Anti-Tumor Effect of Parasitic Protozoans

Subjects: Parasitology

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The immune system may aberrantly silence when against “altered self”, which consequently may develop into malignancies. With the development of tumor immunology and molecular biology, the deepened understanding of the relationship between parasites and tumors shifts the attitude towards parasitic pathogens from elimination to utilization. The antitumor impact implemented by protozoan parasites and the derived products has been confirmed. The immune system is activated and enhanced by some protozoan parasites, thereby inhibiting tumor growth, angiogenesis, and metastasis in many animal models.

anti-tumor protozoon biotherapy immune response

1. Introduction

Cancer is a growing global disease with no borders. It is regarded as one of the leading causes of mortality in humans, with an estimated 19.3 million new cancer cases and almost 10.0 million cancer deaths occurring in 2020, according to Global Cancer Statistics [1]. The development of efficient therapeutic strategies is important. In addition to traditional cancer treatments including surgery, radiotherapy, and chemotherapy, more recently, cancer biotherapies such as cancer vaccines, monoclonal antibodies, gene therapy drugs, and immunomodulators have been used clinically and studied in clinical trials. By killing cancer cells or inhibiting their growth, the mechanism of biotherapy mainly induces defensive immune responses against tumors in multiple targets and directions, in contrast to traditional treatment [2]. In recent years, some parasites, especially intracellular protozoan parasites, have been considered effectors to induce anti-pathogen and anti-tumor immune responses so as to overcome tumor escape and active tumor surveillance system. The persistent antigen release caused by the chronic infection of protozoan parasites leads to a long-term specific immune response, which may have broader advantages for promoting cancer treatments.

2. Tumor Therapy with the Injection of Parasite

Recently, the use of non-pathogenic live protozoan parasites as anticancer therapeutic approaches has drawn attention worldwide, of note is the long history of antitumor effect by *Trypanosoma cruzi.* At the very beginning in 1946, the work from the former Soviet Union reported that cancer is rare in patients infected with *T. cruzi* once before [3], which opened possibilities for research on cancer biotherapy. Later, researchers developed an anticancer experiment in which *T. cruzi* extracts were directly inoculated in peritumoral areas over different tumors, and all of the results showed a reduction in tumor size [4]. However, the work was hampered by controversial results and the complicated international political situation, and so the molecular basis of this phenomenon has

remained elusive [5]. Moreover, several live-attenuated protozoan parasites including *Leishmania infantum* and *L. tropica* [6], *Neospora caninum* [7], and *Toxoplasma gondii* [8] have all lately been employed as antitumor biological agents via intratumoral injection, all of which decreased tumor development and local inflammation. The injection of live *N. caninum* tachyzoites either in or remotely from the tumor, successfully treated murine thymoma EG7 by strongly activating the natural killer cell- (NK cell-) and CD8-T cell-dependent protective antitumor response associated with interferon (IFN)-γ secretion in the tumor microenvironment, resulting in the lysis of the cancer cell [7]. Researchers from Ege University Medical School first reported that intratumoral administration of attenuated *Leishmania* strains in 4T1 breast cancer-bearing mice promoted M1 dominant activation of macrophages in spleen and tumor tissues with induction of proinflammatory cytokines that helps the generation of protective Th1 responses [6][9]. It is a pity that the survival rate and other antitumor effects were not recorded. Further studies on the molecular basis and the effect on different tumors needs to be carried out to deepen the understanding and application of antitumor biotherapy with *Leishmania* strains.

The parasite *T. gondii*, on the other hand, attracts more attention. The characteristic of *T. gondii* that can infect nearly all types of cells and modify the immune response of the host opens up a wide range of clinical possibilities for *T. gondii* as an oncolytic protozoan in human medicine [10]. Considering that the toxoplasma virulence is cell type-independent, researchers in 1985 performed an intralesional injection with 10^7 formalin-fixed *Toxoplasma* tachyzoites one day after the syngeneic Lewis lung carcinoma cell inoculation and discovered some mice (2/6) completely rejected the growth of the implanted tumor [11]. It is conceivable that live organisms could elicit a much more robust antitumor effect than dead ones. Thus, what followed was gamma radiation-attenuated Toxoplasma [12], and gene-edited-attenuated Toxoplasma such as *ΔCPS*, *ΔOMPDCΔUP*, *ΔGRA17* strains, and *Δldh1-Δldh2* strains used for tumor biotherapy, which all presented the ability to repress the growth of established tumors and to help the inhibition of lethal tumor development in the mice [8][13][14]. Overall, these protozoan parasites are proven to have effective antitumor effects, while the precise targets of tumor cells need further investigation. Referred to as the chimeric antigen receptor (CAR)-T method, whether the combination of protozoan parasites and tumor-specific antigens would promote the target invasion and antitumor effects requires further research data.

