Clinical Cooperation Unit Skin Cancer (D0900)

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The Clinical Cooperation Unit Skin Cancer is a division of the DKFZ 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 dermato-oncological outpatient 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 DKFZ, the University of Heidelberg and the Mannheim Clinics, the unit also maintains 4 beds in the dermatology ward for inpatient treatment.

Patients with malignant melanoma in all stages will be the main focus of our efforts, but our unit also provides comprehensive in- and outpatient 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 dermatoncological conferences which are held every Wednesday afternoon (appointments should be made in advance: Tel. +49-621-383-2127).

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 considered to be the main obstacle to successful treatment of melanoma patients. On the other hand, experimental immunotherapeutic approaches are steadily gaining more ground.

Since in recent years so much progress has been made in tumor immunology in general and especially in melanoma research, it has been possible to identify a number of melanoma-associated tumor antigens which are recognized by specific T-lymphocytes. Furthermore, a great deal of knowledge has been gained on the immunological mechanisms which are involved in the recognition and destruction of tumor cells. These discoveries have, in turn, led to the development of several new and interesting experimental therapy concepts for the treatment of melanomas. Some of these have already reached the clinical testing stage.

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

H. Heimbach, B. Gschwendt, E. Rossmann, B. Wasser

In cooperation with: Dr. Hermann Lage, Prof. Manfred Dietel (Charité, Pathology Berlin) und Prof. Dr. Pranav Sinha (Charité, Clinical Chemistry, Berlin), Prof. Bernd Kaina (Applied Toxicology, Mainz), Prof. Annemarie Pousta (DKFZ, Div. H0600), Prof. Peter Krammer (DKFZ, Div. G0300), Prof. Peter Lichter (DKFZ, Div. H0700), Dr. Martina Schönitzer (DKFZ, R0800).

Chemotherapy is an extremely ineffective and unsatisfactory means of treating malignant melanoma due to the drug resistance which is characteristic of this disease and is either intrinsic at onset or develops with application of cytostatic drugs. Alternative treatment methods are presently being developed, but have not reached the stage where they could routinely be applied in clinical practice. For this reason, chemotherapy will continue to be a primary treatment method and urgently needs to be improved and made more effective. This could be achieved if the mechanisms underlying drug resistance could be discovered and examined in order to develop strategies for countering them. An important step in this direction is a detailed analysis of the reasons for the fact that the same drug is able to effectively kill sensitive tumor cells but is ineffective against others. When this phenomena can be explained it will be possible to improve existing treatment methods and to develop new therapeutic strategies.

We want to contribute to this effort by studying drug-resistant and drug-sensitive melanoma cell lines in an in vitro model system (Table 1) (Kern et al. 1997). The mechanisms brought to light by these comparative studies will then have to be pursued further in regard to their relevance and means of functioning. This knowledge, in turn, can be applied to improving chemotherapeutic regimens.

The following cytostatic drugs with different working mechanisms were used:

I. cisplatin: DNA-damage, induces cross-links

II. etoposide: Inhibits DNA-Topoisomerase II, induces DNA-strand breaks

III. fotemustin: DNA-alkylating agent; which induces DNA-strand breaks

IV. vindesin: Mitosis inhibitor, inhibits polymerization of microtubuli.

Table 1: In vitro Model System for Analysis of Drug Resistance in Melanoma. The drug-resistant cell lines are given along with the corresponding cytostatic drugs which were used for selection. The cells were cultured continuously in the presence of the given cytostatic drug and the various concentrations of the drugs used represent the various grades of resistance (Kern et al. 1997).

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Cytostatic Drug Used for Selection</th>
<th>Drug Levels of Resistance (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeWoVIND</td>
<td>Vindesin</td>
<td>0.05 1 2.5 5</td>
</tr>
<tr>
<td>MeWoFOTE</td>
<td>Fotemustin</td>
<td>1 16 40 100</td>
</tr>
<tr>
<td>MeWoETO</td>
<td>Etoposide</td>
<td>0.01 0.1 0.5 1</td>
</tr>
<tr>
<td>MeWoCIS</td>
<td>Cisplatin</td>
<td>0.01 0.1 0.5 1</td>
</tr>
</tbody>
</table>

Once the cell culture models have been established, the search begins for differential expression of genes and proteins in studies comparing sensitive and resistant cell lines which possibly point to specific mechanisms.

