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Meeting Highlight

Sixth Charles Heidelberger Conference on "Control of Cell Proliferation and Differentiation: Molecular Targets in Carcinogenesis and Cancer Therapy", 9–13 July 1995, Essen, Germany*†

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THIS CONFERENCE, the sixth in a prestigious series founded in commemoration of Dr Charles Heidelberger (1920–1983), was held for the first time in Europe, following the previous Conferences, organised by former students, post-doctoral fellows and colleagues of Charles Heidelberger in 1984 (Chicago) [1], 1988 (Honolulu) [2], 1990 (Kyoto) [3], 1991 (Los Angeles) [4] and 1994 (Honolulu) [5]. This had been much awaited by many of Charles Heidelberger's former European associates with whom he had maintained close links and collaboration. As in the previous Conferences of this series, Mrs Patricia Heidelberger was able to attend and to refresh memories of Charles Heidelberger with her lively accounts of past events and episodes, both scientific and personal.

Charles Heidelberger was an international leader in research on carcinogenesis and cancer therapy. The Conferences bearing his name have aimed at discussing both these areas in parallel, since they form two interrelated aspects of the same problem.The Scientific Programme thus focused on molecular mechanisms in cell type-specific carcinogenesis and tumour progression, based on current rapid advances in molecular genetics and cell biology, and on novel approaches and strategies that could result in enhanced effectiveness and selectivity of therapeutic tumour targeting.

CONTROL OF CELL PROLIFERATION AND DIFFERENTIATION

Molecular mechanisms controlling cell cycle progression and cell cycle-regulated transcription were discussed by **Drs R. Bernards** (Amsterdam, The Netherlands) and **R. Müller** (Marburg, Germany). Among the cellular substrates phosphorylated by cyclin cdk (cyclin-dependent kinase) complexes is pRb, the product of the retinoblastoma gene. Phosphorylation in late G_1 releases the transcription factor E2F from pRb, resulting in transcriptional activation of E2F-responsive genes. Little is known about the regulation of the two pRb homologues, p107 and p130. **Dr Bernard's** group has cloned two new E2F family members that interact with p107 (E2F-4) and p130 (E2F-4 and E2F-5). Enforced expression of E2F-4 induces $G_1 \rightarrow S$ progression and co-transfection with an activated *RAS* oncogene results in oncogenic transformation of rat embryo fibroblasts. Both p107 and p130 were found to be phosphorylated by cyclin D1–cdk4, but not by cyclin E–cdk2, and a p107-induced cell cycle block can be released by cyclin D1–cdk4, but not by cyclin E–cdk2. In contrast, pRb is phosphorylated by both cyclin–kinase complexes. There are thus important differences in substrate specificity between G_1 cyclins. **Dr Müller** showed that transcription of S/G_2-specific genes, such as *CDC2*, *CYCLIN-A*, and *CDC25C*, is largely mediated by novel *cis*-acting repressor elements (*CDE* and *CHR*) located close to major transcrip-

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tion initiation sites. The *CDE–CHR* module may thus represent an interesting target in connection with gene therapeutic approaches. The formation of *CDE–CHR* protein complexes in both G_0 and G_1 cells, and their dissociation in S/G_2 was demonstrated by genomic footprinting. Cell cycle regulation of the *CDC25C*, *CDC2* and *CYCLIN-A* promoters is strongly impaired by mutation of the *CDE* or *CHR* and leads to high expression in G_0/G_1. Cell cycle regulation is lost upon removal of the enhancer region located immediately upstream of the *CDE*, but is largely restored when an enhancer-less minimal promoter fragment is linked to the constitutive SV40 early promoter/enhancer. This indicates that the *CDE CHR* functions by blocking the activation of transcription by the glutamine-rich activators Sp1 and NF-Y, which bind to the *CDC25C*.

The Ser/Thr-specific kinase Raf is part of a Ras-dependent signalling pathway, with Ras lying upstream of Raf. Ras moves its effector Raf to the plasma membrane where it becomes activated by an as yet unknown mechanism. **Dr N. Nassar** (Dortmund, Germany) reported on the X-ray crystal structure of the complex between residues 1–167 of the human Ras-related GTP-binding protein 3Rap1A and RBD (Ras-binding domain) expressed from human c-Raf1. The topology of RBD strongly resembles that of ubiquitin. The Rap1A/RBD complex was purified in the presence of the GTP analogue GppNHp prior to crystallisation. The structure of complexed Rap1A resembles uncomplexed Ras–GppNHp. Tight interactions provide for the inhibitory effect of RBD on guanine–nucleotide dissociation. The structure of the Rap1A/RBD complex provides information on the question of whether the observed mode of interaction is also used by other GTP-binding proteins (e.g. Rho, Ran) and other effector molecules (e.g. GAP). The small size of the Rap1A (Ras)/RBD interface may permit the design of inhibitory 'anti-Ras drugs'.

The expression of a number of genes is downregulated in the presence of a mutationally activated *RAS* gene. **Dr R. Schäfer** (Zürich, Switzerland) has identified such potential suppressors of neoplastic phenotypes (*H-REV* genes) in primary rat fibroblasts, and in non-tumorigenic (rat 208F, mouse 3T3) and transformation-resistant rodent cell lines (REF-52 and EK-3). In phenotypic revertants of *RAS*-transformed cells, the expression of genes encoding extracellular matrix components (e.g. collagen type I) and the matrix-modifying enzyme lysyl oxidase, was at least partially restored. Moreover, expression of the angiogenesis inhibitor, thrombospondin, was downregulated in *RAS*-transformants, probably contributing to the neovascularisation of tumours originating from these cells. However, upregulation was not consistently seen in phenotypic revertants. Two novel genes, *RIL* (*H-REV18*), coding for a protein shared by the homeodomain proteins Lin-II, Isl-1 and Mec-3 (LIM) gene, and *H-REV107*, encoding an 18 kDa polypeptide without similarity to known proteins, were isolated by subtractive hybridisation. The ectopic expression of *H-REV107* in *RAS*-transformed cells suggests that this gene may serve as a suppressor of transformation. Preliminary analyses indicate the absence of *H-REV107* expression in tumorigenic cell lines and experimental and human tumours.

Since epithelial tumours are by far the most frequent cancers in humans, the molecular cell biology of carcinogenesis in epithelial cell systems is a high priority. **Dr N.E. Fusenig** (Heidelberg, Germany) used the human skin keratinocyte cell line, HaCaT, and *C-HA-RAS* transfected HaCaT-derived clones (which in nude mice give rise to benign or malignant tumours, respectively) to characterise genetic and phenotypic changes at different stages of skin carcinogenesis. Both tumorigenic phenotypes exhibited an altered response to growth factors and autonomous growth potential *in vitro*. Malignant (invasive) cells could only be discriminated *in vivo* by their progression to squamous cell carcinomas; benign cells formed stationary cysts, and immortal cells were growth-inhibited followed by terminal differentiation, suggesting a decreasing sensitivity to mesenchyme-mediated growth control. In a surface-transplantation assay, in which tumour and host cells are initially separated by an acellular collagen matrix, HaCaT cells and benign sub-clones formed organised and differentiating epithelia, whereas malignant cells rapidly elicited mesenchyme activation and directed angiogenesis, preceding the onset of invasive growth.

Dr T. Kuroki (Tokyo, Japan) clarified several steps in a possible signal transduction pathway mediating squamous cell differentiation, a negative regulator of carcinogenesis. The Ca^{2+}-independent isoform η of PKC (protein kinase C) was highly expressed in epithelial tissues at the mRNA and protein level, in close correlation with differentiation. Cholesterol sulphate, a potent inhibitor of tumour promotion and of the proliferation of keratinocytes and their malignant counterparts, was found to act as a second messenger activating the η isoform. Upon activation, transglutaminase 1 is transcribed, resulting in the cross-linking of structural proteins of keratinocytes.

