Heparanase: a universal immunotherapeutic target in human cancers

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Heparanase has been identified as a particularly important player in metastasis, and its expression directly correlates with the metastatic spread of various tumors. Ideal targets for immunotherapy are gene products that are silenced in normal tissues but overexpressed in cancer, and that are directly involved in tumor cell survival and progression. Metastasis is the culmination of neoplastic progression. The importance of the role of heparanase in metastasis implies that immune escape by downregulation of heparanase expression could reduce the mortality of the cancer. These characteristics of heparanase make it an attractive universal target for cancer immunotherapy. Here, we review current knowledge about heparanase and its involvement in tumor metastasis, with an emphasis on recent results from heparanase-targeted cancer immunotherapy studies.

Introduction

The malignant tumor is one of the most lethal diseases for humans. Therapy of malignant tumors still relies mainly on surgical exeresis, radiotherapy and chemotherapy. Although surgical exeresis is a preferred choice for early-stage solid tumors, the processes of invasion and metastasis that often occur in advanced-stage solid tumors and hematologic malignancies make surgical therapy for these tumors unsuitable. In addition, treating such tumors with radiotherapy and chemotherapy is undesirable, owing to their side effects. During the past few years, immunotherapy has emerged as a new modality for cancer treatment because of its weak side effects and applicability; this therapy harnesses the immune system to recognize and specifically eradicate tumor cells [1].

One of the most well-studied cancer immunotherapy approaches is the use of dendritic cells (DCs) loaded with tumor-associated antigens (TAA) to induce antigen-specific antitumor immunity. Particularly important in DC-based immunotherapy for cancer is the selection of suitable TAA [2]. Currently, dozens of TAA have been described [3]. Although the expression of most TAA is restricted to a few tumor types and to a fraction of patients with these types of tumor, the appearance of antigen-loss mutations in tumor cells in response to immune pressure is well described [4,5]. To circumvent this issue, a class of TAA termed 'universal tumor antigens' has been proposed that is hypothesized not only to induce antitumor immunity against a broad range of tumor types, but also to have crucial functional roles in tumor growth and development.

Heparanase is the only endogenous endoglycosidase found, to date, that can degrade the heparan sulfate proteoglycans (HSPGs) in the extracellular matrix (ECM) and basement membrane (BM) [6]. Unlike most other TAA, heparanase is found in most malignant tumors, and its expression has been linked to tumor metastasis and angiogenesis [7–9]. With the exception of lymphoid organs, placenta and platelets, it is either not expressed or expressed at very low levels in normal tissue [10–12]. Activation of heparanase is a determining factor in metastasis that enables tumor cells to break through the ECM and BE barriers, release multiple types of cytokine and cause the formation of new vessels and local permanent planting. Some tumor cells can downregulate TAA expression to evade immune surveillance [13]. However, because of the crucial role of heparanase in tumor progression, downregulation of its expression as a means of immune escape might itself have deleterious effects on the proliferation and

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metastasis of tumor cells. T-regulatory cells usually accumulate at the tumor site, where they suppress the function of effector T cells, resulting in tumor growth despite the presence of TAA-specific cytotoxic T lymphocytes (CTLs) [14]. Interestingly, T-regulatory cells against heparanase were not found in patients with (colorectal) cancer [15]. Heparanase-targeted immunotherapy is thus expected to be prolonged and more efficient owing to the absence of T-suppressor cells. Based on the above features, heparanase is therefore considered to be an ideal universal immunotherapeutic target in human cancers.

