

Review

Survivin - a universal tumor antigen

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Summary. Tumor-associated antigens recognized by cellular effectors of the immune system are potential targets for antigen-specific cancer immunotherapy. These antigens are classified as tissue (melanocyte)-specific proteins, cancer-testis antigens (proteins expressed in normal testis and various cancers), tumor-specific peptides derived from mutations in tumor cells, and others. Clinical studies with peptides and proteins derived from these antigens have been initiated to study the efficacy of inducing specific cytotoxic T lymphocytes (CTL) responses *in vivo*. However, most of the peptide epitopes used in these vaccination trials are melanocyte-specific, and these peptides cannot be applied for tumors of non-melanocyte origin. Furthermore, the expression of most tumor antigens is heterogeneous among tumors from different patients and can even vary among metastases obtained from one patient. Immune selection of antigen loss variants may prove to be an additional obstacle for the clinical applicability of most of the known CTL epitopes. Recently, a new tumor antigen, survivin, has been identified on the basis of spontaneous CTL responses in different cancer patients. Survivin is expressed in most human neoplasms, but not in normal, differentiated tissues. Importantly, downregulation or loss of survivin would severely inflict the growth potential of the tumor cell. Since survivin is expressed by a variety of different tumors MHC-restricted survivin epitopes may serve as important and widely applicable targets for anti-cancer immunotherapeutic strategies.

Key words: Survivin, Tumor antigens, T-cells, Cancer, Immunotherapy

The cellular immune system

The immune system is a defense system, which has evolved in vertebrates as a protection from invading pathogenic microorganisms (Tada, 1997). It consists of a large variety of cells and molecules that in a dynamic

network are capable of specifically recognizing and eliminating a huge variety of antigens. Once a pathogen has been recognized, the immune system engages a large number of different cells and molecules to mount an appropriate response to eliminate or neutralize the intruder. Immune responses involve two major groups of cells: lymphocytes and antigen-presenting cells. Lymphocytes are produced in the bone marrow, circulate in the blood and lymphatic system, and reside in various lymphoid organs (Fu et al., 1999). B-lymphocytes mature within the bone marrow. A mature cell expresses a unique antigen-binding receptor; the membrane-bound antibody molecule. When a naive B cell encounters an antigen that matches its antigen receptor, it starts to divide rapidly. Its progeny differentiate into memory and effector B cells, i.e. plasma cells. Although plasma cells only live for a few days, they secrete enormous amounts of antibodies, which are the major effector molecules of humoral immunity.

Cellular immunity is largely based on T lymphocytes. Like B cells, T cells also arise from the bone marrow. However, unlike B cells they migrate to the thymus for maturation. A T cell expresses a unique antigen-binding molecule called the T-cell receptor (TCR) on the cell surface. In contrast to membrane-bound antibodies on B cells, which can recognize antigen alone, the majority of TCR recognize a complex ligand, comprising an antigenic peptide bound to a protein called the major histocompatibility complex (MHC) molecule (Moss et al., 1992). When a T cell encounters antigen in the context of a MHC molecule, it undergoes clonal expansion and differentiates into memory and various effector T cells; helper (Th) cells and cytotoxic T lymphocytes (CTL).

While the T_h cells provide help to activate B cells, antigen-presenting cells and CTL, only the latter has a vital function in monitoring the cells of the body and eliminating any cell that displays foreign antigen; primarily virus infected cells. However, in addition to providing protection against infectious agents, CTL are thought to provide some degree of protection against spontaneous tumors, by virtue of their ability to detect quantitative and qualitative antigenic differences in transformed cells (Castelli et al., 2000). Tumorigenesis is characterized by both genetic and epi-genetic changes in

the tumor cell. As a result, the protein repertoire inside the tumor cell is altered compared to the normal counterpart. Class I MHC molecules sample peptides from protein-degradation inside the cell and present these at the cell surface to CTL. Hence, this enables CTL to scan and detect these intra-cellular alterations.