Embryonic mouse skin undergoes drastic morphological changes between days 13 and 16 of gestation (formation of rudimentary hair follicles; stratification and cornification of interfollicular epidermis). **Dr N. Huh** (Tokyo, Japan) described a culture system on floating membrane that allows skin tissue isolated from 12- or 13-day embryos to develop in a manner similar to the process *in vivo*. Corresponding to their sequential activation *in vivo*, differentiation markers of epidermal keratinocytes, including cholesterol sulphotransferase and cytokeratin K1, were induced during the cultivation period. Epithelial growth factor (EGF), transforming growth factor α (TGFα), and keratinocyte growth factor (KGF) specifically inhibited hair-follicle formation, with marginal effects on interfollicular epidermis. The inhibitory action by EGF was reversible at an early stage of the development of hair rudiments. Exogenous genes introduced via an adenovirus-based expression vector were successfully expressed in almost all epidermal cells in the histological architecture, providing a system for the study of molecular mechanisms in morphogenesis.

To decipher the steps in the signal transduction pathway leading to macrophage differentiation, **Dr E. Huberman** (Argonne, Illinois, U.S.A.) used human promyelocytic HL-60 cell variants susceptible or resistant to differentiation induction by 5 phorbol 12-myristate 13-acetate (PMA). PMA-resistant variants exhibited discrete deficiencies in the expression of genes encoding cellular receptors, PKC isoforms, and/or ligands. By treatment with the respective peptides and proteins, or by transfection of suitable expression vectors, PMA resistance could be mimicked in the susceptible HL-60 cells, or the wild-type phenotype of

PMA-resistant cells could be restored. The role of specific gene products (deficient in PMA-resistant cells) in signal transduction, resulting in the macrophage phenotype, was thus substantiated. A specific protein complex (PC) composed of a 10 kDa and a 14 kDa protein may regulate (suppress) cell multiplication during terminal differentiation of myelomonocytic cells. In HL-60 cells, these proteins are expressed during terminal differentiation as in mature peripheral blood granulocytes and monocytes. Purified PC inhibits the multiplication of HL-60 cells and related cell types. Exposure of cells to an agent or condition that does not induce differentiation but suppresses cell multiplication does not result in PC expression.

Dr N. Colburn (Frederick, Maryland, U.S.A.) reported that mouse JB6 cells that are sensitive (P^+), but not those that are resistant (P^-), to tumour promoters show activation of the AP-1 transcription factor in response to phorbol esters and EGF. If AP-1 activation is required for transformation, then pharmacological or gene blockers of AP-1 should inhibit phorbol ester-induced promotion. Indeed, both retinoids and glucocorticoids, and a dominant-negative *JUN (TAM 67)* gene stably expressed in P^+ cells, inhibited both induced AP-1 activation and transformation of JB6 P^+ cells. One or more effector genes transcriptionally regulated by AP-1 thus drive(s) the process. In a keratinocyte model (the 308 mouse papilloma cell line), expression of *TAM 67* or a *KERATIN 14* promoter blocked phorbol ester-induced activation of AP-1 as well as matrigel invasion. A subset of matrix metalloproteinases has been implicated in the latter process. *TAM 67* also inhibited activation of the transcription factor NFκB, suggesting that (i) cross-talk of Jun with p65 to activate NFκB may be relevant to progression, and (ii) dominant-negative Jun may target both AP-1 and NFκB when preventing transformation. MAP (mitogen-activated protein) kinases or Erks (extracellular signal-regulated protein kinases) have been implicated in the activation of the AP-1 complex in JB6 cells as well as in other models. Erks may thus serve as alternative targets for the prevention of carcinogenesis.

Intercellular communication via gap junctions is involved in the control of cell proliferation and differentiation. As discussed by **Dr E. Winterhager** (Essen, Germany), trophoblast invasion of the uterus during embryo implantation is the only naturally occurring event where normal cells exhibit invasion properties similar to malignant cells. However, in contrast to tumour cells, the trophoblast invasion process is strictly controlled. The proliferative trophoblast cell population of human placenta is characterised by the expression of cx40. Different malignant variants of the trophoblast, the choriocarcinoma cell lines BeWo, Jeg-3 and Jar, were investigated for cx-expression. Jeg-3 cells, which lack communication properties, were used for cx-transfection. All cell clones stably transfected with cx26, cx40 and cx43 exhibited decreased proliferation rates.

Dr W. Schmiegel (Bochum, Germany) found an inverse correlation of pRb levels and p16 status in pancreatic carcinoma cells. In cells undergoing proliferation arrest, the G_1-inhibitor p16 interferes with G_1–cyclin complexes and blocks Rb phosphorylation. Dysregulation of G_1 cell-cycle factors was investigated in pancreatic tumour cell lines ($n = 18$). Analysis of the *P16* gene revealed either homozygous deletions or mutations leading to loss of, or altered, expression. No viral (SV40, E1A [Ad12]) sequences were detected that could affect the recognition site of pRb. Changes in cellular expression of either p16 or Rb may lead to a proliferative advantage in pancreatic tumour cells. Cyclin D1 was expressed weakly in asynchronously grown pancreatic carcinoma cells, whereas mammary carcinoma cells and NIH3T3 fibroblasts exhibited normal levels. Surprisingly, the absence of cyclin D1 expression was accompanied by strong expression and exclusive cytoplasmic localisation of cdk4. Overexpression of cdk4 and its cytoplasmic localisation in pancreatic carcinoma cells points to a dysregulation of this kinase.

DNA LESIONS AND MUTATIONAL PATTERNS AS INDICATORS OF EXPOSURE TO DNA-REACTIVE AGENTS

Genes involved in cell-cycle control, e.g. *TP53* and *RB*, *CYCLIN D1*, and the cyclin–kinase inhibitor *P16/CDKN2*, are altered with various frequency in many forms of human cancer. As described by **Dr R. Montesano** (IARC, Lyon, France), the nature and functional consequences of these alterations may be informative in tracing the molecular pathogenesis of neoplasia and in providing clues to the aetiology of specific cancers. Mutation of the *TP53* gene was analysed in 63 oesophageal squamous cell carcinomas (SCC) from various geographical areas for which data on alcohol and smoking habits were available. *TP53* mutations were found at high frequency and were preferentially associated with exposure to tobacco and alcohol. Mutational analysis of *P16/CDKN2* was performed by sequencing exons 1, 2 and 3. In 24 oesophageal cancers, two inactivating mutations were found in exon 1, but no mutations, homozygous deletions or detectable gene rearrangements were detected in exon 2, where most of the sequence changes reported have so far been located. Thus, alterations in *P16/CDKN2* did not appear to play a major role in this group of patients.

Dr M. Bignami (Rome, Italy) reviewed her recent studies on cellular tolerance to DNA methylation damage and genome instability. Methylation-tolerant cell lines generally displayed elevated mutation rates and microsatellite instability associated with defective mismatch repair (MR). Tolerance was found to be a recessive phenotype and two complementation groups were identified. Clones of group I exhibited microsatellite instability, and mutation rates were increased up to 8-fold. In contrast to wild-type cells, extracts from group I clones were unable to perform O^6-methylguanine [O^6-MeGua]-dependent repair synthesis on a methylated plasmid. This failure correlated with the inability to correct single mismatches *in vitro*. Human colorectal carcinoma cell lines with defects in MR were also unable to carry out this repair synthesis, providing the first evidence that O^6-MeGua in DNA can be processed by MR.

