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Meeting report

7th International Symposium of the Division of Experimental Cancer Research (AEK) of the German Cancer Society

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Introduction

The Seventh International Symposium of the AEK, the Division of Experimental Cancer Research of the German Cancer Society, was held on 24–26 March in Heidelberg. The symposium was attended by more than 400 participants. It was a highly successful meeting, providing 3 days of expert information on recent achievements and new perspectives in experimental cancer research. The following topics were covered in five main symposia and eight minisymposia:

1. Molecular basis of proliferation and differentiation
2. Induction of differentiation as a therapeutic concept
3. Genetic risk factors for malignant transformation and tumor progression
4. Possibilities and problems associated with the evaluation of carcinogenic potency
5. Tumor immunology.

In the symposia and in corresponding poster sessions current research in biochemistry, immunology, carcinogenesis and epidemiology, molecular genetics and cytogenetics, pathology, therapy and pharmacology, virology, cell and developmental biology and metastasis were covered. Abstracts were published in this journal {J Cancer Res 119 [Suppl 2]: S 36–96 (1993)}. In this Meeting report the chairpersons cover the plenary lectures presented in the five symposia.

Symposium I. Molecular basis of proliferation and differentiation

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The discoveries of oncogenes and tumor-suppressor genes represent a major breakthrough in experimental oncology. However, this knowledge remains limited without further information about the biological role of these genes, the corresponding gene products and the biochemical pathways that are affected. In recent years it has become clear that both oncogenes and suppressor genes encode elements of regulators of signal-transduction systems involved in either growth regulation or differentiation. Thus, a detailed knowledge of the corresponding signal-transduction pathways and their mutual interaction is imperative for an understanding of the function of oncogene and suppressor gene products. Therefore, molecular mechanisms of signal-transduction cascades, leading to proliferation and differentiation, represented a dominant section of the annual meeting of the Biochemistry Branch of the Division of Experimental Cancer Research of the German Cancer Society.

At the symposium devoted to this topic, *Axel Ullrich* (Martinsried, Germany) presented recent insights in the regulation of growth factor receptors. Many growth factors stimulate cellular proliferation by interaction with cell-surface receptors that possess an intrinsic, ligand-sensitive, protein-tyrosine kinase activity. These receptor tyrosine kinases (RTK) comprise an extracellular ligand-binding domain that is linked directly to a cytoplasmic catalytic domain, which is thought to generate a biochemical signal that results in a specific cellular response. While this response is, under normal circumstances, cell division or differentiation, it is now well established that abnormalities at the level of RTK structure or expression, or within the RTK-initiated signalling pathway, are involved in oncogenesis. This is because polypeptides that are known to play important roles in the control and transmission of cellular growth and differentiation signals have been found to be identical or closely related to oncogene products. The receptors for epidermal growth factor (EGF), and colony-stimulating factor I were found to be proto-oncogene counterparts of v-*erb*B and v-*fms;* and *HER2*, the human homolog of the chemically induced rat *neu* oncogene, is a growth factor receptor for a recently identified EGF-like ligand.

For cancer in humans, currently available evidence indicates that overexpression, rather than structural alterations, is involved in tumor progression in cancers connected to RTK abnormalities. In vitro, it has been shown that increased expression levels in conjunction with activation by the ligand result in efficient transformation by EGF-R and *HER2/neu*. *HER2/neu* gene amplification and overexpression are frequent characteristics of human mammary carcinomas and

correlate, statistically with patient survival and tumor recurrence.

The necessity for RTKs to dimerize, thereby allowing inter-receptor transphosphorylation and activation, offers a means by which these receptors can be inhibited. EGF-induced receptor dimers have been detected both in cell-free systems and in living cells. The purified EGF receptor extracellular domain alone undergoes ligand-induced oligomerization, which indicates that this function is an intrinsic property of the ligand-binding domain. In addition, the A431-cell-derived, purified extracellular domain was shown to inhibit EGF-R phosphorylation activity in vitro, a result that suggests that receptor dimerization is not only a consequence of ligand interaction, but is also essential for biological signal generation. This requirement has not yet been demonstrated for the mutated EGF-R derivative v-*erb*B.

Recent studies have shown that an EGF-R mutant lacking most of the cytoplasmic domain is able to form heterodimers with wild-type receptors when expressed in the same cell. These heterodimers are not subject to EGF-induced tyrosine autophosphorylation. Furthermore, coexpression of mutant and wild-type receptors diminished the number of high-affinity EGF binding sites and reduced the rate of ligand-induced receptor degradation. In other studies a kinase-negative EGF-R point mutant was coexpressed in cells with an active EGF-R mutant that lacked carboxy-terminal sequences. While the kinase-negative mutant was transphosphorylated, its expression in NIH-3T3 cells increased the ligand requirement for EGF-stimulated DNA synthesis. These experiments demonstrate extensive cross-talk and transactivation between EGF receptor monomers, properties that render this signalling pathway potentially sensitive to inactivation by expression of receptor mutant polypeptides.