Tumor antigens

It was demonstrated in the nineteen seventies, that naturally induced tumors in rodents were largely non-immunogenic. In addition results from clinical studies were disappointing. This led to the notion that tumors are not sufficiently distinct from normal tissue in order to activate the immune system. Subsequently, it was concluded that immunological intervention in cancer treatment would be futile (Hewitt et al., 1976). However, in 1982 van Pel and Boon showed that a protective immune response could be generated against a 'non-immunogenic' murine tumor. Thus, they provided the first experimental evidence that lack of immunogenicity could be due to the tumor's inability to activate the immune system rather than the absence of tumor antigens (Van Pel and Boon, 1982). This observation was subsequently confirmed and extended to human cancers showing that by proper manipulation the tumor antigens expressed by the tumor cells can be recognized by the immune system. This may generate an immune response, which is capable of eradicating the tumor. A number of studies have shown that the CD8+ CTL arm of the immune response, alone or sometimes in combination with CD4+ T_h cells, constitutes the primary anti-tumor effector arm of the adaptive immune response (Castelli et al., 2000). This notion is supported by the frequent correlation seen between tumor progression and loss of the human MHC (HLA) class I expression in cancer patients (Hicklin et al., 1999) strongly indicating that progressing tumors in cancer patients escape an apparently effective class I MHC-restricted immune response by means of class I loss. The revelation that tumors after all could be sufficiently 'foreign' to be recognized by the immune system has renewed the efforts to identify and characterize tumor antigens. Until this day the focus has mainly been on the CTL arm of the immune response, in part for technical reasons. However, it should be emphasized that it is becoming more and more clear that the CD4 T cell response plays an essential role in tumor rejection, especially in the induction phase or in the extension of a persisting CTL response *in vivo* (Zajac et al., 1998, 1999; Toes et al., 1999). Subsequently, the incorporation of class II-restricted tumor antigens into effective tumor vaccination protocols is probably very important.

Tumor antigens can be divided into four different groups. It should be noted, however, that the distinction between some of the groups is somewhat arbitrary and is more a matter of degree.

Group I. A number of patient-specific antigens have arisen as a result of somatic mutations in normal gene

products. Unique tumor antigens recognized by CTL have been isolated from tumor-bearing mice and from cancer patients. Mut-1 was the first murine tumor antigen recognized by CTL isolated from the spontaneously induced Lewis lung carcinoma line (Mandelboim et al., 1994). The Mut-1 epitope was generated by a point mutation in the connexin 37 protein generating a novel class I (H-2K^b)-restricted epitope. Immunization of mice with Mut-1 peptide elicits CTL that recognize the tumor of origin and initiates a response that can lead to the regression of established metastases in tumor-bearing mice. Tumor antigens resulting from point mutations in normal gene products recognized by CTL from cancer patients have also been identified, although they only represent a small fraction of the human tumor antigens isolated so far (Boon et al., 1996; Rosenberg, 1999).

Group II. This group consists of tumor-specific antigens that nevertheless are shared among cancer patients. The group can be further subdivided into two subgroups; one subgroup consisting of antigens that have arisen as a result of mutations related to the oncogenic process and therefore is shared among some patients. For example mutated oncogenes (like p53 (Ciernik et al., 1996) or ras (Bergmann-Leitner et al., 1998)) or translocated oncogenes (like bcr/abl (Yotnda et al., 1998)) provides new epitopes. The other subgroup consists of viral antigens present in cancers of viral etiology such as Epstein Barr virus (EBV)-induced lymphoma and human papilloma virus (HPV)-associated cervical cancer (Boon and van der Bruggen, 1996).

Group III. The largest number of antigens identified so far are shared tumor antigens that correspond to normal tissue-specific gene products, also called "differentiation antigens". Such antigens have been isolated from melanoma and include MART-1/Melan A, gp100 and tyrosinase; all antigens expressed by normal melanocytes (Van den Eynde et al., 1997; Castelli et al., 2000).

