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A1

The *in vivo* cell kinetics in breast carcinogenesis

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Background: Disruption of the balance between apoptosis and proliferation is considered to be an important factor in the development and progression of tumor. In this study we determined the *in vivo* cell kinetics along the spectrum of apparently normal epithelium, hyperplasia, preinvasive lesions and invasive carcinoma, in breast tissues affected by fibrocystic changes in which preinvasive and/or invasive lesions developed, as a model of breast carcinogenesis.
Materials and method: A total of 32 areas of apparently normal epithelium and 135 ductal proliferative and neoplastic lesions were studied. More than one epithelial lesion per case was analyzed. The apoptotic index (AI) and the proliferative index (PI) were expressed as the percentage of TUNEL (TdT-mediated dUTP-nick end-labelling) and Ki-67 positive cells, respectively. The proliferative/apoptotic index (P/A) was calculated for each case.
Results: Statistical analysis demonstrated significant differences among the tissue groups for both indices ($P<0.0001$). The AIs and PIs were significantly higher in hyperplasia than in apparently normal epithelium ($P=0.04$ and $P=0.0005$, respectively), in atypical hyperplasia than in hyperplasia ($P=0.01$ and $P=0.04$, respectively) and in invasive carcinoma than in *in situ* carcinoma ($P=0.0001$ and $P<0.0001$, respectively). The two indices were similar in atypical hyperplasia and in *in situ* carcinoma. The P/A index increased significantly from normal epithelium to hyperplasia ($P=0.01$) and from preinvasive lesions to invasive carcinoma ($P=0.04$), whereas it was decreased (NS) from hyperplasia to preinvasive lesions. A strong positive correlation between the AIs and the PIs was found ($r=0.83$; $P<0.0001$).
Conclusion: These findings suggest accelerating cell turnover along the continuum of breast carcinogenesis. Atypical hyperplasias and *in situ* carcinomas might be kinetically similar lesions. In the transition from normal epithelium to hyperplasia and from preinvasive lesions to invasive carcinoma, the net growth of epithelial cells results from a growth imbalance in favour of proliferation. In the transition from hyperplasia to preinvasive lesions there is an imbalance in favour of apoptosis.

A2

Rescue of HER-2-positive breast carcinoma cells from dormancy by growth factors produced during wound healing

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Background: Clinical and experimental data have raised the possibility that surgical removal of the primary tumor promotes the growth of metastatic lesions.
Purpose: This study was undertaken to determine the effect of wound healing drainages and postsurgical sera obtained from breast carcinoma (BC) patients on proliferation of dormant BC cells and to assess the role of HER2 oncoprotein in this proliferation.
Method: Proliferation of dormant BC cells was evaluated *in vitro* by SRB colorimetric assay. Growth factors were identified by inhibition with specific antibodies and displacement of ^{125}I-EGF from its receptor. Cellular damage was measured by creatine phosphokinase level. The role of HER2 was analyzed by removal of HER2 from the membrane and inhibition by the anti-HER2 monoclonal antibody herceptin.
Results: Healing wound drainages and postsurgical sera from BC patients stimulated the *in vitro* growth of BC cells. Removal of the HER2 oncoprotein from BC cell membrane led to a dramatic decrease in the induced proliferation. Drainage-induced proliferation was around 50% inhibited by antibodies directed against EGF-like factors, including HB-EGF and TGF-α. Levels of these growth factors in postsurgical sera, as well as the level of drainage-induced proliferation, were directly correlated with the entity of surgery ($r=0.8$, $P=0.0007$ and $r=0.64$, $P=0.009$, respectively). Treatment of the tumor cells with herceptin, abolished the patients' drainage-induced proliferation when added to cultures before the growth stimulus.
Conclusion: HER2 overexpression by BC cells plays a major role in the postsurgery rescue of metastatic BC cells from dormancy. Herceptin appears to inhibit this growth induction. A prospective randomized clinical trial of perioperative treatment with herceptin of BC patients is starting.

A3

Asynchronous LOH analysis of ductal carcinoma *in situ* from patients who subsequently developed invasive ductal carcinoma

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Management of women with ductal carcinoma *in situ* (DCIS) is currently a major concern. Biological characteristics in the light of progression from DCIS to invasive ductal carcinoma (IDC) remain unknown. Our previous study [1] investigating synchronous lesions demonstrated higher LOH frequencies in parallel with the tumor progression from atypical ductal hyperplasia (ADH) to DCIS and IDC [1]. We report here an asynchronous LOH analysis of DCIS from patients who subsequently developed IDC.

We collected 88 biopsy specimens, originally diagnosed benign, from the patients who subsequently developed IDC in the ipsilateral breast. Seven asynchronous lesions of initial biopsy (re-evaluation was DCIS) and the respective IDC were subjected to LOH analysis in this study. Thirteen microsatellite markers, which were mapped to and/or very close to the tumor suppressor genes or regions with frequent LOH in breast cancer, were used.

LOHs were observed in parallel with the tumor progression from DCIS to IDC in all cases except for one that developed IDC in another quadrant. The six patients developed IDC near the initial biopsy, and presented similar or identical histopathologic features. LOH analysis of biopsy specimens from patients who subsequently developed IDC demonstrated acquisition of genetic change at an earlier stage, as the same allele at the same genomic locus was lost in DCIS.

Our results suggest that genetic alternations accumulate during cancer progression from DCIS to IDC, and DCIS presents a high risk of developing invasive transformation.

Reference

1. Amari *et al. Oncol Rep* 1999, **6**:1277.

A4

Phagocytic activity of monocytes in patients with breast cancer at different clinical stages

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The investigation was designed to evaluate numerical and functional properties of peripheral blood monocytes (PBMo) in patients with breast cancer at different clinical stages. Monocyte phagocytosis test was performed in 19 patients with benign breast tumor, 29 patients with breast cancer and 10 healthy subjects. Cancer patients were divided into three groups on the basis of the clinical stage of disease: group A, patients with localized disease; group B, patients with regional lymph node metastasis; and group C, patients with distant metastasis. Patients with advanced disease (group C) showed an increase in neutrophils, but no differences in the total count of leukocytes and absolute number of lymphocytes as compared with healthy individuals, patients with benign breast tumor or patients with lower stage. However, the mean number of monocytes decreased in patients with benign disease and further decreased in cancer patients, reaching the significantly lowest value in patients with distant metastasis. Phagocytic activity of PBMo was found to be significantly lower in patients with benign tumor, and it became further reduced in the cancer group, related to the clinical stage. Thus, we noted a sixfold decrease in the capacity of phagocytosis, fourfold decrease in percentage of phagocytosis and twofold decrease in phagocytic index in patients with advanced stage (group C). The alterations in number and function of PBMo in patients with benign and malignant breast tumor were observed in close association with clinical stage of disease, and thus they could be considered as indicators of tumor progression. However, further studies are required to determine whether monocyte dysfunction could provide additional prognostic information in the case of breast cancer diagnosis and therapy.

A5

The CC chemokine RANTES as a potential contributor to breast cancer progression

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In breast carcinoma, high levels of tumor-associated macrophages are correlated with lymph node metastases and clinical aggressiveness. Potential candidates that may support the recruitment of monocytes from the circulation into breast tumors are the members of the CC subfamily of chemokines. In the present study we evaluated the expression of the CC chemokine RANTES in sections of breast cancer patients diagnosed in different stages of disease. Our results indicate that high incidence and intensity of RANTES expression were directly correlated with a more advanced disease, suggesting that the chemokine may be involved in breast cancer progression.

