**Division Viral Transformation Mechanisms (F0300 / F030)**

**Head:** Prof. Dr. Frank Rösl

**Scientists**
- Dr. Adriana Aguilar (-12/01)
- Dr. Anastasia Bachmann
- Dr. Patrick Finzer
- Dr. Elke Kehm
- Prof. Dr. Karl-Werner Knopf
- Dr. Martin Müller
- Dr. Andrea Patzelt (-10/01)
- Dr. Ulbaldo Soto
- Prof. Dr. Rainer Zawatzky

**Guest Scientists**
- Vladimira Durmanova (6 - 7/02)

**PhD and MD Students**
- Sabine Baars (-2/01)
- Johanna de Castro Arce (12/01)
- Marcel Jung
- Christian Kunzten (cand. med.) (-12/01)
- Heike Lindegger (4/01)
- Stephanie Mattil
- Nico Michel
- Tina Rüter
- Ralf Spannagel
- Alfonso Vega (09/02 - )
- Robert Ventz (cand. med.) (-5/01)

**Diploma Students**
- Monika Hann (-04/02)
- Petra Galmacher (2/02 - )
- Georg Pougialis (-12/01)
- Christina Weber (-10/01)
- Heibert Wurmbäck
- Johanna de Castro Arce (cand. med.) (-10/01)
- Johanna de Castro Arce (cand. med.) (-10/01)

**Technicians**
- Petra Galmacher (2/02 - )
- Georg Pougialis (-12/01)
- Christina Weber (-10/01)
- Heibert Wurmbäck

**Secretary**
- Diana Steiner

**Supporting Personal**
- Alexandra Lichtenwald
- Sabine Boite
- Janina Görnemann (-4/02)
- Markus Klehr
- Diana Mas (11/02 - )
- Henrich Michaely (-5/01)
- Katja Parsche (-2/02)
- Sabine Schenk
- Jan van Riggelen

**Applied Tumor Virology**

**Division F0300**

**Viral Transformation Mechanisms**

**Composition of the transcription factor AP-1 in HPV16/18 positive cells and derived somatic cell hybrids**

U. Soto, P. Finzer, J. de Castro, J. van Riggelen, E. Goeckel-Krzikalla, F. Rösl

In cooperation with: Dr. Peter Angel, Prof. Dimitri Komitowski, DKFZ

Although AP-1 is considered as a positive regulatory protein in all HPV types investigated so far, our recent experiments strongly suggest that AP-1 seems to be also a central key element of an intracellular surveillance mechanism negative controlling HPV transcription and cell proliferation (Soto et al., 1999; Oncogene 18: 3187-3198) [1]. AP-1 consists of different nuclear proteins (e.g. c-jun, junB, junD, c-fos, fosB as well fra-1 and fra-2) which can form either homo- or heterodimers to build up a functional transcription complex. The corresponding binding sites are located within the HPV upstream regulatory region and are indispensable for the expression of the viral oncoproteins E6 and E7. As previously shown, AP-1 seems also to be involved in negative regulation of viral transcription, because treatment of HPV-positive cells with the antioxidant pyrrolidine-dithio-carbamate (PDTC) selectively reduced the amount of viral mRNA at the level of initiation of transcription, which was accompanied by an enhanced affinity of jun-members with the fos-related antigen-1 (fra-1) (Rösl et al. 1997; J. Virol. 71: 362-370). This was of particular interest, since fra-1 can abrogate AP-1 activity under certain experimental conditions (Yoshioka et al., 1995; Proc. Natl. Acad. Sci. USA 92: 4972).

Furthermore, using non-tumorigenic and tumorigenic somatic cell hybrids made between the HPV 18-positive cervical carcinoma cell line HeLa and normal human cells (Stanbridge, 1984; Cancer Surv. 3: 335) as model system, we found that AP-1 composition differed considerably between these cell lines. Monitoring nuclear extracts from non-tumorigenic hybrid cells, jun-family members (in the order c-jun > junD > junB) were again mainly heterodimerized with fra-1. In contrast, fra-1 concentration was low in extracts from tumorigenic segregants of the same hybrids and in parental HeLa cells. Conversely, c-fos the canonical dimerization partner of jun proteins was expressed in malignant HPV-positive cells, but was absent within AP-1 complexes from non-tumorigenic hybrids. Similar findings were obtained in other cervical carcinoma cell lines and freshly HPV 16 immortalized human keratinocytes. Changes in AP-1 composition and stoichiometry have profound effects on non-malignant HPV-positive cells: ectopical expression of c-fos under the control of a heterologous promoter induces tumorigenicity, which was preceded by a change of a jun/fra-1 ratio towards a constellation initially detected in the malignant counterparts. Furthermore, malignant conversion was accompanied by a resistance against TNF-α, capable to...
selective suppress HPV 18 transcription in formerly non-malignant cells. c-fos overexpression also abrogated the transcription of particular chemokines (= chemotactic cytokines), which regulate the network of infiltrating immunomolecular effector cells necessary to control persisting viral infections (for review, see Miller and Krangel, 1992; Crit. Rev. Immunol. 12: 17) (see also Soto et al., 1999; Oncogene 18: 3187-3198) [1, 2].