The methylation of cytosine residues in vertebrate DNA predisposes these pyrimidines to become mutational hotspots in the germline and in somatic cells. Deamination of 5-methylcytosine gives rise to thymine, which is inherently more difficult for the cell to recognise and repair than uracil, the deamination product of cytosine.

Dr P.A. Jones (Los Angeles, California, U.S.A.) and his colleague **Dr C. Schmutte** have, for the first time, directly compared the abilities of human cell extracts to repair G:U and G:T mismatches in DNA by excision repair. G:U

mispairs were repaired by excision at 600-fold greater rates than those seen for G:T mismatches, emphasising the importance of cytosine methylation in DNA in the generation of mutations associated with specific human cancers. The frequencies of somatic mutations at CpG sites in the *TP53* gene vary markedly in different human tumours, depending on the type of tumour and the suspected aetiological agent.

Dr D.E. Brash (New Haven, Connecticut, U.S.A.) and **Dr A. Ziegler** (Zürich, Switzerland) presented recent results on the roles of sunlight and the *TP53* gene in human skin carcinogenesis. The distinctive pattern of mutations induced by UV light, i.e. C → T changes at sites of adjacent pyrimidines, including CC → TT, was found in the *TP53* gene in skin cancers and precancers. To determine how p53 prevents skin cancers, *TP53* knockout mice were exposed to UV light and their skin was examined for apoptotic keratinocytes generated by UV overexposure ('sunburn cells'). Sunburn cells were much rarer when one or both *TP53* alleles had been inactivated. This indicates that the *TP53* gene participates in a 'cellular proofreading' mechanism, which is evidently important early in skin carcinogenesis: UV-damaged cells recognise their abnormality and self-destruct by apoptosis, thereby removing precancerous cells from the skin. Because a *TP53* mutation will make the cell resistant to apoptosis, a population of sun-damaged cells will become enriched for *TP53*-mutants.

In his studies on UV-induced *TP53* mutations in non-melanoma skin cancer, **Dr J. Pontén** (Uppsala, Sweden) found two distinct patterns of response, termed reactive and clonal, respectively. The clonal pattern was specified by (i) point mutations at known *TP53* hot-spots and (ii) its morphology, with sharp borders against the surroundings. The clonal pattern was seen in 146/236 biopsies from normal facial skin of people greater than 50 years of age; it was strongly apparent in premature cells and typically vanished upon differentiation. 120/180 cases of DPL (dysplasia)/CIS (carcinoma *in situ*)/SCC showed a clonal *TP53* pattern (DPL, 78%; CIS, 64%; and SCC, 42%). 15 cases with bands, DPL, CIS and/or invasive SSC were analysed in detail for the topography of *TP53* mutations in multiple areas of synchronous immunohistochemically p53-positive lesions. Preliminary data suggest that (i) immunohistochemical positivity is mostly accompanied by ⩾1 mutation of the *TP53* gene; (ii) regardless of cell morphology, these mutations have the signature of UV damage; (iii) mutations occur early in the sequence DPL → CIS → SCC and do not increase in number with morphological progression; clonal bands may contain unique mutations compared to mutations of adjacent CIS or SCC; (iv) CIS and SCC coexisting topographically have the same *TP53* mutation; (v) *TP53* mutations may exist in morphologically normal epidermal cells and persist in mature immunohistochemically negative cells, proving that the latter derive from the former; and (vi) loss of the remaining *TP53* allele is a common feature of the development of CIS or SCC.

GENES AND PROTEINS INVOLVED IN THE REPAIR OF SPECIFIC DNA DAMAGE: RELEVANCE TO CARCINOGENESIS AND CANCER THERAPY

Dr T. Lindahl (South Mimms, U.K.) described the current state of knowledge regarding base-excision repair, a major pathway required for correction of spontaneous hydrolytic and oxidative DNA damage as well as DNA lesions inflicted by alkylating agents. Most DNA replication and repair enzymes in mammalian cell nuclei (e.g. DNA polymerases α, β, δ and ε) have direct counterparts in yeast. In contrast, the abundant mammalian enzymes that bind to, and are specifically activated by, DNA strand interruptions, poly(ADP-ribose)polymerase and DNA-dependent protein kinase, have not been detected in yeast (nor has p53, which is elevated in response to DNA strand-breaks). A family of four distinct DNA ligases are present in human cell nuclei, whereas only a single ligase has been detected in yeast. The cellular responses to DNA strand-breaks may thus differ markedly between higher and lower eukaryotes. Biochemical analyses with human cell extracts and purified enzymes have elucidated several key processing steps occurring at DNA strand-breaks, and may allow the identification of protein defects in inherited human syndromes associated with hypersensitivity to DNA-damaging agents.

The studies of **Dr K. Tanaka** (Osaka, Japan) suggest that the XPA protein (missing or altered in patients of xeroderma pigmentosum [XP] group A) is involved in the damage recognition step of nucleotide excision repair (NER). The XPA protein preferentially binds to DNA damaged by UV, cisplatin or osmium tetroxide, via a DNA-binding domain containing a C4 type zinc finger motif. XPA also binds to the ERCC1 repair protein and to replication protein A (RPA), enhancing the damaged DNA-binding activity of XPA and suggesting that a specific interaction between these proteins is required for an early step of NER. XPA null mice are defective in NER and—contrary to wild-type or heterozygous mice—developed SCC on their back skin at high frequency after irradiation with low doses of UVB.

Dr J.-M. Egly (Strasbourg, France) reported on TFIIH, a multisubunit protein complex previously identified as a basal transcription factor required for the initiation of gene transcription. Four of the subunits of TFIIH, p80/ERCC2, p89/ERCC3, p62 and p44, are implicated in NER; three others, p40/MO15, p38/cyclin H and p32, have been identified as cell-cycle regulators. TFIIH, as well as its yeast counterpart, contains several enzymatic activities. DNA-dependent ATPase activity reflects the potential DNA helicase activities of p89 and p80; the cyclin-dependent kinase complex, containing at least MO15 and cyclin H, utilises the carboxy-terminal domain of the large subunit of RNA polymerase II as a substrate. Micro-injection of highly purified TFIIH into cells from patients deficient in ERCC2 and/or ERCC3 restores the DNA repair reaction. Clinical symptoms such as dwarfism, microencephaly, deafness, "wizened facial appearance", mental retardation, infantile sexual development, or atrophy of the optic nerve, cannot, however, be explained on the basis of DNA repair deficiency, but point to dysfunctioning of TFIIH in the regulation of cell survival, transcription and cell proliferation.

Dr L. Samson (Boston, Massachusetts, U.S.A.) focused on the response of cells to the toxic effects of alkylating agents. Several eukaryotic DNA repair genes, which provide resistance to alkylation damage, have been cloned by their ability to functionally complement alkylation-sensitive strains of *E. coli*. These include yeast, rodent and human O^6-alkylguanine–DNA alkyltransferases (AGTs) and

3-MeAde (3-methyladenine) DNA glycosylases. Studies using mutant yeast strains altered in their capacity for repair of DNA alkylation damage, revealed that AGTs, which repair mutagenic O^6-alkylguanine residues in DNA in a one-step suicide mechanism, protect *S. cerevisiae* against both the mutagenic and cytotoxic effects, and 3-MeAde DNA glycosylases only protect against the cytotoxic effects of alkylating agents. A human *AGT* transgene was expressed in mouse bone marrow cells, and shown to confer substantial resistance to the toxic effects of N-nitroso-N,N'-bis(2-chloroethyl)urea (BCNU). Murine embryonal stem cells (ES-cells) bearing homozygous null mutations in a *3-MeAde DNA glycosylase* gene have become highly sensitive to the toxic effects of various alkylating agents.