Analyses performed by using the T47D and MCF-7 human breast adenocarcinoma cells indicated that RANTES expression is tightly regulated by cytokines. Furthermore, the results of our study indicate that T47D-derived RANTES partially contributes to monocyte migration, and suggest that *in vivo* this chemokine may be involved in inducing monocyte infiltration to breast tumor sites. In the present study we further characterized the paracrine and autocrine mechanisms by which RANTES may support breast cancer progression. The results suggest that RANTES may be involved in a complex process, in which a crosstalk between infiltrating monocytes and the tumor cells may affect tumor progression.

A6

The relevance of translational research for radiotherapy in breast cancer

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In several EORTC trials the role of radiotherapy in breast cancer has been examined. It has been shown that patients with DCIS radiotherapy have a reduced risk of both invasive and noninvasive ductal cancer recurrences. For patients with early breast cancer we demonstrated that a boost of 16 Gy reduces the risk of recurrence in the breast by nearly a factor of 2, and is especially clinically relevant for patients younger than 50 years. In locally advanced breast cancer patients, a similar reduction in the local recurrence rate was seen when chemotherapy or hormontherapy was added to radiotherapy.

Despite these achievements, we are not able to select enough patients with the need for radiotherapy, and the required dose in this patient population. New techniques, such as comparative genomic hybridization assay, DNA microarrays, functional DNA screens and functional yeast assays, may guide us more precisely toward the optimal treatment strategy in individual patients. Furthermore, these research lines offer the possibility to investigate the mechanism of action, and therefore lead to the development of new drugs that will potentiate the cell-killing effect of radiotherapy. This lecture focuses on the integration of these new techniques in relation to the obtained results from the above-mentioned clinical trials.

A7

Monocyte phagocytic function in patients with breast cancer during therapy

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The present study was designed to elucidate phagocytic function of peripheral blood monocytes in patients with breast cancer during surgery and chemotherapy. Absolute and relative number of peripheral blood leukocytes and monocyte phagocytic function (percentage of phagocytosis [PP], phagocytic index [PI] and capacity of phagocytosis [CP]) were determined in 29 patients with breast cancer and 10 healthy individuals. These parameters were determined at the time of diagnosis, following surgery and after chemotherapy. The total count of circulating leukocytes, and absolute and relative counts of polymorphonuclears and lymphocytes were not significantly different between investigated groups, before and after therapy. The mean number of monocytes was significantly lower in cancer patients at diagnosis, but increased following surgery reaching the control value. There were no significant postchemotherapy changes in the number of monocytes. PP, PI and CP were decreased at the time of diagnosis. PP and CP recovered to normal values following surgery, but PI remained decreased. Following chemotherapy PP and CP remained stable, whereas PI further decreased reaching the values significantly lower than those found before the start of chemotherapy. However, 3 months after last cycle of chemotherapy, all tested parameters returned to normal values. These results showed that phagocytic activity of cancer patients' monocytes, decreased at diagnosis, returned within the normal range after surgical therapy. However, we need time to determine whether the alteration in PBMo phagocytic activity may provide additional prognostic information when monitoring surgically treated breast cancer patients.

A8

Spontaneous apoptosis of circulating T-lymphocytes and its correlation to their prolactin receptor expression and prolactin plasma levels in patients with breast cancer

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We have previously shown that a higher percentage of circulating $CD3^+$ T lymphocytes undergo spontaneous apoptosis in cancer patients as compared with normal controls. Prolactin (PRL) has been reported to inhibit apoptosis in various cell types, including a Nb2 rat lymphoma cell line. In addition, there is evidence that the human PRL-antagonist hPRL-G129R induces apoptosis in breast cancer cell lines. We investigated a possible relationship between prolactin receptor (PRL-R) expression and apoptosis of $CD3^+$ T lymphocytes, as well as PRL plasma levels, in patients with breast cancer. Peripheral blood mononuclear cells of patients ($n=11$) and sex-matched normal controls ($n=12$) were stained with Annexin V, anti-Fas mAb (CD95), mouse antihuman PRL-R mAb B6.2, anti-CD3 mAb and respective isotype control mAbs. Multicolor flow cytometry was used to compare expression of these markers on T cell. In patients, $37 \pm 19\%$ (median $\pm$ SD) of $CD3^+$ cells bound Annexin V, marking early apoptosis of T lymphocytes compared with $17 \pm 10\%$ in controls ($P<0.004$). Furthermore, $82 \pm 15\%$ of the $CD3^+$ T cells were Fas^+ in patients, compared with $51 \pm 9\%$ in controls ($P<0.0001$). All $CD3^+$ T lymphocytes were positive for PRL-R expression in breast cancer patients, as well as in normal control individuals. The mean fluorescence intensity of PRL-R on T lymphocytes of breast cancer patients was 106–172 (median 119) compared with 87–176 (median 123), suggesting no difference in PRL-R expression on T lymphocytes in patients versus controls. PRL plasma levels were comparable in patients and normal controls (4.8 ± 3.4 ng/ml versus 9.8 ± 4.6 ng/ml). In concordance with these findings, PRL was not able to inhibit the onset of apoptosis of Jurkat cells, a thymic lymphoma cell line, incubated with Fas cross-linking CH-11 mAb. These results indicate that PRL/PRL-R might not be involved in modulating Fas/Fas ligand interactions, which are, in part, responsible for apoptosis of T lymphocytes, leading to excessive turnover of T cells in the circulation of patients with breast cancer.

A9

Mutation detection in familial and sporadic breast cancers by denaturing high-performance liquid chromatography (DHPLC)

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Objective: Denaturing high-performance liquid chromatography (DHPLC) is a recently developed method for detection of mutation that is gaining importance as a screening method for analyzing familial breast cancers, as well as heterogeneous tumor material.
Method: DHPLC was established for mutation detection in *BRCA1/2* diagnostic, using more than 200 different positive controls. Up until now, 64 DNA samples from patients with familial background for breast cancer (BC) were analyzed by DHPLC for *BRCA1/2* mutations. An additional 136 sporadic BC were examined for p53 mutations, analyzing exons 5–8 by DHPLC. Positive results were confirmed by direct DNA sequencing.
Results: The analysis of 64 DNA samples from patients with familial background for BC revealed several mutations and unclassified variants (UVs). Twenty-three different p53 mutations could be detected in 138 sporadic BC. Dilution of mutant DNA by wild-type DNA revealed the high sensitivity of this method: 5% mutant DNA is sufficient to achieve a positive DHPLC result. However, confirming a positive DHPLC result by DNA sequencing is difficult in heterogeneous tumor material.
Conclusion: DHPLC is a reliable, high-throughput technique for detection of mutation in familial breast cancers, as well as in heterogeneous tumor material.

A10

Specific immunotherapy of MUC1-positive adenocarcinomas with a recombinant vaccinia virus expressing MUC1 and IL-2

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Current therapies for most types of cancer focus on either surgical or radiotherapeutic eradication of the primary tumor as the best opportunity for cure. Therapy of disseminated disease has focused on chemotherapy, but, with the exception of certain rarer types of tumors, few patients are cured by chemotherapy, and even improvements in survival have been difficult to demonstrate. TRANSGENE's current approaches to oncology focus on the stimulation of the body's own immune system to induce rejection of tumors. One of these approaches is antigen-specific therapy. The first product candidate for antigen-specific therapy expresses the tumor-associated MUC1 antigen, stimulating a cellular immune response that may be useful in treating breast cancer and various epithelial cancers, such as lung, pancreatic and ovarian cancers. The product developed is a recombinant vaccinia virus containing sequences that code for human MUC-1 antigen and interleukin-2. A phase I trial in nine women with breast cancer was performed, in which the potential product was well tolerated without serious side effects, and MUC1-specific immune responses were observed. Phase II trials in breast and in prostate cancer began during the second quarter of 1998. A phase I trial in lung cancer patients is also in progress. During the same period, a second-generation product was developed. The new construct has been put into a highly attenuated vaccinia virus (modified virus Ankara), the safety of which was tested in a clinical trial in MUC1-positive cancer patients. Based on these results, phase II studies are in preparation to assess the clinical efficacy of this product in different populations of patients whose tumors express the MUC-1 tumor antigen.

