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REVIEW ARTICLE

SANTAVAC™: A Novel Universal Antigen Composition for Developing Cancer Vaccines

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Abstract: ***Background:*** Development of a universal cancer vaccine for the prevention of all cancers has been under development for many years. Antiangiogenic cancer vaccines elicit immune responses with the potential of destroying tumor vasculature endothelial cells without affecting vasculature integrity in normal tissues. The methods used in the development of antigen compositions comprising these vaccines have been recently improved and described in this report in the context of SANTAVAC™ development - the first cancer vaccine based on endothelial cell heterogeneity.

Methods: The present report summarizes data related to SANTAVAC™ development, including technical key points associated with optimal SANTAVAC™ production, a description of the composition required for preparing cancer vaccines with the highest predicted efficacy and safety, and a strategy for SANTAVAC™ large-scale implementation. Patents related to SANTAVAC™ and other universal cancer vaccines are also described.

Results: SANTAVAC™ was shown to be the most promising antigen composition for anti-cancer vaccination, allowing for immune targeting of the tumor vasculature in experimental models with a high predicted efficacy (up to 60), where efficacy represents the fold decrease in the number of endothelial cells with a tumor-induced phenotype and directly related to predicted arrest of tumor growth.

Conclusion: The use of SANTAVAC™ as a universal antigenic composition may spur vaccine development activities resulting in a set of therapeutic or prophylactic vaccines against different types of solid cancers.

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1. ANTIANGIOGENIC CANCER VACCINES

Effective treatments for most types of cancers have yet to be developed, and cancer remains the second leading cause of death. Therefore, novel and effective cancer-preventative therapies including cancer vaccines are crucially needed [1]. Destruction of the tumor endothelium as consequence of vaccination has advantages over vaccines that elicit immune responses specific to cancer cells because the tumor endothelium is genetically stable and is less likely to develop escape mutations [2]. Moreover, the endothelial cell (EC) to cancer cell ratio in tumors is approximately 1:100 and because vascular integrity is essential to tumor growth and metastasis, destruction of only a few ECs can lead to vascular obstruction resulting in arrest of tumor growth [3-6]. For these reasons, vaccines targeting the tumor vasculature represent a promising approach for preventing solid tumor growth and metastasis, and can justifiably be referred to as 'universal cancer vaccines' (UCV).

The simplest way to target the immune response against the tumor vasculature is by immunizing with antigens derived from ECs resulting in the elicitation of immune responses specific for a comprehensive array of target cell antigens [7-9]. The degree of antigenic composition similarity between a vaccine derived from tumor ECs and non-tumor ECs would directly affect the vaccine efficacy and the chances of eliciting side effects

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resulting in damage to healthy vasculature. Therefore, EC heterogeneity is considered critical to the design of EC-based cancer vaccines because of their ability to induce immune responses targeting tumor vasculature ECs.

EC heterogeneity has been well described [10, 11], including morphology, gene expression profiles, and antigen composition differences between different organs and even tissues within the same organ. Moreover, the gene expression profile of ECs is significantly influenced by growth factors released by tumors that induce angiogenesis required to build the tumor vasculature [12-19]. These data suggest that EC heterogeneity was the cornerstone for EC-based vaccine design capable of eliciting immune responses selectively targeting the tumor vasculature, thereby preventing undesired damage to other vessels that may lead to systemic vasculitis with internal hemorrhage and destruction of internal organs. This is a significant safety concern since it has been shown that experimental autoimmune vasculitis developed following immunization with ECs [20, 21] and several cell-based immunizations have been associated with the elicitation of autoimmunity in animals and humans [22-27].