Dr M. Sekiguchi (Fukuoka, Japan) has used gene targeting to establish mouse lines deficient in the *AGT* gene. Contrary to wild-type mice, *AGT* knock-out mice suffered early death upon administration of N-methyl-N-nitrosourea (MeNU) (50 μg/g body weight). The bone marrow of these animals became hypocellular, and leucocyte and platelet counts decreased strongly, reflecting a reduced reproductive capacity of haematopoietic stem cells and the protective action of *AGT* towards the cytotoxic effects of N-nitroso compounds.

Dr S.L. Gerson (Cleveland, Ohio, U.S.A.) reported that transgenic mice overexpressing the *AGT* transgene were protected from the development of MeNU-induced thymic lymphomas and other tumours induced by methylating agents. The AGT repair protein also protects cells from the cytotoxic effect exerted by the anticancer agents, temozolomide and chloroethylnitrosoureas, which attack at the O^6-atom of guanine in DNA. Most recently, he utilised a mutant *AGT* gene, described by **Dr A.E. Pegg**, and retroviral gene transfer to express the mutant *AGT* in human haematopoietic CD 34+ bone marrow progenitor cells. The mutant *AGT* is resistant to inhibition by the *AGT* inhibitor, O^6-BeGua. Marked potentiation of protection is observed in transduced cells expressing the mutant *AGT* gene against the cytotoxic effect of the combination of O^6-benzylguanine (O^6-BeGua) and BCNU. Animal and other preclinical studies are underway to verify the potential utility of this approach in patients receiving O^6-BeGua and BCNU.

Dr J. Thomale (Essen, Germany) reported on the immuno-analytical quantification of the overall and gene-specific formation and repair of defined DNA alkylation products in mammalian cells. The rates of O^6-alkylguanine repair in rat cell DNA differed distinctly as a function of the size of the alkyl group. O^6-MeGua was repaired with similar efficiency in active and in non-transcribed genes, predominantly by *AGT*, whereas O^6-ethylguanine (O^6-EtGua) climination from transcribed genes appears to be complemented by excision repair. Notably, (O^6-EtGua)-derived *H-RAS* gene mutations were not detected in N-ethyl-N-nitrosourea (EtNU)-induced rat mammary tumours compared to a high frequency of G:C → A:T transitions in the MeNU-induced counterpart tumours. Correspondingly, transgenic surplus AGT repair capacity in mammary epithelia resulted in a substantially reduced incidence of mammary tumours after exposure to MeNU, but not to EtNU, emphasising the significance of differential gene-specific repair in carcinogenesis.

Because 8-oxo-7,8-dihydro-2'-deoxyguanosine triphosphate (8-oxo-dGTP), produced by reactive oxygen species in the cellular nucleotide pool, can be incorporated into DNA and cause mutations, mammalian cells contain an enzyme activity that hydrolyses 8-oxo-dGTP to 8-oxo-dGMP thereby preventing misincorporation. **Dr K. Sakumi** (Fukuoka, Japan) has cloned the cDNAs of the mouse and human genes encoding 8-oxo-dGTPase (human *mutT* homologue, *MTH1*). *mth1* knock-out mice are being produced. **Dr H. Hayakawa** (Fukuoka) reported that 8-oxo-dGTP is not only generated by direct oxidation of dGTP but also via phosphorylation of 8-oxo-dGDP by nucleoside diphosphate kinase. 8-oxo-dGMP, derived from 8-oxo-dGTP, is further degraded to excretable 8-oxo-deoxyguanosine by a nucleotidase. This nucleotidase, whose most preferred substrate is 8-oxo-dGMP, was partially purified from an extract of human Jurkat cells and its mode of action was elucidated.

Dr J.H. Miller (Los Angeles, California, U.S.A.) described his analyses of several mutator genes in *E. coli*. Two of these, *mutY* and *mutM*, prevent mutations resulting from 8-oxo-7,8-dihydroguanine (OH^8Gua) in DNA. A human cDNA clone containing a large segment of the *mutY* homologue, and the genomic clone containing the entire gene, have been isolated and characterised. Two additional mutators in *E. coli*, *mutA* and *mutC*, which operate via a novel mechanism by encoding mutator tRNAs, were cloned and sequenced.

Dr M. Radman (Paris, France) discussed recent advances concerning mismatch repair (MR) in relation to cancer and other genetic diseases that could be affected by mutator genes. Over two decades of studies with bacterial systems have shown that MR is a DNA editing system for both DNA replication and recombination. Whereas bacteria prosper with one efficient MR system, editing both replicational and recombinational heteroduplexes, higher organisms appear to require multiple systems, including DNA editing by MR, selective cell suicide by heteroduplex-induced apoptosis, and perhaps even selective cell killing by specific lymphocytes as a result of the presentation of 'non-self' peptides from mutant proteins.

hMSH2 (human MutS Homologue), hMLH1 (human MutL Homologue), and hPMS1 and hPMS2 (human Post Meiotic Segregants), belong to highly conserved families of MR (and recombination fidelity) proteins first described in bacteria. As pointed out by **Dr R. Fishel** (Philadelphia, Pennsylvania, U.S.A.), mutation frequency increases up to 1000-fold (mutator effect) when the corresponding genes are defective and recombination between only partially homologous DNA sequences is enhanced (homologous recombination). hMSH2 purified to apparent homogeneity was found to bind specifically to mismatched nucleotides. Aberrant recombination or a mutator phenotype could explain the occurrence of multiply mutated proto-oncogenes/tumour suppressor genes in cancer cells. Alterations of *hMSH2* and *hMLH1* are responsible for cancer susceptibility in 90% of hereditary non-polyposis colon cancer (HNPCC) families, accounting for 15% of all colon cancers or 23,000 new cases per year in the U.S.A. Several interesting mutations of these genes have been identified in both spontaneous and hereditary cancers. Functional changes in the hMSH2 and hMSH1 proteins are under study.

Instability in microsatellite sequences, as observed in 20–30% of most tumour types, especially in HNPCC, is correlated with defective MR. These results and the observation of microsatellite instability early in tumorigenesis suggest that *hMSH2*, *hMSH1* and other mutator genes may play a central role in the development of many tumours.

Dr J. Jiricny (Pomezia, Italy) pointed out that mutations in the *hMSH2* gene on chromosome 2p16, with extensive sequence conservation from bacteria through yeast to humans, have been linked to more than 50% of HNPCC cases. However, a number of tumours linked to the HNPCC locus were found to carry no mutations in *hMSH2*. A new member of the *MSH* gene family, *GTBP*, identified at the same locus, encodes a 160 kDa protein that binds mismatches in the form of a heterodimer with hMSH2. Several tumours and tumour-derived cell lines exhibited *GTBP* mutations. Interestingly, microsatellite instability in *GTBP*-mutant tumours was less pronounced than in *hMSH2*-mutant tumours. Further to its role in mismatch correction in the form of GTBP/hMSH2 heterodimers, hMSH2 is likely to be involved in the recognition and correction of replication-associated structures not addressed by the GTBP/hMSH2 complex.

DNA DAMAGE-INDUCED GENES

DNA damage-induced programmes of cellular gene expression may be either beneficial (DNA repair, cell survival, apoptosis of overmutagenised cells) or detrimental, probably depending on the net outcome of induced signal flow and of reactions to the stimuli. As pointed out by **Dr P. Herrlich** (Karlsruhe, Germany), DNA-damaging chemicals or radiation can induce a cellular response only if their primary interaction with informational molecules is translated into "physiological language", involving signal transduction and post-translational modification of regulatory proteins. One of two identified pathways induced by UVC irradiation originates from DNA lesions; however, it is not yet clear how transcription factors are reached and modified. The other pathway involves the activation of cell surface growth factor receptors, apparently involving downregulation of dephosphorylation of receptor protein tyrosine kinases. The extensive changes in gene expression induced by DNA-damaging agents may be explained by the activation of different cell surface receptors, and other, as yet, unclarified pathways. Activation of the transcription factor CREB is mediated by a UVC-induced p108 CREB kinase. Methyl methane sulphonate (MMS) or N-methyl-N-nitro-N-nitrosoguanidine (MNNG) activate *JUN* and *ATF-2* through Jun kinases; however, kinetics and other features suggest the existence of another, undefined pathway triggered by alkylating agents.