A11

Novel morphoregulatory functions for the adhesion receptor Ep-CAM in the mammary epithelium

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Ep-CAM, an epithelial cell–cell adhesion receptor, is often overexpressed in association with proliferation and remodeling in epithelial tissues. Development of the mouse mammary gland during pregnancy is associated with a progressive upregulation of Ep-CAM expression, eventually reaching very high levels at day 16 of pregnancy. This phenomenon is paralleled by a concomitant branching of the mammary ductal tree and a sustained epithelial cell proliferation. Using a MMTV-LTR/Ep-CAM transgenic mouse model, we demonstrate that forced expression of Ep-CAM in the mammary epithelium leads to an induction of budding and secondary branching of the glandular tree in virgin females. Interestingly, a complete cycle of gestation in the Ep-CAM transgenic mice results in extreme ductal hyperplasia/ductectasia and lobular hypoplasia, in combination with partially decreased differentiation of both ductal and alveolar (lobular) epithelial cells. Surprisingly, mammary gland involution is affected because of a decreased frequency of apoptotic figures and increased rate of cell proliferation. These results support novel morphoregulatory functions for the adhesion receptor Ep-CAM in epithelial tissue development and homeostasis.

A12

Transcriptional regulation of apoptosis in mouse mammary gland

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Postlactational regression of the mammary gland is characterized by extensive apoptosis of the epithelial compartment. Involution occurs in two phases: an early reversible phase and a later phase accompanied by breakdown of the extracellular matrix and remodeling of the gland. We have used both knockout mice and a cell-culture model to identify the transcription factors that regulate the early phase of involution. Conditional deletion of Stat3 results in diminished apoptosis and delayed involution, whereas, in contrast, loss of IRF-1, a downstream target of Stat1, accelerates the first phase of involution. We have begun to analyze in more detail the molecular events associated with the activation of these transcription factors. Downstream targets have not been identified, although IGFBP-5 may be an indirect target of both Stat3 and IRF-1. In the absence of Stat3, elevated levels of p21, p53 and Stat1 are observed. Using a mammary epithelial cell culture model, KIM-2, and inducible activation of Stat3 and Stat5, we have shown that dimerization of Stat3 alone is sufficient to induce apoptosis of KIM-2 cells. Furthermore, apoptosis can be significantly increased by blocking a survival pathway. In contrast, dimerization of Stat5 provides a differentiation signal and, subsequently, a survival signal for differentiated KIM-2 cells. Interplay between Stat3 and Stat5, identification of downstream targets, and crosstalk with other pathways is now being investigated.

A13

Association of the Epstein–Barr virus with breast cancer: *in vivo* and *in vitro* studies

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Epstein–Barr virus (EBV) may be a cofactor in the development of different malignancies, including several types of carcinomas. We demonstrated the presence of EBV in human breast cancers. We detected the EBV genome by PCR in 51% of the tumor biopsies. In 90% of the cases studied, the virus was not detected in healthy tissue. The presence of the EBV genome in breast tumors was confirmed by Southern-blot analysis. The EBV latent protein EBNA-1 was observed in a fraction (5–30%) of tumor epithelial cells. Expression of the EBV genes BNLF1 and BARF0 will be reported.
A statistical relationship was established between the presence of EBV and several poor prognostic factors. EBV may be a cofactor in the development of a subset of breast cancers.
Latently EBV infected breast undifferentiated human epithelial cell line, MDA-MB-231, was obtained and injected into nude mice. Tumors were obtained in which EBV persists. The persistence of EBV in nude mice tumors, in the absence of any selection, suggests that mammary epithelial cells could be a natural host for EBV. These models will be used for the elaboration of specific therapeutic targets.

Normal breast mammary epithelial cells are now being infected by EBV in order to investigate the oncogenic potential of EBV in those epithelial cells.

A14

Bovine leukemia virus in human breast tissues

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Bovine leukemia virus (BLV) is an oncogenic retrovirus that commonly infects cattle and causes a B cell leukemia/lymphoma in ‰ of 1% of infected cattle. BLV is present in much of marketed beef and dairy products, and breast cancer incidence is greatest in countries with high consumption of bovine foodstuffs. We were therefore interested in determining whether humans were infected with BLV, and whether it might play a role in breast cancer. In previous studies we found that many humans had antibodies to BLV envelope glycoprotein (gp51) and capsid protein (p24), suggesting humans might possibly be infected with BLV. We used immunohistochemistry (IHC) and *in situ* PCR (IS-PCR) to detect viral protein and proviral DNA, respectively, as signs of infection in surgically excised human breast tissue sections. IHC utilized a monoclonal antibody to the BLV p24 capsid protein. IS-PCR utilized primers from the tax region of the BLV genome to amplify a product with directly incorporated digoxigenin-11dUTP tags, which were then detected with a peroxidase-conjugated antibody to digoxigenin. The majority of the breast tissues had evidence of BLV proviral genome and four out of 27 were positive for BLV capsid protein. We are working to accumulate data on enough samples to determine whether infection of breast tissue is associated with the pathologic classification of the tissue. This research was supported by funds from the California Breast Cancer Research Program.

A15

Restored expression of Fhit protein in Fhit-minus breast cancer cells

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The gene FHIT, encompassing the FRA3B fragile site, is located in a region of chromosome 3p14.2 that is often deleted in several types of epithelial cancers and, therefore, it has been investigated as a candidate tumor suppressor. In breast cancer inactivation of FHIT occurred in 70% of the patients, and it is caused by both alterations in the regulation of Fhit expression and by deletions of the gene. Moreover, analysis of 500 cases of breast carcinomas with 20 years of follow up demonstrated that loss of Fhit protein is associated with high proliferative, large and undifferentiated tumors, even though Fhit is not a prognostic factor. In order to elucidate the possible role of FHIT as a tumor suppressor in breast cancer and to identify its mechanisms, Fhit protein-negative breast cancer cell lines lacking endogenous protein expression were stable transfected with FHIT cDNA. Stable transfectant clones showed no alteration in cell morphology and in *in vitro* anchorage-dependent and independent proliferation. A significant delay in the tumor growth in nude mice was observed for some Fhit-positive clones; in an additional case the outgrowing of the tumor was due to loss of Fhit expression *in vivo*. Interestingly, some Fhit-positive clones are able to develop tumors *in vivo* despite high stable Fhit expression, prompting us to investigate the different mechanisms between the two types of Fhit-expressing clones.
Supported by AIRC.

A16

Identification of HER2-positive breast carcinomas as a particular subset with peculiar clinical behaviours

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A large series of 2000 primary breast carcinomas was analyzed for HER2 overexpression, and its prognostic potential. A subset analysis, considering HER2-positive tumors as an independent subset of breast carcinomas, was conducted. In our series, HER2 positivity was not associated with nodal status, unless the number of infiltrated nodes was considered, whereas it was strongly associated with large tumors ($P > 10^{-4}$), grade III tumors ($P > 10^{-4}$), lymphoid infiltration ($P > 10^{-4}$) and absence of hormone receptor expression ($P > 10^{-4}$). HER2 overexpression was a strong prognostic indicator in N^+ patients ($P < 10^{-7}$), whereas its prognostic impact was weak and not statistically significant in the N^- patients. Analysis of the hazard ratio of relapse in relation to time from surgery indicate that the poor prognosis associated with HER2-positivity in N^+ patients was found to be due to a peak of relapses in the first 3–4 years from surgery. Multivariate analysis of different prognostic factors in $HER2^+$ and $HER2^-$ subsets indicated that grade is the most important factor, followed by nodal status, lymphoid infiltration and tumor size in HER2-negative breast carcinomas, whereas nodal status was the most important prognostic factor, with tumor size showing only borderline significance, in the HER2-positive group. Together, the results indicate that HER2-positive breast carcinomas represent a particular subset of tumors with peculiar clinical and pathological behaviours. Thus, conclusions drawn from clinical trials, which serve as the basis for clinical management of breast carcinomas, might not always be valid for this low-frequency subset.
Supported by AIRC.