The potential link of fra-1 in negative regulation could be of functional relevance, since the gene is located on chromosome 11 (11q13) (Sinha et al., 1993; Genomics 18: 165), a chromosome, which has tumoursuppressive function on cervical carcinoma cells (Saxon et al., 1986; EMBO J. 5: 3461; Koi et al., 1986, Mol. Carcinogenesis, 2: 12). Moreover, RFLP analyses have shown that this region is often found to be deleted or structurally rearranged in cervical carcinoma (Jesudasan et al., 1995; Am. J. Hum. Genet. 56: 705). In fact, all cervical carcinoma cells tested so far either lack or express very low levels of fra-1. To establish complementation groups between different cervical carcinoma cell lines on the basis of AP-1 composition, the technique of somatic cell hybridization was applied. The resulting hybrids were examined for their in vivo phenotype in immunocompromized animals. Following up these studies, we could show that suppression of tumorigenicity again correlated with reconstitution of an AP-1 composition pattern, which mainly consisted of jun/fra-1 heterodimers (1:1 ratio). In contrast, fra-1 was even diminished in malignant hybrids, while c-fos remained expressed or was even up-regulated. The findings propose a novel role for AP-1 as an essential component of inter- and intracellular surveillance mechanisms controlling HPV transcription, cytokine sensitivity, chemokine expression (e. g. MCP-1) as well as cell proliferation in vivo [2; 5].

TNF-α, interferon and chemokine signalling in malignant and non-malignant HPV-positive cells

A. Bachmann, R. Zawatzky, U. Soto, P. Finzer, J. van Riggelen, T. Ritter, F. Rösl

In cooperation with Dr. Philippe Delvenne, Dept. Pathology, University of Liege, Belgium (European Network Program).

Beside disturbance of a functional T-cell surveillance, dysregulation of chemokine expression may represent another important event during the multi-step progression to cervical cancer. Chemokines (e. g. "monocyte-chemoattractant-protein-1" MCP-1) are responsible for the recruitment and activation of T-cells, NK cells and macrophages (for review, see Miller and Krangel, 1992; Crit Rev. Immunol. 12: 17). Inappropriate chemokine expression may also explain the often observed depletion of immunomolecular effector cells within malignant HPV-positive lesions (Spinillo et al., 1993; Gyn. Oncol. 48: 210). Conversely, spontaneous regression of benign warts is consistently accompanied by an enhanced macrophage infiltration and TNF-α synthesis (Hagari et al., 1995; J. Invest. Dermatol. 104: 526). In fact, TNF-α seems to be a key cytokine with pleiotropic functions, since it can both suppress viral transcription and induce chemokine (MCP-1) gene expression, but only in non-malignant cells (Rösl et al., 1994; J. Virol. 68: 2142). It is therefore likely that TNF-α may not only be involved in wart regression, but could play also an important role in the immunological control of HPV-positive dysplastic cervical lesions. Another interesting property of TNF-α is its ability to confer an antiviral state through induction of the interferon- (IFN-β) gene (Mestan et al., 1986; Nature 323: 816). Type I interferon (IFN) production is the most rapid host immune response against a whole variety of viral infections (for review, see Guidotti and Chisari, 2000; Virolgy, 273: 221).

Using somatic cell hybrids as model system, we demonstrated that the conversion to a malignant phenotype was accompanied by the loss of interferon-(IFN)-β synthesis. IFN-β is inducing the “delayed interferon response” and is responsible for signal amplification and finally for the actual antiviral/growth-inhibitory properties of type I interferons. Similar to MCP-1, IFN-β gene could be only induced by TNF-α in non-malignant HPV-positive cells. Biological activity of TNF-α induced IFN-β was directly confirmed in functional assays by protecting the cells against encephalomycarditis virus (EMCV) infections. The addition of neutralizing antibodies against IFN-β block this effect, clearly excluding the possibility that other interferon types were involved. Conversely, all cells independent from the in vivo phenotype could be protected against EMCV lysis when either IFN-α/β or IFN-γ was supplemented exogenously into the tissue culture medium. These data indicate that TNF-α induced interferon signalling (= “immediate-early” IFN response) is perturbed in cervical carcinoma cells. Testing 13 different HPV 16/18 positive cell lines, EMCV protection was only restricted to non-malignant cells, indicating that the antiviral effect correlates with the growth inhibitory and virus-suppressive properties of TNF-α. In addition, we could provide evidence that the “interferon-regulatory factor-1” (IRF-1) and the DNA-binding subunit of the “interferon-stimulated gene factor 3” (ISGF3) p48 (responsible for the “delayed” interferon response) can only be induced by non-tumorigenic cells. This data strongly suggest that the mere viral onconogene expression is not necessarily abrogating IRF-1 and p48 function, but strikingly depends on the genetic context and the in vivo properties of the cells [8].