2. SANTAVAC™ - THE FIRST ANTIGENIC COMPOSITION FOR VACCINATION BASED ON EC HETEROGENEITY

Recently, EC surface profiling experiments have been performed demonstrating that EC heterogeneity was important to vaccine design approaches. Tumor type-specific changes were observed on the surface of cultured human microvascular endothelial cells (HMECs) in the presence of tumor-conditioned medium collected from different cancer cell types [28-35]. Changes in cell surface antigen profiles were characterized by cell proteomic footprinting (CPF), an advanced proteomics approach used to define cell phenotypes via mass spectrometric analysis of the extracellular surface (Fig. **1A**, **1B**) [36]. A patent related to CPF was issued in 2007 [37]. Findings from previous studies have motivated the use of primary HMECs (Fig. **1C**) for these experiments since these cells are involved in tumor angiogenesis, and microvasculature-derived ECs exhibited functional differences compared to large vessel-derived ECs [38, 39], including differences in responses to growth stimulators [40, 41] and extracellular protein expression patterns [42-44]. Examining the relationships between surface profiles within the HMEC group by principle component analysis (PCA) (Fig. **1D**) of corresponding CPF demonstrated that tumors induced reproducible tumor type-specific changes to the HMEC surface profile, which ranged from relatively insignificant to pronounced, and that tumor-induced changes to the cell surface profile directly defined HMEC escape from cytotoxic T lymphocyte (CTL)-mediated cell death in an *in vitro* model of human antiangiogenic vaccination [28, 30]. Taken together, these findings provided useful information regarding the design of SANTAVAC™ (Set of All Natural Target Antigens for Vaccination Against Cancer) - the compositions of specifically derived HMEC surface antigens divergent from those expressed by ECs in normal tissues that prevents the elicitation of autoimmune reactions. SANTAVAC™ can be prepared with different adjuvants to produce vaccines specific against different types of solid cancers that can be tested *in vivo* for immunogenicity and safety. Between 2007-2015, patents related to SANTAVAC™ development were issued [32-35].

3. SANTAVAC™ COMPOSITION

SANTAVAC™ design processes have focused on using cells as the source of native EC antigens for the elicitation of immune responses against target cells [9, 32-35, 45, 46]. Whole cells possess a set of cell-surface antigens critical to vaccine efficacy [47, 48], in contrast to many ubiquitous intracellular antigens that could elicit various adverse autoimmune responses (Fig. **1A**) [31]. To exclude intracellular content from SANTAVAC™, the original approach for the collection of cell surface antigens is used [32]. That is, cell surface targets are accessible to proteases whose enzymatic byproducts can be isolated after *in vitro* proteolytic cleavage [29, 32, 49-52]. While trypsinizing the surface of live cancer cells yielded a digest containing less than 1% of the total cell protein content, this trypsin digest was more effective than whole cells at eliciting an antitumor immune response [49]. Thus, the digest's composition, comprised of proteolytically cleaved cell surface targets, was directly related to the killing rate of target cells in cytotoxicity assays (CTA) [36, 50]. These findings suggested that a set of proteolytically cleaved cell surface targets represented the cell's 'antigenic essence' fit for use in vaccine