A17

Prevention of thymic atrophy in mammary tumor bearers by IFN-γ

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Development of the *in vivo* transplantable D1-DMBA-3 mammary tumors results in an alteration of several cytokines in the host, and IFN-γ is one of the most severely downregulated. Notably, the thymuses of these mice display a profound atrophy that is associated with a severe depletion of $CD4^+8^+$ thymocytes. Investigations into the possible mechanisms that lead to this thymic atrophy revealed that the levels of proliferation assessed by *in vivo* labeling with 5′-bromo-2′-deoxyuridine (BrdU) were similar in control and tumor-bearing mice. However, our studies implicated a modest increase in apoptosis, coupled with an arrest at the triple negative stage of differentiation in the thymic hypocellularity in tumor bearers. We have transfected the DA-3 mammary tumor cell line, derived *in vitro* from the *in vivo* D1-DMBA tumors, with the IFN-γ gene and showed the

production of high levels of IFN-γ protein by the transfected cells. Inoculation of hosts with IFN-γ transfected cells 4 days prior to challenge with the D1-DMBA-3 tumor resulted in a blockage of the thymus involution in these mice. In contrast, using in the same protocol untransfected DA-3 cells, the progressive atrophy observed in animals with D1-DMBA-3 tumors was observed. These results suggest that the lack of IFN-γ may be an important factor in the thymic atrophy that occurs during mammary tumorigenesis.

A18

Immunotoxins: experimental design

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One of the major goals of tumor immunotherapy is to overcome immune escape and tumor anergy mechanisms. The identification of (relatively) tumor-specific epitopes is more important for adoptive immunotherapy strategies than their immunogenicity. In the immunocellular approach, D44v-epitope-specific T-cells were cloned introducing a fusion gene encoding the single chain Fv-fragment of CD44v-specific mAb and the zeta-chain of the TCR complex. MHC-independent retargeted cytotoxicity could be shown toward antigen-expressing tumor cells *in vitro* and *in vivo*. In a humoral approach, the fusion gene for a Her2neu-specific scFv and a bacterial toxin was expressed in *E coli*. After purification the fusion toxin showed significant activity in animal experiments using Her2-neu-expressing tumors. Meanwhile, the first six patients suffering from Her2-neu-expressing cancers have been treated topically so far. No significant systemic or local side effects could be detected. Four out of six patients had a local PR/CR. Further clinical studies are warranted and ongoing.

A19

Three-dimensional ultrasound-guided biopsy of breast lesion: a new diagnostic support in the preoperative diagnosis

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A safe and precise preoperative histologic diagnosis is the goal in the modern treatment of breast cancer, to optimize the surgical radicality and to reduce unnecessary mutilation without increasing the risk of residual cancer and later recidives and to optimize the degree of surgical radicality. Ultrasound-guided procedures are useful in biopsying US-detectable breast lesions. In recent years many bioptic procedures have been developed; each shows advantages and disadvantages, but until now none has been defined as the optimal one.

The aim of our study was to improve and to optimize the reliability of the high-speed breast core biopsy, using three-dimensional ultrasound guidance.

From September 2000 to March 2001, we performed 57 high-speed breast core biopsies (Bard Instrument) under 3D US guidance (three dimensional representation of the needle in the lesion): 13 lesions had a diameter >2 cm and 44 had a diameter ≤2 cm; in 13 of the latter the diameter was ≤1 cm. From each tumor we obtained only 2 to 3 bioptic cores (in at least one core we demonstrated the presence of the needle central or marginally in the lesion). All of the patients underwent breast operation after the bioptic histologic diagnosis (at biopsy approximately 90% had breast cancer, and approximately 10% did not have malignant lesions). The diagnosis of malignancy or benignancy was confirmed in 97% of cases (55/57); two false-negative bioptic results indicated hyperplasy and suspected adenosis, but the successive postoperative diagnosis showed clear malignancy.

With 3D US support we were able to reduce the number of biopsies for each lesion (two to three) without reduction of the histologic results, also reducing the costs and the possible complications (haematomas, infections and malignant cell spreading).

A20

Genetic modifiers of cancer risks conferred by *BRCA1* and *BRCA2*

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Approximately 15% of all breast cancer patients have a positive family history of the disease. *BRCA1* and *BRCA2* are two genes that explain major proportions of families with multiple cases of early-onset breast and/or ovarian cancer. Despite the high risks of breast and ovarian cancer conferred by deleterious *BRCA1* and *BRCA2* mutations, a strong variability in phenotype has been observed among families segregating the same mutation. This can range from early-onset breast cancer and ovarian cancer, to late-onset breast cancer without ovarian cancer. Even within a single pedigree, ages of onset of cancer can vary substantially. These observations support the idea that disease outcome in carriers is codetermined by other factors. Different risk estimates for *BRCA* mutations, depending on the type of population studied, also attest to this point. Risk estimates derived from families with multiple cases of early-onset breast cancer, used for linkage analysis to detect *BRCA1* and *BRCA2*, came out substantially higher than those from population-based studies, and risks also appear to differ between populations.

Both genetic and environmental factors are thought to interact with *BRCA1* and *BRCA2*. The influence of nongenetic factors is demonstrated by the finding that even identical carrier twins may differ in disease history. Simple chance may determine age of onset, because multiple genetic mutations are required for full tumorigenesis. Thus far we are not even sure whether modifiers of *BRCA*-conferred risk actually exist, but some suggestive associations have been reported, which will require independent confirmation. Rare alleles at HRAS1 were found to increase risk of ovarian cancer in *BRCA1* carriers, whereas breast cancer risk has been found to be modified by rare alleles at the androgen receptor. Among Ashkenazi Jewish women, a polymorphism in the 5′UTR of the *RAD51* gene increased risk of breast cancer fourfold, but only in carriers of the *BRCA2*-6174delT.

A21

A novel sodium phenylacetate-dextran derivative ester inhibits the growth and angiogenesis of MCF-7ras breast cancer xenografts

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We previously showed that sodium phenylacetate (NaPa) and carboxymethyl benzylamide dextran (CMDB) are both able to block the tumor growth of the breast cancer cell line MCF-7ras in athymic mice. In this study, we studied the effect of a new molecule: a CMDB

esterified by phenylacetic acid (NaPaC). *In vitro*, NaPaC can inhibit threefold to fourfold more MCF-7ras proliferation than NaPa alone. Furthermore, we showed that the antiproliferative activity of NaPaC was dependent on phenylacetate substitution. *In vivo* studies showed that a very low dose of NaPaC (15 mg/kg) inhibited the MCF-7ras tumor growth of 60% without animal toxicity. The inhibition of tumor growth was concomitant with a reduction in angiogenesis and an increase in necrosis. Moreover, we demonstrated that NaPaC inhibited the paracrine mitogenic effect of MCF-7ras conditioned medium (CM) on fibroblasts and endothelial cells proliferation.