Group IV. A group of antigens are shared tumor antigens that correspond to normal gene products, which are expressed in cancer cells but not in normal tissue (except in some cases testis). Prototypes of this group are the MAGE, GAGE, and BAGE families (Boel et al., 1995; Boon and van der Bruggen, 1996; Van den Eynde and Boon, 1997). In addition, a number of oncogenes are overexpressed in particular cancers, e.g., the HER-2/neu oncogene, which is frequently overexpressed in adenocarcinomas of breast, ovary, and colorectum. CTL isolated from cancer patients or generated *in vitro* against HLA-A2 binding peptides of HER-2/neu were found to specifically kill antigen-positive HLA-A2-matched cancer cell lines (Rongcun et al., 1999). Interestingly, two antigens were recently identified, which are expressed in most of the common human malignancies, but not in normal tissue. Vonderheide et al. demonstrated that human CTL against the widely expressed telomerase could be raised *in vitro*, which lysed a number of tumor cell lines of different origin

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(Vonderheide et al., 1999). Recently, two groups detected T cell responses against the anti-apoptotic protein survivin independently. Schmitz et al. described that survivin is capable of inducing specific CTL in vitro, when the protein is processed and presented by dendritic cells (Schmitz et al., 2000). In addition, we described spontaneous T-cell reactivity in leukemia, melanoma, as well as breast cancer patients (Andersen et al., 2001a,b).

Antigen-specific vaccination against cancer

After promising preclinical trials in murine models, the efficacy and safety of tumor-specific vaccination strategies is currently being evaluated in cancer patients. A large number of different types of vaccination are being performed. In very general terms, some vaccinations are based on the use of targeting defined antigens, whereas others are based on vaccines utilizing whole tumor cells (Melief et al., 2000). The advantage of tumor cell-based vaccines, especially those consisting of autologous tumor cells, is that these in principle comprise all tumor antigens expressed by the tumor cells from that particular patient. As a consequence, there is - at least with respect to vaccine design - no need for prior identification of the tumor antigens. However, since the antigenic composition of tumor cell-based vaccines is very complex and not fully characterized, it is very difficult to understand their therapeutic effect, or lack thereof, on the disease. In contrast, the use of vaccines comprising defined antigens provides the means to monitor the induced response and subsequently enable the development of improved vaccine strategies based on empirical findings.

The identification of tumor-associated CTL epitopes led to the first defined antigen-specific vaccine: a single synthetic peptide representing a class I MHC-restricted CTL epitope emulsified in incomplete Freund's adjuvant. Promising results were obtained with such vaccines in a variety of murine tumor models (Velders et al., 1998), thus setting the stage for testing of similar peptide-based vaccines in cancer patients. The first wave of Phase I clinical studies revealed that such vaccines do not induce significant adverse side-effects and are capable of inducing peptide-specific T-cell responses even in end-stage cancer patients (Rosenberg et al., 1998; Lee et al., 1999; Marchand et al., 1999; Turner et al., 1999; Weber et al., 1999; Melief et al., 2000; Scheibenbogen et al., 2000). Significant anti-tumor effects have only been observed in a fraction of the patients and truly therapeutic effects have been extremely rare. This is not unexpected considering the significant tumor burden these patients are usually suffering from, as well as the fact that in many of these patients the immune system is partially compromised. However, these initial trials have cleared the way for vaccination studies in patients with less progressed cancer, such as those in whom the primary tumors have recently been resected and who receive vaccinations as

an adjuvant therapy against minimal residual disease.

The appropriate delivery of tumor antigens in anti-cancer vaccines is currently under intense investigation. This includes cell-based (dendritic cells) or molecularly defined adjuvants, inflammatory cytokines, agents that modulate APC and T cell function, as well as the use of DNA- and virus-based vaccine vectors (Melief et al., 2000). Another driving factor in the development of vaccines with a more complex composition is the desire to target multiple tumor antigens either by designing a vaccine comprising or encoding a collection of carefully selected CTL and T_h cell epitopes or a vaccine comprising or encoding one or more entire tumor antigens. This is likely to increase the magnitude and flexibility of the vaccine-induced anti-tumor response and will prevent tumor escape that otherwise occurs through selective loss of single target antigens. The advantage of the use of long peptides or proteins in future anti-cancer vaccination trials is that all potential MHC epitopes within the delivered protein can be presented to host T cells. To some extent, such vaccines can be administered to subjects independent of their HLA type.