In order to investigate the biological consequences of dominant-negative EGF-R mutants, a series of recombinant retroviruses that encode EGF receptors bearing point or deletion mutations were generated and their growth-inhibitor and anti-oncogenic potential upon coexpression with wild-type EGF-R or the v-*erb*B oncogene product in NIH 3T3 cells was examined. The results indicate that both kinase-negative point mutants and C-terminally truncated EGF-R efficiently inhibit the oncogenic activity of overexpressed wild-type EGF-R. This dominant-negative strategy opens a variety of new avenues towards a better understanding of the role of RTKs in cancer and offers the possibility for gene therapy of malignant neoplasias.

Studies devoted to molecular mechanisms of differentiation are currently focussing on the regulation of tissue-specific gene expression. *R. Cortese* (Rome, Italy) discussed the transcription factor LFB1/HNF1, which regulates hepatocyte-specific transcription of several genes. The effector binds as a dimer to cis-acting elements that match the inverted palindrome GTTAATNATTAAC. The DNA-binding domain of LFB1/HNF1 is characterized by a unique tripartite structure that includes an unusually long homeodomain (domain C), a region related to the POU-specific A-box (domain B) and a short N-terminal dimerization domain (domain A). Cortese reported, that a recombinant peptide, corresponding to the isolated homeodomain of LFB1/HNF1, binds as a monomer to a half-palindrome binding site, but shows diminished sequence specificity. Domain B, in addition to the homeodomain, is required and sufficient for proper recognition of LFB1/HNF1-responsive sites. A protein consisting of only these latter two domains is a monomer in solution, but forms dimers upon DNA binding. The protein-protein contacts established within the bound dimer restrain the orientation of the two homeodomains with respect to one another, thus contributing in a critical fashion to the recognition of the dyad-symmetry-related LFB1/HNF1 sites. The DNA-independent dimerization domain (domain A) is required to increase the affinity of DNA binding, but does not influence the dimer geometry.

The frequently observed antagonism between proliferation and differentiation represents an essential biological feature. An imbalance of the corresponding pathways may lead to uncontrolled (transformed) growth. An interesting new model for this mechanism was described by *T. Graf* (Heidelberg, Germany). The E26 acute leukemia virus encodes the nuclear oncogenes v-*myb* and V-*ets* in a single fusion protein and is unique in its capacity to transform both myeloblasts and multipotent progenitor (MEP) cells in vitro and in vivo. Graf and collaborators studied the role of v-*ets* in MEP cell transformation by employing a temperature-sensitive (ts) mutant of E26 called ts1.1, which contains a single point mutation in the Ets DNA binding domain. They found that this lesion renders the DNA binding domain of Ets thermolabile for binding specific DNA sequences and that mutant-transformed progenitor cells can be induced by temperature shifts to differentiate along both the erythroid and myelomonocytic pathways. In addition, mutant, but not wild-type, transformed cells show a significant level of spontaneous differentiation into eosinophils, which cannot be further increased by temperature shifts. The results therefore indicate that the Ets domain of the fusion protein blocks lineage-specific commitment events in transformed multipotent progenitors. They also suggest that the inhibition of erythroid and myeloid cell commitment is dependent on the DNA-binding capacity of the Ets domain whereas inhibition of eosinophil commitment appears to be independent of DNA binding. Thus, the ts1.1/E26 system provides a useful tool to search for genes regulated by v-*ets*. It could also be shown that the DNA-binding capacity of ts1.1/Ets can be inactivated in a reversible fashion. Graf et al. succeeded in identifying a first candidate gene that is activated upon back-shift of ts1.1 MEP cells previously shifted to the nonpermissive temperature, and its further characterization is in progress.

Symposium II. Induction of differentiation as a therapeutic concept

(By G. Eisenbrand, Fachbereich Chemie, Lebensmittelchemie und Umwelttoxikologie der Universität Kaiserslautern, Erwin-Schrödinger-Strasse, D-67663 Kaiserslautern, and K. Havemann, Abteilung Hämatologie/Onkologie, Zentrum für Innere Medizin, Baldingerstrasse, D-35043 Marburg)

Induction of cell differentiation in experimental tumors by a variety of agents has been known for many years and has been of interest as a potentially promising treatment modality for human cancer. All-*trans*-retinoic acid, is a well known inducer of cell differentiation in experimental tumors. In acute promyleocytic leukemia it has been shown to achieve

complete remission in the great majority of patients without development of the severe side-effects inherent to conventional therapy, although the remissions that follow all-*trans*-retinoic acid therapy are not durable. Induction of differentiation has gained much attention, and research into the underlying mechanisms and the exploration of its potential as a therapy for other human malignancies is being pursued with great intensity.