3. Antitumor Effect of Parasitic Products

Some parasitic products have been demonstrated to have specific antitumor effects. It is well known that the levels of aberrant chondroitin sulfate proteoglycans (CSPGs), a protein family that displays one or multiple chondroitin sulfate (CS) side chains, are upregulated in many cancers but the variability of this protein within different tumor tissues is striking, due to the great diversity of structure cores assembled by numerous enzymes regulated, based on tissue and cell type [15][16]. The CS side chains or the protein core of CSPGs can bind extracellular matrix components or a growth factor receptor complex to transmit pro-oncogenic signaling through which the proteins are implicated in cancers. The identification and targeting of CS in cancers remained a technical challenge until the specific CS structure, termed oncofetal CS (ofCS) was found to share a high affinity among cancer cells [17]. A refined malaria protein called rVAR2 was discovered to bind with the distinct ofCS from a variety of different cancer cell lines [18], with remarkably high specificity and affinity (KD ~15 nM) [19]. This discovery was further supported by

the observation that rVAR2 intravenously injected, adhered to the tumors in in vivo xenograft animal models [20]. The high efficiency targeted tumor characteristic of rVAR2 makes it a potentially ideal carrier for anti-cancer drug delivery. Indeed, it has been proven that a hemiasterlin analog (KT886) conjugated rVAR2 (VDC886), formed as the complex which carried an average of three toxins per rVAR2 molecule, effectively kill a total of 33 cancer cell lines in vitro with inhibitory concentration 50 (IC50) values ranging from 0.2 pM to 30 nM [18]. Remarkably, the VDC886 treatment significantly slowed the growth of both non-Hodgkin's lymphoma (Karpas299) and prostate cancer (PC-3) in mouse models, indicating a potential role in clinic therapy.

Unlike malaria VAR2, rhoptry and dense granule proteins secreted by *T. gondii* play advantageous roles in regulating the growth of tumors. Two key organelles, the apical rhoptry [21] and dense granule [22] are essential for host invasion and immune escape. Through a reverse genetic approach to target complete gene deletions, particular proteins including rhoptry protein 5 (ROP5), ROP17, ROP18, ROP35, and ROP38, the dense granule protein 2 (GRA2), GRA12, and GRA24 are demonstrated to effectively activate antitumor immune responses involving CD4+ and CD8+ T cells and the interleukin-12 (IL-12)/IFN-γ T_H1 axis, while deletion of GRA3, GRA15, GRA16, ROP16, or ROP21 did not affect the antitumor activity [23]. Coincidentally, the secreted GRA15 was confirmed to localize to the endoplasmic reticulum and to activate innate immune and stimulator of interferon genes (STING) responses by promoting polyubiquitination at Lys-337 and oligomerization in a tumor necrosis factor (TNF) receptor-associated factor (TRAF) protein-dependent manner [24]. In addition, the GRA16 displayed the ability to break the chemoresistance of irinotecan by inhibiting nuclear factor kappa B (NF-κB) via a PP2A-B55/AKT/NF-κB p65 pathway against non-small-cell lung carcinoma (NSCLC) [25]. Recently, the fact that the small intestine (SI) exhibited low tumorigenesis or metastatic growth from distant tumors attracted attention. Concomitantly, a protein named *Eimeria* antigen (EA) that acts as a robust stimulator to promote IL-12 releasing from dendritic cells (DC) and to upregulating inflammatory modulators (Monocyte chemoattractant protein-1 (MCP-1), IL-6, IFN-γ, and TNF-α) for the defending of sarcoma tumor in mice was discovered [26]. Perhaps the vast immunologic compartment induced by EA in SI also provides a potent tumor immunosurveillance. These findings collectively point to a deeper understanding of fundamental mechanisms of host cell pathways manipulated by these secreted proteins, which could provide novel targets for developing effective therapies against aggressive solid tumors.