Inhibitors of histone-deacetylase can bypass HPV oncogene function

P. Finzer, C. Kuntzen, U. Soto, R. Ventz, F. Rösl

In cooperation with Dr. H. Cederberg, “Triple Crown America Inc”, Perkasie, PA, USA.

Modulation of histone acetylation is a regulatory process, which controls the nucleosomal organization of bulk cellular DNA and in turn gene expression. Changes in the chromatin architecture is coordinated by a concerted interplay between histone acetylases (HAT) and deacetylases (HDAC), which are also targets of the viral oncoproteins E6 and E7 (Patel et al., 1999; EMBO J. 18: 5061; Brehm et al., 1999; EMBO J. 18: 2449). The present
The role of apoptosis in the development of cervical cancer
A. Aguilar-Lemarroy, A. Vega, F. Rössl

In cooperation with: Prof. Patricio Gariglio, Cinevestav, Mexico-City, Mexico; Prof. P.H. Krammer, DKFZ.

Besides the above mentioned immunological escape mechanisms, accumulation of cervical intraepithelial cell abnormalities could be also favoured by disturbances in apoptotic signal transduction (Nair et al., 1999; Pathol. Oncol. Res. 5: 95). Considering tumor formation simply as the result of a numerical dysbalance between cell loss (e.g. through immunological elimination) in favor to cell gain (via uncontrolled proliferation and ignorance of cellular homeostasis signals), there is in fact substantial evidence that certain human pathogenic viruses can selective interfere with apoptosis. Apoptosis can be achieved by a variety of external signals such as UV light, nutritional depletion, growth factor withdrawal or chemotherapeutic drugs. The most physiological way, however, to trigger programmed cell death is the engagement of CD95 receptor to its ligand (CD95L) (for review, see Krammer 2000, Nature 407: 789). Whether immortalized or malignant HPV-positive cells are sensitive to such natural apoptotic stimuli is an important question to-wards our understanding of immunological surveillance of persisting “high-risk” HPV infections.

To unravel the function of the individual oncogenes of “high risk” HPV 16 in modulating the cellular response to apoptogenic signals, we used human keratinocytes, which were separately immortalized either with E6, E7 or E6/E7 oncoproteins of HPV16 via retroviral gene transfer. Applying agonistic CD95 antibodies (APO-Ab) or recombinant CD95 ligand, only E7-immortalized cells undergo strong apoptosis. In contrast, E6- and E6/E7-expressing keratinocytes were resistant under the same experimental conditions. The dominance of E6 correlated with a significant down-regulation of p53, c-myc and p21 on protein level, but without significant alterations of Bcl-2 or Bax.

CD95 was only slightly reduced in resistant cells, while there were no quantitative differences in the expression levels of “Fas-associated death domain” (FADD), caspase 8 or caspase 3.

CD95-resistant HPV 16 E6-and E6/E7 positive keratinocytes, however, can be sensitized to agonistic CD95 antibodies or CD95 ligand (CD95L) after short-term inhibition of proteasome-mediated proteolysis. Cells undergo apoptosis already 3 hours after combined administration of MG132/APO-Ab (or CD95L), which was preceded by a strong re-expression of p53 and c-myc. MG 132 itself was not able to induce apoptosis even after incubation for 8-12 hours. Reactivation seems to be specific for these molecules, since the levels of other known proteasome-controlled proteins such as Bax, p21 or IkB-α were not affected. Conversely, carrying out the same experiments with cervical carcinoma cells harbouring a mutated p53 protein (C33a) or p53 “null” lung carcinoma cells (H1299), no resensibilization occurred, despite c-myc was induced. These data indicate that the reduced bioavailability of p53 is the major regulatory event in perturbing the CD95 response in HPV 16 immortalized cells [6; 7]. Hence, therapeutic strategies which selectively re-activate p53 may help to sensitize HPV16 positive cells to CD95-mediated apoptosis, render them more susceptible to immunological control.

In contrast to immortalized non-malignant cells, tumori-genic HPV16 and HPV 18-positive cervical carcinoma cells were refractory against agonistic CD95 antibodies or CD95L addition even when p53 was present and expressed in moderate levels. Furthermore, only HPV 18-expressing cells can be resensibilized to CD95-induced apoptosis when treated in combination with cycloheximide (CHX), while HPV 16-positive cervical carcinoma cells remained resistant under equivalent conditions. Somatic cell hybridizations between CD95a and CD95a cells revealed that CD95 sensitivity was a dominant trait. Although CD95a cervical carcinoma cells expressed even higher level of p53 and CD95 receptor at their surface than their counterparts, resistance was attributed to the inability to form a functional “DISC”. On the other hand, “DISC” formation could be complemented in CD95a × CD95a hybrids. These data indicate that resistance to CD95-mediated apoptosis may not only represent an additional immunological escape mechanism during HPV-induced carcinogenesis, but may also explain the differ-
ent incidence rates of HPV 16 and HPV 18 types in cancer patients [6, 7].