A22

Nongenomic effects of estrogens on signal transduction pathways in estrogen-sensitive carcinoma cell lines

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Research thus far has concentrated mainly on the classical steroid hormone (SH) receptors and on the underlying mechanisms of antihormone interactions with these receptors. This is mainly because, in the conventional view, estrogen (E) and progesterone produce most of their effects through interaction with cellular/nuclear receptors with subsequent alteration of the gene-regulating machinery. However, emerging data suggest that these lipophilic hormones are also able to produce rapid effects within several seconds, which cannot be adequately explained through the classical mechanism. Further investigation has recently led to the discovery of membrane-bound forms of E-receptors, which are coupled to cytosolic signal transduction proteins. These rapid responses have been observed in several tissues such as myometrial cells, neurons, endothelium, osteoblasts, granulosa cells and some breast cancer cell lines. The binding of E to these cell-surface forms of E-receptors is thought to activate several second messenger systems via the activation of G-proteins, resulting in the activation of different protein kinases. One such kinase is the mitogen-activated protein (MAP) kinase, which may serve as a stimulus for cell proliferation.

We report preliminary results showing the functional existence of such receptors in the human breast carcinoma cell line MCF-7. Using spectrofluorometry to measure the intracellular calcium concentration, evidence has been collected that the addition of E to these cells causes a rapid rise in the intracellular calcium concentration. This mechanism may prove to be an important initial signaling pathway, leading to the activation of specific protein kinases and subsequent proliferation.

A23

Prognosis and treatment of locally recurrent breast cancer

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Even in case of adequate curative treatment of the primary tumor, approximately 10–20% of all patients will develop a locoregional recurrence in the course of disease. At the time of diagnosis of the recurrence, one-third of patients already has distant metastases. The 5-year survival for patients without metastases is approximately 40%. Prognostic factors are the initial lymph-node status and the disease-free interval. Whether in-breast recurrences (IBTR) carry a better prognosis than chest-wall recurrences after mastectomy is not definitively clear; the better survival after IBTR might be due to a selection bias for breast preservation.

A major goal of treatment for locoregionally recurrent breast cancer is to achieve local control at the recurrent site. This includes surgery and/or radiotherapy. For local control, the patterns of local spread (scar or outside scar, multifocality, size, site) are important. In irresectable lesions, the addition of hyperthermia to radiotherapy yields improved local control. Encouraging local control rates have also been reported from some phase II studies with concurrent radiochemotherapy. In general, patients with local control at the recurrent site have a significantly better long-term prognosis as compared with patients with re-recurrence.

Local recurrence is often associated with subsequent occurrence of distant metastases. Prophylactic ('adjuvant') systemic treatment is theoretically justified, but its impact on prognosis is unclear. Hormonal treatment is recommended if the recurrence is positive for ER/PR receptors. The use of chemotherapy is currently being investigated in several multicentre studies.

A24

Telomerase activity and bcl2 expression in human breast cancer

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Background: Telomerase is a ribonucleoprotein that synthesizes telomers and plays an important role in cellular immortalization. Bcl2 gene encodes for a mitochondrial protein that is thought to prevent apoptosis of normal cells. We previously reported telomerase activity in 74% of human invasive breast cancers, and detected a significant association between telomerase activity and prognostic parameters such as nodal status, tumour size and cellular proliferation. We hypothesized that telomerase reactivation in human breast cancer was associated with reduced immunohistochemical expression of bcl2.

Materials and method: Bcl2 immunohistochemical expression was determined in 25 infiltrating breast carcinomas with known telomerase activity (17 telomerase-positive and 8 telomerase-negative). The percentage of strongly and moderately stained tumour cells for bcl2 was determined by a breast pathologist who was blinded to telomerase data. Fisher's exact test was used to examine the association between telomerase activity and bcl2 expression.

Results: The median percentage of strongly stained tumour cells was 50% for telomerase-positive tumours (range 0–100%) and 45% for telomerase-negative tumours (range 0–100%). Twelve (70%) out of 17 telomerase-positive tumours expressed strong or moderate bcl2 staining in more than 50% of tumour cells, compared with six (75%) out of eight telomerase-negative tumours ($P=1.0$).

Conclusion: Telomerase reactivation appears to be independent of bcl2 protein expression in human breast cancer.

A25

Protection against growth of MUC1/sec transfected mammary tumor cells is mediated by an effector cell with perforin-dependent cytotoxicity

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We have previously found that DA-3 mammary tumor cells transfected with the secreted form of the MUC1-gene (DA-3/sec) resulted in no tumor growth, whereas transfection with the

neomycin vector alone (DA-3/neo) or with the transmembrane form of MUC-1 (DA-3/TM) did not change the growth characteristics of the DA-3 cells. Implantation of the DA-3/sec in nude mice resulted in tumor development, indicating that the immune response is a major cause of the lack of growth of these cells in immunologically intact mice. *In vitro* activated spleen cells from DA-3/sec-injected mice showed strong cytotoxicity against DA-3/sec, but not to DA-3, DA-3/neo or DA-3/TM cells. This cytotoxicity was clearly neutralized by *in vivo* administration of anti-CD3 monoclonal antibody, but not by anti-CD4 or anti-CD8 antibodies. Analysis of the mechanism of killing of the effector cells revealed that anti-Fas antibody did not affect the reaction. Furthermore, FasL-transfected EL-4 cells were not able to kill the DA-3 cells. In contrast, concanomycin A, a perforin-specific inhibitor, greatly reduced the cytotoxicity of spleen cells from DA-3/sec-injected mice. These data suggest that a cell with a phenotype compatible to that of a NK T cell, may be responsible, at least in part, for the protection against the growth of DA-3/sec cells in immunocompetent mice.

A26

Regulation of episialin/MUC1 expression in breast carcinomas: a complex interplay between stimulatory and inhibitory factors

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Episialin/MUC-1 is an epithelial mucin-like transmembrane glycoprotein, which is highly overexpressed in a majority of human carcinomas, in particular breast and ovarian carcinomas. We and others have shown that this overexpression results in reduced adhesion and a higher metastatic potential of the tumor cells [1]. The overexpression of episialin originates mainly at the transcriptional level (10-fold or more increased levels of episialin mRNA are found in breast tumor specimens and breast carcinoma cell lines [2,3]). Consequently, information on the regulation of the episialin promoter may provide a clue to the regulation of metastasis. Examination of the episialin promoter revealed several putative regulatory elements.

(1) We have shown that the episialin promoter is positively regulated by STATs (signal transducers and activators of transcription) in T47D breast and other carcinoma cell lines, using several established inducers of episialin expression (eg IFN-γ and IL-6) as STAT-activating ligands [3]. IL-6 (an activator of STAT3) can stimulate the episialin promoter, and binding of STAT3 to the STAT element in the episialin promoter is observed in bandshift assays [3]. The possible involvement of STATs in tumor progression (eg via episialin expression) is also indicated by the increased levels of activated STAT3 that are found in breast carcinoma cell lines with a high episialin expression [4]. Similar results are found *in vivo*, where constitutive activation of STAT1 and/or STAT3 is found in breast tumors [5].

(2) Glucocorticoids also can stimulate episialin expression in T47D cells through binding of the glucocorticoid receptor (GR) to GRE half sites present in the episialin promoter [4].

(3) In addition, we report that glucocorticoids can attenuate the effect of IL-6/STAT3, using reporter, bandshift and FACS assays [4]. The effect of glucocorticoids on STAT3-mediated episialin expression occurs both through direct interactions between STAT3 and GR, as well as via indirect pathways. Conversely, addition of IL-6 can augment GR-mediated episialin expression [4].

(4) We have also identified a 200-bp sequence fragment far upstream in the episialin promoter that may bind a negative regulator of episialin expression. The identity and exact binding sequence of this putative inhibitor has not yet been determined.

We conclude that the expression of episialin/MUC1 is determined by a balance between positive and negative regulators, which is distorted in tumors, leading to a higher episialin expression and thus a more aggressive tumor type.

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A27

Characterization of a hormonally induced reverse transcriptase (RT) from the human breast cancer cell line T47D: a possible involvement in human breast cancer

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Since the discovery of the mouse mammary tumor virus (MMTV), which was shown to be involved in mouse mammary carcinoma, there has been an attempt to discover similar viruses associated with human breast cancer. We have already shown that the human mammary carcinoma cell line T47D releases retrovirus-like particles in response to steroid treatment.

