Clinical Cooperation Unit Skin Cancer (D0900 / D070)

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The Skin Cancer Unit is a Division of the German Cancer Research Center located in the Dermatology Clinic of the University of Heidelberg in Mannheim. The Skin Cancer Unit is focussed on the prevention, diagnosis and treatment of skin tumors. This includes squamous cell carcinomas of the skin and the skin appendages, lymphoma of the skin as well as malignant melanomas which account for ¾ of all deaths caused by skin disease.

Optimal care of tumor patients requires interdisciplinary cooperation with other clinical departments such as surgery, pathology, radiology and hematology/oncology. The fact that we are part of a university medical school provides especially favorable conditions for such cooperative efforts. The goal of the Skin Cancer Unit is to achieve a closely coordinated interlocking between the newest experimental developments in the laboratory and clinical practice. The unit (established 1997) includes a modern, spacious laboratory and a special dermo-oncological out-patient clinic in which chemotherapy as well ultrasound scans of the lymph nodes can be performed. An important service provided by the unit is a special program for the aftercare of tumor patients. In addition, as provided for by the cooperation contract between the German Cancer Research Center, the University of Heidelberg and the Mannheim Clinics, the unit also maintains 4 beds in the dermatology ward for in-patient treatment.

Patients with malignant melanoma in all stages will be the main focus of our efforts, but our unit also provides comprehensive in- and out-patient care for patients with advanced squamous cell carcinomas and lymphomas of the skin. Furthermore, the unit offers an opportunity for concerned doctors to present their patients (case histories) and receive additional advice and analysis in the dermo-oncological conferences which are held every Wednesday afternoon (appointments should be made in advance: Tel. +49-621-383-2127).

Homepage: http://www.dkfz-heidelberg.de/melanom

Background
Melanoma is a curable disease if it is diagnosed on time and properly removed surgically. The treatment of choice for a primary malignant melanoma is surgical excision with a wide safety margin, depending on the tumor penetration depth and the anatomical location. Surgical treatment is also preferred and recommended for in-transit and satellite metastases. In cases where surgical methods cannot be applied, treatment is very problematic and patients and physicians are confronted with difficult choices. In the advanced stages of the disease there are only limited possibilities for therapy, since conventional methods such as radiation and chemotherapy are not very effective. Consequently, new treatment methods are urgently needed. Although there have been promising reports on new bio-modulating and immunological therapeutic approaches to treatment of the malignant melanoma, chemotherapy still plays a major role. Response rates are generally low. This well-known resistance to chemotherapy is generally con-
Chemoresistance

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The prognosis for melanoma in the advanced stages is very poor primarily because chemotherapy is so ineffective in fighting this disease. For reasons that are still unclear melanoma cells usually prove to be very resistant to systemic treatment with antineoplastic agents.

In the search for the specific mechanisms responsible for the resistance observed in the treatment of cancer patients research has been done on the effects of various cytotoxic agents on tumor cells and on the processes that lead to cell death [1]. Several studies confirmed that mechanisms conferring drug resistance in hematological tumors are not operative in the same way in solid tumors such as human melanoma. Thus, comprehensive analysis must be performed to gain a better understanding of the specific forms of drug-resistance which are typical in human melanoma. Induction of apoptosis is widely considered to be one of the most important mechanisms by which cytotoxic agents kill tumor cells. Therefore, defects in the function of the apoptotic cascade should lead to a drug-resistant phenotype. The apoptotic pathway is still under intensive investigation and analysis concerning apoptosis deficiency as a relevant mechanism in drug-resistance has just begun. It is also reasonable to assume that further resistance mechanisms exist which have not yet been discovered. In our work we have employed several molecular biological approaches have been employed in order to identify these, as yet, unknown molecules possibly involved in resistance to therapeutic drugs [1, 2].

Since most experimental observations of the drug-resistance phenomenon cannot be performed directly on the patient, we have developed an in-vitro model system which is designed to achieve very specific and reproducible analyses of these mechanisms. [Kern et al., Anticancer Research 17 (1997) 4359-4370].

As various melanoma cell lines were examined it became evident that a cell line which is resistant in the presence of a certain drug also displays cross-resistance to other drugs with similar working mechanisms. This indicates the existence of certain general resistance mechanisms which have not yet been discovered and which account for the drug resistance displayed by malignant melanomas.