Dr V. Rotter (Rehovot, Israel) discussed the role of the *TP53* gene in cell-cycle regulation. p53 transactivates *WAF-1*/p21, is spatially regulated during the cell cycle, and can induce proliferation arrest at G_1/S or G_2/M cell cycle checkpoints. Overexpression of p53 may force the cell to exit the cell cycle through either apoptosis or cell differentiation, depending on cell type and lineage. There is considerable evidence suggesting that p53 is involved in mediating the response to DNA damage, by orchestrating both an arrest of cells in G_1 to permit prereplicative repair and the decision to undergo apoptosis. Levels of p53 rapidly increase upon DNA damage, mainly through stabilisation of the p53 protein. p35 may also be involved in B-cell differentiation. 70Z3 pre-B-cells expressed wild-type p53 and could be induced to undergo cell differentiation (expression of the kappa light chain gene). γ-irradiation of 70Z3 pre-B cells induced increased p53 levels and a change in the cell-cycle pattern followed by cell differentiation. This response was abrogated by overexpression of mutant *TP53*. Exposure to DNA-damaging agents led to the accumulation of wild-type p53, and overexpression of wild-type p53 induced cells to undergo apoptosis or differentiation.

Only a moderate level of *TP53* mRNA and hardly any p53 protein are found in G_0 lymphocytes. As shown by **Dr W. Deppert** (Hamburg, Germany), triggering of these cells into the cell cycle results in a rapid rise in *TP53* mRNA during G_1, but p53 protein levels remain low. With the onset of DNA synthesis, the *TP53* mRNA level declines, while there is a pronounced increase in p53 biosynthesis. Highly purified p53 protein showed 3′-5′ exonuclease activity. Negative feedback autoregulation of p53 synthesis rapidly increased the level of p53 upon DNA damage. Lethally γ-irradiated G_0 lymphocytes did not show increased p53 protein levels despite the presence of *TP53* mRNA. Irradiated G_0 lymphocytes rapidly underwent apoptosis (apparently p53-independent). In irradiated cells triggered into the cell cycle by concanavalin A, p53 levels rose immediately and apoptosis was drastically delayed. p53 protein expression in G_0 lymphocytes is, therefore, regulated at the translational level, and the response to DNA damage induced by γ-irradiation is primarily directed towards enhanced DNA repair rather than apoptosis.

Dr H.R. Herschman (Los Angeles, California, U.S.A.) reported the identification of a mitogen-induced prostaglandin synthase (PGS), the rate-limiting enzyme in the synthesis of prostanoids, in mitogen-treated fibroblasts. PGS-2 is also induced by inflammatory stimuli in macrophages, epithelial cells and endothelial cells. Using antisense oligonucleotides and pharmacological inhibitors, PGS-2 expression was shown to be necessary for prostaglandin E-2 (PGE2) production in mitogen-induced fibroblasts and in endotoxin (LPS)-induced macrophages, despite the presence of competent, constitutive PGS-1. The data suggest a discrete arachidonate pool that is available only to PGS-2. In contrast, mast cells produce prostaglandin in response to IgE receptor aggregation in both a PGS-1 and a PGS-2 dependent manner. In mast cells, distinct phospholipases release distinct arachidonate pools, made available to the two distinct PGS enzymes.

SEQUENTIAL EVENTS IN CELL TYPE-SPECIFIC CARCINOGENESIS, TUMOUR HETEROGENEITY AND TRANSGENIC MODELS

Malignant progression of human cancer may arise by clonal evolution and selection of deviant population subsets. Dr W.K. Cavenee (La Jolla, California, U.S.A.) described systematic tests of the significance of genetic defects occurring during the progression of astrocytic brain tumours. Analysis of mutations of the *TP53* gene in matched low-grade and recurrent high-grade tumours indicates clonal evolution. Retroviral transfer of wild-type *TP53* into glioblastoma cells led to cessation of cell proliferation, morphological changes and the release of an angiogenesis inhibitor.

Amplification and truncation of the EGF receptor (*EGFR*) gene occurs during the transition of low- to high-grade disease. Retroviral transfer of such genes substantially enhanced tumorigenicity *in vivo* without affecting cell proliferation *in vitro*.

A systematic study of 300 human brain tumours was presented by **Dr A. von Deimling** (Bonn, Germany). These analyses have permitted a more precise molecular classification of astrocytic gliomas and meningiomas, thus establishing new grounds for clinical neuro-oncology. Pilocytic astrocytomas apparently represent a genetically distinct entity. Progression from astrocytoma (predominant lesion: loss of heterozygosity (LOH) at chromosome 17p) to anaplastic astrocytoma seems to involve a gene on chromosome 19q; progression to glioblastoma multiforme may be influenced by gene(s) on chromosome 10. However, most of the latter tumours do not exhibit LOH at 17p and may thus arise *de novo*. Oligoastrocytomas and oligodendrogliomas frequently exhibit LOH at 19q and 1p (not seen in astrocytomas). LOH at 19q and 1p was observed in both the astrocytic and oligodendroglial portions of oligoastrocytomas, suggesting a monoclonal origin. The highly significant association of LOH22 and *NF2* gene mutations in meningiomas suggests that *NF2* is a tumour suppressor gene commonly defective in meningioma formation. However, different molecular pathways may be involved since distinct histopathological meningioma subtypes are characterised by significantly different frequencies of *NF2* mutation (fibroblastic/transitional meningiomas, 80%; meningiotheliomatous meningiomas, 25%).

A frameshift mutation at codon 1309 of the adenomatous polyposis coli (*APC*) gene is very often observed in familial adenomatous polyposis coli (FAP) families. **Dr T. Noda** (Tokyo, Japan) has developed a mouse model for FAP by introducing this mutation into the *APC* gene together with a carboxy-terminal haemagglutinin A (HA) epitope tag. The mutant APC protein is predominantly localised in the cytoplasm and forms a heterodimer with wild-type APC protein. Multiple adenomas or adenocarcinomas, showing LOH of the *APC* gene, developed along the intestinal tracts of all heterozygous mutant mice. A 'second hit' affecting the remaining wild-type *APC* allele is apparently required for malignant transformation. When the *APC* mutants were crossed with *TP53* mutant mice, tumour progression was not affected, but the incidence of colon tumours was significantly increased.

Binding of the E6 and E7 oncoproteins of human papilloma virus (HPV) 16 to p53 and pRb leads to p53 degradation and pRb alteration. **Dr C.A. Reznikoff** (Madison, Wisconsin, U.S.A.) has found that in cultured human uroepithelial cell(s) (HUC), E6 and/or E7 increase the number of precrisis cell doublings and delay senescence. These cells rarely become immortalised, but if so, this is always accompanied by chromosome alterations. Additional events may thus be required for E6- or E7-transformed cells to escape senescence. Four of five independent E6-immortalised clonal HUC lines showed loss of 3p, with the smallest common region lost at 3p14-pter, while 3/3 E7-HUCs exhibited clonal 20q gains (20q11-qter region over-represented). The significant associations of E6 with 3p loss and E7 with 20q gain, and between the frequency of random chromosome anomalies in all E6- compared to all E7-immortalised HUC lines, implicate functional alterations of p53 (but not of pRb) in generating chromosome instability. Over-representation of gene(s) on 20q may thus complement pRb alteration, while deletion of 3p gene(s) may synergise with loss of p53 in overcoming senescence and initiating tumorigenesis. 3p losses and 20q gains have also been observed in human cancers, including bladder carcinomas.