A. Apoptosis deficiency

A number of studies have implicated apoptosis (programmed cell death) as an important mechanism by which chemotherapeutic agents kill susceptible cells. Tremendous progress has been made in understanding apoptosis as a result of molecular identification of the key components of this intracellular suicide program. Here it has been suggested that inhibition or dysregulation of apoptosis might be responsible for resistance to anticancer drugs (Eliopoulos et al. 1995; Los et al. 1997). 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 in human melanoma and deficiency in apoptotic pathways leading to drug resistance.

We have analyzed the apoptotic pathway in cisplatin- and etoposide-induced cell death in sensitive MeWo cells in comparison to pathways leading to cell death in resistant melanoma cells derived from MeWo [1]. Etoposide and cisplatin induced apoptotic cell death in drug-sensitive MeWo cells as indicated by dose-dependent DNA-fragmentation and cleavage of poly(ADP-ribose) polymerase (PARP). Further detailed investigations of the pathway revealed that apoptosis is mediated by mitochondrial processes and death-receptor/ligand-systems do not play an essential role. Analog analysis in etoposide-resistant cells demonstrated marked apoptosis-deficiency associated with a block in the signal transduction between Bcl-2 and cytochrome c. Cell death in cisplatin-resistant cells is char-
characterized by an altered apoptotic pathway which results also in final apoptotic features (DNA-fragmentation, caspase-activity), but not however, by the same mechanism (Helmbach et al., 2001, submitted) (Fig. 1).

B. Identification of differentially expressed genes by DDRT-PCR

It is reasonable to assume that various resistance mechanisms do exist which have not yet been discovered. Several molecular biological approaches were chosen in order to identify so far unknown molecules possibly associated with a drug-resistant phenotype. By applying CGH-analysis (comparative genomic hybridization) to drug-resistant and sensitive MeWo cells a high number of additionally acquired genetic imbalances were detected [7]. In order to get further insight, we characterized the sensitive and resistant MeWo cell variants by differential display reverse transcription-polymerase (DDRT-PCR).

In two independent DDRT-PCR approaches differential expression of 114 mRNA fragments could be detected. After at least two independent Northern blot analyses on all MeWo variant cell lines 11 cDNA clones have been confirmed as differentially expressed genes. These cDNA clones DSM-1-DSM-11 (Drug-Resistance- associated Sequence in Melanoma) include 3 Gene (DSM-1, DSM-3, DSM-5) of known function, 4 previously sequenced genes (DSM-2, DSM-4, DSM-6, DSM-7) of uncharacterized function and four novel genes (DSM-8 - DSM-11) without match in Gene Bank. All 11 genes showed altered mRNA expression in high-level etoposide-resistant cells, whereby seven genes (DSM-1-6, DSM-8) were found to be decreased in their transcription rate in these cells. The mRNA-expression of the remaining genes (DSM-7, DSM-9-11) was enhanced in etoposide-resistant cells. The expression of 5 of the genes (DSM-5, DSM-7-10) was modulated in various independently established resistant cell lines suggestive of an association with drug resistance [5]. However, functional assays need to be performed to prove the concept.

All genes found to be associated with drug-resistance need to be characterized concerning their functional relevance for drug-resistance. For some of the genes plasmids with the corresponding nucleotide-coding sequences and relevant antibodies are already available. By transfection of appropriate plasmids stable cell lines will be generated which allow functional testing regarding drug-resistant phenotype. Studies have already started at the DKFZ.

By 2-D-gel analysis we detected over 70 differentially expressed proteins (pH 2.8–pH 10) comparing sensitive and resistant MeWo-Melanoma- cell lines. The identification of the proteins has begun and a marked up-regulation in resistant cells has been detected for the following proteins: TCTP (translationally controlled tumor protein) hEFC-1-δ (human Elongation Factor 1-δ) TRP (tetrapeptide repeat protein) 14-3-3γ (isof orm of the 14-3-3 family).

None of the identified proteins have been associated with chemoresistance up to now. Their function and relevance in the development of drug-resistance is going to be investigated by inhibitor studies and transfection of the corresponding genes. Furthermore, the identification of the remaining proteins should be continued. This will be performed by analyzing existing 2-D-protein-data bases of other cell types or by excision of protein spots and further microsequencing. Subsequent functional assays will require most of the time calculated for these studies.