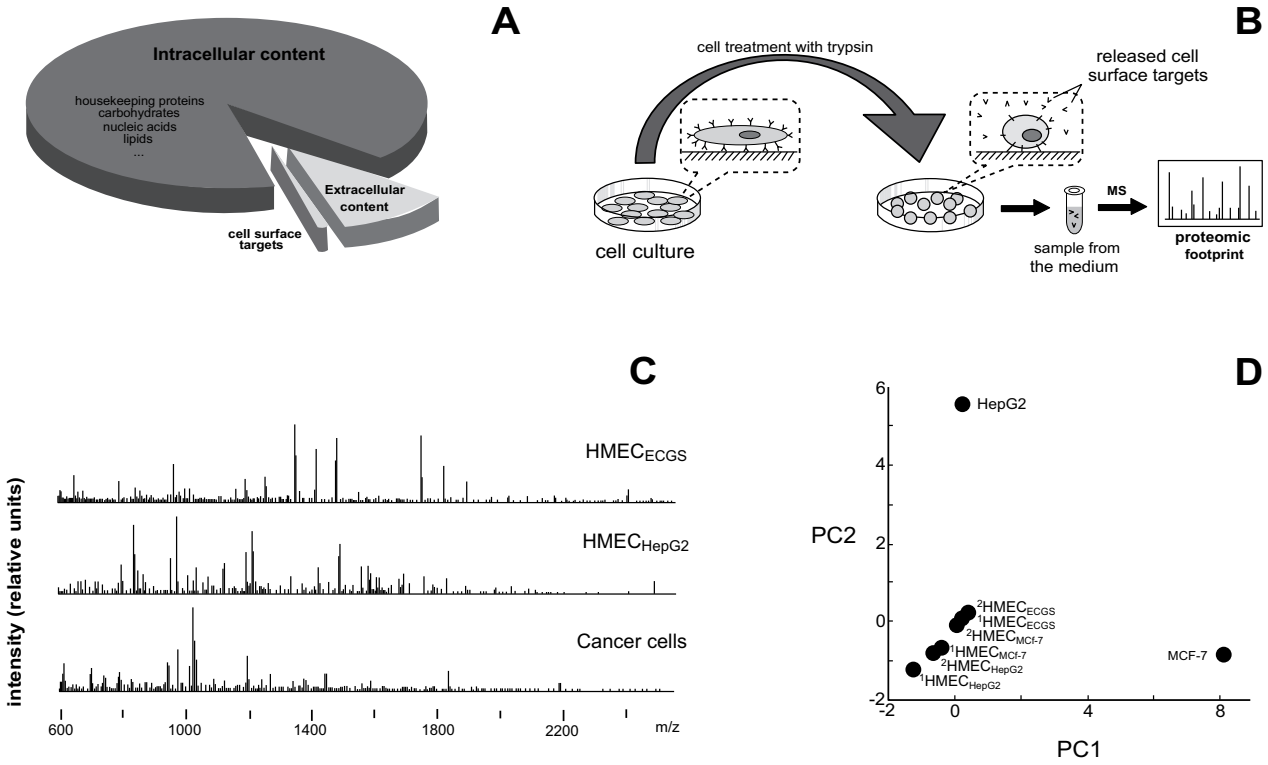

Fig. (1). Profiling of critical cell surface vaccine targets identified by cell proteomic footprinting. (**A**) Depiction of the relative ratios of surface targets accessible to the immune system and the remaining undesired cellular content. Adapted from [31]. **(B)** Cell proteomic footprinting. Adherent cell cultures were washed to remove traces of culture medium and subsequently treated with a protease. Released cell surface protein fragments were collected and subjected to mass spectrometry analysis. The set of peptides obtained represents the proteomic footprint. (**C**) Examples of cell proteomic footprints for non-ECs (HepG2) and HMECs induced to grow in the presence of non-tumor stimuli provided by EC growth supplement (HMEC$_{ECGS}$) or HepG2 cancer cells (HMEC$_{HepG2}$). Adapted from [28, 36]. (**D**) Principle component analysis (PCA) of cell footprints obtained from HMECs and control non-ECs (HepG2 and MCF-7) that were projected in the space of the first two principal components. PCA shows the degree of difference between cell surface profiles. Cell surface profiles are shown for HMECs stimulated to grow in the presence of EC growth supplement (^{1}HMEC$_{ECGS}$ and ^{2}HMEC$_{ECGS}$), human breast adenocarcinoma MCF-7 cell-conditioned medium (^{1}HMEC$_{MCF-7}$ and ^{2}HMEC$_{MCF-7}$), LNCap human prostate adenocarcinoma cell-conditioned medium (^{1}HMEC$_{LNCap}$ and ^{2}HMEC$_{LNCap}$), or HepG2 human hepatocellular carcinoma cell-conditioned medium (^{1}HMEC$_{HepG2}$ and ^{2}HMEC$_{HepG2}$). Superscript numbers correspond to different HMEC primary cultures. Adapted from [28].

formulations. SANTAVAC™ represents the 'antigenic essence' of HMEC following tumor-induced changes [32-35].

The induction of HMEC needed to produce SANTAVAC™ requires cancer cells to influence the HMEC surface antigen profile. We previously demonstrated that tumor-induced HMEC heterogeneity was a result of differences in the strength of tumor-derived growth signals (independent of the tumor) [28]. Based on these observations, it was hypothesized that tumors would affect the HMEC surface expression profile in the same manner *in vivo*, and that tumor-induced changes to the HMEC antigenic profile would be a consequence of the magnitude of the growth stimulus. Since stimuli of different strengths can be delivered simultaneously by tumors *in vivo* depending on the distance from the tumor cells, it can be expected that HMECs with different target surface profiles would also be present in the tumor-associated vasculature. Obviously, destruction of any type of HMEC at any location in the tumor vasculature or in vessels near the tumor would lead to vessel obstruction and arrest of tumor growth. Therefore, this assumption was used to define the optimal SANTAVAC™ composition that most effectively targeted the immune re-

sponse. This was measured using CTAs of tumor-induced HMECs and the predicted efficacy *in vivo* was measured for respective compositions.