Publications (* = external co-author)


Tumorvirus-specific vaccination strategies
In collaboration with Prof. Dr. L. Gissmann, Dr. H.W. Zentgraf, Dr. J. Kleinschmidt, all DKFZ, Prof. Dr. H. Müller, Universität Leipzig, Dr. M. Kast, Loyola University Chicago, Prof. Dr. U. Sonnewald and Dr. S. Biemelt, IPK Gatersleben, Prof. Dr. H. Will, Heinrich-Pette Institut Hamburg

15-20% of all human tumors worldwide are associated with viral and bacterial infections. Cervical cancer alone represents the second most common cancer among women with half a million of new cases every year. The prime risk factor for cervical cancer is the infection with human papillomaviruses (HPV). It is one major objective of the research team to develop new virus-specific vaccination strategies and to improve the efficacy of existing strategies. Preference is made for therapeutic vaccination approaches which are addressed in a number of different projects.

Improved induction and detection of HPV 16 E7-specific cytotoxic T-lymphocyte responses after DNA- and protein-based vaccination
N. Michel, M. Müller

DNA vaccination is a promising approach for inducing both humoral and cellular immune responses. For immunotherapy of human papillomavirus type 16 (HPV16) - associated disorders the HPV 16 E7 protein (E7) is considered a prime candidate, as it is expressed in all HPV-16-positive tumors. Unfortunately, the E7 protein is a very poor inducer of a cytotoxic T-cell response, when being used as antigen in DNA vaccination. We could demonstrate that certain recombinant E7 proteins possess a dramatically increased potential to induce E7-specific cytotoxic T-lymphocyte responses after DNA vaccination of mice [1,2,6]. The increased immunogenicity was observed with E7 proteins that were modified in several alternative or cumulative ways: (i) fusion with signals that mediate the classical or non-classical export of the protein out of DNA transfected cells, (ii) fusion with a carrier protein that leads to a more than 1000-fold higher intracellular steady-state Level as compared to unmodified E7 protein, (iii) redirecting the nuclear E7 protein into the lumen of the endoplasmic reticulum (ER). Our data suggest that the molecular mechanisms that control induction of an E7-specific immune response after DNA vaccination are rather complex. It is possible that the increased transfer of antigen from DNA transfected cells to professional antigen-presenting cells (APCs) (cross priming) contributes to the enhanced immunogenicity of the modified E7 proteins. This cross priming might be mediated by the function of the export signals, that were
fused to the E7-protein, or by the higher steady state level of the proteins in the cells, ending up in the release of higher amounts of protein after apoptotic death of such cells. Alternatively, the modified E7 proteins might have lost their specific immune suppressive potential, that was recently described in the literature, leading to an enhanced direct priming of immune responses by directly transfected APCs.

In an independent approach we intended to improve the induction of an E7-specific CTL responses after vaccination of mice with bacterially expressed and purified E7 proteins. To this end, three different “cell penetrating peptides” (CPPs) were tested for their ability to import fused E7-proteins into the cytoplasm of cells. We hypothesized that this may lead to an enhanced MHC-I presentation of E7-epitopes by the cells and finally allowing the induction of a more potent E7-specific CTL response after protein-based vaccination in vivo. Unfortunately, the CPPs showed neither in vitro, nor in vivo any significant impact on the immunogenicity of the fused E7-proteins. Because it is reasonable, that the full length E7 protein hampers the CPP-mediated transport, several modified E7-proteins were subsequently designed that may demonstrate better transport capabilities. The immunogenicity of these proteins is currently under investigation. Furthermore, we generated two potent monoclonal antibodies against E7, both of which are detecting the protein in a sensitive and specific manner in western blot and immunofluorescence. Moreover, this led us to a new vaccination strategy in which bacterial expressed E7 proteins will be applied to mice in the from of immune complexes formed by the newly produced antibodies.

During our studies, we set up four different detection methods for murine E7-specific CTL responses in our laboratory. In a comparative analysis we were able to define conditions that allow the alternative usage of each of the four assays leading to an identical interpretation of mice in positive or negative for E7-specific CTLs.