Structural and functional features of heparanase

The human heparanase gene (*HPR1*) maps to chromosome 4 at band 4q21.3 and is linked to the genetic marker D4S400 [16]. *HPR1* is expressed as 5-kb and 1.7-kb mRNA species, generated by alternative splicing. In the human immune system, including the spleen and peripheral blood leukocytes, the 5-kb form of the mRNA, HPSE 1a, is the major transcript of *HPR1*, whereas the 1.7-kb form of the mRNA, HPSE 1b, is the major transcript in human placenta and platelets [11,12,16]. The two mRNA transcripts for heparanase have the same open reading frame and encode the same polypeptide of 543 amino acids with a molecular weight of 61.2 kDa [16]. The mature, active 50-kDa enzyme, isolated from placenta, platelets and various cell lines, was found to lack the N-terminal 156 amino acids from the initiation codon, suggesting post-translational processing of the heparanase polypeptide [10]. In fact, heparanase was subsequently found to be a heterodimer consisting of a 50-kDa C-terminal subunit (Lys^{158}-Ile^{543}) associated non-covalently with an 8-kDa N-terminal subunit (Gln^{36}-Glu^{109}), with an intervening 6-kDa peptide (Ser^{110}-Gln^{157}) excised by proteolysis [17–19]. The sequence also contains a 35-amino acid N-terminal signal sequence (Met^{1}-Ala^{35}) and might have a C-terminal transmembrane domain (Pro^{515}-Ile^{534}), which is consistent with the observation that a fraction of heparanase activity is membrane bound and can only be solubilized by detergents [11,20]. Recently, several splice variants of heparanase and a heparanase homolog, termed heparanase-2, were identified, but their biological significance and function remains to be determined [21–23].

HSPGs have a key role in the self-assembly, insolubility and barrier properties of the ECM and BM. Therefore, cleavage of heparin–heparan sulfate (HS) affects the integrity and functional state of tissues and, therefore, fundamental normal and pathological phenomena involving cell migration and responses to changes in the extracellular microenvironment [6,7]. Given that heparanase is the only endogenous endoglycosidase identified that can degrade HS, it is now generally understood that heparanase is involved in many biological and pathological processes [8]. Activated immune cells, including T cells and macrophages, express active heparanase, which upon liberation facilitates diapedesis by cleavage of HS in the ECM and BM. Heparanase is also essential for embryo implantation, where its invasive properties, similar to properties of metastatic tumor cells, enable attachment of the pre-implantation embryo to the maternal uterine epithelia [24,25]. Platelets are also a rich source of heparanase, and their aggregation with tumor cells is believed to facilitate tumor cell metastases and ECM disassembly following platelet degranulation [9,26].

As well as its crucial role in cell migration, heparanase can liberate HS-binding growth factors from ECM depots and make them available for growth factor-dependent processes, such as angiogenesis and wound healing [18,27]. Moreover, independent of its enzymatic activity, heparanase stimulates phosphatidylinositol 3-kinase (PI3K)- and p38-dependent endothelial cell migration and invasion through enhancing Akt signaling [28]. It also promotes vascular endothelial growth factor (VEGF) expression via the Src pathway [29]. These effects appear to be mediated by as yet unidentified heparanase receptors.

Identification of heparanase as a potential target for advanced tumor therapy

Involvement of heparanase in tumor metastasis

It is well known that both the mRNA and protein levels of heparanase are increased in most of the human tumors examined [30]. Given that heparanase produced by tumor cells can mediate the degradation of HS in the ECM and BM, which act as barriers to tumor cell invasion and spread (Fig. 1), it can be hypothesized that heparanase is a key enzyme involved in the metastasis of malignant tumors. Indeed, it was demonstrated many years ago that the metastatic potential of tumor cells is correlated with heparanase expression. Evidence for a direct role of heparanase in tumor metastasis was provided by the conversion of tumor cells, such as T-lymphoma and melanoma cells, from a non-metastatic to metastatic phenotype following stable transfection and overexpression of *HPR1* [11]. Additional evidence for heparanase involvement in tumor metastasis comes from *in vivo* studies of heparanase inhibitors, in which dramatic reductions in the incidence of tumor metastasis have been reported in several tumor models of animals treated with these inhibitors [19,31–33].

Intravital video microscopy for direct *in vivo* observation of the early steps in metastasis has improved the understanding of the involvement of heparanase in tumor metastasis. These studies proposed for the first time that extravasation might not be the rate-limiting process, and that metastatic inefficiency depends, to a large extent, on the inhibition of growth of a subset of extravasated cells [34,35]. Heparanase enables the release and activation of HS-binding factors such as fibroblast growth factors (FGFs) and VEGF from HSPGs, which can facilitate endothelial cell migration and sprouting toward the angiogenic stimulus. As such, heparanase is thought to be crucial for the proper seeding and proliferation of the disseminated cells by creating a more favorable tumor-supportive microenvironment (Fig. 1) [36–38].