H. Michna and *M. Schneider* (Berlin) reported on the induction of terminal differentiation by progesterone antagonists in experimental mammary carcinomas. The most important endocrine stimulants for proliferation of mammary (carcinoma) epithelial cells is provided by estrogens: On the other hand, the physiological role of the progesterone receptor in epithelial cells is to mediate differentiation and, in this way, an additional growth control is achieved. A new therapy for progesterone-receptor-positive tumors may thus be the treatment with progesterone antagonists. Onapristone, belonging to a new class of antiprogestins, has been shown to induce differentiation in progesterone-receptor-positive mammary carcinoma and epithelial cells. This results in growth inhibition by a progesterone-receptor-mediated effect, leading to the induction of terminal differentiation with a blockade of the cell cycle in a variety of experimental tumor models, such as MXT, DMBA, MNU and T61 tumors. After treatment periods of 2–6 weeks tumor size, mitotic index and the grade of malignancy decreased, whereas the volume fraction of glandular structures and appearance of apoptosis increased threefold, compared to controls. Mammary glands showed treatment-induced morphological features of differentiation with the appearance of secretory activity. The staining pattern of lectins, especially for α-fucose-specific lectin binding, showed a strong negative correlation with the degree of tumor differentiation. Growth inhibition and differentiation of experimental mammary carcinomas were accompanied by a strong stimulation of immunolocalized transforming growth factor β_1 ($TGF\beta_1$) secreted by epithelial cells, whereas no effect was detected in the surrounding stromal tissue. Since TGFβ is known to promote differentiation of most epithelial cells, this might be relevant to the mode of action of these antiprogestins. The results suggest that onapristone offers an innovative new treatment strategy for hormone-dependent breast cancer by induction of terminal differentiation. Clinical studies have been initiated.

R. Lotan (Houston) reported on retinoids as modulators of squamous carcinoma growth and differentiation. The epithelium of the oral cavity, tracheobronchial tract and endocervix is mostly nonkeratinizing. However, the cells have the potential to undergo an abnormal squamous differentiation with keratinization during vitamin A deficiency, exposure to carcinogens or tumor promoters, or after malignant transformation to squamous cell carcinomas (SCC). The squamous metaplasia that develops in various epithelial tissues during vitamin A deficiency in vivo is restored to the normal nonkeratinizing state by vitamin A or some of its analogs (retinoids). Furthermore, retinoids inhibit squamous differentiation in cultured keratinocytes and tracheobronchial epithelial cells, and modulation of squamous differentiation markers by retinoids is useful to measure the response of aberrantly keratinizing cells. Treatment of head and neck SCC cell lines (HNSCC) with β-all-*trans*-retinoic acid resulted in inhibition of 8/9 cell lines and suppressed colony formation in semisolid medium. All-*trans*-retinoic acid treatment suppressed expression of various squamous differentiation markers to various degrees. The results indicated that some of the malignant HNSCC cells recapitulate the main characteristics of keratinocyte squamous differentiation in culture and that all-*trans*-retinoic acid suppresses this differentiation as it does in normal keratinocytes. Nuclear retinoic acid receptors (RAR and RXR) are thought to mediate the effects of retinoids on growth and differentiation of normal and tumor cells, acting as DNA-binding transcription-modulating factors like steroid receptors. Cellular retinoic-acid-binding proteins (CRABP-I and CRABP-II) have been implicated in controlling the level of retinoic acid available for interaction with nuclear receptors. RAR-α, RAR-γ and RXR-β were found in all of 4 HNSCC lines examined, RAR-β and CRABP-II in 3 out of 4 lines. All-*trans*-retinoic acid treatment increased levels of RAR-β and RAR- in 3 out of 4 cell lines. An inverse relationship between expression of RAR-β and squamous differentiation markers suggested a role for RAR-γ in suppression of squamous differentiation. In situ hybridization with antisense RNA to detect mRNAs for nuclear retinoic acid receptors in surgical specimens from premalignant and malignant lesions of 31 patients with head and neck cancer revealed a significant decrease in the expression of RAR-β in dysplastic and malignant tissues relative to normal or hyperplastic tissues. No such differences were found for RAR-α and RAR-γ. RAR-β was present in all 5 biopsies of normal and buccal mucosa but only in 6 out of 10 tongue leukoplakia and in 4 of 6 buccal mucosa leukoplakia specimens. The RAR-β level increased in 2/4 tongue leukoplakia and in 4/6 buccal mucosa leukoplakia lessions after months of 13-*cis*-retinoic acid treatment.

The fascinating results implicate loss of RAR-β expression during development of head and neck cancer and indicate that retinoic acid can restore RAR-β expression. This might open new possibilities for therapy of squamous carcinoma.