An RT transcript from T47D cells was isolated, using RT-PCR with primers based on the published T47D endogenous retroviral *pol* sequences. The PCR product encodes a 372-amino-acid long protein. The new T47D RT is almost identical to both previously described RTs from T47D cells, as well as to the enzymatically active RT from human bone marrow cells (95 and 97% identity, respectively). The DNA product was cloned into a bacterial expression system. A 42-kDa RT-related fragment was expressed, purified, and used to immunize rabbits. The antibodies recognize a 60–70 kDa hormonally induced protein specifically in T47D cells. Moreover, steroid hormones induce the apperance of RT protein foci in the cell cytoplasm, as demonstrated by confocal laser microscopy. A parallel hormonal induction of the RT activity in cell supernatants was observed. Expression of the RT-related protein was also detected in tumor cells of breast cancer biopsies sections. These results support the idea that retroviruses may be associated with human breast carcinoma.

A28

Antitumor potential of bisphosphonates

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Bisphosphonates (BPs), especially those with a nitrogen-containing substituent, are potent inhibitors of osteoclast-mediated bone resorption. They have found extensive clinical use for the treatment of both benign and malignant bone disease. BPs bind to hydroxyapatite and rapidly accumulate in bone where they inhibit the mevalonate biosynthetic pathway, thereby preventing the post-translational prenylation of small GTP-binding proteins and inducing apoptosis in osteoclasts. Recent *in vitro* studies indicate that BPs also inhibit proliferation, reduce viability and induce apoptosis in several human tumor cell lines. In addition, BPs reduce the invasion of tumor cells through extracellular matrix and impair the

binding of tumor cells to bone *in vitro*. This growing body of evidence suggests that BPs may have the potential to exert direct antitumor effects *in vivo*, particularly in bone metastases where the local BP concentration is elevated by the enhanced osteoclastic resorption of BP-loaded bone. Several experiments with zoledronic acid (a highly potent BP with an imidazole substituent) administered to mice injected with mammary, prostate or myeloma cancer cells indicate not only inhibition of the tumor-induced osteolysis, but also a reduction in the growth of bone metastases, accompanied by the induction of tumor cell apoptosis. Moreover, zoledronic acid has recently been shown to potently inhibit endothelial cell proliferation *in vitro* and angiogenesis in mice bearing subcutaneous implants loaded with growth factors. Overall, these findings provide a rationale for testing the antitumor potential of the more potent nitrogen-containing BPs in pilot clinical trials.

A29

Transcriptional regulation through the estrogen receptor (ER)-α and splice variants of ER-β via classical and nonclassical signal transduction pathways

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The aim of this study was to analyze the transcriptional regulation of different ERs via alternative cis-elements. For this purpose we performed transient transfections with a luciferase reporter plasmid for estrogen-responsive elements (ERE) and AP-1 elements, and expression plasmids for ER-α, ER-β1 and ER-β2. Cells were left unstimulated or stimulated with E2, tamoxifen or raloxifen. We found that, in the breast cancer cell line SKBR3, ER-β1 lead to a significant inhibition of AP-1 activity by E2, whereas through ER-β2 E2 lead to a stimulation of transcriptional activity. Antiestrogens inhibited transcription through ER-β1 but did not exhibit an effect through ER-β2. When transfecting ER-β1 in SKBR3 cells the basal transcriptional activity increased, in contrast to the results obtained when transfecting the human osteosarcoma cell line U2OS with the same receptor. E2 in SKBR3 cells leads to a significant transcriptional inhibition, whereas this effect is not seen in U2OS cells. Also the antiestrogens tamoxifen and raloxifen exhibit in SKBR3 cells via the same receptor a transcriptional inhibition, but in contrast in U2OS cells they lead to a stimulation of transcription. In summary, splice variants of ER-β are able to regulate the actvity of the AP-1 complex differentially, indicating that the relative expression of these variants in a tumor could modulate its hormonal sensitvity.

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A30

Late radiation sequelae in women after breast-conserving cancer therapy: effects of hyperbaric oxygen therapy

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Background: Persisting symptomatology after breast-conserving surgery and radiation is frequently reported. In most cases symptoms in the breast resolve without further treatment. In some instances, however, pain, erythema and edema can persist for years and can impact on the patient's quality of life. Hyperbaric oxygen therapy was shown to be effective as treatment for late radiation sequelae. The objective of this study was to assess the efficacy of hyperbaric oxygen therapy in symptomatic patients after breast cancer treatment.

Patients and method: Forty-four patients with persisting symptomatology after breast-conservation therapy were prospectively observed. Thirty-two women received hyperbaric oxygen therapy in a multiplace chamber for a median of 25 sessions (7–60). One hundred per cent oxygen was delivered at 240 kPa for 90-min sessions, five times per week. Twelve control patients received no further treatment. Changes throughout the irradiated breast tissue were scored before and after hyperbaric oxygen therapy, using modified LENT-SOMA criteria.

Results: Hyperbaric oxygen therapy patients showed a significant reduction in pain, edema and erythema scores as compared with untreated controls ($P < 0.001$). Fibrosis and teleangiectasia, however, were not significantly affected by hyperbaric oxygen therapy. Seven out of 32 women were free of symptoms after hyperbaric oxygen therapy, whereas all 12 patients in the control group had persisting complaints.

Conclusion: Hyperbaric oxygen therapy should be considered as a treatment option for patients with persisting symptomatology following breast-conserving therapy.

A31

Sentinel lymph node biopsies in breast cancer

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Introduction: Lymph node biopsy is not only important as a prognostic factor, but also influences therapy. However, axillary lymphadenectomy is often accompanied by high morbidity. The sentinel lymph node biopsy (SLN) should reduce the morbidity, but give the same prognosic value. In 97.5% cases metastasis occurs in lymph nodes (LN) of level I first, and only less than 3% directly in LN level II. The first draining LN can be identified either by radioactive material or colouring technique.

Method: Patients with primary breast cancer (41 cases, age 30–80 years), 1 day before surgery, received peritumoral 1–3 ml nanocolloid containing 99m-technetium, with scintigraphy performed 30–120 min later. Alternatively 2 ml Patentblau (2.5% Byk Gulden) was applied during the surgery 15 min before the axillary lymphadenectomy. The marked LN was separated and sent to pathology together with the other LN.

Results: Seventeen patients received Tc-nanocolloid, 21 Patentblue. Three patients were treated with both identification methods. Out of 41 patients, 14 had an axillary metastasis. Comparison of radioactive labelling showed no false-negative results, but 7.3% false negativity was obtained with the colour method.

A32

Organochlorines and breast cancer: effect of exposure to dieldrin on risk and survival

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Some organochlorines have weak estrogenic activity, and may therefore interfere with breast cancer risk and survival. We assessed prospectively risk and prognosis of breast cancer in relation to serum concentrations of several compounds, which have shown to be estrogenic *in vitro* and *in vivo*. Study participants (7712 women)

donated blood twice (1976–1978 and 1981–1983) and were followed for 17 years with regard to development of breast cancer. Information on potential breast cancer risk factors and prognostics were obtained through standardized questionnaires and by linkage to the Danish Breast Cancer Cooperative Group.

Breast cancer risk associated with baseline exposure in 1976–1978, and repeated measurements of organochlorines (average concentration of the two measurements) was examined in two cohort-nested case–control studies, including 240 cases and 477 controls, and 155 cases and 274 controls, respectively. The cases served as a cohort in the survival analysis, in which the average duration of follow up to death was 86 and 79 months after the first and second sampling.

