the surrounding tissue. Even 24 hours later, a fluorescent marker extravasated at the same spot indicating that these tumor vessels remain leaky and functional. Additionally, we observed no effect of this low dose TNF on several microvessel parameters, like branching, density and diameter, indicating that the observed effect is not the result of vascular destruction but rather results from further enhancement of the already leaky properties if the tumor vasculature to allow passage of molecules with a certain size (90). Apparently to achieve vascular destruction higher levels of TNF, like those administered in the ILP, are needed. Further investigation into the exact mechanism of this TNF effect is ongoing.

Also, encapsulating TNF into long circulating liposomes reduce the TNF associated side effects, shows similar biological activity and results in an increased localization of the cytokine in the tumor (91-93). Systemic injection with long circulating liposomal TNF in combination with Doxil resulted in an improved tumor response in soft tissue sarcoma bearing-rats (94) and enhanced the effects of radiation against human colon cancer xenographs due to increased lymphocyte infiltration (96,96).

5.3. Sensitizers for TNF

Intravenous administration of a low dose TNF (3 – 5 µg) in Meth A bearing mice results in thrombosis, increased vessel permeability, leukostasis, and haemorrhage in the tumor vessels leading to necrosis and regression of the tumor. *In vitro*, Meth A cells are relative insensitive to TNF, further indicating a host-mediated effect (2). From the supernatant of Meth A culture medium several factors were isolated that modulate the endothelial properties; vascular permeability factor, nowadays known as VEGF, and endothelial monocyte activating polypeptides I and II (EMAP) (97-99). EMAP has the ability to induce tissue factor procoagulant activity in ECs and it was speculated that over-expression of EMAP-II might predispose the tumor vasculature to the procoagulant effects of TNF and increase sensitivity to the cytokine. B16BL6 melanoma and HT-1080 human fibrosarcoma-bearing mice underwent hemorrhage after intratumoral EMAP-II administration followed by systemic injection of TNF (100). Also upregulation of EMAP-II using retroviral gene transfer shows that initially TNF-resistant tumors become sensitive to systemic TNF therapy (101) and therefore EMAP-II could be of additional benefit in the ILP. To investigate the role of EMAP-II in the ILP retroviral gene transfer was used to generate EMAP-II expressing BN175 and secondly wild-type tumor-bearing rats were pre-treated intravenously with recombinant EMAP-II. The results form these studies showed that EMAP II renders the otherwise resistant tumor responsive to TNF (102, 103).

Tumor biopsies taken from patients with IT-mets revealed that the upregulation of EMAP-II strongly correlates with complete response. No correlation was found in the STS patients due to an overall low expression of proEMAP and EMAP-II in this type of tumor (104). Therefore EMAP-II could potentially be a prognostic factor for TNF-treatment of patients with IT-mets. *In vitro* experiments show that EMAP-II facilitates TNFR1 apoptotic signaling in the ECs (105, 106). However, little is known about the TNFR1 expression profile within tumor tissue before and after ILP but co-staining for ECs and TNFR1 shows that TNFR1 is mainly expressed by cells closely associated with ECs but not by ECs themselves, in contradiction to EMAP-II that co-localizes with ECs (104). This is very surprising and suggests that another cell type beside the ECs in the tumor vasculature is responsible for the TNF-induced anti-tumor effects. This might be a first indication that ECs are indirectly involved in the TNF-induced effect and could explain why ECs *in vitro* are resistant to TNF alone and need other cytokines like interferon-gamma (IFN), EMAP-II, interleukine-1beta (IL-1ß) or blood cells to induce changes in macromolecule permeability, morphological changes and apoptosis (6, 107). Apparently, the underlying mechanism of TNF-based therapy is more complicated then a simple binding of TNF to TNFR1 expressing ECs and is currently under investigation.

5.4. Transformation of TNF

Besides local treatment or drug encapsulation to reduce the toxicity or improve delivery, the chemical structure of the compound can be altered. Corti *et al.* coupled TNF with a cyclic CNGRC peptide, a CD13 ligand, to better target the tumor vasculature (NGR-TNF). Mice bearing B16F3 melanoma or RMA-T lymphomas were pretreated with NGR-TNF intraperitoneal followed 2 hours later by interperitoneal injection of doxorubicin or melphalan. Although the LD50 values of mTNF and NGR-mTNF are similar, mTNF was inactive at doses lower than 100 to 1,000 ng while NGR-mTNF inflicted anti-tumor effects with doses as low as 0.001 – 0.1 ng without any toxic side effects (108). This low dose is sufficient to improve the anti-tumor effects of doxorubicin or melphalan in lymphoma and B16F1-bearing mice as a result of a better penetration of the chemotherapeutic drug in the tumor tissue (108, 109). Second, coupling TNF with RGD, also induced a delay in tumor growth without toxic effects (110).

Mayumi and colleagues chemically modified TNF with water-soluble polymers like PEG or polyvinylpyrollidone (PVP). This increase of the steric hindrance and protection from proteolytic degradation resulted in an increased drug stability and circulation time. However, the increase in size limits the distribution from blood to target tissue and steric hindrance can inhibit binding to the receptor. Therefore an optimal modification was designed to find the balance between clearance, toxicity and anti-tumoral activity (111-113), which proved to be effective in treatment of S-180 tumor-bearing mice (114). Shibata *et al.* designed a pegylated lysine-deficient mutant TNF (sp-PEG-mTNF-K90R) with higher affinity for both TNF-receptors with an anti-tumor activity 60-fold higher than native TNF in the Meth A mouse model (115). Also binding of TNF at the surface of gold nanoparticles (cAu-TNF) improved the safety of TNF while retaining the

anti-tumor efficacy by selectively altering the permeability of the tumor vasculature (116).