Publications (* = external co-author)

Tumorantigens/Lymphome

S. Eichmüller, D. Usener, F. Rellerberg, T. Hartmann, J. Bartels, A. Jochim

In cooperation with: PD Dr. Reinhard Dummer (Zurich, Switzerland); Dr. Hermann Lage (Charité Berlin); Dr. Karl Güte (HNO Clinic, Mannheim); PD Dr. Stefan Duebel (Immunology Institute, Heidelberg)

The work of our project group is centered around identifying and characterizing specific tumor antigens, in particular those specific to cutaneous T-cell lymphomas (CTCL) with...
the more far-reaching goal of applying this knowledge to improving existing or developing new therapeutic and diagnostenic methods. In the process of characterization the tumor antigens (TA) are examined at the following levels:

**Screening and expression analyses (Topic I)**
By employing the SEREX method, a serological screening procedure, and by means of DNA hybridization using cDNA phage libraries, new tumor antigens or homologues of previously discovered TA are identified and then examined more closely in regard to their serological specificity (reaction against antibodies from the blood sera of patients and control persons) as well as in regard to their expression in various tissues (RT-PCR and Northern blot analysis). We have been able to identify a number of TAs which reveal serologically interesting aspects. Furthermore, we were able to show that one of these TAs which had previously been identified (SCP-1) and a newly discovered TA (cTAGE-1) display an expression pattern in tissue which is tumor-specific [1]. In order to draw an antigen profile of CTCL which is as comprehensive as possible we also studied the expression of known TAs, especially those belonging to the “cancer-germline-antigen” group, in various CTCL tissues and cell lines. In this manner, for example, LAGE-1 could be identified as a TA which is also expressed in CTCL. Particularly the newly discovered TAs are subjected to extensive RT-PCR analysis in order to study their expression in the tissue and cell lines of various tumors so that their relevance for these tumor entities can be evaluated.

**Synthesis and evaluation of new tumor markers (Topic II)**
All of the new TAs which in the described screening processes (Topic I) proved, at least in their serological reactivity, to be tumor-specific are subjected to further analysis at the protein level. For this purpose proteins are synthesized recombinantly and isolated (His-Tag) so that, on the long term, an ELISA can be established. Tumor-specific antigens, such as the cTAGE-1, are presently being examined in regard to their immunological relevance by means of epitope-mapping experiments in an cooperative effort with Dr. Stefan Dübel (University of Heidelberg).

**Generation of cytotoxic T-Cells against CTCL tumor antigens (Topic III)**
In a final step, tumor-specific TAs are examined in view of their possible usefulness as a target structure for cytotoxic CD8 T-cells. The cytotoxic T-cells generated in this manner are then tested to see if they can be used to kill specifically HLA-matched, TA-positive CTCL lines. For this purpose, TAs are selected which either have been found and described by our group (see Topic II) or which have previously been identified and are known to be expressed in CTCL. In the first series of experiments the generally established and recognized computer programs were used to predict HLA-dependent peptides for LAGE-1 and then to synthesize them and test their binding affinities so that the peptides with strong binding qualities could be used to generate cytotoxic T-cells. These experiments are being performed in cooperation with Dr. Sun of our unit (subgroup “Cellular Immunity”)

Publications (* = external co-author)

**Immunotherapy**

Y. Srõ, A. Stein, U. Hofmann, M. Song, W. Eickelbaum

In cooperation with: Prof. Georgio Parmiani, Dr. M. Sensi (National Cancer Institute, Milan, Italy), Prof. Dr. Marcus Maeurer (Microbiology, Mainz), Prof. Dr. H.-G. Rammensee (Tübingen), PD Dr. Reinhard Dummer, PD Dr. Frank O. Nestle (Dermatology, Zürich, Switzerland), PD Dr. Carmen Scheibenbogen, Prof. Dr. Ulrich Keilholz (FU Berlin); PD Dr. Harald Krophofer (Basel Institute of Immunology, Switzerland).

Over the last decade, the incidence and mortality of malignant melanoma have increased continuously throughout the world. One of the major challenges to clinicians in order to cure this disease is to early detect and eliminate it, since once disseminated, melanoma is incurable due to its high resistance to conventional therapies. Thus, it is imperative to develop new therapeutic strategies to treat or prevent from the micrometastasis. Previous attempts of immunotherapy employing biological reagents (e.g. cytokines, BCG) alone or in combination with the intact tumor cell-based vaccine therapy have not shown any clinical benefit for the patients. Depending on tumor volume immunosuppression might differ from clinical stage to stage and thus it might be difficult (if not impossible) cured (advanced) melanoma by immune therapy solely. This demonstrates that more than one method is needed to treat melanoma in all different clinical stages.