The equine sarcoid: a model for the development of a therapeutic, papillomavirus-specific vaccine

S. Bolte, S. Mattil, L. Gissmann, H. Müller, M. Müller

Human Papillomaviruses cannot be propagated in vitro. This circumstance has hampered the development of vaccines based on e.g. inactivated or attenuated HPV virions. It was demonstrated a couple of years ago, however, that upon expression in a number of different experimental systems the viral structural proteins assemble spontaneously into so called virus-like particles (VLPs). These VLPs are structurally and functionally similar to infectious virions. They are able to bind to the cellular receptor for the Papillomaviruses and even enter into cells. Since VLPs lack the viral genome, this interaction does not lead to a viral infection. Upon immunization with VLPs, animals develop anti-capsid antibodies that are able to neutralize infectious virions. These animals are, therefore, protected against challenge with papillomaviruses making VLPs ideal candidates for a prophylactic vaccination against HPV-associated diseases.

By fusion of the papillomavirus major capsid protein L1 with viral tumor antigens we were able to extend the applicability of VLPs to the tumor therapy. Similar to L1, the L1 fusion proteins assemble into so called chimeric virus-like particles (CVLPs). We were able to demonstrate that CVLPs induce cytotoxic T-lymphocytes, directed against the viral tumor-antigen in immunized mice. In a mouse tumor model, animals were protected specifically against challenge with tumor cells. In addition, it was possible to cure the mice from existing, experimentally induced tumors. In parallel to the rather artificial mouse tumor model it was desirable to test the therapeutic properties of CVLPs on naturally induced PV-associated tumors. In collaboration with the Veterinärmedizinische Fakultät der Universität Leipzig we initiated a clinical phase I study for the therapy of equine sarcoïds, a frequent skin tumor among horses induced a the bovine papillomavirus. Sarcoïds are difficult to treat by conventional means and they are therefore a serious problem for veterinarians, horse-breeders and -owners. Based on our earlier results on HPV 16, we developed and produced BPV 1 CVLPs consisting of a BPV 1 L1/E7 fusion protein. Initially, this vaccine was tested on a total of 15 horses suffering from BPV-associated sarcoïds. Aim of the study was to determine whether the vaccine is tolerated by the horses and whether a specific immune response against the BPV antigens can be induced. In addition we recorded the tumor status of the animals.

The clinical trial revealed that the animals tolerated the vaccination well. Even after repeated vaccination using dosages up to 2.5 mg CVLPs no unwanted side reactions were noticed. All but one animal developed high titer antibodies directed against BPV virions. In some animals positive effects on the tumor status were recorded ranging from complete loss of tumors to halt of tumor growth. In other animals, however, the existing tumors continued to grow. This might be explained by the fact that the study group comprised animals with a severe stage of disease, some animals carrying more than 60 tumors. However, we cannot rule out at this time, that MHC-I haplotype plays a role in the observed clinical responses. In a follow up study, we started to immunize animals with a more moderate disease status and preliminary results indicate that CVLPs are a promising vaccine for the treatment of equine sarcoïds. Currently, we are planning a placebo-controlled phase II study in collaboration with the University of Leipzig and INTERVET. To be able to measure cellular immune responses in vaccinated horses, we developed an ELISA to quantify production of equine interferon gamma. We produced monoclonal antibodies that will be used in an ELISPOT assay system for the detection of virus-specific cytotoxic T-cells.
HPV transgenic plants for prophylactic vaccination against papillomaviruses
S. Biemelt, U. Sonnewald, M. Müller

Cervical cancer is linked to infection with human papillomaviruses (HPV) and is the third most common cancer among women worldwide. There is a strong demand for the development of an HPV preventive vaccine. Transgenic plants expressing the HPV major capsid protein L1 could be a system to produce virus-like particles for prophylactic vaccination or could even be used as edible vaccines to induce an L1-specific prophylactic immune response. In close collaboration with the IPK Gatersleben we generated transgenic tobacco and potato plants carrying the HPV 16 major structural gene L1 under the control of the cauliflower mosaic virus 35S promoter. All attempts to express either the original, unmodified L1 gene or an L1 gene with a codon usage optimized for expression in plants failed. Surprisingly, small amounts of the protein were detected using an L1 gene optimized for expression in human cells. However, northern blot analysis revealed that most of the L1 transcripts were degraded. Introduction of the translational enhancer Ω derived from the tobacco mosaic virus strongly increased transcript stability and resulted in accumulation of L1 protein to 0.5% and 0.2% of total soluble protein in transgenic tobacco and potato plants, respectively. The plant-derived L1 protein displayed conformation-specific epitopes and assembled into virus-like particles. Furthermore, we did not find any indications of protein modification of the L1 protein produced in plants. Most importantly, plant-derived L1 was as immunogenic as L1 expressed in baculovirus-infected insect cells. Currently, L1-transgenic potato tubers are being fed to mice to determine, whether the animals develop a humoral immune response against papillomavirus capsids. Early results from these experiments indicate that oral uptake of transgenic plant material leads to an induction of a humoral immune response against the viral capsid and this response seems to be dependent on the co-administration of an oral adjuvant.