Heparanase as a potential target for cancer drug development

Given that overexpression of heparanase has been observed in most human tumors and heparanase enhances tumor metastasis and angiogenesis by degrading HS and releasing HS-binding factors, heparanase could be a suitable therapeutic target for advanced cancers and/or for the prevention of metastasis. In fact, attempts to inhibit heparanase enzymatic activity were initiated soon after research on heparanase began, at the same time that the clinical relevance of heparanase activity was being realized. Recently, with the availability of recombinant heparanase and the establishment of high-throughput screening methods, several heparanase inhibitors have been discovered. These include heparin and low-molecular-weight heparin, polysulfated polysaccharides, neutralizing antibodies and others [9,31].

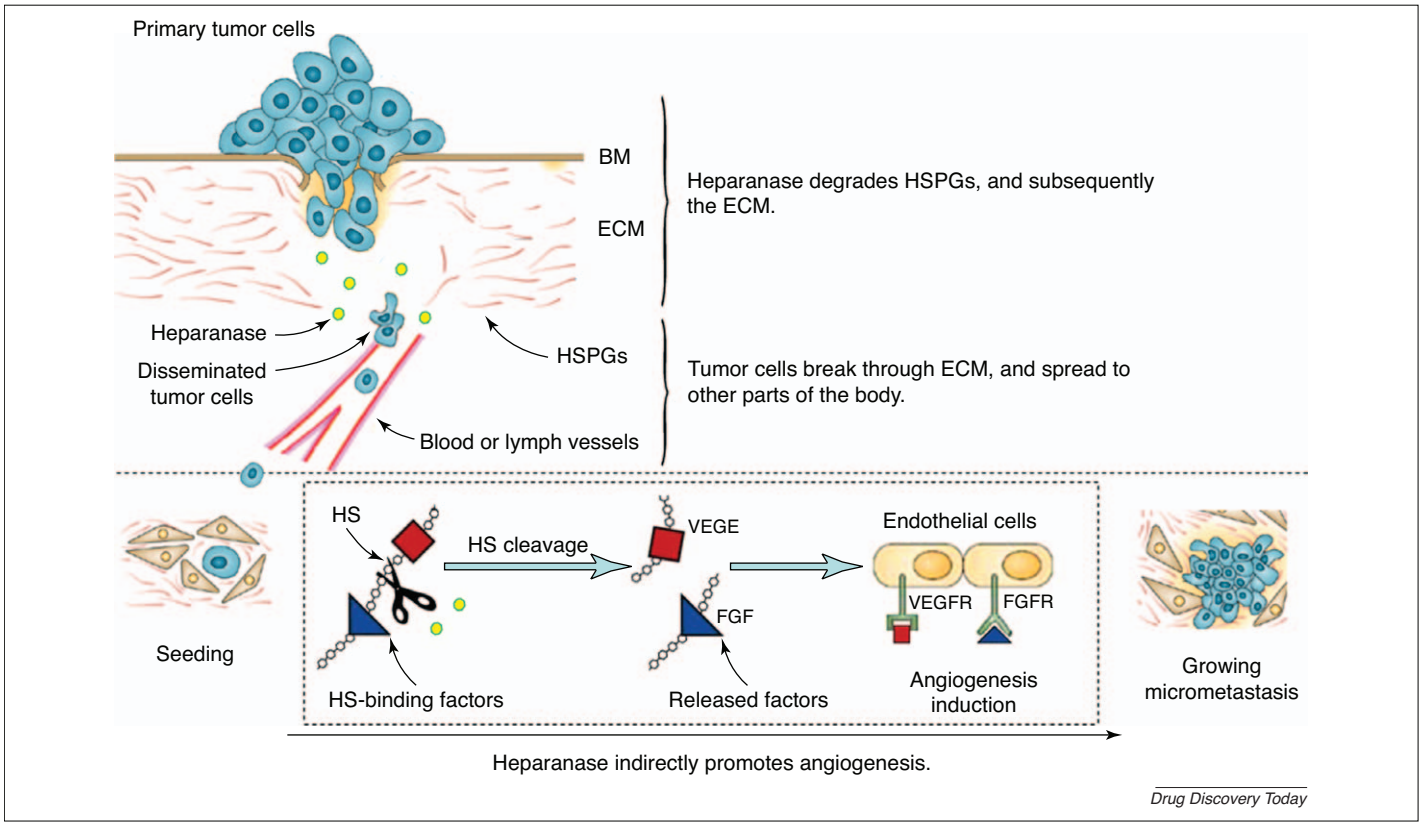


FIGURE 1

Heparanase enhancement of tumor metastasis by degradation of the ECM and the release of ECM resident factors. Heparanase degrades HSPGs and, subsequently, the ECM, an essential step that enables tumor cells to penetrate the tissue barrier and become metastatic. Heparanase can also liberate HS-binding factors, such as VEGF and FGFs, from ECM depots, making them available for angiogenesis.

Targeting heparanase for cancer immunotherapy

The concept of cancer immunotherapy is based on the manipulation of the host immune system to fight the cancer. The primary advantages of active immunotherapy are its relative lack of side effects, its specificity against target tumor cells and the generation of a long-lasting memory response against tumor-specific antigens [39,40].

DCs, the most efficient antigen-presenting cells (APCs), have been increasingly used in cancer immunotherapy to improve immune responses. DCs have a central role in the initiation and regulation of tumor-specific immune responses, as they have the unique ability to activate antitumor effector T and B lymphocytes. In addition, they are capable of promoting natural killer (NK) cells or NK T cell activation, which also have important antitumor effects. These actions have been extensively exploited during the past decade, leading to the development of DC-based cancer immunotherapy. Evidence that antitumor immunity can be generated by vaccination with TAA-loaded DCs has been demonstrated in numerous studies in recent years [39,40].