4. PREDICTED EFFICACY OF ALLOGENEIC AND AUTOLOGOUS SANTAVAC™

A critical key to finding the optimal SANTAVAC™ composition with maximum efficacy was based on the observation that HMEC targets that had a surface profile induced by human prostate adenocarcinoma (LNCap) cells were effectively killed by a SANTAVAC™ formulation generated from HMECs induced by human hepatocellular carcinoma (HepG2) cells [28]. These findings supported the *in vitro* design of autologous SANTAVAC™ with a targeting efficacy of 1.7 (*i.e.*, 1.7 tumor ECs were destroyed before one EC in normal tissue was destroyed) [28, 53]. This efficacy provides a therapeutic window in which tumor HMEC cells could be killed by SANTAVAC™-induced immune responses before normal tissue HMECs (including ECs involved in angiogenesis) are adversely affected.

Because the obtained experimental data were limited by the availability of specific antigen compositions, gaps in the experimental data were filled by approximation of experimental data. Analysis of the data suggested that the efficacy of the autologous SANTAVAC™ may exceed 18 (Fig. **2C**). To reach such an efficacy level, the SANTAVAC™ composition and the profile of target HMECs should be quite similar (correlation coefficient for their CPF should exceed 0.82) [30]. Additionally, it was concluded that specific experimental points were required to directly observe the maximum efficacy for SANTAVAC™ in experiments designed to establish the maximum allogeneic SANTAVAC™ efficacy.

Although alloantigens elicit lower target cell killing rates than autoantigens, the use of alloantigens allows for the use of the patient's own biomaterial to be excluded from vaccine preparation, thereby simplifying research, development, and facilitating vaccine implementation in clinical practice. Moreover, correctly prepared alloantigen compositions that induce low target cell killing rates also exhibited low killing rates of HMECs stimulated to proliferate by normal cells, thereby providing a therapeutic window similar to one elicited by autoantigens. For example, high killing rates were observed for HepG2-stimulated target HMECs, where allogeneic SANTAVAC™ was prepared from MCF-7-stimulated HMEC cells and the observed targeting efficacy equaled 4 (Fig. **2A, 2B**) [28, 30]. This promising observation led to subsequent experiments that filled experimental gaps that better estimated the maximum efficacy of alloantigens. In this study, two types of efficacy were described in relation to the allogeneic SANTAVAC™ vaccine: i) Efficacy I allowed for an *in vitro* estimation of the number of tumor vasculature endothelial cells that would be destroyed before one normal tissue endothelial cell (even if a cell is actively proliferating and involved in angiogenesis) was destroyed, and ii) Efficacy II allowed for an *in vitro* estimation of vaccine efficacy in the context of suppression of HMEC proliferation of the tumor vasculature and is a reflection of the potential for the vaccine to arrest tumor growth, *i.e.* describes the vaccine's therapeutic efficacy. It was found that moderate tumor-induced changes to the HMEC surface antigen profile would be preferable in the production of allogeneic SANTAVAC™ resulting in an efficacy I equal to 17.3 (predicted safety) and efficacy II equal to 60 (predicted capacity to arrest tumor growth) (Fig. **2D, 2E**) [54]. This optimal cell surface profile of HMEC was induced by a 15% HepG2 cell conditioned medium preparation. This allogeneic SANTAVAC™ composition (final composition) currently is considered the most efficient and efficacious for further vaccine development activities and clinical trials [54].

5. KEY POINTS PERTAINING TO SANTAVAC™ PREPARATION

Several important technical points pertaining to vaccine preparation include the use of proteolytically cleaved cell surface antigens. Although the protease-based isolation of cell surface molecules [55-58] and the use of cancer cells for vaccination have long been described, this approach has some important limitations in terms of protease purity and the isolation of cell surface components using protease-mediated mechanisms versus the use of fluid shearing approaches.