Genetic vaccination for the prevention and treatment of HPV-associated diseases
C. Leder, J.A. Kleinschmidt, D. Kuck, M. Müller

The objective of this project was to determine whether vectors based on the Adeno-associated virus or naked DNA is suitable as genetic vaccination against HPV 16 infections. As antigen we used the HPV 16 L1 gene alone or fused with parts of the E7 gene. This approach should allow both, prophylactic (against L1) and therapeutic vaccination (against E7). For an efficient genetic vaccination it was necessary to produce an L1 gene with an altered nucleotide composition. We synthesized a humanized version of L1 in which 78% of the codons were modified to achieve higher expression levels in human cells. We could demonstrate that this gene expressed at least 10000 fold better compared to the un-altered L1 gene. We also observed a significantly improved immunogenicity of the codon-optimized genes. Using the L1/E7 fusion proteins in the vaccination experiments revealed that nuclear localization of the capsid proteins, which is abolished for the fusion protein, is an important factor in the induction of a humoral immune response. Sedimentation analysis revealed differences in the ability of the unfused L1 protein and the different fusion proteins to assemble into higher complexes such as capsomers or virus-like particles and these observed differences seem to be dependent on nuclear localization of the various proteins. On the other hand, the induction of E7-specific cytotoxic T-cells was independent of nuclear localization.

Identification and characterisation of cellular proteins as interaction partners of the minor capsidprotein L2 of human papillomaviruses
J. Görnemann, T. Hofmann, H. Will, M. Müller

Two structural proteins form the Papillomavirus (PV) capsids. While the functions of the major structural protein L1 are well established, the exact functions for the minor structural protein L2 are much less well defined, except for some information on a role in viral entry and maturation of infectious virions. To gain more insight in the function of L2 we used the yeast two hybrid system with the HPV 11 L2 and HPV 16 L2 as bait proteins to isolate putative cellular interaction partners. We identified four proteins interacting with L2 proteins of at least two different HPV types and this interaction was confirmed in vitro by pull down assays [5]. Further evidence for this interaction was obtained by in vivo localization studies. Two of the proteins, the previously described PATZ and a novel protein, designated PLINP, were localized in discrete nuclear domains and colocalized with L2. The third protein, designated PMSP, is a newly identified cytoplasmic protein which was recruited to nuclear dots (PODs, PML oncogenic domain) when coexpressed with L2. The fourth protein interacting with HPV16, 11 and 1 L2, the tubular-nephritis antigen related protein (TIN-Ag-RP) shows a cytoplasmic as well as a membrane bound subcellular distribution. Taken together, our data indicate that L2 of HPVs with different phenotypes interacts with several cellular host proteins, recruits one of them to the nucleus, and is complexed with at least three cellular proteins in specific nuclear domains. These findings suggest an HPV type-independent modulatory function of L2 on host cell functions that involves discrete nuclear domains and alteration of the subcellular distribution of cellular proteins. The interacting cellular proteins identified may play a role in the viral life cycle and establishment of viral persistence.

Publications (* = external co-author)
Regulation of constitutive interferon (IFN) synthesis in macrophages

R. Zawatzky, A. Weyland, H. Wurmbeack

Interferons (IFN) are secreted glycoproteins with pleiotropic activities. IFN-α and IFN-β - often referred to as type I IFN - are mainly induced by viral infections in a wide variety of different cell types whereas IFN-γ - type II IFN - is released from activated T-cells upon stimulation with mitogens or specific antigen. Both type I and type II IFN have pronounced antiviral activities and are indispensable for the recovery from viral infections. Although in vitro nearly all cells are capable of generating an IFN-α/β response following viral infection, macrophages are considered the most important IFN producing cells. They are preferentially located near potential entry sites for pathogens and start a pronounced IFN synthesis within 3-4 hours after contact with virus, well before other cells respond. For the defense against invading microorganisms macrophages are dependent on the presence of IFNs. The term IFN-macrophage alliance was coined to stress that macrophages are both producers and targets of type I IFNs. Our intention is to identify the biochemical mechanisms for induction and maintenance of constitutive IFN synthesis in primary macrophages.

We designed experiments to find out the reason for the early and vigorous onset of virus induced IFN production in macrophages. We suspected an autocrine priming effect due to the continuous secretion of IFN-β by infected cells. Due to the constitutive release of IFN-β, macrophages also have elevated levels of IRF-1. We have therefore planned to study IFN-β expression in IRF-1 deficient mice. Finally, it is planned to study the influence of nitric oxide (NO) on the expression of constitutive IFN-β in macrophages. NO is released by a variety of cells and can act as a positive and/or negative regulator of IFNs and other cytokines.