The involvement of cell death by apoptosis in the control of tissue growth has been largely neglected but is now attracting much more attention. Apoptosis has been described as cellular "suicide", the functional opposite of mitosis, and has been defined on the basis of morphological features. Condensation of chromatin at the nuclear membrane, apparently occurring within a few minutes, followed by nuclear fragmentation, are typical characteristics of apoptosis.

H. G. Mannherz (Marburg) presented evidence that DNA degradation, resulting in the typical apoptotic DNA ladder of 200 base pairs, is effected by deoxyribonuclease I (DNase I). Experimentally this DNA degradation can be induced by incubation of isolated nuclei of rat thymocytes and lymph node cells in the presence of Ca^{2+} and Mg^{2+}, also essential cofactors of the well-characterized endonuclease DNase I. Since a very low endogenous Ca^{2+}/Mg^{2+}-dependent endonuclease activity was found in the $CD4^+$ T-cell hybridoma RG-17, nuclei of RG-17 cells were used as substrates to estimate the DNA degradation with nuclear extracts of rat thymocytes and lymph node cells. Both extracts were able to digest RG-

17 chromatin in the presence of Ca^{2+} and Mg^{2+} but not in the presence of Mg^{2+} and EDTA at internucleosomal sites. No fragmentation was seen after the nuclease activity had been removed by preincubation of the extracts with immobilized rat anti-(DNase I). Identical results were obtained after incubation of RG-17 nuclei with culture supernatants of COS cells transfected with pSG6 plasmids containing the rat parotid DNase I insert. Immunostaining of rat thymocytes, lymph node cells, fibroblasts ans transfected COS cells with rat anti-(DNase I) gave a clear perinuclear signal, most likely corresponding to the nuclear envelope and the endoplasmic reticulum. The similarity between the endonuclease activated during apoptosis present in nuclear extracts of a number of different cells and the culture supernatants of transfected COS cells strongly suggests that the nuclease present in these extracts and in supernatants and nuclear extracts of transfected COS cells are identical. This endonuclease is constitutively present in thymocytes and lymph node cells, but appears to be up-regulated in NIH-3T3 fibroblasts after serum withdrawal and in the rat prostate after testosterone ablation. However, endonuclease constitutively expressed in intact, non-apoptotic nuclei might also become activated by mechanisms unrelated to apoptosis, and the scientific debate on whether endonuclease activation may be considered a general marker of apoptosis is not settled yet.

Symposium III. Part A. Genetic risk factors for malignant transformation and tumor progression

(By H. M. Rabes, Pathologisches Institut der Universität, Thalkirchner Str. 36, D-80337 München, and K. Mölling, Max-Planck-Institut für Molekulare Genetik, Ihnestr. 63–74, D-14195 Berlin)

Genetic risk factors may be involved at various stages of the multistep process of carcinogenesis. At the earliest stages genetically determined profiles of the metabolism of chemical carcinogens may play a role in the generation of active carcinogenic reactants and thus in the rate of formation of promutagenic DNA adducts. At this level DNA repair is critical for the extent to which promutagenic lesions, induced either by chemicals or physical factors, give rise to mutations. Several DNA-repair-deficiency syndromes are examples of increased genetic risk for transformation and predisposition to cancer, e.g. xeroderma pigmentosum (XP) and Cockayne syndrome (CS), which exhibit UV sensitivity. The molecular defect in these genetically heterogeneous diseases resides in the nucleotide excision repair pathway. *Geert Weeda,* Rotterdam, reported on the isolation of complementary human repair genes involved in these syndromes. One of these genes is the ERCC6 gene encoding a protein of 1493 amino acids with a putative chromatin- and nucleotide-binding domain and seven helicase signatures. From cytogenetic and Southern blot analyses of cells from a CS patient it became evident that one of the ERCC6 alleles is deleted, suggesting a possible role of ERCC6 in CS. Transfection of a functional ERCC6 cDNA to cells of CS patients revealed a specific correction of the UV sensitivity and RNA synthesis recovery of CS-B cells.

The ERCC3 gene encodes a DNA helicase. The recent cloning of the yeast and *Drosophila* homologs (SSL2/RAD25 and Haywire respectively) and the unexpected finding that the ERCC3 protein is one of the components of the basal transcription-initiation factor BTFII/TFIIH of class II genes suggest that the ERCC3 protein plays a role in both nucleotide excision repair and transcription. The involvement of ERCC3 in transcription initiation probably defines the hitherto unresolved essential role of the gene.

For gene targeting in mouse embryonal stem cells, replacement vectors were constructed with *neo* expression cassettes in coding exons to create null alleles. To circumvent the problem of possible lethality, targeting constructs were also made in which "subtle" mutations found in patients are mimicked. Homologous recombinants were obtained with a frequency of up to 15% of all dominant marker-containing transformants. After these had been implanted into pseudopregnant foster mothers several chimeric mice were generated. These mice should help increase the understanding of mutagenesis and carcinogenesis in the absence of a specific DNA repair pathway.