Although earlier work demonstrated a possible loss of cell mass after trypsinization without any apparent change in cell viability [59], subsequent studies demonstrated that protease treatment affected cellular integrity [60-64] at levels sufficient

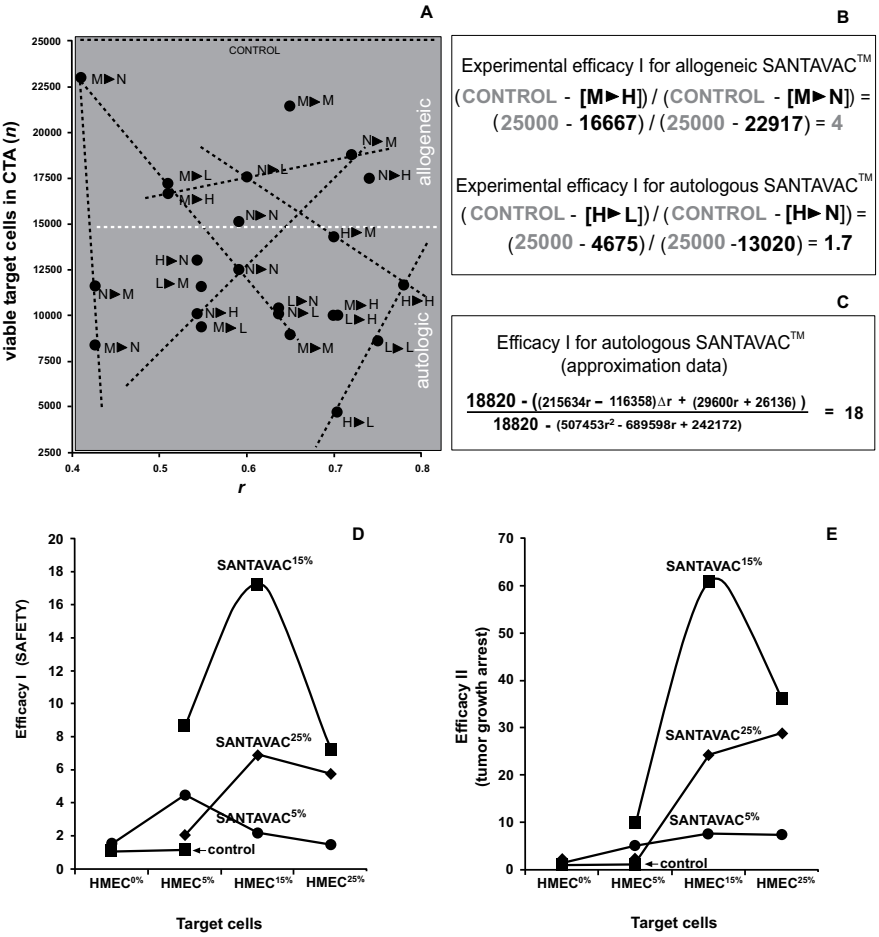

Fig. (2). Prediction of the optimal SANTAVAC™ composition by cell surface profiling and cytotoxicity assays. (**A**) Results of cytotoxicity assays (CTAs) and cell surface profiling. Data represent the cytotoxicity of effector CTLs against target HMECs *versus* the similarity between surface profiles of target HMECs and HMECs used to produce SANTAVAC™ used in the CTAs. Data represent the mean value of three independent measurements. The similarity between cell surface profiles is presented as the correlation coefficient ***r*** between corresponding proteomic footprints. 1►2 - First letter corresponds to HMECs used as a source of SANTAVAC™ and the second letter corresponds to the target HMECs used in the same CTA. 'Autologous' and 'allogeneic' correspond to areas of the autologous and allogeneic antigens, respectively. Different letters are used to identify HMECs stimulated to grow by EC growth supplement ('G'), by MCF-7 cells ('M'), by LNCap cells ('L'), or HepG2 cells ('H'). Data were scaled to bring all controls to equal values (25,000 cells, see CONTROL line). Dashed lines show examples of linear dependence between CTA data. Adapted from [28] (**B**) Predicted efficacy I for allogeneic and autologous SANTAVAC™ measured on the data depicted in plot 'A'. (**C**) Maximal efficacy I for autologous SANTAVAC™ calculated by data approximation from the plot 'A'. Adapted from [30]. (**D**) Efficacy I of target cell killing by allogeneic SANTAVAC™ in CTA. Efficacy I was calculated as a ratio of the number of *non-stimulated cells in control wells* (*i.e*., $HMEC^{0\%}$) to the number of tumor-stimulated cells in experimental wells. Efficacy I allows *in vitro* estimation of SANTAVAC™ efficacy by demonstrating how many endothelial cells were destroyed in the tumor vasculature before 1 endothelial cell was destroyed in healthy tissue (used to establish vaccine safety). Adapted from [54]. (**E**) Efficacy II of target cell killing by allogeneic SANTAVAC™ in CTA. Efficacy II was calculated as a ratio of the number *of tumor-stimulated cells in control wells* (*i.e*., $HMEC^{5\%}$, $HMEC^{15\%}$, or $HMEC^{25\%}$) to the number of tumor-stimulated cells in experimental wells, *i.e*., the percentage of tumor-conditioned medium in control wells was same as in the experimental wells. Efficacy II allows *in vitro* estimation of the SANTAVAC™ efficacy by demonstrating the degree of HMEC proliferation suppression in the tumor vasculature and used to establish the degree by which the vaccine can arrest tumor growth (vaccine therapeutic effect). '**Control**' indicates the data related to the control (■) in CTA where fibroblast-associated antigens were used to simulate CTL. Percent values indicated by superscripts correspond to the percentage of tumor-conditioned medium used to stimulate target HMEC or HMEC used to generate SANTAVAC™. The most effective final SANTAVAC™ composition intended for clinical trials is indicated (√). Adapted from [54].