At present, knowledge of TAA in patients with cancer is still limited to markers in only a few cancers, such as melanoma antigen recognized by T cell-1 (MART-1), which is specific for melanoma [41], and carcinoembryonic antigen (CEA), which is specific for gastrointestinal tumors [42]. These tumor-specific antigens, also called autoantigens, are shared among patients with the same type of tumor. Therefore, immunization with these autoantigens can only induce an immune response for the identical type of tumor that expresses the self-antigens. It has thus been a challenge for researchers to identify universal TAA for immunotherapy of multiple types of tumor. An ideal universal TAA should have the following characteristics: (i) expressed by most human cancers but rarely so in normal tissues; (ii) required in the process of tumorigenesis to avoid antigen variation or depletion; (iii) the potential to include peptide sequences that bind to major histocompatibility complex (MHC) molecules; and (iv) recognized by the T-cell repertoire in an MHC-restricted fashion to elicit a specific T lymphocytes response [43].

The rationale for exploring heparanase as a universal TAA for cancer immunotherapy is based on the findings over the past 10 years that heparanase has a crucial role in the progression and metastasis of human tumors. Currently, immunotherapy-targeting heparanase research is focused mainly on developing DC-based therapeutic vaccines. Theoretically, these vaccines should elicit heparanase-specific T lymphocyte immune responses, especially in CTL cells, which can specifically kill heparanase-positive tumor cells. More attractively, heparanase-specific T-regulatory cells are absent in some patients with cancer [15], suggesting that the CTL response induced by heparanase might result in more efficient tumor suppression than that induced by other TAAs.

Heparanase recombinant virus-based immunotherapy

Genetic modification of DCs with recombinant viruses encoding the TAA gene is a practical strategy for directly activating DCs for cancer immunotherapy [39,40]. To investigate whether heparanase