Many studies analyzing the genetic basis of malignant transformation and progression are focussed on modifications of cellular proto-oncogenes. The human neuroblastoma is a wellknown example used to investigate stage-dependent genetic alterations. Recent developments were summarized by *Manfred Schwab,* Heidelberg. N-*myc* is amplified between five- and several-hundredfold frequently in neuroblastomas and, at lower frequency, in other human cancers with neuronal qualities. In neuroblastomas N-*myc* amplification is significantly correlated with poor prognosis. Amplification is associated with elevated expression or mRNA and protein. N-*myc* encodes two polypeptides of relative masses 62 kDa and 64 kDa, which are phosphorylated, at least in vitro, by casein kinase II and are localized in the nucleus, where they can associate in vivo with another protein, Max, through a C-terminal dimerization motif, one leucine zipper. An N-terminal portion of N-*myc,* referred to as a "Myc box", can substitute for the transcription-activating function of the yeast transcription factor Gal 4, thus raising the possibility that N-*myc* itself may act as a transcription factor, either alone or in association with other factors.

Chromosome 1p is the site of the most frequent genetic alterations in neuroblastoma. Originally identified as deletions, chromosome 1p alterations have more recently turned out to involve reciprocal translocations also. The finding of constitutive 1p alterations in patients with neuroblastoma favors the idea that this genomic region harbors genetic information involved in neuroblastoma development. Chromosomal in situ hybridization has allowed molecular probes to be defined flanking translocation breaks within an estimated 5×10^6 base pairs. Employing walking technology with yeast artificial chromosomes it should be possible soon to identify and characterize in detail the genetic information altered at chromosome 1p.

In recent years, tumor-suppressor genes have become a main focus of tumor research. Using the RB gene as an example, *Hein te Riele,* Amsterdam, studied the loss of function of this gene during development and the cascades of synergizing mutations in tumorigenesis in mouse model systems. RB-1 encodes a nuclear protein with a cell-cycle-dependent phosphorylation pattern, the hypophosphorylated form being associated with growth arrest in G_0/G_1. During mouse embryonic de-

velopment, the murine homolog Rb-1 was found to be expressed predominantly in differentiating cell layers.

In the first mouse model, an inactivated Rb allele was introduced into the mouse germline, using embryonic stem-cell technology, generating heterozygous animals carrying *one* inactive Rb copy in *all* cells. For the second model, both Rb copies were inactivated by homologous recombination in embryonic stem cells, which on fusion to wild-type blastocysts generated chimeric mice carrying *two* inactive Rb copies in *some* cells. Both types of animals have a high risk of containing cells with a complete lack of Rb function in susceptible tissues.

Intercrossing of $Rb^{+/-}$ heterozygotes revealed the functional requirement of Rb-1 in embryonic development: $Rb^{-/-}$ embryos failed to reach term and showed a number of abnormalities in neural, hepatic and/or erythropoietic development. Notwithstanding the embryonic lethality of nullizygosity of Rb-1 in heterozygous crossings, double-knock-out $Rb^{-/-}$ ES cells efficiently contributed to most tissues in chimeras. These included lung, thymus and spleen, which were expected to require a functional Rb protein, on the basis of the high expression of Rb-1 in wild-type adults, and tissues that were severely affected in homozygous $Rb^{-/-}$ embryos.

No retinoblastomas were observed, however, $Rb^{+/-}$ heterozygotes and double-knock-out $Rb^{-/-}$ chimeras developed tumors of the pituitary gland. In the heterozygotes, loss of the wild-type Rb-1 allele was observed in most cases, whereas in the chimeras the tumors were exclusively derived from $Rb^{-/-}$ cells. These tumors, although large in size (3–5 mm) are relatively benign as they did not infiltrate the adjacent brain tissue, whereas most displayed characteristics of well-differentiated melanotrophs of the pars intermedia, as judged from the positive immunostaining for melanocyte-stimulating hormone α and β-endorphin.

Symposium III. Part B. Genetic risk factors for malignant transformation and tumor progression

(By M. Schwab, Deutsches Krebsforschungszentrum, Abt. 0130, Im Neuenheimer Feld 280, D-69120 Heidelberg)

Genetic risk factors are being recognized at increasing rate as being intimately related to the process of tumorigenesis. Radiation and other genotoxic agents induce the expression of genes by causing the posttranslational modification of transcription factors (*Peter Herrlich*, Karlsruhe). In the transcription factor AP-1, both subunits are phosphorylated. For Jun, mutation of two of these sites (in the transactivating domain) at least partially prevents UV-induced AP-1 activation. In addition to nuclear events, UV irradiation induces several post-translational changes in cytoplasmic proteins such as Raf-1, MAP-2 kinase and NFkB. Modification is triggered by UV absorption in the nucleus. This is suggested by the finding that the cytoplasmic changes caused by UV (not by growth factors or phorbol esters) cannot be induced in thrombocytes and cytoblasts. Interestingly extranuclear signalling events are required for the activation of AP-1, shown by the blocking effect of a negative-dominant Raf-1 kinase mutant and by suramin, which interferes with the interaction of growth factors with growth factor receptors. It thus appears that cells possess rapid communication pathways from the site of UV absorption in the nucleus to the plasma membrane level (reverse signalling). Preliminary experiments on the nature of the growth factor receptors suggest the participation of interleukin-1-receptor and basic fibroblast growth factor receptor.