Research Topics:
Apoptosis-Deficiency

A number of studies have implicated apoptosis (programmed cell death) as an important mechanism by which chemotherapeutic agents kill susceptible cells. These studies suggest that inhibition or dysregulation of apoptosis may be responsible for resistance to anticancer drugs. It has been shown in some tumor systems that induction of apoptosis by several cytostatic drugs was mediated either by death-receptor/ligand-systems (such as the CD95-system) or by mitochondrial processes. There is very little known about drug induced apoptosis and disturbances in apoptotic cascades causing drug resistance in the human melanoma tumor system.

Results:
We have analyzed the apoptotic pathway in cisplatin- and etoposide-induced cell death in drug-sensitive MeWo cells in comparison to pathways leading to cell death in resistant melanoma cells derived from MeWo [Kern et al., 1997]. Etoposide and cisplatin induced apoptotic cell death is mediated by mitochondrial processes. Analogous analysis in etoposide-resistant cells revealed a different pattern; marked deficiency in the apoptosis processes with characteristically strongly reduced release of cytochrome c from the mitochondria and down-stream signal transduction. Cell death in cisplatin-resistant cells is also characterized by changes in the apoptotic pathway (as compared to non-resistant cells), but different mechanisms are involved [2].

Effect-specific mechanisms:
DNA Repair

Increasing evidence has been provided that DNA damage induced by cytostatic drugs is counteracted by a corresponding compensatory modulation of DNA repair mechanisms. This mechanism which is essential for the protection and survival of normal cells may prevent cell death in drug-treated tumor cells. In the past few years it has been proven that DNA-mismatch repair (MMR) deficiency, as well as increased DNA-repair results in drug-resistance. We were able to show that in melanoma cells exhibiting resistance to cisplatin-, etoposide- and vindesine the nuclear content of various DNA-MMR-proteins (hMLH1, hMSH2 und hMSH6) was reduced. In melanoma cells that demonstrate resistance to fotemustine the amount of nuclear MMR-proteins was nearly unchanged, whereas the activity of O6-Methylguanine-DNA-methyltransferase (MGMT) was considerably enhanced [Lage et al., Int J Cancer 80 (1999) 744-750]. In cooperation with Prof. Wießler’s group at the DKFZ, inhibitors of MGMT have been designed and proven to be capable of partially over-
coming drug resistance in fotemustine-resistant MeWo-cells [3].

Identification of differentially expressed molecules
It is reasonable to assume that there are more resistance mechanisms which have not yet been discovered. In order to get further insight, we characterized MeWo cell variants by differential display reverse transcription-polymerase (DDRT-PCR) as well complex cDNA hybridisation. In order to further expand the scope of investigations on unknown mechanisms in drug-resistance of human melanoma, we analyzed the global protein-expression (proteom analysis) using two-dimensional gel electrophoresis [Sinha et al., *Electrophoresis* 21 (2000) 3048-3057].

By performing DDRT-PCR and Northern Blot analyses we were able to identify 11 cDNS clones as differentially expresses genes. These cDNS clones DSM-1-DSM-11 (Drug-Resistance- associated Sequence in Melanoma) contain 4 genes (DSM-1, DSM-3, DSM-5, DSM-7), with known functions, 3 genes (DSM-2, DSM-4, DSM-6) that have already been sequenced without characterizing their functions, and 4 new genes (DSM-8 - DSM-11) which could not be matched with a gene in the gene bank [Grottke et al., *Int J Cancer* 88 (2000) 535-546]. Functional analysis has shown that DSM-2 (ICERE) is involved in resistance to etoposide [4]. Furthermore, another 120 differentially expressed genes could be identified by means of complex cDNA-hybridisation [5].

By employing 2-D-Gel analysis we were able to identify more that 70 differentially expressed proteins (pH 2.8-pH 10) and make comparisons between sensitive and resistant MeWo melanoma cell lines [Sinha et al., 2000; Sinha et al., *Electrophoresis* (2003) in revision].