Tumour heterogeneity, irreversible or reversible, represents the sum of multiple genetic and non-genetic alterations in key systems regulating cell proliferation and morphogenesis. **Dr S. Hirohashi** (Tokyo, Japan) showed that the cadherin system, which mediates Ca^{2+}-dependent homophilic cell–cell adhesion, is inactivated by multiple mechanisms in poorly differentiated, highly invasive carcinoma cells. Mutations have been found in the genes encoding E-cadherin and its undercoat proteins, α and β catenins, which connect E-cadherin to actin filaments and establish firm cell–cell adhesion. Transcriptional inactivation of E-cadherin caused by CpG-methylation in the E-cadherin promoter also plays a significant role. Tyrosine-phosphorylation of the E-cadherin–catenin complex might subvert cell adhesion; an association of c-erB-2 with β-catenin was demonstrated.

Dr F. Mitelman (Lund, Sweden) pointed out that the presence of karyotypically unrelated clones within epithelial neoplasms is being reported with increasing frequency, in particular in tumours of the skin, head-and-neck, breast and colon. Such clones are often small but may be numerous and they are usually pseudodiploid with only a single or few but balanced aberrations. At least two interpretations of this type of cytogenetic intratumour heterogeneity are possible. Either an invisible primary genetic alteration preceded the chromosomal aberrations, and the visible aberrations are secondary changes accrued during tumour progression, or the unrelated clones originated from different cells. The former interpretation seems less likely since no examples exist of similar massive heterogeneity in tumours with a well-established primary chromosomal abnormality.

As described by **Dr L. Luzzatto** (New York, New York, U.S.A.), haemolysis in paroxysmal nocturnal haemoglobinuria (PNH) is due to an acquired, intrinsic abnormality of red cells. These cells have a greatly increased sensitivity to complement, due to a deficiency of all proteins normally anchored to the cell membrane by a glycosylphosphatidyl inositol (GPI) anchor, and specifically of a membrane inhibitor of reactive lysis (CD59). The PNH abnormality has now been shown to arise through a somatic mutation in a multipotent stem cell in the X-linked gene *PIG-A*, which encodes a protein required for an early step in GPI biosynthesis. Thirty-four different somatic mutations in the *PIG-A* gene, spread throughout the entire coding region, the majority of which cause frameshifts through small deletions, small insertions and deletion–insertions, were identified in 26 PNH patients. The close relationship between PNH and aplastic anaemia (AA) suggests that a survival or selective advantage is conferred by *PIG-A* mutations to cells of PNH clones in the context of bone marrow failure. This is supported by the new finding that approximately 30% of patients with PNH show two independent mutations. In rare cases, PNH patients develop acute myeloid leukaemia (AML). PNH may thus be a preleukaemic clonal disorder

with only a relative proliferative advantage of PNH cells compared to normal stem cells; in AML, by contrast, the proliferative advantage is absolute.

Dr H. Zarbl (Seattle, Washington, U.S.A.) discussed recent evidence suggesting that activating *Ha-ras-1* mutations arise as background mutations in epithelial cells of the developing rat mammary gland. A novel topological structure within the c-*Ha-ras-1* promoter ('toposwitch') was found in mammary epithelial cells, but not in liver cells, suggesting developmental regulation of its formation. When the fraction of *Ha-ras-1* alleles that retained the 'toposwitch' was measured in mammary epithelial cells as a function of time after carcinogen exposure, the results demonstrated that MeNU, but not dimethylbenzanthracene (DMBA), initiated the 'toposwitch' mechanism. MeNU-initiated loss of the toposwitch might induce the progression of pre-existing *Ha-ras-1*-mutant clones towards malignancy by increasing intracellular Ha-ras protein levels above the threshold required for the phenotypic expression of transformed phenotypes.

Dr Zhao-Qi Wang (Vienna, Austria) has analysed the function of *c-fos* proto-oncogene in development and oncogenesis in transgenic and knock-out mice. Mice lacking *c-fos* develop osteoporosis due to a block in osteoclast differentiation. Macrophages, an osteoclast-related cell type, are also affected, indicating an essential role for c-fos in osteoclast differentiation and osteoclast/macrophage lineage determination. Transgenic mice overexpressing c-fos develop osteosarcomas (transformation of osteoblasts). This phenotype is specific for c-fos, since transgenic mice overexpressing other AP-1 members (e.g. c-*jun*, *jun*B and *fos*B) exhibit no phenotype. Bone tumour formation in *c-fos* transgenics was drastically reduced in a genetic background in which one *c-fos* allele is disrupted (*c-fos*$^{+/-}$), and was almost absent in a *c-fos*$^{-/-}$ background, suggesting that the threshold level of c-fos and the presence of osteoblasts are important in osteosarcoma formation. The functional co-operativity between c-fos and c-jun in [c-fos-]induced oncogenesis was studied using bitransgenic mice overexpressing both c-fos and c-jun. These animals exhibited enhanced bone tumour development, most likely due to increased osteoclast activity and bone remodelling, indicating that overexpression of both c-fos and c-jun may affect other cell types in addition to osteoblasts. Putative AP-1 responsive genes are thus important regulators of osteosarcoma formation.

To assess how different proto-oncogenes can contribute to the development of malignant lymphomas, **Dr A.W. Harris** and colleagues (Melbourne, Australia) have established strains of transgenic mice expressing oncogenes (linked to the immunoglobulin heavy chain (Eμ) enhancer) specifically in lymphoid cells. Constitutive expression of the Eμ-*myc* transgene in the B-cell lineage predisposed the animals to pre-B- and B-cell lymphomas. Lymphomagenesis was greatly accelerated when other transgenes such as *N-ras, abl, bmil, bcl2* or *cyclin D1*, were combined with Eμ-*myc* by an interstrain cross. Such experiments helped to establish *CYCLIN D1* as the oncogene in the *BCL1* locus translocated in mantle cell lymphoma. In Burkitt's lymphoma, mutations in the *TP53* gene commonly occur together with a deregulated *MYC* gene. To test the co-operativity of the two altered genes, mice were generated that inherited both an Eμ-*myc* transgene and a disrupted *TP53* allele. Lymphomas developed within the first 6 weeks of life. Most had lost the wild-type allele of *TP53*, implying that *TP53* deletion and *MYC* deregulation suffice to transform lymphocytes to malignancy. Recent experiments with *TP53*$^{-/-}$ haematopoietic cells suggest that *TP53* loss contributes to malignancy by removing a barrier to indefinite cell proliferation.

The clonality of T-cell lymphomas that originate in *myc/pim-1* bitransgenic mice indicates the requirement for additional events in the progression to full malignancy. To isolate genes that co-operate with both *myc* and *pim-1*, **Dr T. Möröy** (Essen, Germany) used provirus tagging in Eμ-*L-myc/pim-1* bitransgenics. Accelerated tumour formation was observed in infected animals. The *gfi-1* gene on mouse chromosome 5 was transcriptionally activated by proviral integration in a large number of these tumours. Forced expression of the *gfi-1*-encoded zinc finger protein in [IL-2]-dependent T-cells provoked increased survival upon IL-2 depletion. These data suggest that *gfi-1* is a proto-oncogene co-operating with both *myc* and *pim-1* genes in T-cell lymphomagenesis, possibly via mediation of growth factor independence.