Myc proteins have been implicated in the regulation of cell growth and differentiation (*Bernhard Lüscher*, Hannover). The identification of Max, a basic-region/helix-loop-helix/leucine-zipper protein, as a partner for Myc has provided insights into Myc's molecular function as a transcription factor. Recent evidence indicates that the relative abundance of Myc and Max is important to determine the level of specific gene transcription. Two major in vivo phosphorylation sites in Max (Ser-2 and Ser-11) can be modified in vitro by casein kinase II. Phosphorylation at these sites modulates DNA binding by increasing both the on and off rates of Max/Max homodimers as well as Myc/Max heterodimers. In addition, the shorter version of Max (p21) binds with similar affinity to DNA yet its rate of dissociation is faster than that of the longer version of Max (p22). These data argue that different Max complexes have different kinetic properties and that these can be modified by casein kinase II phosphorylation. This could be a biologically relevant mechanism involved in the efficient exchange of different dimeric complexes on DNA. A set of phosphorylation sites near the amino terminus of the Myc protein within a region possessing transactivating activity appears of importance for the transforming potential of the protein. Furthermore, this region is often mutated in v-*myc* and in several c-*myc* genes from Burkitt lymphoma cells. At least one of these sites can be modified by glycogen synthase kinase 3 (CSK3) in vitro, another by a mitogen activated protein (MAP) kinase. When the amino-terminal phosphorylation sites were mutated for analysis of their biological importance for Myc function, little difference in the ability to transactivate synthetic promoter constructs containing 5 -CACGTG motifs was observed between the mutants. To test for altered growth properties Rat1A cells stably transfected with wild-type c-*myc* and the mutant constructs were analyzed for their ability to support colony formation in soft agar. Individual mutations of the phosphorylation sites or combinations there of influenced the growth behavior of the Rat1A cells. However, no clear pattern of the importance of specific sites has emerged so far.

The mechanism of chromosomal translocations in lymphoid tumors has been intensely studied in recent years (*Thomas Boehm*, Freiburg). Lymphocytes are particularly prone to such chromosomal damage because they rearrange their antigen-receptor genes during the assembly of functional immunoglobulins and T cell receptors respectively. This process is mediated by specific recombination signal sequences, which flank variable (V), diversity (D) and joining (J) elements in these loci and is executed by an as yet ill-defined enzyme (complex) referred to as recombinase. The finding of ectopic "pseudo"-recombination signals at the junction of chromosomal rearrangements in lymphoid tumors suggested the involvement of an aberrant recombinase activity in the chromosomal abnormalities; however, examples are also known where no pseudo-recombination signals can be found. In principle, three categories of genetic lesions are possible. First, chromosomal damage may be innocu-

ous to the afflicated cell and thus no selection ensues. Second, a chromosomal lesion may be lethal; consequently, the afflicted cell is eliminated. Third, and probably most importantly, a genetic aberration may initiate tumorigenic conversion. It is this latter change that is selected in vivo and becomes apparent in clinical disease. In broad outline, proto-oncogenes may be associated with either the proliferation or differentiation of cells. Proto-oncogenes, such as *myc*, p53 and others, known to be involved in cell-cycle control, become mutated in the course of tumor development. Other proto-oncogenes, many of which are known transcription factors, generally become transcriptionally deregulated without suffering structural damage to their coding regions (although exceptions exist). These changes have a "knock-on" effect and eventually result in full tumorigenic conversion.

Symposium IV. Possibilities and problems associated with the evaluation of carcinogenic potency

(By W. K. Lutz, ETH Zürich, Institut für Toxikologie, Schorenstrasse 16, CH-8603 Schwerzenbach, and J. Wahrendorf, Deutsches Krebsforschungszentrum, FS 3, Im Neuenheimer Feld 280, D-69120 Heidelberg)

The risk of cancer from exposure to a carcinogen is a function of dose and potency of the carcinogen in a given individual. An evaluation of carcinogenicity data in a qualitative manner (± carcinogen versus noncarcinogen) is therefore of little use with respect to cancer risk assessment.

In this symposium, three sources of data were discussed with regard to a quantitative evaluation: (a) the animal bioassay for carcinogenicity, (b) short-term tests for mutagenic activity, and (c) epidemiological data on cancer incidence. Finally, as a case study, the common mushroom was evaluated as a putative carcinogenic hazard in the diet.