The project group “Cellular Immunity, Immune Therapy & Gene Therapy” is therefore striving to develop a combination of immune- and gene-therapeutic methods with the main focus on experimental work towards improving already established methods, developing new treatment strategies and evaluating their safety and effectiveness for general clinical application.

A therapy method which we want to improve is the vaccination of melanoma patients with peptide-pulsed, autologous dendritic cells [*Nestle FO et al. *Nat. Med.* (1998) **4**: 328-332] [8, 9]. So far this treatment can only be applied to a limited range of patients with a defined HLA-haplotype for which antigenic peptides derived from tumor associated antigens have already been characterized. In order to widen this spectrum it is necessary to identify more of the relevant protein epitopes that can be integrated into therapy methods. Therefore the primary goal of our work is to identify as many of these peptide epitopes as possible [3]. The studies performed thus far have concentrated on characterizing HLA-class I binding peptides which arise from the processing of the differentiation antigens TRP1, TRP2 and tyrosinase and from the cancer-testis antigen LAGE 1.

A number of *in vitro* studies have shown that cytotoxic T-lymphocytes (CTL) are able to lyse melanoma (tumor) cells with certain variations depending on the HLA and tumor antigens in question [1-3,5]. The goal of palliative as...
well as curative adjuvant melanoma therapies is to achieve a specific stimulation of the immune system through the induction of CTLs. This approach is currently being evaluated in preliminary experimental therapy studies and will also be applicable to other tumor types [2,4,7]. The prerequisites and conditions which determine the success of this kind of therapy approach are manifold and, at this time, not completely understood. The following steps, among others, are important for achieving a better understanding of these phenomena:

1. It is still not known what kind of immunization is the most effective for achieving an efficient CTL induction in vivo. Along with determining the “right” antigen, the following questions must be answered: What is more effective; the peptide (which one?) by itself or the peptide + the adjuvant (which one?) or the peptide + dendritic cells (DC generated how?) or gene-modified DC or the whole protein etc.? These questions will be addressed in small clinical studies in cooperation with the EORTC (European Organization for Research and Therapy of Cancer) and with the “ADO” (project group for dermatological oncology).

At present a large, multi-center prospective randomized clinical study is being carried out in which patients with metastasized melanoma are either immunized with peptide-loaded dendritic cells or receive the standard chemotherapy with DTIC. The study was initiated in December 2000 and is financed by a grant from the German Cancer Aid Society (Deutsche Krebshilfe). The treatment and statistical evaluation of 240 patients will take from 2 to 3 years.

2. Monitoring the reaction to vaccination: Here great deal of work is necessary to establish and standardize the optimal method of monitoring the immune response during and following immunization. As yet, there are no simple and reliable techniques available for this purpose. Complicated and time-consuming analysis procedures - such as T-cell frequency analysis by means of limited dilution - are not feasible for larger numbers of patients. Several different monitoring procedures are now being - and will also in the future be - evaluated in cooperation with the EORTC Melanoma Cooperative Group and with other immunological research groups.

Publications (* = external co-author)


**Gene Therapy**

A. Paschen, A. Sucker, H. Rothfels, D. Thomas, E. Gelen, S. Christmann, C. Sterzik, C. Holmsee, R. Heber

In cooperation with: Dr. Jürgen Kleinschmidt (DKFZ Div. F0100), Dr. Wolfram Osen (DKFZ, Div. F0200) Prof Dr. Trinad Chakraborty, PD Dr. Eugen Domann (Microbiology, Gießen), PD Dr. Gerd Sutter/Dr. Ingo Drexler (GSF Munich), Prof. Dr. Uwe Haberkorn (Nuclear Medicine, University of Heidelberg), Dr. Siegfried Weiss (GBF Braunschweig).

Experience in the treatment of melanoma patients with IL-7- and IL-12-gene-modified tumor cells has already been gained in pilot studies in Berlin [2, 3]. Studies are now being planned in which the immunization of melanoma patients with gene-modified tumors cells will be carried out in a manner analogous to the pilot studies.

Furthermore, in vitro work is now being done on developing a concept for performing in vivo gene transfers. Before this can be done, a vector has to be developed which takes into consideration the safety measures which are necessary for it to be applied directly in vivo. For this purpose, the tentative plan is to integrate several safety steps which include, among others, tissue-specific promoter elements, pharmacologically inducible regulator elements and so-called suicide genes. The development of the vector in cooperation with competent research groups here in the Heidelberg area will be tested in various cell culture models in vitro and then, subsequently, in tumor animal models which have already been established.