The most consistent finding observed was dieldrin's adverse effect on breast cancer risk as well as prognosis. More than a twofold increased risk was found among women with the highest baseline concentration compared with those with the lowest, and a significant trend was apparent. A high serum dieldrin concentration was also significantly associated with an increased overall mortality, being threefold for baseline measurements and almost sixfold when repeated measurements was assessed. Similar results were obtained when using breast cancer recurrence and/or death caused by breast cancer as end-point.

A33

Fhit loss in familial breast cancer: is loss of DNA repair function linked to alterations at chromosome fragile sites?

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The *FHIT* gene at 3p14.2 encompasses the common fragile site, FRA3B, and is involved in frequent chromosome rearrangements in human cancers. Fhit protein expression is reduced or lost in the majority of esophageal, lung, gastric, cervical, pancreatic, kidney and bladder cancers, and a large fraction of other cancers. Fhit expression in sporadic breast cancers has been studied by several groups, and reported to show alteration in expression of Fhit in 30–50%. Because familial breast cancers were reported to show a higher frequency of LOH at 3p14.2 than sporadic breast cancers, we were interested in whether common fragile regions might be targets for repair by the Brca1 and Brca2 proteins, and might thus be a downstream target in *BRCA1*- and *BRCA2*-induced familial breast tumors. We studied a panel of Brca2-deficient breast tumors and showed that only 18% expressed Fhit strongly, compared with 48% of sporadic tumors ($P=0.002$). Very recently we completed a similar study of *BRCA1* familial tumors, and observed that only 9% of these tumors showed strong expression versus more than 40% of sporadic breast tumors ($P<0.001$, odds ratio 0.09). We conclude that loss of *BRCA1* and *BRCA2* functions affect stability of the FHIT/FRA3B locus and possibly other fragile loci.

A34

ErbB family of receptors in breast cancer

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The ErbB2 receptor tyrosine kinase is overexpressed in many human breast tumors, a phenomenon correlating with more aggressive tumor characterisitcs and a worse patient prognosis. ErbB2 is considered, therefore, as a target for cancer therapy. In this respect, a growth inhibitory antibody (4D5) directed against the extracellular domain of ErbB2 has been raised. Furthermore, the humanized version (Herceptin™) is now being used in the clinic to treat metastatic breast cancer patients whose tumors overxpress ErbB2. In our work, 4D5 has been applied as a selective inhibitor of ErbB2 function. Treatment with 4D5 blocks G1/S phase progression in breast carcinoma cells that overexpress ErbB2. This block correlates with a rapid reduction in ErbB2 phosphotyrosine content, downregulation of signal transduction pathways, a reduction in the expression of proteins involved in the sequestration of the cyclin-dependent kinase inhibitor p27, and relocalization of p27 onto Cdk2 complexes. Strikingly, the 4D5-induced G1 block can be rescued by treatment with various ErbB ligands. The degree of rescue is ligand-related and is associated with activation of specific signaling pathways. Additionally, through comparison with an ErbB2-overexpressing gastric carcinoma cell line (MKN7) that proliferates normally in the presence of 4D5, we have demonstrated that decreased ErbB2 phosphotyrosine levels do not necessarily lead to growth inhibition in response to 4D5. These data imply that ErbB2 overexpression alone is insufficient to predict cellular response to ErbB2-directed therapies. The possible contribution of other ErbB receptors to the process of malignant transformation will be discussed in relation to the evolution of ErbB-directed treatment strategies.

A35

Effect of anthracyclin-based neoadjuvant chemotherapy on disseminated tumor cells in breast cancer patients: an immunocytologic and molecular approach

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Objective: The leading cause of death from epithelial cancer is metastatic tumor relapse due to early dissemination of tumor cells. Cytokeratins are specific markers of epithelial cancer cells in bone marrow. As previously shown, these epithelial cells in bone marrow seem to be resting 'in dormancy'. This biological behaviour might be an explanation for the resistance to cytotoxic agents. In the present study, we evaluated whether primary chemotherapy in locally advanced, nonmetastatic breast cancer can eliminate cytokeratin-positive cells in bone marrow. Furthermore, we investigated the influence of primary chemotherapy on the tumor-associated gene expression.

Method: Twenty-one breast cancer patients underwent bone marrow aspiration before and after neodjuvant chemotherapy. For immunocytologic tumor cell detection we used the monoclonal antibody 5D3 (Biogenex), which is directed against common epitope on cytokeratin polypeptides, including cytokeratin 8/18/19. For the molecular approach, after isolation of RNA, the reverse transcription with Superscript II Reverse Transkriptase (Gibco/BRL) and Oligo $(dT)_{12-18}$ Primers (Gibco/BRL) was performed, followed by the amplification procedure with nested-RT-PCR for β_2-microglobulin, muc-1, CK-20 and carcinoembryogenic antigen (CEA).

Results: Fifteen patients met the inclusion criteria. Before chemotherapy, five out of 15 (30%) had cytokeratin-positive cells in bone marrow. Four out of five were still tested positive after finishing chemotherapy. With the RT-PCR procedure, all bone marrow aspirates showed a signal for β_2-microglobulin (positive control). Expression of cytokeratin-20 was absent, whereas muc-1 was expressed in all aspirates. Two out of five showed expression of the CEA before chemotherapy, which was absent in one and only slight

in the other case after chemotherapy. Before chemotherapy immunocytologic tumor cell detection and RT-PCR procedure for CEA was concurrently negative in 10 out of 15 patients.
Conclusion: (1) A negative result in immunocytochemistry of bone marrow of breast cancer patients seems to agree on the RT-PCR for CEA. (2) Tumor cells could develop a different pattern of gene expression under primary chemotherapy. (3) RT-PCR procedure for muc-1 and CK-20 seems not to be useful for investigating disseminated tumor cells in bone marrow.

A36

Cloning of novel mammary tumor progression and metastasis genes

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Crucial to the prognosis of cancer patients is not growth of the primary tumor, but rather dissemination of neoplastic cells to other organs As the process of the activation and inactivation of genes that are involved in tumorigenesis and metastasis is still poorly understood, the aim of this study is to identify novel mammary cancer progression and metastasis genes *in vivo*. Proviral insertions of mouse mammary tumor virus (MMTV) in mammary epithelial cells are able to activate flanking oncogenes, leading to mammary tumor induction. Classical examples of MMTV-induced oncogenes are Wnt1 and Fgf3. Full neoplastic transformation to an invasive and metastasizing tumor requires activation of collaborating onco/metastasis genes. Thus, additional proviral insertions may lead to metastasis-inducing genes. In this study we used a BALB/c^{+} mouse strain (ie a BALB/c substrain that aquired C3H-MMTV by forster-nursing), and compared extra proviral integrations in a series of sets of independent primary tumors and metastases. As a first step, the isolated tumor sets (primary tumor and metastases) were analyzed by Southern blotting using a MMTV-LTR specific probe. A number of the lung metastases indeed carried additional MMTV integrations, which were not found in the primary tumor. These additional integrations might activate genes being responsible for the lung metastases. To analyze the flanking sequences more efficiently, an adaptor ligation-mediated PCR (Splinkerette-PCR) was modified for the metastasis-related proviral MMTV integrations. To this end, genomic DNA was digested and ligated to a suitable splinkerette linker. The subsequent PCR gets its specificity by using a unique MMTV-LTR-related oligonucleotide and a splinkerette-specific oligonucleotide, which is only able to bind to the DNA if extension of the MMTV oligonucleotide occurs. BLAST/NIX (DNA analysis software) analysis of the derived additional sequences from 23 tumor sets resulted in the discovery of a novel common integration site. The effect of this putative metastasis gene on the metastic potential of mammary tumor cells is presently being investigated.