to affect cell viability [59, 62, 65, 66]. More recent studies have utilized a more highly purified trypsin preparation with an activity of 15,000 U/mg resulting in cell lysis rates of less than 0.1% [49]. These data demonstrated that mammalian cells could be treated with trypsin without inducing lysis if a sufficiently pure trypsin preparation was used, thereby providing the foundation for preparing SANTAVAC™ formulations consisting of pure cell surface targets (*i.e.*, without contamination by intracellular content released by damaged cells). Moreover, the use of highly pure trypsin prevents contamination of the SANTAVAC™ preparations by trypsin admixtures and by trypsin autolysis products [32, 36].

Animal cells are sensitive to fluid shearing in serum-free medium [67-71]. A study by Lau and Tchao (2007) demonstrated that susceptibility of mammalian cells to damage caused by fluid shearing depended on the growth conditions (up to 56% cell death) [68]. To obtain pure cell surface antigen preparations, cells were treated with protease preparations in serum-free medium, however, damage to the cell membrane resulted in the release of the intracellular contents. As shown in Fig. **1A**, the amount of cell surface antigens obtained from 100 cancer cells is comparable to the amount of intracellular molecules contained within a single cell. Therefore, a critical aspect to consider in the preparation of cell surface antigen preparations is minimizing cell destruction as a consequence of fluid shearing. When protocol conditions were optimized and careful manipulation of the HMEC cells was maintained, an observed cell death rate of less than 0.1% was achieved after HMEC trypsinization [49], confirming the capacity of SANTAVAC™ production by careful treatment of live cells using a highly pure protease.

6. ANIMAL MODELS FOR SANTAVAC ™ VACCINES

Although animal models can be developed to confirm the efficacy of SANTAVAC™ final composition to arrest tumor growth, these models would provide little supporting evidence regarding the efficacy of SANTAVAC™ preparations in humans. For example, is has already been established that mice vaccinated with ECs had delayed tumor growth [32, 72-75]. However, using SANTAVAC™ in mice would not be very efficacious because results obtained as a consequence of xenovaccination [76] would be a poor reflection of SANTAVAC™ efficacy following administration to an allogeneic recipient. To achieve results in mice that paralleled the effect of SANTAVAC™ in humans, vaccines using mouse cells would have to recreated and a 'mouse-specific' SANTAVAC™ would have to be generated. However, developing such a formulation for use in a mouse model would not support results for a 'human-specific' SANTAVAC™. Therefore, testing of SANTAVAC™ in animals would not further support the efficacy of this vaccination approach for use in humans since SANTAVAC™ is a result of the 'sharp tuning' of human allogeneic HMEC phenotypes to produce an efficient antigenic composition efficacious only in humans.