Another well-known anti-tumor protein is the calreticulin from *T. cruzi* (TcCRT), which is secreted into the extracellular milieu to induce immune modulations [27][28]. TcCRT consists of an N-terminal vasostatin-like domain (aa 20-193) that can directly bind with endothelial cells through a scavenger-like receptor and acts as a potent angiogenesis inhibitor [29][30]. It is well known that angiogenesis-focused therapy is frequently utilized in conjunction with other treatments for a variety of tumor types [31][32]. Most likely, by directly interacting with endothelial cells, the N-terminal vasostatin-like domain inhibits vascular endothelial growth factor (VEGF)-induced cell proliferation and induces cell apoptosis [33]. TcCRT makes use of its anti-tumor properties in this manner.

4. Activating the Cellular Immune System

4.1. Immune Cells

Cells of innate immunity and adaptive immunity, including NK cells, DC, Innate lymphoid cells (ILC), cytokine-induced killer cells (LAK), specific T lymphocytes, etc., play important roles in tumor immunotherapy by controlling tumor growth and killing tumor cells.

Acting as toxic immune cells, activated NK cells managed by a suite of activating, co-stimulatory and inhibitory receptors [34], can directly kill tumor cells, especially those that lack major histocompatibility complex (MHC; also known as human leukocyte antigen (HLA)) class I [35], such as tumor cells from metastatic and blood tumors, and indirectly improve the response of antibodies and T cells [36]. By the stimulatory infection of *Plasmodium*, NK cells can be activated to kill some lung cancer cells which in return can release tumor antigens resulting in activation of the systemic response of tumor antigen-specific T cells in peripheral blood, spleen, and lymph nodes, as the consequence of additional cancer cell death [37]. Possibly, the toxic activation of NK cells via IFN-γ expression is induced by the infection of Plasmodium through the up-regulated expression of CD69 and CD25 [38]. As reported by Chen, et al. [39], three clinical trials based on *Plasmodium* immunotherapy against advanced cancers have been approved and are underway with clinical safety guaranteed. In addition, the infection of *N. caninum* [7], *Leishmania amazonensis* [40], and *T. gondii* [41] increased the levels of NK cells mainly through an IFN-γ-dependent pathway. Moreover, toxoplasma infection can induce the conversion of NK cells into ILC1-like cells which are Eomes-dependent and the changes appear permanent [42], which may help defend against tumors. However, the molecular mechanism of how these protozoans activate NK cells is not well studied. While the role of NK cells in controlling tumor growth is well established, the function of newly discovered ILCs in defending tumors remains poorly understood. Nevertheless, their ability to produce large amounts of cytokines [43][44] indicates that ILCs may contribute to tumor-associated inflammation, and the interaction between protozoan parasites and ILCs may shed light on the development of new antitumor therapies. Genetic modification with superior activation or decreased inhibitory signals in NK cells enhances their tumor cell killing ability [45]. It is conceivable when using the specific parasitic molecular, such as VAR2 form *Plasmodium*, as the chimeric antigen receptor (CAR) in NK cells, the most effective CARs used to redirect T cells also work well for NK cells [46][47].

Undoubtedly, T cells play crucial roles against tumors [48]. Activation of naïve T lymphocytes requires T cell receptor (TCR) signaling, costimulatory signaling, and cytokine support [49][50]. A neoantigen peptide, uniquely encoded by mutated DNA of tumor cells, has distinct epitopes from those of normal cells and will be processed and displayed on the surface of tumor cells and antigen-presenting cells (APC) as the form of neoantigen peptide-major histocompatibility complexes (pMHC) [51][52]. During T cell activation, the expressed membrane proteins CD4 on T helper cells and CD8 on cytotoxic T lymphocytes, bind to MHC class II (MHC-II) or MHC-I molecules, respectively [53]. However, recent evidence revealed that the multifaceted suppressive signals resulted in an exhausted phenotype or dysfunctional state of T cells [54][55]. Hence, effective therapeutic immunity against tumors can be stimulated by reversing tumor-associated immunosuppression. So far, a few studies have indicated that this goal can be addressed using the infection of protozoans, which may reverse T cell suppression and activation by providing exogenous antigens. Mice immunized with live-attenuated *Leishmania* parasites presented a higher percentage of CD4+ and CD8+ T cells, indicating a robust cellular response was generated [56][57], consequently these T cells infiltrated the 4T1 breast cancer resulting in the decrease of tumor volume and prolonged the survival period of mice [6]. Similarly, vaccination with *T. cruzi* significantly inhibited the growth of breast and colon tumors

through the activation of CD4+ and CD8+ T cells and the increase of macrophages and dendritic cells. A diverse cell-mediated (CD4+ and CD8+ T cells) immunity lasted more than 200 days in mice that were triggered by an attenuated *T. gondii* against pancreatic cancer recurrences [58].