For melanoma, the tumor for which the largest number of CTL-defined tumor antigens has been characterized, powerful CTL responses against antigens have been induced by vaccination and some patients experienced a complete remission of their disease (Rosenberg, 1996; Marchand et al., 1999). However, most of the peptide epitopes used in these vaccination trials are melanocyte-specific, and these peptides cannot be applied for tumors of non-melanocyte origin. Furthermore, the expression of most tumor antigens is heterogeneous among tumors from different patients and can even vary among metastases obtained from one patient. Immune selection of antigen-loss variants may prove to be an additional obstacle for the clinical applicability of most of the known CTL epitopes. These epitopes are derived from proteins, which are not essential for the survival of the tumor cell. Thus, if powerful CTL responses are induced by therapeutic measures such as vaccinations it is likely that tumor cells lacking the expression of these antigens will have a pronounced growth advantage. Thus, only CTL epitopes derived from proteins, which are either linked to the neoplastic transformation such as mutated tumor suppressor genes, or genes essential for the survival of tumor cells, would not be inflicted by immune selection. However, only the latter would have a broad immunotherapeutic potential.

Survivin

Survivin is a recently described member of the inhibitor of apoptosis protein gene family. It mediates suppression of apoptosis by direct inhibition of caspase 3 and 7, which are the main effector proteases of the apoptosis pathways (LaCasse et al., 1998). Survivin is expressed in a cell-cycle-regulated manner in the G2/S

phase of proliferating cells, and is rapidly downregulated by cell-cycle arrest in the G1 phase. Inhibition of survivin expression results in increased apoptosis and inhibition of cell proliferation (Ambrosini et al., 1998). Survivin is undetectable in normal adult differentiated tissues but is expressed in several human cancers including lung, colon, breast, pancreas, and prostate cancer as well as hematopoietic malignancies (Ambrosini et al., 1998; Adida et al., 2000). Furthermore, a series of melanoma and non-melanoma skin cancers have also been reported invariably survivin-positive (Grossman et al., 1999a,b). A large analysis of human transcripts revealed survivin as the fourth most highly expressed protein in human cancer tissue compared to normal tissue (Velculescu et al., 1999). The overexpression of survivin in human cancers suggests a general role of apoptosis inhibition during tumor progression. This notion is substantiated by the observation that expression of survivin was associated with an unfavorable prognosis in colorectal and bladder cancers, as well as neuroblastoma (Kawasaki et al., 1998; Swana et al., 1999; Islam et al., 2000). Survivin overexpression induces an increased resistance to apoptotic stimuli from chemotherapeutic agents, whereas anti-sense targeting against survivin induces apoptosis and sensitizes tumor cells to chemotherapy (Olie et al., 2000). Thus, the universal expression of survivin in cancer and the fact that the protein plays a prominent role for the survival of the cancer cell suggests that survivin might be eligible to serve as a universal tumor antigen for both diagnostic and therapeutic purposes.

The first evidence for this hypothesis was provided independently by two groups. Schmitz et al. described that survivin is capable of inducing specific CTL *in vitro*, when the protein is processed and presented by dendritic cells (Schmitz et al., 2000). Thus, DC incubated with soluble recombinant survivin were shown to induce specific MHC class I-restricted CTL. In addition, they were capable of inducing a CTL line against a HLA-A2-restricted survivin-derived peptide epitope.

Our approach was to use 'reverse immunology', i.e. to scan the survivin protein for the presence of HLA-A2-binding motifs and to use these epitopes to search for specific T cell responses in tumor patients. We were indeed able to detect spontaneous T-cell reactivity in leukemia and melanoma patients by ELISPOT assay (Andersen et al., 2001a). Recently, we extended these data by the identification of specific T-cell reactivity against this antigen in peripheral blood from breast cancer patients. The presence of spontaneous CTL responses against the HLA-A2-restricted peptide antigens derived from survivin in patients suffering from three completely unrelated tumor types, i.e. melanoma, breast cancer and CLL, strongly indicates that CTL-defined epitopes from survivin are of substantial immunotherapeutic value. Furthermore, we were able to directly isolate such survivin-reactive cells *ex vivo* by means of magnetic beads coated with MHC/peptide