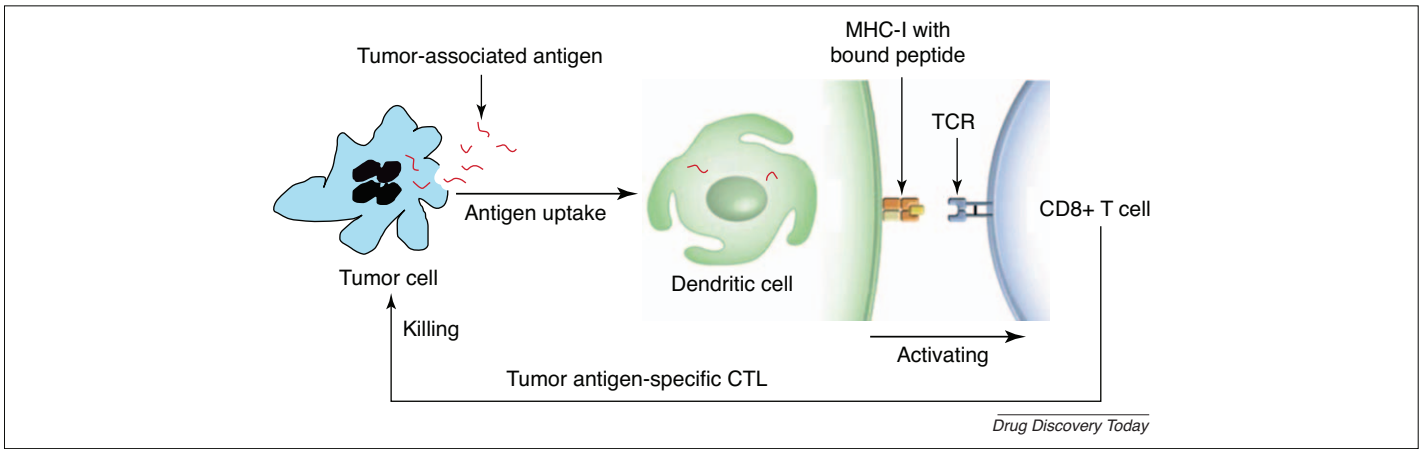


FIGURE 2

Antigen presentation and T cell activation. DCs take up antigens from tumor cells and subsequently process and present antigenic peptides via MHC molecules to CD8+ T lymphocytes. This elicits a tumor antigen-specific CTL response.

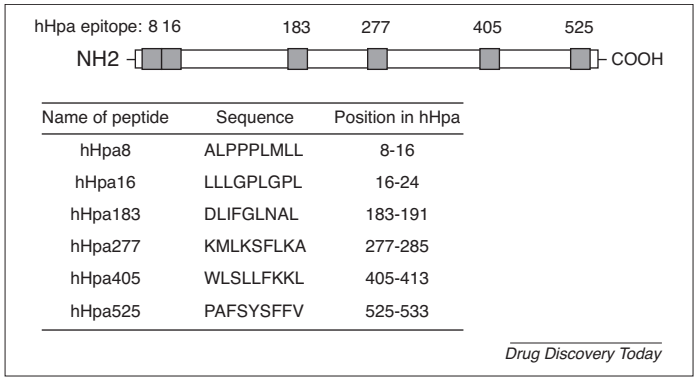
could be used as a target to induce specific antitumor immunity, a recombinant adenovirus vector was constructed that contained full-length heparanase cDNA; the vector was transfected into DCs that were isolated from peripheral blood mononuclear cells of healthy HLA-A2-positive donors [44]. The study demonstrated that the modified DCs activate heparanase-specific CTLs, resulting in potent, specific lysis of human gastric cancer KATO-III cells that were heparanase positive and HLA-A2 matched. Furthermore, the heparanase-specific CTLs could not lyse SGC-7901 cells that were heparanase positive but not HLA-A2 matched. Further studies revealed that the modified DCs can also promote interferon (IFN)-γ secretion of the effector CTL cells, which enhances non-specific immunological functions of the tumor host [44].

Heparanase peptide-based immunotherapy

CTLs are considered the chief mediators of tumor immunosurveillance via recognition of TAA as cognate peptides bound to MHC molecules on the surface of tumor cells. A major achievement in the field of tumor immunology over the past 20 years has been the clear demonstration that CTL epitopes binding to MHC, rather than integral TAA, induce CTL reactions (Fig. 2) [45]. These epitope peptides are usually eight to ten amino acids long, with two to three primary anchor residues that interact with the MHC-I molecules and two to three amino acid residues that bind to the T cell receptor (TCR) [46]. Therefore, identification of CTL epitopes from TAA has become a crucial step in the development of antigen-targeted immunotherapy for cancer.