Perspectives
• Since an in vitro model has already been established and the first protein-biochemical and molecular biological studies are in progress, we should have a sufficient basis for developing DNS and protein chips with resistance-associated genes/proteins in cooperation with the research groups in DKFZ with the necessary expertise in this field. This would provide us with an invaluable tool for finding out more about these molecules.
• In a combined effort with other members of our unit working on experimental therapy approaches we plan to accumulate a comprehensive collection of various drug-resistant cell lines as well as fresh tumor tissue from a large number of patients with detailed and complete medical files and documentation.
• We will use this extensive data to create a “resistance profile” for individual melanoma patients which predicts potential reactions to antineoplastic agents, thus enabling us to select the most effective therapy regimen.

As soon as we know which molecules are involved in drug resistance we can find ways of manipulating them so that pharmaceutical substances will be able to realize their full therapeutic potential. The desired modifications could be achieved by using molecular biological or gene therapy strategies (e. g. ribozyme, antisense) or by developing new forms of chemotherapy.

Tumorantigens/Lymphome
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The work of this project group is centered on the identification of tumor-associated antigens (TAAs) and the evaluation of their applicability for diagnostic and therapeutic purposes. The various projects are embedded into three main topics: (1) the search for tumor antigens using screening methods (2) evaluation of the newly discovered TAA, cTAGE-1 und GBP antigens, and (3) the development of diagnostic tools.

We use various screening methods such as SEREX, a serological method for screening phage banks, to discover new tumor-associated antigens of homologues and to more clearly define the TAA’s that have already been discovered. The screening efforts were focused first primarily on the cutaneous T-cell lymphoma (CTCL), a lymphoproliferative disease of the skin for which only a limited number of tumor-specific antigens have been identified [6] but now more emphasis is being placed on human melanoma. In addition to looking for new antigens we have also studied the expression of known tumor-associated antigens, particularly those belonging to the “cancer-germline-antigen” group, in various tissues and cell lines (CTCL, Melanom). The most important result of this work was that the Antigen LAGE-1 and the antigen group GAGE were expressed profusely in the CTCL samples, thus providing promising new starting points for developing new methods of therapy and diagnosis [7]. Recently, we have begun to look for testis antigens which are expressed by malignant melanoma cells [8].

In a series of new experiments for detecting CTCL antigens we were able to identify several tumor-associated antigens which exhibited an interesting serological pattern. Furthermore, we were able to show that one of the tumor-associated antigens which were already known (SCP-1) and two newly discovered TAA (cTAGE-1 und GBP-TA) exhibited a differential expression pattern which could make them promising targets for immunotherapy [6, 9, 10]. Such tumor-specific antigens could be used for cell-based immunotherapy or - if they are expressed on the cell surface - for antibody treatment. For this reason achieving a complete analysis of the newly identified tumor antigens by means of expression analysis (RNA and protein) and immunohistology is a focal point of our research efforts along with the examination of antibody reactivity in sera derived from patients. Regarding cTAGE-1, the first TAA detected in CTCL, we have been able to show that this antigen is a member of a larger family to which even a second tumor-specific antigen belongs (cTAGE-5A) [10].

We will be performing further studies at the protein level on all of the new tumor-associated antigens which in the screening process proved at least in their serological reac-
tivity to be tumor-specific. The goal of this undertaking is to develop new diagnostic tools by generating antigen-specific antibodies and by developing an ELISA system for detecting tumor-specific antibodies in patient blood.

**Immuno- and Gene Therapy**


In cooperation with: Prof. Pierre Coulie (Ludwig Institut, Brussels, Belgium); Prof. Georgio Parmiani (National Cancer Institute, Mailand, Italy), Prof. Dr. Marcus Maueuer (Microbiology, Mainz), Prof. Dr. G. Rammensee (Tuebingen), Prof. Dr. Reinhard Dummer, PD Dr. Carmen Scheibenbogen (FU Berlin), Prof. Frederico Garrido (Granada, Spain), PD Dr. Gerd Sutter & Dr. Ingo Drexler (Virology, GSF Munich), Prof. Lutz Gissmann (DKFZ, Abt. F0200), Prof. Dr. Harald Klüter & Dr. X.D. Nguyen (Transfusion Medicine, Mannheim), Dr. Siegfried Weiss (GBF Braunschweig) Prof. T. Chakraborty & PD Dr. E. Domann (Microbiology, Giessen), PD Dr. Harald Kropshofer and PD Dr. Anne Vogt (Roche Institute for Molecular Genetics, Basel, Switzerland).