Regarding potential preclinical trials, SANTAVAC™ is a natural product that does not include any non-human substances and only traces of whole proteins derived from the intracellular compartment. Therefore, it is reasonable to test SANTAVAC™ safety in preclinical trials in the context of different adjuvant formulations.

7. OTHER RECENT UCV DEVELOPMENT APPROACHES

There have been several attempts to prepare UCVs, including the better-known vaccines that target telomerase and mucin. Telomerase activity is indispensable for tumor immortalization and growth; therefore, the catalytic and rate-limiting subunit of telomerase (hTERT) is an attractive UVC candidate [77]. Recently, the TeloVac trial in the U.K. assessed whether adding GV1001 (a peptide vaccine representing a 16-aa hTERT sequence [78]) to gemcitabine and capecitabine chemotherapy regimens was beneficial. The Kael-Gemvax Company filled a world patent application in 2013 [79] and now has pending patents in several countries, including the U.S. and E.U.

ImMucin is another immunotherapeutic approach often referred to as a UCV. The Vaxil Biotherapeutics company holds patents [80-82] related to ImMucin, a 21-mer synthetic vaccine composed of the entire signal peptide domain of cell surface-associated mucin 1 [83]. Overexpression of mucin 1 is associated with many cancers and for this reason is considered a cancer therapy target and a candidate for developing a UCV [84]. However, according to 'The Human Protein Atlas'

(http://www.proteinatlas.org), melanoma, glioma, and skin cancers (among others) have no or low mucin 1 expression levels. Furthermore, it is of concern that there are high mucin 1 expression levels in normal tissues including lung, gastrointestinal tract, liver, female tissues, and kidneys. These high and varied mucin 1 expression levels between cancer cells and normal cells significantly limits ImMucin-based immunotherapy approaches.

Even though the TeloVac and ImMucin vaccines were designed to target different types of cancers and can be considered UCVs, their efficacy in treating primary cancers or in the treatment of relapses would be limited. One study found that specific cancer cell surface antigens were substantially modified under the selective pressures of drug treatment [50] that may be sufficient to allow cancer cells to escape the immune response. This fact, together with poor induction of specific antitumor immune responses may be a reason for the negative or poor results observed during the TeloVac [85] and ImMucin [86] clinical trials.

CURRENT & FUTURE DEVELOPMENTS

Cancer is one of the leading causes of death worldwide. In 2012, 14 million new cases were diagnosed and 8.2 million cancer-related deaths were reported worldwide, accounting for 13% of all deaths according to the WHO. It is expected that the number of cancer-related deaths will continue to grow and that by 2030 about 13.1 million new cases will be diagnosed. We predict that introduction of a UCV into clinical practice today in all countries would save millions of human lives.

Due to the large number of different tumor types (at least 30 are common) a clinical trial testing the efficacy of SANTAVAC™ to each cancer type would be required. Furthermore, a significant number of different SANTAVAC™ vaccine preparations differing in distinct compositions would need to be tested as either preventive or therapeutic vaccines. Additionally, cohorts of human subjects in clinical trials may be stratified by gender, age, race, life styles, or concomitant therapies. It is therefore expected, due to the significant heterogeneity between clinical trials, that some trials may yield positive results (from weak to pronounced delay of tumor growth depending on the tumor's requirement for vasculature, total tumor growth arrest, or even its destruction) while others do not, suggesting that SANTAVAC™ development and clinical trials testing its efficacy be started simultaneously in numerous countries following local regulatory requirements for the treatment and prevention of different tumors in the context of different adjuvants, vaccination regimes, and patient cohorts. Delaying the development of this universal antigen composition for cancer vaccines may result in millions of lost lives.

To support this strategy, the SANTAVAC™ developer and its committed partners currently provide the final composition of SANTAVAC™ to scientific organizations at no cost for use in vaccine development activities followed by clinical trials in accordance with the local regulations. The set of clinical trials are expected in the near future to provide data regarding the efficacy of SANTAVAC™-based cancer vaccines on human.

CONFLICT OF INTEREST

PGL declares that SANTAVAC™ preparation is protected by Eurasian, Japanese, Korean, and European patents and the U.S. patent is pending. The SANTAVAC™ trademark is protected in Russia and protection is pending in the E.U., U.S., China, Korea, and Japan.

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