The first group of immunogenic epitopes in heparanase was discovered by Sommerfeldt *et al.* using the SYFPEITHY algorithm nonapeptides of the heparanase amino acid sequence, which were predicted to exhibit a high binding capacity to HLA-A*0201 (HLA-A2). HLA-A2 is the most frequent MHC-I molecule expressed at the cell surface, and it is expressed in nearly 50% of Caucasians, Asians, and Hispanics, and 33% of African-Americans. *In vitro* experiments showed that DCs loaded with the three predicted HLA-A2-restricted peptides of human heparanase (hHpa) containing residues 8–16 (ALPPPLMLL, hHpa8), 16–24 (LLLGPLGPL, hHpa16), and 183–191 (DLIFGLNAL, hHpa183) all promoted heparanase-specific CTLs to lyse breast cancer cells (Fig. 3) [47].

In another study [48], HLA-A2-restricted epitope prediction algorithms based on supermotif and quantitative motif methods were used to predict 30 HLA-A2-restricted heparanase epitopes, representing another group of immunogenic CTL epitopes in hHpa Interestingly, hHpa8 and hHpa16, which were discovered by Sommerfeldt *et al.*, were included in the 30 predicted epitopes, and another epitope discovered by Sommerfeldt *et al.*, hHpa183, was located close to the predicted epitope hHpa184 (184–192, LIFGLNALL) from this second study. This overlap indicates that the computer-based epitope prediction methods used are effective and feasible. Five predicted epitopes consisting of nine amino residues with the highest binding score for the HLA-A2 molecule were selected for further analysis in this study. By using molecular modeling and peptide-binding assays, all the candidate peptides were deemed to be suitable HLA-A2-restricted CTL epitopes, with high affinity for the HLA-A2 molecule. Using a standard four-hour ^{51}Cr release assay, hHpa277 (277–285, KMLKSFLKA), hHpa405 (405–413, WLSLLFKKL), and hHpa525 (525–533, PAFSYSFFV) were found to elicit HLA-A2-restricted CTL responses (Fig. 3). These responses were specific for heparanase-positive tumor cells, including KATO-III gastric cancer cells, SW480 colorectal cancer cells and U2OS osteogenic sarcoma cells. Antibody blocking assays further



Name of peptide	Sequence	Position in hHpa
hHpa8	ALPPPLMLL	8-16
hHpa16	LLLGPLGPL	16-24
hHpa183	DLIFGLNAL	183-191
hHpa277	KMLKSFLKA	277-285
hHpa405	WLSLLFKKL	405-413
hHpa525	PAFSYSFFV	525-533

FIGURE 3

hHpa peptides able to elicit HLA-A2-restricted CTL responses. These peptides include hHpa8, hHpa16 and hHpa183, identified by Sommerfeldt *et al.* [47], and hHpa277, hHpa405 and hHpa525, identified by Chen *et al.* [48].

demonstrated that these heparanase-specific CTLs are mainly derived from CD8+ T lymphocytes. In addition, the study revealed that these three peptides could also increase the frequency of IFN-γ producing CTL cells compared with a negative control peptide. Considered together, these results suggest that hHpa277, hHpa405 and hHpa525 peptides are novel HLA-A2-restricted epitopes that are capable of eliciting specific CTL responses against heparanase-positive human tumor cells *in vitro* [48].

To investigate the *in vivo* immune response elicited by heparanase CTL epitopes, similar experiments were performed with predicted CTL epitopes derived from the mouse heparanase (mHpa) protein [49]. *In vitro* experiments showed that the predicted mHpa398 (398–405, LSLLFKKL) and mHpa519 (519–526, FSYGFFVI) peptides could activate heparanase-specific CTLs to lyse various tumor cells expressing both heparanase and H-2K^b (mouse MHC-I molecule). *In vivo* experiments further indicated that the mHpa398 and mHpa519 peptides offered the possibility of not only immunizing against tumors, but also treating tumor-bearing hosts successfully. Recently, using HLA-A2 transgenic C57BL/6 mice as the experimental model, the *in vivo* immune response elicited by hHpa CTL epitopes hHpa277, hHpa405 and hHpa525 was tested [50]. Results showed that these selected peptides could be presented naturally *in vivo* and also elicited the heparanase-specific lysis of various *in vivo* grown tumor cells expressing both heparanase and HLA-A2.