The growing evidence that tumor-associated antigens (TAA) can be specifically recognized by the immune system made the concept of mobilizing and directing the immune system to attack tumor cells by means of vaccination seem very promising and led to the development of various tumor-specific vaccination strategies. [Sun et al., J Mol Med 77 (1999) 593-60]. However, when evaluated in clinical phase II/III studies these treatment approaches exhibited therapeutic effects only in a small group of cancer patients. [Müller et al. Cancer Gene Therapy 7 (2000) 976-984]. This could be due to the fact that most clinical trials have been performed with cancer patients in the final stages of the disease, but the failures could also be attributed to the fact that most vaccination strategies have been focused exclusively on the induction of a tumor-specific CD8+ T cell (CTL) response. Although CTL are capable of directly killing tumor cells, the induction and maintenance of their effector function heavily depends on the participation of antigen-specific CD4+ T helper cells. Consequently, cancer immunotherapy has to target both, antigen-specific CD8+ and CD4+ T cells which, in turn, means that the design and implementation of these therapies must place the primary emphasis on finding and characterizing the various immunogenic TAA-specific T cell epitopes presented on tumor cells. Consequently, the work in this project group centers around two main goals: (I) Identification of antigen-specific immunogenic T cell epitopes as a prerequisite for (II) development of effective antigen-specific T cell-directed immunotherapies.

(I) Up to now, the studies on T cell epitope identification performed in our group have concentrated on the characterization of immunogenic HLA-class I restricted peptides derived from melanoma associated antigens (MAA). The experimental strategy applied to identify these peptides was based on the concept of “reverse immunology”, i.e. in vitro stimulation of T lymphocytes with predicted, potential CTL epitopes. Using this approach we could identify immunogenic peptide epitopes underlying qualitative differences in antigen processing in the tumor cells. [11-13]. In addition to the reverse concept, we now include direct strategies of epitope identification using global tumor antigens in vitro and in vivo. Dendritic cells (DC) are loaded with antigen-encoding RNA or recombinant protein and immunization of HLA-transgenic mice with recombinant DNA, protein or recombinant microbial vector vaccines. By applying the direct and the reverse strategies simultaneously we take the possibility into account that tumor cells and antigen-presenting cells (APC) may exhibit differences in the spectrum of immunogenic peptides generated from a given tumor antigen. Knowledge of both sets of peptides is of potential interest for the development of cancer vaccines. Taking into consideration the essential role that CD4+ T helper cells play in the induction and maintenance of CTL-mediated tumor immunity, we have extended our analysis to the identification of immunogenic TAA-derived peptides presented on HLA-class II molecules. In analogy to the HLA-class I system, both experimental strategies (reverse and direct in vitro / in vivo) will be employed. The studies on epitope identification will also include new TAA-specific antigens, identified by the “Tumor Antigen” project group.

(II) Analysis of the immunogenic nature of these TAA is a prerequisite for the design of effective T cell directed cancer vaccines, the second major goal of this project group. To achieve this aim we follow two routes (1) optimization of already established vaccines and (2) development of alternative vaccination strategies. We believe that dendritic cell based vaccines have great prophylactic and therapeutic potential as an immunological tool. In the treatment of melanoma patients with peptide-loaded autologous dendritic cells we were able to achieve impressive therapeutic effects. [Nestle et al. Nature Medicine 4 (1998) 328-332]. We hope to improve the therapeutic effects even further by loading DC with antigen-specific CD4+ and CD8+ T cell epitopes identified in the experimental studies described above.