Another mutant, TNF-SAM2, with increased N-terminal basicity has a higher biological activity and milder toxicity than conventional TNF (117, 118) and has similar anti-tumor activity in the melphalan- or doxorubicin-based ILP in BN175-rats (119). It is being tested in the clinical ILP because of its potential decreased toxicity and is potentially applicable in regional perfusions that are not leakage free.

5.5. Alternatives for TNF

As mentioned, TNF targets the tumor-associated vasculature and improves intratumoral drug delivery. So agents with identical vaso-active properties could potentially be an alternative for TNF-based therapy. Histamine is found to be a excellent candidate. It is an inflammatory modulator that is predominantly formed and stored in the granules of mast cells and basophils and causes edema by promoting gaps between ECs and in so doing increases the permeability in venules (120-122). Replacing TNF with histamine in the ILP in combination with either melphalan or doxorubicin results in a tumor response comparable to TNF-based ILP. Histopathology of the tumor showed vasodilatation of the tumor vasculature, damage to the ECs and extravasation of erythrocytes into the tumor interstitium. As a consequence edema and massive hemorrhage occurred. Similar to TNF-based ILP, the major anti-tumor effect could be explained by an indirect effect through a histamine-mediated accumulation of chemotherapeutic drug in the tumor tissue (64, 123). Although, histamine is only slight cytotoxic to ECs *in vitro*, within 15 minutes after histamine exposure gap-formation between the cells resulted in increased permeability (64). These histamine-induced changes are also found by others and are believed to act through changes in VE-cadherin expression (124-126).

Second, interleukine-2 (IL-2) is a cytokine produced by activated T-cells to maintain their growth and cytotoxic response (127) and has been widely used in the treatment of solid tumors (128-130). A high dose of IL-2 causes serious side effects, like hypotension and vascular leakage syndrome causing intravascular liquid entering the organ interstitial space (131). Because of its ability to induce vascular permeability this cytokine was speculated to be a good candidate for ILP. Indeed, we found a synergistic anti-tumor response in BN175-bearing rats after perfusion with IL-2 and melphalan correlating with an increased melphalan concentration in the tumor. However, in contradiction to TNF and histamine no apparent vascular damage is seen after ILP although scattered erythrocytes are observed between the tumor cells indicating an increased vascular leakage. Interestingly, no increased extravasation of macrophages was found in the tumor tissue after ILP but there was a clear difference in distribution of these cells. In control and melphalan-treated animals an even distribution of macrophages throughout the tumor tissue was observed, while a clear clustering of these cells was found in the IL-2 plus melphalan group (132).

As histamine and IL-2 are tested in combination for the treatment of melanoma (133), we further investigated the triple combination histamine, IL-2 and melphalan. Histamine or IL-2-based melphalan perfusion resulted in an overall response of respectively 66% and 67%. Surprisingly, histamine and IL-2 together with melphalan led to on overall response of only 29%. Immunohistology revealed that the histamine-induced haemorrhage and vascular destruction was abolished by the addition of IL-2. Because of these poor results investigation on the triple combination therapy was discontinued (134).

Interaction and adhesion of sprouting endothelial cells with the ECM is mediated by endothelial transmembrane receptors or integrins like αVß3 and αVß5. Quiescent cells shown no luminal expression of these receptors while enhanced exposure of these integrins is found on tumor ECs making these integrins an potential target for anti-tumor therapy (135, 136). In preventing interaction between the integrins αVβ3 and αVβ5 and their ECM ligands apoptosis of ECs take place. Cilengitide (EMD 121974) is a cyclic RGD containing peptide with high affinity for αV-integrins. Intraperitoneal injection with cilengitide resulted in tumor growth arrest of glioblastoma, a highly vascularized invasive tumor, in mice (137) and improved, in combination with radioimmunotherapy, the treatment of breast cancer xenographs (138). The potential contribution of Cilengitide in the melphalan-based ILP to improve solid tumor response is currently under investigation in our laboratory.

6. GENERAL CONCLUSION

Although anti-angiogenic or vascular disrupting strategies are more generally known, manipulation of existing tumor vasculature, strategies to enhance vascular leakage and fluid flow into the tumor, also have been demonstrated to be useful. So, next to vascular normalization, as is shown with for instance anti-VEGF-based therapy, we would like to propose vascular abnormalization as a powerful alternative in solid tumor combination chemotherapy. We and other demonstrated that vaso-active agents like TNF or histamine further enhance these abnormal features in the tumor-associated vessels and as such augments the accumulating of conventional chemotherapy into the tumor tissue, ultimately resulting in an enhanced tumor response.

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Abbreviations: tumor necrosis factor alpha: TNF, methylcholanthrene A fibrosarcoma: Meth A, vascular endothelial growth factor: VEGF, fibroblast growth factor: FGF, endothelial cells: ECs, extracellular matrix: ECM, basal membrane: BM, angiopoietin-2: Ang2, Tie receptor tyrosine kinase: Tie-2, transforming growth factor-1: TGF-1, matrix metalloproteinases: MMPs, platelet-derived growth factor B: PDGFB, angiopoetin-1 Ang1, transforming growth factor-ß: TGF-ß, tumor-associated vasculature: TAV, vascular disrupting agents: VDAs, isolated limb perfusion: ILP, soft tissue sarcoma: STS, melanoma in transit metastasis: IT-mets, polyethylene glycol: PEG, endothelial monocyte activating polypeptides I and II: EMAP-I and II, TNF-receptor 1: TNFR1, interferon-gamma: IFN, interleukine-1beta: IL-1ß, polyvinylpyrollidone: PVP

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