Strategies for improving the immunogenicity of peptide vaccines targeting heparanase

Antigens derived from tumor cells represent suitable targets for developing immunotherapies and vaccines for cancer. Synthetic epitope peptides offer advantages for therapeutic use: they are easy to produce even at clinical grade, free from pathogen contamination, have minimal oncogenic potential and are chemically stable [51]. However, owing to their small molecular weight and single structure, the immunogenicity of synthetic peptide vaccines is weak and thus usually cannot generate an ideal immune response in the body. Traditionally, the method of coupling peptides to a carrier protein has been used to improve the immunogenicity of peptide vaccines. However, given that carrier proteins are also exogenous antigenic stimuli to the host, the induced immune responses are often against the carrier proteins instead of against the target peptides [52]. In recent years [53], the multiple antigen peptides (MAP) design plan has been proposed to overcome the limitations of conventional conjugation approaches. The MAP system does not require a carrier protein for conjugation and, because of the high molar ratio and dense packing of multiple copies of the antigenic epitope in a MAP, it has been shown to stimulate better immune responses than single-chain peptides [52]. In a recent study, MAPs containing multiple copies of hHpa277, hHpa405 or hHpa525 were synthesized, and the immunity generated by these MAPs was evaluated by both *in vitro* and *in vivo* methods. Results showed that this vaccine strategy elicited a more potent CTL response, with an improvement of approximately 20% compared with the corresponding peptides. Using a similar strategy, Yang *et al.* demonstrated that MAPs containing B cell epitope peptides derived from the hHpa protein are capable of inducing a high titer of neutralizing antibodies in sera, further indicating the feasibility of using MAPs to improve the immunogenicity of peptide vaccines targeting heparanase [54]. Using an endoplasmic reticulum retrieval signal, Wang *et al.* designed a new heparanase epitope vaccine and reported that vaccination with DCs pulsed with the modified peptide elicited a robust, specific CTL response. The vaccine also significantly inhibited tumor growth and prolonged the lifespan of the experimental mice, indicating that this strategy could be used to improve the immunogenicity of heparanase CTL epitope peptides [55].

Safety concerns of the heparanase-targeted antitumor immunotherapy

Safety concerns will still limit the use of cancer vaccines in the near future. Although high-level heparanase expression is restricted to tumor cells, it can be detected at low levels in most normal tissues, including activated immune cells, such as T lymphocytes and DCs. Consequently, any heparanase-based cancer vaccine therapy will require thorough assessment of the potential side effects associated with autoimmunity to heparanase-positive tissues. To evaluate the side effects of the immunity elicited by heparanase epitope peptides or recombinant adenovirus expressing full-length heparanase, heparanase-specific CTLs were tested for their ability to lyse autologous lymphocytes and DCs [44,48,49]. Results showed that this heparanase-targeted vaccine did not markedly lyse either these lymphocytes or DCs. Moreover, no CTL-mediated toxicity against normal tissues was observed in animal models. It is possible that the level of heparanase expression in normal cells is below the threshold needed for recognition by these heparanase-specific CTL clones.

Conclusions and perspectives

Several HLA-A2-restricted CTL epitopes in the hHpa protein have been shown to elicit specific antitumor immunity *in vitro* and *in vivo* against various tumor cells. The immunogenicity of these CTL epitopes might be further enhanced by creating MAP vaccines. In addition, there have been no obvious toxicity or autoimmune reactions observed in the evaluation of this strategy in any experimental models so far. These studies provide evidence that heparanase is an ideal universal target antigen for cancer immunotherapy.

Recently, the first therapeutic cancer vaccine, Provenge®, which is used to treat advanced prostate cancer, was approved by the US Food and Drug Administration, heralding a major breakthrough in the struggle for immunotherapy treatments for cancer [56]. Although there are still many hurdles to overcome in heparanase-targeted immunotherapy, we believe that this strategy for inducing specific antitumor immunity, for example, by heparanase CTL epitope peptides, will offer a new universal approach for the treatment of cancer. This is particularly attractive for the prevention of metastasis and recurrence of the primary tumor after surgery. Naturally, before entry into clinical trials, animal studies will be needed to determine the vaccination time, dose and indications, and to further evaluate the possible side effects of this promising anticancer treatment.

Acknowledgements

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