Dr. Krewski (Ottawa) introduced the carcinogen bioassay. Current practice normally calls for exposure of experimental animals to doses up to the maximum tolerated dose (MTD; expected to produce some minor signs of general toxicity). Such studies have been used to compute measures of carcinogenic potency such as the TD_{50}, i.e., the daily dose administered for a standard life span that halves the fraction of tumor-free animals. TD_{50} values span more than seven orders of magnitude, indicating the enormous range of carcinogenic potencies. Recent studies have revealed that this measure of carcinogenic potency is highly correlated with the MTD. This is a consequence of the fact that the range of TD_{50} values that can be found once the MTD is set, can lie only within a 30fold range. Since an MTD is, by definition, close to toxicity, TD_{50} values also correlate with the acute lethal dose LD_{50}. For setting priorities in carcinogen regulation, therefore, a first step might be to determine the MTD without performing a carcinogen bioassay. The TD_{50} value of the compound will be relatively close to the MTD, so that some ranking can be made.

E. Vogel (Leiden) related chemical reactivities of different mutagens to DNA-adduct profiles, to mutation spectra, and to carcinogenic potency. Astonishing differences were seen between various test systems. These were most often related to differences in DNA repair. Numerous repair-deficient and repair-proficient mutant systems have become available in which to study the differences mechanistically. The data also explain differences between species: different mutation spectra are obtained in different test systems with the same genotoxicant. Chemical reactivity can be used as a quantitative indicator of mutagenic potency for simple alkylating agents only; crosslinking agents do not follow the same rules. Their genotoxic potencies appear to be independent of electrophilic potency. Within one test system or animal species, mutation spectra are carcinogen-specific to some extent. This "footprinting" might offer a possibility of correlating carcinogen exposure with tumor induction in an individual or, on the other hand, demonstrate noncorrelation and hence the unlikelihood of a causal relationship.

J. Wahrendorf (Heidelberg) showed that a carcinogenic potency estimate can also be derived from epidemiological data. On the basis of data from occupational cohort studies with arsenic he showed that the potency estimates derived from different studies fall within about the order of magnitude. Carcinogenic potencies of carcinogens in the air are expressed in terms of a "unit risk", which gives a particular exposure the additional (above background) probability of causing (or dying of) a disease under life-long exposure to 1 $\mu g/m^3$ of the risk factor.

For arsenic, for instance, the median unit risk derived from all available studies is found to be about 5×10^{-3}. Combination of the unit risk with the actual concentration of various carcinogenic air pollutants allows the estimation of the cancer risk associated with each carcinogen and priorities to be set for regulatory measures.

S. Shephard (Zürich) presented a comprehensive overview of the data available to assess the mutagenic and carcinogenic potential of the common mushroom *Agaricus bisporus,* known to contain the mutagenic hydrazine derivative agaritine. She showed that the evaluation of a complex food item poses numerous problems on a variety of levels: the concentration of agaritine (and other mutagens?) in the mushrooms, questions about metabolism in humans, genotoxicity without DNA adduct formation, mutagenicity of proximate and ultimate electrophiles, synergistic and antagonistic effects of other mushroom constituents. Some apparently contradictory results could be shown to be due merely to the use of different exposure dose levels. The final risk assessment included unpublished data on the mutagenic activity of agaritine in the new *lac I* transgenic mouse model. It was concluded that one additional cancer case could be expected per 10^6 lives from the consumption of 0.4 g raw mushroom/day.

This symposium showed that numerous tools are available to evaluate carcinogenic potency. Epidemiological data have the important advantage that interspecies extrapolation is not required and that the heterogeneity of the human population with respect to genetics and lifestyle is taken into account to some extent. However, such studies are not easily available with environmental carcinogens so that occupational settings might have to be analyzed instead.

Symposium V. Tumor immunology

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A. Plückthun (Munich) reported on the production of bivalent or bispecific "mini-antibodies" self-assembling in *E. coli.* In these constructs single-chain Fv fragments were connected via a hinge peptide to an amphipatic helix motif. The helix was taken either from a coiled-coil region of a leucine zipper or from an antiparallel four-helix bundle. Surprisingly, the four-helix bundle, a completely artificial protein, gives rise to antibodies with identical avidity to a surface to that of a whole antibody while having only about one-third of the molecular mass, and it performs better than the leucine zipper version. The clearance rates of these mini-antibodies was examined in mouse serum, and it was found that they lie between whole and fast clearance rates; they should, therefore, have characteristics very desirable for tumor imaging or treatment. When improved vectors are used, they can be produced at very high levels in high-density fermentation in *E. coli* in fully functional form. It is notable that the assembly to the dimer occurs in the bacterial periplasm. Yields of 200 mg/ml mini-antibody have been obtained.