Molecular cloning and characterization of interferon-induced genes in mouse macrophages

R. Spannagel, R. Zawatzky

In cooperation with: Dr. Martin Müller and Dr. Peter Krieg, DKFZ

In vivo type I interferons (IFN-α/β) are mainly produced following infection of cells with virus. The release is very rapid in order to induce an antiviral state in neighbouring cells not yet attained by progeny viruses. Besides direct inhibitory effects on virus replication, IFN are involved in stimulating the non-specific host defense and in building up a host immune response. A large set of genes are known to be activated by IFN and to participate in the host response to infection. Among these genes are MHC-class I, C1q and the dsRNA dependent genes 2’,5’oligo-adenylat-synthetase, RNase L and p68 kinase. We were interested to identify novel genes induced by IFN in macrophages since these cells play a central role in organizing the host immune response and during infection they are both producer and target cells of IFN-α/β.

A cDNA library was constructed from mRNA of IFN-α treated primary mouse bone-marrow derived macrophages (BMM) and 17 cDNA clones were isolated by differential screening. On RNA level all clones showed inducibility by IFN. Sequencing revealed that 5 clones coded for genes known to be stimulated by IFN; another 5 cDNAs harbored known genes not yet known to be induced by IFN, and 7 cDNAs coded for unknown genes. We first focussed on a 1.8kb cDNA that was expressed in macrophages only. Due to the constitutive release of IFN-β, the mRNA was detectable in untreated BMM; its expression was, however, blocked in the presence of either monoclonal anti IFN-β or by IL-4 treatment whereas upon addition of exogenous IFN-α for 5h, we observed a strong increase in expression in these cells. However, IFN-β expression could be induced by pretreatment of IFNAR-/- BMM with 5IU of IFN-γ for 4h. These results indicate that constitutive IFN-β gene expression in macrophages is not stimulated by the growth factor M-CSF but is rather maintained by an autocrine feedback.

Further analysis on the molecular mechanism now focusses on the involvement of the Interferon-Regulatory-Factors 3 and 7 (IRF-3 and IRF-7) which are members of a family of transcription factors that regulate both IFN synthesis and signalling. IRF-3 is expressed constitutively in many cell types and is activated by phosphorylation following virus infection whereas IRF-7 is induced by IFN and has been shown to direct the bulk of the „late“ IFN response in the two-step model of virus-induced IFN synthesis. Due to the constitutive IFN-β secretion, macrophages also have elevated levels of IRF-1. We have therefore planned to study IFN-β expression in IRF-1 deficient mice. Finally, it is planned to study the influence of nitric oxide (NO) on the expression of constitutive IFN-β in macrophages. NO is released by a variety of cells and can act as a positive and/or negative regulator of IFNs and other cytokines.


duction of the gene. The ORF encoded a protein of 26kD containing two Ig-like domains and one Fc-binding domain. The cDNA was cloned into the prokaryotic expression vector pET30b, the corresponding protein was expressed in E.coli and purified. Mononclonal antibodies were generated in rats and used for the detection the cellular homologue in cell extracts and supernatants. A protein of the predicted size was found exclusively in cell supernatants of BMM. The secreted protein could be precipitated from supernatants not only by the specific antibodies but also by mouse IgG2a, IgG2b and IgG3 antibodies of irrelevant specificities. Accordingly, we named this gene product Interferon-induced Ig-binding protein (IIBP).

Database-assisted sequence homology searches revealed a pronounced similarity (>60%) of IIIBP to the extracellular domains of the SIRPα1 transmembrane protein – also known as SHPS-1, p84/BIT, MyD-1 and MFR – which binds to CD47. Interestingly, interaction of macrophages that constitutively express SIRPα1 with CD47+ cells has been shown to inhibit various macrophage functions. We have evidence that IIIBP is expressed in at least two different splice variants containing either three or only the two C-terminal Ig domains and a short transmembrane sequence. The longer variant also represents a possible ligand for CD47 since this protein interacts with the N-terminal Ig domain of SIRPα1. Binding of the ligand results in phosphorylation of the intracellular inhibitory ITIM motif of SIRPα1 which then associates with SHP-1 and SHP-2, two phosphatases involved in growth factor signalling. Since IIIBP, the gene isolated in our group lacks cytosolic domains, it represents a putative non-signalling ligand for CD47 which competes for binding to SIRPα1. In collaboration with Dr. M. Müller we are currently constructing Baculovirus vectors for expression of recombinant IIIBP. SIRPα1 and CD47 in insect cells to set up in vitro binding assays with isolated proteins and whole cells to prove our hypothesis. In addition, we are planning to establish models to study the effector functions of IIIBP on macrophages and other cells that also express SIRPα1. Finally, it is our goal to study the genomic organization of IIIBP and to identify the regulatory regions of the gene. The IIIBP promoter will be of special interest since the gene is selectively induced by IFN in macrophages.