Dr W.J. Muller (Hamilton, Ontario, Canada) presented recent results concerning events in development of human breast cancer. Overexpression of *c-erbB-2/HER2/neu* appears to be an important determinant in mammary tumour progression. In mammary adenocarcinomas of *neu*-transgenic mice somatic mutations in the transgene lead to a catalytic activation of neu. Sequence analyses showed in-frame deletions of 7–12 amino acids leading to constitutive tyrosine phosphorylation of neu. The molecular basis for the sensitivity of mammary epithelia to the oncogenic action of neu remains to be elucidated, but c-scr tyrosine kinases have been found to be implicated in neu-mediated signal transduction. Activation of c-scr occurs through its direct physical association with tyrosine phosphorylated neu via an SH2 domain. This interaction is neu-specific since c-scr is incapable of binding the closely related EGFR.

The importance of 5-cytosine methylation in gene control and its subversion in cancer has remained controversial because of the indirect or correlative nature of most studies. Using gene targeting, Dr R. Jaenisch (Cambridge, Massachusetts, U.S.A.) has generated mouse strains carrying a defective DNA methyltransferase (*MTase*) gene. While mice heterozygous for the mutation were apparently normal, homozygotes died at the postgastrulation stage with their genomic DNA partly demethylated, indicating that the maintenance of normal DNA methylation levels is essential for embryonic development. A combination of genetics and pharmacology was used to assess the effects of reduced MTase activity on *APC*^{Min}-induced intestinal neoplasia in mice. Reduced MTase activity due to heterozygosity of the *MTase* gene, in conjunction with weekly administration of the MTase inhibitor 5-aza-deoxycytidine, lowered the average number of intestinal adenomas from 113 (control) to only 2 polyps in the treated heterozygotes. Hence, MTase activity contributes substantially to the development of intestinal neoplasia in this mouse model. These results question the oncogenic effect of DNA hypomethylation and are consistent with a role for MTase in the generation of the C:G → T:A transitions seen at high frequency in human colorectal tumours.

NOVEL APPROACHES TO TUMOUR-SELECTIVE THERAPY AND RATIONAL DRUG DESIGN BASED ON MOLECULAR GENETICS AND CELL BIOLOGY

Dr P. Workman (Macclesfield, Cheshire, U.K.) pointed out that the present revolution in our understanding of the molecular genetics and biochemistry of multistep-oncogenesis is opening up a host of new therapeutic targets for rational, mechanism-based drug discovery and gene therapy. Such approaches will be accelerated further by advances in genome analysis and bioinformatics. The currently most promising targets include genes involved in the control of cell proliferation, signal transduction, apoptosis, invasion, angiogenesis, metastasis and immunomodulation, as well as genes mediating drug resistance, including DNA repair genes.

Currently available delivery systems generally suffer from a lack of specificity. Referring to the utilisation of the 5'-promoter sequence of the murine *tyrosinase* gene to target gene expression to cells of the melanocytic lineage, **Dr I.R. Hart** (London, U.K.) showed that incorporation of such promoter elements into Ela-deleted adenoviral expression constructs resulted in cell type-specific targeting of heterologous genes. The targeting of genes, which were either immunostimulatory (the *IL-2* gene) or represented so-called suicide genes (the herpes simplex virus gene (*HSVtk*)), resulted in therapeutic responses in murine models of malignant melanoma. Combination of these two genetic approaches, possibly in bicistronic vectors, could enhance therapeutic efficacy. Antitumour effects obtained with *HSVtk*/gangciclovir combinations were more pronounced in immunocompetent than in immunoincompetent, athymic animals. Thus, the release of tissue antigens, as a consequence of tumour cell killing mediated via the toxic effects of gangciclovir, may stimulate an immune response capable of dealing with even a weakly immunogenic tumour such as the B16 melanoma.

Dr W.F. Benedict (The Woodlands, Texas, U.S.A.) discussed the use of documenting *TP53* and *RB1* mutations as potential prognostic markers for various human cancers, including bladder and lung cancer. When mutations occurred in both genes within the same tumour, overall survival was particularly poor, although in several cohorts studied, each mutant gene was an independent determinant of prognosis. The possible use of tumour suppressor genes for gene therapy was detailed, as well as the fact that the *TP53* gene was already being used in phase I studies. Also considered for phase I clinical trials are constructs incorporating the *RB1* gene initiated at the second start codon. The resulting 94 kDa protein (p94) had significantly more growth and tumour suppressor activity than wild-type p110. This was also the case in tumours that had retained wild-type *RB1* function. The increased potency of p94 may be due to its prolonged half-life and to the fact that it remains in its underphosphorylated active form for an extended period.

Activation of some oncogenes induces apoptosis, interpretable as one of the host defence mechanisms counteracting neoplastic development. Because cell death mechanisms may be inhibited in cancer cells, molecular analyses of apoptosis could prove useful in relation to cancer prevention and therapy. **Dr Y. Tsujimoto** (Osaka, Japan) focused on the *BCL-2* and *ICE/CED-3* family genes which, respectively, function against apoptosis and drive the cellular death machinery. A widely accepted mechanism involves *BCL-2* activity on reactive oxygen species (ROS). In a system of cell death in hypoxia, however, *BCL-2* or *BCL-XL*, a related gene with similar death-sparing activity, exhibited an anti-apoptotic function by a mechanism other than through regulation of ROS activity. ROS are not common mediators, but rather trigger apoptosis. Several novel interleukin-1,β-converting enzyme (*ICE*)-related genes (*RICS*) have recently been identified. One of these, *RIC-2* (*TX*), induces apoptosis when overexpressed. *[RIC-2]*-induced apoptosis is inhibited by the ICE inhibitor crmA, but not by YVAD-CHO, a tetrapeptide ICE inhibitor, indicating that the *RIC-2* product has a substrate specificity similar to, but distinct from that of ICE.

To determine whether wild-type *TP53* could reverse the loss of chemosensitivity in p53-negative cells, **Dr P.V. Danenberg** (Los Angeles, California, U.S.A.) studied the effects of drugs on HL-60 cells stably expressing, respectively, transfected wild-type *TP53* or *TP53* genes mutated at codon 248 and 143 (controls). Cells expressing the wild-type *TP53* transgene were more sensitive to a number of anticancer drugs with different mechanisms of action than either the parent HL-60 cells or the mutant *TP53* controls. The thymidylate synthase (TS) inhibitor 5-fluoro-2'-deoxyuridine (FdUrd) was greater than 10-fold more cytotoxic to wild-type *TP53* transfectants than to the parent cells or controls. Whereas all of the *TP53* wild-type transfectants could be killed by FdUrd, subpopulations of both the parent and mutant p53 cells survived very high FdUrd concentrations and resumed growth upon removal of the drug. Increased sensitivity of wild-type *TP53* cells to FdUrd correlated with proportionately greater inhibition of TS activity *in situ*. At each concentration of FdUrd, the extent of apoptosis was considerably increased in wild-type *TP53* cells. Moreover, wild-type *TP53* cells underwent arrest in G_1 upon FdUrd treatment, whereas the parent cells did not. Vector-driven expression of exogenous wild-type *TP53* in *TP53*-negative cells thus restored many of the responses of naturally *TP53*-positive cells.