In addition, different methods for applying the nucleic acids will be tested in the animal model in order to achieve an efficient transduction and expression in the tumor cells. In addition to the evaluation of various viral vehicles, non-viral systems such as liposomes will also be tested. This effort should be supported as far as possible by know-how which is already available here in the DKFZ.

Although impressive clinical results have been achieved with immune therapies based on peptide-pulsed dendritic cells in early clinical phase I/II studies, the in vitro generation of autologous DC and synthesis of peptides are ex-
pensive and time-consuming. Consequently an alternative vaccination strategy should be developed by which the anti-tumor vaccine can be delivered directly in vivo to the immune system's antigen-presenting cells. This effort comprises the work of our group on the topic area “Immune Therapy”, concentrating on the development of new vaccine vectors for in vivo tumor therapy. We focus on the use of bacteria as live vector systems capable of infecting dendritic cells, thus bringing the vaccine directly to its ideal targets. The live vectors we use are attenuated variants of gram-positive and gram-negative bacteria: Listeria monocytogenes (gram+) is employed as a vehicle for synthesis and transfer of melanoma-associated antigens to DC whereas Salmonella typhimurium (gram-) serves as carrier system for an anti-tumor DNA vaccine. At the moment, most of the work is being done on two main subjects: (1) study of the general interaction of bacteria with in vitro generated human DC; (2) analyses of bacteria-mediated vaccine transfer to in vitro generated hDC. This will be followed by in vivo experiments in order to estimate the efficacy of the bacterial vaccine vectors in mouse models transgenic for HLA-class I and HLA-class II, respectively.

As mentioned at the beginning, immune therapeutic treatment modalities seem to be rarely effective in inducing an effective anti-tumor immunity in the disease stage of a high tumor load. For this reason, our research on the topic area “Gene Therapy” is aimed towards developing a gene therapeutic method to reduce tumor mass. This strategy is based on the principle of a self-destruction of tumor cells which is achieved through the endogenous expression of “suicide genes”. Therefore corresponding DNA sequences encoding these therapeutic genes must be efficiently delivered into and selectively expressed by the tumor cells. So far our on-going laboratory experiments mainly focus on the last aspect: we want to achieve a high rate of tumor cell restricted “suicide gene” expression by employing specific regulatory DNA elements (promoters and enhancers) controlling gene transcription. These therapeutic DNA constructs will then be packaged into a viral vector system which will allow an efficient transfer of the genetic material into the tumor cells and will finally be tested in the mouse system for their capability to induce a reduction of tumor mass in a transplantable melanoma tumor model.

In the course of our work we will use bacteria (L. monocytogenes and S. typhimurium aroA) as vector systems for in vivo delivery of antitumor vaccines to dendritic cells. L. monocytogenes variants will be employed for synthesis and transport of melanoma-associated antigens and an attenuated auxotrophic mutant of the bacterial strain S. typhimurium (S. typhimurium aroA) will be applied as a carrier for DNA vaccines. While in the first application bacterially synthesized antigens (no post-translational modification) should induce an anti-tumor immune response, in the second one induction should be brought about by an antigen which, after vaccine uptake, is synthesized by the infected host cell itself (antigen in the “native state”). Consequently, we have the possibility to test two different bacterial vector systems in combination with two alternative vaccine forms for their efficiency to target dendritic cells.

Although Listeria was successfully used as a carrier for model tumor antigens in the mouse system, these bacteria, unfortunately, have a negative effect on the process of antigen presentation by murine dendritic cells to CD4+ T-cells (induction of apoptosis and T-cell-anergy through infections). For this reason the interaction of Listeria with human DC generated from peripheral blood monocytes was tested first. It could be shown that immature dendritic cell, in contrast to mature DC, were able to efficiently phagocytose Listeria and that bacterial infection induced maturation of the DC. Most important, under the experimental conditions used infection of hDC with Listeria did not have any negative effect on antigen presentation to specific CD4+ T-cells [1]. These results further contribute to the conception of employing Listeria as vaccine vector for targeting dendritic cells. In order these bacteria particularly suitable as carrier for melanoma-associated differentiation antigens (TRP1, TRP2) listeria-specific expression plasmids were constructed which regulate antigen expression through bacterial DNA control elements. These expression plasmids were electroporated into the L. monocytogenes EGD wild strain as well as into 2 other attenuated variants. These mutants are restricted in their ability to infect neighboring cells and are therefore optimized vaccine carriers. After plasmid electroporation bacterial transcription of the heterologous genes could be demonstrated by means of RT-PCR

Publications (* = external co-author)