Mechanisms of the antitumoral activities of IFN
R. Zawatzky, H. Würmbäck, A. Weyland

In cooperation with: Prof. Dr. Schirmacher, Abt. Zelluläre Immunologie, DKFZ, Prof. Dr. Männel, Patholog. Institut, Universität Regensburg, Dr. Kroeger und Dr. Hauser, Abt. Genregulation und Differenzierung, GFB Braunschweig

Unmethylated CpG-containing oligodeoxynucleotides (CpG ODN) are strong immune stimulators and induce a pronounced cytokine response in macrophages and professional antigen-presenting cells that in turn activate Th1 lymphocytes. In a murine tumor model we investigated the antitumoral properties of CpG ODN. We found that i.v injection of 5µg CpG ODN induced high levels of circulating IFN-α and activation of natural killer cells. When applied three days prior to tumor cell inoculation CpG ODN strongly reduced lung metastasis measured by counting tumor colonies [3]. In syngeneic IFN receptor-deficient (IFNAR1−/−) animals, however, CpG ODN had no effect on the establishment of tumor cell metastasis and these results demonstrate the importance of type I IFN as integral part of the innate immune system.

In future experiments using tumor-vaccinated IFNAR1−/− animals we are planning to study whether IFN is also essential for the development of long-living tumor-specific memory T-cells.

Publications (* = external co-author)

Improvement of herpes simplex virus 1 vectors for gene and immune therapy
K.-W. Knopf, E. Kehm, S. Schenck

In cooperation with Prof. Dr. L. Gissmann, Dr. M. Müller, Dr. H. Delius, DKFZ; Dr. A. Epstein, University of Lyon, France; Prof. Dr. R. Manservigi, Dr. P. Marconi, University of Ferrara, Italy; Prof. Dr. T. Brocker, University of Munich; Prof. Dr. J. Rajcani, Slowak Akademy f Sciences, Bratislava, Slovakia.

During the report period the group was mainly engaged in the collaborative EUROAMP project, within the 5th European Framework Programme 1999-2003, aiming to develop and evaluate novel and safer herpes simplex virus 1 (HSV-1) vectors for human gene and immune therapy. In this program amplicon vectors, that derived from HSV-1 defective vectors, are used as vector vaccines. Ideally, or rather with the appropriate helper systems, amplicon vectors can be packaged without wild-type virus contamination such that the resulting virus particles display unique features as follows. The amplicon particles harbor a genome with several copies of the antigen gene whose number is limited only by the packaging capacity of the HSV-1 nucleocapsid. The amplicon par-
articles readily infect cells from different tissues and species but, in the absence of helper virus, they cannot replicate. Consequently, expression of the desired antigen occurs only in the initially infected tissue cells. As viral model antigens for the assessment of the amplicon vectors, structural and non-structural proteins from onco-genic human papillomavirus 16 (HPV-16) were chosen. The intended immunological studies in mice are backed up by cooperations with experts within the Research Program ATV. Of further advantage for the carrying out of the project is the long-standing experience of the group in the field of HSV-1 DNA replication [1, 2]. With the objective to improve antigen expression as well as immune response via MHC-I and MHC-II molecule presentation, several novel amplicon vectors were engineered for expression of selected HPV-16 antigens, and the transcription units of those were optimized for human codon usage (E7, L1, and L2). In other amplicon constructs, fusions between codon-optimized HPV-16 E7 gene sequences and HSV-1 structural genes were performed in order to display the known antigenic sites of E7 on the surface and/or in the tegument of the amplicon virus particle. Antigen expression from the different ampiclon constructs in transiently transfected and infected cells was compared by Western blot analysis and confocal immunofluorescence microscopy. Results showed that cells infected with amplicon particles exerted a higher level of antigen expression, and furthermore, that the antigen expression was significantly enhanced by using codon-optimized transcription units of HPV-16. On the other hand, it was found that the finally achieved amplicon stocks were still contaminated and that with different amounts of HSV-1 helper virus. Therefore, at the end of the report period, the novel HSV-1 BAC helper system of Y. Saeki (Massachusetts General Hospital, Boston) was introduced in the lab. Testing is ongoing whether or not this system qualifies for the helper virus-free packaging of amplicons, that is a prerequisite to perform the intended immunological studies in animals.

Another cooperative project with Slowakia (WTZ Project #SVK00/006), sponsored by the BMBF, focussed on the generation of attenuated herpes simplex virus vectors for human gene therapy and vaccination. Initial aim was the identification of the role of specific mutations in the essential herpesviral envelope protein, glycoprotein B (gB) for viral pathogenicity. More recent investigations center on the possibly innovative use of such an HSV-1 gB-deletion mutant as a novel gene therapy vector for single-cycle-replication. Strategy of the mutational analysis was the reversion of amino acid residues that were altered in the pathogenic HSV-1 strain ANG (ANGpath) as compared to the apathogenic HSV-1 strain HSZP. The reverted gB gene of ANGpath were planned to be introduced back into the ANGpath virus genome by homologous recombination, applying cotransfection of ANGpath DNA and transfer plasmids containing the modified gene. To ease the identification of the ANGpath gB revertants during plaque purification, the construction of an ANGpath gB deletion mutant was foreseen, in which gB was replaced by the gene of EGFP (Enhanced Green Fluores-