Although DC cannot directly kill tumor cells [59], they are the cornerstone of the anti-tumor immune response due to the ability to activate T cells by extracting and transporting specific tumor antigens making them the foundation of the anti-tumor immune response [60]. However, it is usually insufficient for DC maturation due to the suppressive mechanisms within tumors [61], including suppressing the expression of MHC or costimulatory molecules [62]. Some protozoan parasites may help promote the mutation of DCs, and this notion is supported by studies demonstrating that ligand molecules from parasites bind with Toll-like receptors (TLRs) to stimulate DC activation, such as glycosylphosphatidylinositol (GPI) from *Leishmania major* [63], *T. cruzi* [64], *P. falciparum* [65], and *T. gondii* [66], and Profilin-like protein from *T. gondii* [67]. In mice implanted with murine Lewis lung cancer (LLC) cells, malaria parasite infection promotes the maturation of DCs through up-regulating the expression of CD80 and CD86 resulting in the activation of T cells [37]. Recently, Lantier, et al. [7] found that unlike dead parasites or soluble tachyzoites antigens, live *N. caninum* tachyzoites are able to activate murine or human DCs to secret proinflammatory cytokines, which convincingly suggests that the use of live tachyzoites is a necessary condition for immunotherapeutic treatment. In fact, four days after the injection of live tachyzoites, they observed recruitment of DCs, along with a high increase of IFN-γ and IL-12, indicating a measurable and systemic immune response against EG7 thymoma in mice. Coincidentally, Baird, et al. [14] found that $CD11c^+$ DC antigen-presenting cells from the ovarian carcinoma microenvironment invaded by the infection of toxoplasma cps strain, strongly upregulate the costimulatory molecules CD80 and CD86, which regained the ability to cross-present antigen to prime tumor antigen-specific CD8+ T cell responses.

4.2. Cytokines

Cytokines are major regulators of innate and adaptive immunity, and some play critical roles in tumor cells [68]. Among these molecules, IFN-γ can induce tumor cell cycle arrest and establish tumor cell dormancy [69], and IL-12 can promote Th1 antitumor immune response which may be secreted by the stimulation of IFN-γ and in turn triggers the re-activation of the IFN-γ production cycle [70], while IL-10 and IL-13 can inhibit Th1 cells from secreting IFN-γ [71]. The highly expressed IL-6 can promote inflammation and tumor cell immunosuppression [72], and possibly aggravate tumor cell growth and metastasis by IL-6/Signal Transducer And Activator Of Transcription 3 (STAT3) mediated inhibition of DC and lead to the dysfunction of the immune system [73]. Generally speaking, cytokines dominated by IL-6 are beneficial for tumor proliferation and metastasis, while these inflammation factors mediated by IFN-γ have an anti-tumor effect on blocking tumor progression [74]. For instance, exposure to Toxoplasma tachyzoites, in addition to activating immune cells as mentioned above, can induce the antitumor effect in mice models by increasing the expression levels of IFN-γ [8][13][23][41][75], IL-12 [14][23][41][76], and TNF-α [8][13].

5. Activating the Humoral Immunity System

A long-term, effective anti-tumor immune response is essential for the treatment of tumors, and during this period, some tumor-associated antigens (TAA), which are at very low levels in normal cells, will be recognized and prime the humoral immunity [77]. The auto-tolerance prevents the direct recognition against self-antigen, but the infection of protozoan may help induce the anti-tumor humoral immunity followed by the releasing of antibodies which can affect the biology of the tumor by blocking certain receptors on the surface of tumor cells [78]. Infection of toxoplasma ME49 strains significantly increase the levels of IgG1 and IgG2a in LLC-bearing mice [75], and, live *L. tarentolae* carrying E7 protein induced significant levels of IgG2a against HPV-associated tumors [79]. It would be more convincing if the tumor-specific IgG was detected. The similarity between surface antigens of *T. cruzi* and Ehrlich's adenocarcinoma cells was confirmed by the cross-reaction of indirect immunofluorescence [80]. Later, the sera from *T. cruzi* lysate-vaccinated mice significantly reduced the tumor size of Ehrlich's adenocarcinoma. This could imply that the specific immune profile generated by antigens of *T. cruzi* has a positive effect on the growth of a tumor, at least regarding this type of neoplasm.

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