However, since the production autologous DC vaccines is extremely expensive, we are also working on alternative vaccination methods. By developing vector systems which bring the antigens directly to the dendritic cells in vivo we can avoid the costly time-consuming procedures required to generate and pulse dendritic ex vivo in the laboratory. To achieve this aim we use the facultative intracellular bacteria Listeria monocytogenes as a bacterial vector system for TAA. We have been able to show with in vitro studies that this bacterium is able to efficiently infect human dendritic cells thereby inducing phenotypical DC maturation and release of pro-inflammatory cytokines [Paschen et al. Eur J Immunology 30 (2000) 3447-3456]. In addition to this bacterial system we will use Modified Vaccinia Virus Ankara (MVA) as a viral vector system. For both vector systems recombinant variants carrying melanoma differentiation antigens (MDA) are available. Due to the fact that MDA have been identified as targets for cellular immune responses in human melanoma patients and that these antigens have been explored extensively in animal models we chose MDA as targets for the evaluation of our vaccination strategies. The efficiency of the new vaccine vectors will be tested in human cell culture systems and in ex-
Experimentual animal studies (prophylactic and therapeutic) based on the B16 melanoma mouse model. The outcome of these evaluation studies will be directly applied to the development of new experimental clinical therapy approaches by our unit.

**Experimental Therapy**

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One of the main goals of the group is to apply the methods developed in the laboratory as directly and effectively as possible to clinical practice encompassing diagnostic procedures, e.g. newly detected serum markers, as well as therapeutic approaches, e.g. innovative vaccination strategies. This concept requires a closely knit cooperation between all of the project groups in the Skin Cancer Unit in order to function efficiently. These cooperative efforts are greatly aided by the fact that the laboratory (including a GMP lab for the production of vaccines) and the out-patient treatment facilities are all located in the same building.

Since there is still no effective, generally accepted standard therapeutic regimen for malignant melanoma, a large portion of our work involves conceiving, carrying out and evaluating clinical studies which are designed to test the effectiveness and safety of new therapy approaches [14]. The broad scope and number of the ongoing studies in our unit make it possible to offer innovative treatment with constant and careful monitoring to the majority of patients with cutaneous neoplasms. All of the patients’ reactions to the therapeutic measures and any negative side effects are thoroughly and precisely documented in accordance with the strict criteria required by "good clinical practice" (GCP).

From the very beginning the Skin Cancer Unit has participated in a large variety of clinical trials. These include large-scale completed and ongoing randomized, multicenter studies carried out on the national as well as international level under the auspices of recognized umbrella organizations (ADO, EORTC) as well as smaller cooperative projects with only 2 to 4 other hospitals participating.

The fact that our unit runs its own GMP laboratory in which vaccines for humans can be produced under strictly controlled hygienic conditions promotes prompt transposition from the laboratory to the clinic. This advantage was most prominently evidenced in the first trials on vaccination with autologous dendritic cells in patients with metastasized melanoma [15] which then provided the basis for the large multi-center Phase III trial now running comparing the effectiveness of the vaccination therapy to treatment with chemotherapy. Another vaccination strategy in which peptide epitopes from the melanoma-associated antigens tyrosinase and GM-CSF were applied as adjuvant therapy and in stage IV melanoma in a Phase-II clinical trial was also tested in our unit [16-18].

Several experimental projects carried out by scientists and technical assistants in our laboratory in the framework of basic research have already reached the point where they can be applied in therapeutic systems and tested in phase I/II trials. The great progress achieved in identifying and characterizing new antigens in cutaneous T-cell lymphomas [9] is now being evaluated in regard to its value for application in immunotherapy. Another research group in our unit was able to identify and characterize new tumor-associated antigens for melanoma [12] which could also very well be used for vaccination strategies. Specific T-cell epitopes for both of these malignancies are now going through final analyses and evaluation before being integrated into treatment strategies and tested in clinical trials.

Research in our unit has not only contributed to the knowledge of known prognostic markers but has also lead to the discovery of new serological markers which, as the next step, will be tested and evaluated in a larger number of patients. These studies form a part of an larger cooperative European research effort (consisting of 6 European project groups and financed by a grant from the European Commission). Another cooperative project (in cooperation with Prof. C. Garbe and PD A. Hauschild) is concerned with evaluating various follow-up procedures for melanoma patients. The data gathered in the framework of these three projects will be entered into a high-capacity, computer supported data bank and organized so that relevant clinical and laboratory results can be found called up quickly and accurately to be compared and analyzed. This will provide us with a basis for testing on a large scale the effectiveness of the new serological and tissue-bound marker proteins for predicting the reaction of patients to a specific treatment or the length of their survival time.

**Publications** (* = external co-author)