Angiogenesis is downregulated in the mature brain, but may be reactivated under pathological conditions, such as tumour growth and progression. **Dr K.H. Plate** (Freiburg, Germany) investigated the expression of vascular endothelial growth factor (VEGF) and its cognate tyrosine kinase receptors flt-1/VEGF receptor-1 and flk-1/KDR/VEGF receptor-2 (where KDR is kinase insert domain-containing receptor) during brain development and angiogenesis induced by human glioblastomas and rat cerebral transplants of C6 or GS-9L glioma cells. The data suggest a tightly regulated paracrine control of endothelial cell proliferation and angiogenesis, which is transient during brain development, switched-off in mature brain, and turned on during tumour growth in glioma/glioblastoma cells (VEGF) and in the host vasculature (flt-1 and flk-1). The growth of tumours originating from glioma cell grafts in nude mice or syngeneic rats was inhibited significantly by transfer of a signalling-defective *FLK-1* gene into endothelial cells *in situ*. These data identify VEGF as an angiogenesis factor in human and rodent glial tumours, and the VEGF/flk-1 system as a possible therapeutic target.

As pointed out by **Dr U. Graeven** (Bochum, Germany), melanoma progression is highly correlated with the degree

of vascularisation in the primary tumour. Therefore, the *in vitro* expression of VEGF and its receptors *KDR* and *FLT* was studied in melanoma cell lines and primary human melanocytes. VEGF mRNA expression was detected by polymerase chain reaction (PCR) in all melanomas, in SV40 T-transformed melanocytes and in 1/4 normal melanocytes. As analysed by ELISA for $VEGF_{165}$, none of the normal melanocytes, but all the melanomas and the SV40 T-transformed melanocytes, showed autonomous VEGF secretion. PCR for *KDR* and *FLT* revealed mRNA expression in all melanomas and 1/4 melanocytes. *In vitro* assays failed to show growth stimulation of melanoma cells by VEGF. The role of its receptors in melanoma progression, in addition to a paracrine endothelial cell stimulation, remains to be elucidated.

To identify molecules selectively expressed by angiogenic endothelial cells as potential candidates for therapeutic targeting, **Dr H.G. Augustin** (Göttingen, Germany) has analysed gene expression in migrating versus resting endothelial cells, using differential RNA display and recombinant antibody technology. A number of novel endothelial cell-specific monoclonal antibodies (MAbs) were generated. The gene encoding follistatin, an activin-binding protein, proved to be expressed exclusively by migrating, as opposed to quiescent, endothelial cells. The follistatin/activin system appears to be involved in the autocrine regulation of endothelial cells in angiogenesis, modulating the growth-inhibitory functions of members of the TGF-β growth factor family.

With increasing evidence supporting the notion that effective immune responses can be elicited by cancer cells, the therapeutic exploitation of specific antitumour host defences remains a topical issue. As discussed by **Dr E. Mihich** (Buffalo, New York, U.S.A.), adriamycin (ADM) has multiple immunomodulating effects, such as the activation of macrophages, the stimulation of cytotoxic lymphocytes (CTLs), and the induction of IL-2 production by T-cells. In the syngeneic EL4 lymphoma-C57BL/6 mouse model, treatment of advanced tumours with non-toxic doses of ADM plus IL-2 resulted in a high proportion of long-term survivors (LTS). Tumour necrosis factor (TNF) stimulated CTLs in addition to its multiple other immunomodulating effects. Non-toxic combinations of cyclophosphamide with TNF also induced a high proportion of LTS in the EL4 model, contrary to either drug or cytokine alone. In all cases, $CD8^+$ cells were required for a positive therapeutic outcome. The unique immunomodulating effects of certain anticancer drugs in combination with cytokines may thus be exploited to develop curative strategies.

Type-I/EGF receptor-related tyrosine kinases are overexpressed in various types of human tumours, particularly in mammary and ovarian carcinomas, but expression is low in normal epithelial tissues. These growth factor receptors are thus candidate target molecules for tumour-directed therapy. **Dr W. Wels** (Freiburg i. Br., Germany) reported that the uptake of therapeutic molecules bound to cell surface receptors can be effected either via receptor turnover or by ligand-induced internalisation. The variable domains of MAbs FRP5 and 225, which bind to the extracellular domains of ErbB-2 and EGFR, respectively, were cloned, and fusion genes coding for single-chain antibody molecules (scFv) were produced by joining light and heavy chain variable domains. Recombinant immunotoxin genes were constructed by adding scFv encoding DNA to sequences encoding truncated *Pseudomonas* exotoxin A (ETA) devoid of its own cell-binding domain. Similarly, an immunotoxin specific for ErbB-3 and ErbB-4 was generated by fusion of human cDNA encoding the EGF-like domain of the growth factor heregulin β1 (HRGβ1) with the modified *ETA* gene. The bacterially expressed immunotoxins scFv(FRP5)-ETA, scFv(225)-ETA, and HRGβ1-ETA, bind to the respective receptors and exhibit potent cytotoxicity in cultured human tumour cells. Low-dose treatment specifically inhibited the growth of receptor-positive tumour xenografts in nude mice.

Dr K.D. Bagshawe (London, U.K.) described the antibody-directed enzyme prodrug therapy (ADEPT) approach which aims to overcome the limitations of antidrug conjugates and ultimately to restrict cytotoxic action to tumour sites. Antibody–enzyme conjugates (AECs) have proved to be non-toxic and to localise in tumours efficiently. The enzyme acts as an amplification mechanism. After clearance of the enzyme from blood and normal tissues, a large number of prodrug molecules can thus be activated in the extracellular space of a tumour, to produce a drug which is a 100-fold more toxic than the prodrug. High drug concentrations should, therefore, be attainable and drugs should be able to diffuse through tumours efficiently. In a pilot clinical trial in patients with advanced, drug-resistant colorectal carcinoma, 4 partial and 1 mixed responses were seen in 8 evaluable patients using a benzoic acid mustard prodrug developed in preliminary studies.

Recent data on NB-506, a promising new indolocarbazole anticancer agent targeting topoisomerase I, but also inhibiting DNA polymerase α and RNA polymerase II, were presented by **Dr S. Nishimura** (Tsukuba, Japan). NB-506 showed differential cytotoxicity in various cell lines, with a positive correlation between cellular drug content (accumulation), and cytotoxic effect. No cross-resistance to NB-506 was seen in multidrug-resistant cell lines. In various human cancer xenografts in nude mice, NB-506 not only inhibited tumour growth, but also caused tumour regression. Moreover, NB-506 strongly inhibited the growth of metastases in various murine metastasis models. NB-506 has a wide therapeutic window and very low cumulative toxicity in terms of mouse mortality.

Dr L.A. Loeb (Seattle, Washington, U.S.A.), who concluded the Conference with an impressive discussion on the future impact of molecular biology on understanding cancer, described the selection of biologically active molecules from large populations of random sequences. This provides a method to analyse the relation between enzyme structure and function and a technology to create new molecules, e.g. new enzymes for gene therapy. The approach has been applied to the analysis of promoter sequences, β-lactamases, thymidine kinase (TK), human immunodeficiency virus (HIV) reverse transcriptase, DNA polymerases, and DNA repair proteins. HSV TK, in contrast to the human enzyme, phosphorylates a variety of nucleoside analogues. This difference in substrate specificity has been exploited for the treatment of herpes infections and recently as a suicide target in cancer gene therapy. Oligonucleotides containing random sequences for different segments comprising the putative nucleoside binding site of the *HSV TK* gene were substituted. By transforming *E. coli* and

screening more than 8×10^6 clones, more than 2500 different active *TK* mutants were obtained, many with multiple amino acid substitutions. Some of these mutants encode enzymes with increased heat stability, and both increased and decreased K_ms and V_{max}s for thymidine, or with an increased ability to phosphorylate 3′-azido-3′-deoxythymidine. Using both positive and negative selection, mutants were isolated that preferentially phosphorylate ganciclovir and aciclovir. One mutant contains six amino acid changes within the putative substrate binding site. The evolution of new, more efficient enzymes by random sequence mutagenesis will provide a source of new activities for gene therapy.

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