The above-mentioned production technology in *E. coli* as well as the size and functional integrity of these designer "mini-antibodies" suggest that this is the right way for the generation of cost effective and efficient diagnostic and therapeutic anticancer drugs.

J. Gerdes (Borstel) reported on the molecular and functional characteristics of the monoclonal antibody Ki-67. The nuclear antigen defined by mAb Ki-67 is an ubiquitously expressed human nuclear protein strictly associated with cell proliferation and widely used in routine pathology as a "proliferation marker" to measure the growth fraction of cells in human tumors. Ki-67 detects a double band with apparent molecular masses of 345 kDa and 395 kDa in immunoblots of proteins from proliferating cells. Gerdes et al. cloned and sequenced the full-length cDNA encoding this cell-proliferation-associated protein and identified two differentially spliced isoforms of mRNA. New monoclonal antibodies against different bacterially expressed parts of this cDNA react with the native Ki-67 antigen thus providing evidence that they had cloned the authentic Ki-67 antigen cDNA. The central part of the Ki-67 antigen gene contains a large exon with several tandemly repeated elements, the Ki-67 repeats, each including a highly conserved new motif, the Ki-67 motif, which codes for the epitope detected by Ki-67. Computer analysis of the nucleic acid and the deduced amino acid sequences of the Ki-67 antigen confirmed that the cDNA encodes a nuclear and short-lived protein with no significant homology to any of the known sequences. The evaluation of functional properties revealed that the native antigen has DNA-binding activity and that Ki-67 antigen-specific antisense oligonucleotides inhibit the proliferation of IM-9 cells, indicating that the Ki-67 antigen may be an absolute requirement for cell proliferation. We conclude that the Ki-67 antigen defines a new category of cell-cycle-associated nuclear nonhistone proteins and hypothesize that this protein may serve as a kind of histone substitute during those phases of the cell cycle when nucleosome structure is disintegrated.

O. Roetzschke (Tübingen) reported on the identification of antigenic determinants recognized by antigen-specific receptors of T lymphocytes. In contrast to antibodies which can recognize antigenic epitopes on proteins in solution, T cells recognize antigens on cell surfaces in the form of small processed peptides, which are associated and presented via major histocompatibility (MHC) molecules. MHC class I molecules guide CD8 T lymphocytes such as cytotoxic T cells (CTL) while class II MHC molecules guide CD4 T lymphocytes, which perform a helper function and cytokine release. In the human, class I MHC molecules are coded by alleles from three different genes namely HLA-A, -B and -C, while MHC class II molecules are coded by alleles from the genes HLA-DR, -DQ and -DP. Roetzschke reported the isolation of HLA-A-restricted melanoma-derived tumor antigens which are recognized by autologous CTL from melanoma patients. He also reported and summarized new unpublished results from other research groups who have identified consensus motifs of several peptides from HLA-A and -B alleles. He then presented new data on consensus motifs of peptides from HLA-C alleles and reported that the respective peptide motifs resemble those of HLA-A or -B ligands. He concluded that the processing and origin of ligands appear not to be different from those of ordinary class I molecules. The function of the HLA-C peptides, however, remains speculative. The hypothesis was put forward that HLA-C molecules might serve as sentinals to signal proper antigen processing to NK (natural killer) cells.

Margot Zöller (Heidelberg) summarized recent findings on CD44 splice variants, work that had been performed in collaboration with P. Herrlich and H. Ponter from the Kernforschungszentrum Karlsruhe. CD44, originally described as a lymphocyte homing receptor, comprises a family of membrane-integrated glycoproteins which differ by posttranslational modifications as well as in their primary amino acid structure. By alternative splicing, up to ten additional exons can be inserted in the extracellular part of the smallest isoform of CD44 (CD44s), which is the most abundant one, found not only on the majority of lymphocytes, but on connective tissue and many epithelial cells, while larger isoforms (CD44v) are predominantly expressed on tumor cells. One of these additional exons, exon v6, was shown to be of particular importance in the process of tumor progression. In a variety of rat tumors, exon v6 was expressed on all lines that metastasize via the lymphatic systems, but not on a line that grows non-invasively. The importance of exon v6 for metastasis formation was confirmed by induction of the metastatic phenotype via transfection of non-metastasizing tumor cells with cDNA of CD44v. The functional relevance of exon v6 was further demonstrated by blocking metastatic progression with a monoclonal antibody (1. 1ASML). Further analysis revealed that 1.1ASML apparently inhibits tumor cell embedding and invasion of the lymphatic tissue. Binding studies to a whole array of components of the extracellular matrix as well as to cellular monolayers did not reveal a potential ligand for exon v6 of CD44v; instead, expression of exon v6 weakened the binding qualities of the N-terminal part of CD44s. It was suggested that CD44v may not act via ligand interaction, but by interfering with ligand interactions of the invariant part of the molecule. Exon v6 was found to be involved not only in metastasis formation, but also in lymphocyte maturation and activation.