complexes and to demonstrate that these survivin-reactive T cells were capable of lysing HLA-matched tumor cells of different tissue types (Andersen et al., 2001b). In addition, using multimerised MHC/survivin-complexes survivin-specific CTL could readily be detected *in situ* in the tumor microenvironment. Such cells were depicted in the primary tumor and the sentinel lymph node of a stage III melanoma patient as well as in a primary breast cancer lesion. The detection of survivin-reactive CTL *in situ* (Andersen et al., 2001b) might be of particular importance. There is consensus that the induction of efficient and powerful T-cell responses requires proper priming of the T cell. However, it is equally important that the T cells acquire the ability to home to the site of action. The combined detection of survivin-specific T cells in the blood and in the tumor lesions indicates that these cells are capable of circulating and homing to the effector site. Importantly, this has been shown in both melanoma and breast cancer patients. This is a significant finding, since several clinical reports have indicated the existence of a functional dissociation between local and systemic anti-melanoma T-cell responses. Thus, at least in melanoma, the presence of tumor antigen-specific T cells in the blood may not lead to clinically relevant responses, i.e. responses at the tumor site (Thor Straten et al., 1999).

For the ongoing quest to identify antigens that can trigger tumor-specific CTL responses and which are widely applicable to a range of tumor types - ideally a universal tumor antigen - survivin add the second candidate to meet these criteria. Similar to the first candidate - the catalytic subunit of telomerase - survivin is expressed by cells of the most common human malignancies. Importantly, downregulation or loss of survivin would severely inflict the growth potential of the tumor cell. Thus, if CTL responses are induced by therapeutic measures such as vaccinations it is likely that tumor cells lacking the expression of these antigens will have a pronounced growth advantage (Maeurer et al., 1996; Cormier et al., 1998; Riker et al., 1999). The apparent mandatory expression of survivin and telomerase imply that these antigens are not prone to immune selection since downregulation or loss of their expression would severely inflict the growth potential of the tumor cell (Ambrosini et al., 1998; Swana et al., 1999). Notably, while reactivity of human CTL against telomerase has to date only been demonstrated in T-cell lines after *in vitro* stimulation, spontaneous CTL reactivity against survivin has been shown to occur in cancer patients.

The future of survivin-based vaccination will depend on clinical outcome, and the type of side effects that may follow immunization. When peptides derived from melanocyte differentiation antigens were first used to treat patients with stage IV melanoma it was envisioned that this may lead to pronounced destruction of melanocytes, which in turn might manifest clinically, i.e., vitiligo or retinitis. Fortunately, clinical experience demonstrated that the incidence of vitiligo in patients

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receiving vaccinations was not significantly higher than the incidence of melanoma-associated hypopigmentation in patients receiving other forms of therapy (Becker et al., 1999). Thus, no severe adverse effects in the normal tissues or organs have been reported in the clinical trials of cancer vaccines specific to the MAGE-1, MAGE-3, Melan-A, gp100, tyrosinase, and NY-ESO-1 in melanoma patients, although these molecules are expressed in the normal testis, retina, or melanocytes at both mRNA and protein levels (Jager et al., 2001). For survivin, the odds that no major adverse autoimmune effects will be induced are even better since overexpression of survivin is largely restricted to neoplastic cells. In our studies we did not detect spontaneous immune reactions against survivin in any of the healthy donors included (n=20), clearly indicating that anti-survivin T cell responses is truly tumor specific. Rohayem and colleagues substantiated these findings. They examined the presence of antibodies against survivin in sera from cancer patients, and described antibody responses to survivin in 22% of lung cancer patients and in 8% of colorectal cancer patients (Rohayem et al., 2000). Notably, particularly high levels of survivin expression have been found in lung cancer cell lines (Tamm et al., 1998). Furthermore, data from a recent study demonstrated the presence of survivin-specific antibodies in gastrointestinal cancer patients (Yagihashi et al., 2001). Together, these data substantiate the notion that immune responses against survivin are restricted to cancer patients. Furthermore, the presence of antibodies in the sera of cancer patients suggests that survivin may not only be considered a major T-cell antigen but also a B-cell antigen.

The attractiveness of survivin for vaccination purposes is further substantiated by the fact that downregulation or loss of its expression as some form of immune escape would hamper the progression of the tumor, particularly if subjected to anti-cancer chemotherapy. Since expression of survivin in tumors is correlated with drug resistance and/or shorter survival of cancer patients (Bjorkman et al., 1987; Chicz et al., 1993; Carosella et al., 1999), the combination of a survivin-based immunotherapy with conventional cancer chemotherapy might be an effective way to improve the cancer treatments of today.

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