A37

The association between cyclo-oxygenase-2 expression and cell proliferation and angiogenesis in human breast cancer

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Background: Cyclo-oxygenase (COX) is the rate-limiting enzyme in converting arachidonic acid to prostaglandins. There are two isoforms of COX: COX-1, which is expressed in many tissues; and COX-2, which is the inducible form. COX-2 has been reported to be involved in carcinogenesis and tumour angiogenesis. Therefore, we hypothesized that COX-2 expression was associated with that of vascular endothelial growth factor (VEGF) and proliferating cell nuclear antigen (PCNA) in human breast cancer.
Materials and method: RNA was extracted from 15 human breast carcinomas and adjacent noncancerous tissue (ANCT). COX-2, VEGF 189 and PCNA expressions were estimated by reverse transcriptase-PCR (RT-PCR) and Taqman methodology in the RNA samples. The results were analyzed using Spearman's correlation with Student's *t*-test.
Results: Median mRNA copy number for PCNA mRNA in tumours was 1.65×10^6 (range 3.79×10^5–1.46×10^7). For COX-2, the median mRNA copy number was 4.56×10^5 (range 2.48×10^3–5.10×10^6) in tumours and 2.26×10^6 (range 1.37E+05–4.79E+07) in ANCT. Copy numbers of VEGF mRNA in tumours had a median value of 2.13×10^6 (range 3.42×10^1–3.37×10^7). There was a highly significant correlation between COX-2 and PCNA levels in tumours ($rs = 0.7896$; $P = 0.000001$) and VEGF in tumour samples ($rs = 0.4610$; $2P = 0.0320$).
Conclusion: COX-2 expression is significantly associated with increased cellular proliferation and angiogenesis in invasive breast cancer. The upregulation of COX-2 in ANCT suggests that COX-2 in the host is relevant to mammary carcinogenesis.

A38

Acidification-induced sensitization to thermoradiotherapy in breast cancer

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Hyperthermia is an extensively studied cytotoxic agent, with strong radio- and chemosensitizing potential. Recent positive clinical trials combining superficial or deep heating techniques with radiation therapy strongly support a role for hyperthermia as an adjuvant to radiation. Many *in vitro* and *in vivo* studies have shown that acute extracellular acidification will compromise fundamental protective cellular responses and enhance tumor response to hyperthermia and chemotherapy.
Breast cancers, like most other tumors, exhibit elevated levels of lactate production that provides a basis for selective acidification. A phase I/II clinical trial is underway to test the hypothesis that hyperglycemia-induced acute acidification will sensitize carcinoma of the breast to thermoradiotherapy. Six patients consented to fast for at least 4 h and ingest oral glucose (2 g/kg, 0.44 g/ml) 1.5 h before each hyperthermia treatment (HT) during a course of thermoradiotherapy. Hyperglycemia reduced tumor pH_e before the first hyperthermia session by 0.10 ± 0.04 pH unit (–0.29 to +0.08) from 7.12 ± 0.11 (6.65–7.52); and during the third week of treatment hyperglycemia reduced tumor pH_e in five patients by 0.01 ± 0.04 pH unit (–0.06 to +0.1). The three patients with a CR (60%) exhibited tumor acidification during both sessions, in contrast to the two patients with a PR (40%) who exhibited tumor acidification only during one session. Tumor acidification may indicate tumor response.
Human tumor cells adapted to growth at pH_e 6.7 do not show thermosensitization until pH_e is below 6.3 (pH_i <6.45). Combining an inhibitor of respiration such as MIBG with hyperglycemia blocks mitochondrial respiration and increases lactate production. Thus, tumor oxygenation occurs coincidentally with acute acidification.

Rats bearing the R3230 Ac rat mammary adenocarcinoma were administered 1 g/kg glucose ip, and/or 20 mg/kg MIBG ip. The median pO_2 for glucose plus MIBG was increased from 5.3 to 13.8 mmHg. A single ip injection of glucose or MIBG in rats fasted for 24 h before irradiation did not show an increase in tumor growth delay compared with 5 Gy radiation alone. However, combined treatment with glucose plus MIBG significantly inhibited tumor growth delay. Radiation therapy and glucose plus MIBG was more than additive. These results support our hypothesis that hyperglycemia plus an inhibitor of respiration will sensitize tumors to radiation by oxygenation, in addition to enhanced hyperthermia sensitization by acute acidification.

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A39

A novel phenylacetate-dextran derivative (NaPaC) inhibits breast cancer cell proliferation and modifies their interactions with endothelial cells

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Breast cancer treatments are limited by secondary effects and chemoresistances or hormone resistances. Sodium phenylacetate (NaPa), a nontoxic metabolite, has been shown to induce *in vivo* and *in vitro* antiproliferative effects on various cell types in our laboratory.

We have previously shown that NaPa treatment induced breast tumor cell apoptosis without acquired drug resistance. On the other hand, we have demonstrated that a dextran derivative (CMDB) was not only antitumoral but also antiangiogenic in a MCF-7ras tumor model in animals. Recently, we have synthesized a novel hybrid molecule, CMDB-NaPa ester, called NaPaC, and tested its effect on the proliferation of MDA-MB-231 and MCF-7 breast tumor cells.

NaPaC inhibits, dose dependently, the proliferation of MDA-MB-231 cell (IC_{50} 0.5 mmol/l) and of MCF-7 cell (IC_{50} 1.5 mmol/l). Primary cultured endothelial cells (HUVEC) are weakly affected by NaPaC treatment. Cytostatic effect of NaPaC was evidenced from the accumulation of tumor cells in G0/G1 phase after 96 h of treatment.

As compared with the NaPa parent molecule, this new molecule was 10-fold more efficient on these two tumor cell lines. Moreover, NaPaC induces a strong apoptotic effect, as measured with Annexin V-positive cells, on these tumor cells. In order to understand the interactions between tumor cells and endothelial cells, the conditioned media were prepared and added to HUVEC cells. Our results showed a clear killing effect on HUVEC cells. However, this killing effect can be rescued by adding NaPaC.

Taken together, our results showed that NaPaC is a powerful antitumoral molecule, with cytostatic and proapoptotic effects on MDA-MB-231 and MCF-7 tumor cells. Further studies should be conducted to better understand the mechanism of these mutual interactions between tumor cells and endothelial cells, especially the killing effect on HUVEC cells.

A40

Structural features for peptides binding the class I molecules

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Examination of the MHC crystal structures indicate that key peptide binding positions are defined pockets within the groove of a particular allele of class I molecules; for example, for H-2K^b, the high-affinity binding of ovalbumin peptide SIINFEKL requires P5 to be occupied F/Y, and P8 to have L/I, and other peptides that lack these anchoring amino acids would bind with such a low affinity that would be unable to induce, or be effective targets for CTLs. Using MUC1 8 or 9mers as model peptides, we were able to demonstrate the following: (1) these bind with low affinity; (2) the binding is unusual and the peptides loop out of the groove – indeed, the 9mer loops out so much it can be detected by monoclonal anti-MUC1 peptide antibody; and (3) crystallization structures of K^b with the 8mer (SAPDTRPA) demonstrates that MUC1 and SIINFEKL have an identical shape within the groove, the only difference being imposed by the side chains, which are selectively recognized by T-cell receptor. The amino acids occupied in the specific anchoring pockets are small hydrophobic, rather than long/large hydrophobic F/Y or L/I residues, thus the low affinity of the MUC1 peptide. The implication of the studies is that the rules for high-affinity binding in generation of CTL hold, but that these are not fixed rules: low-affinity peptides bind, and indeed with the same general conformation and shape as high-affinity peptides in one dimension; in another they are clearly more 'flexible'.

A41

The standardized mistletoe preparation Lektinol has antitumoral potencies

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Extracts of Viscum album L have been used for decades for nonspecific stimulation of the immune system in cancer therapy. Mistletoe lectins have been identified as the active components, with cytotoxic and immunomodulatory activities. New experimental data demonstrate that the special extract preparation Lektinol® (Madaus AG, Cologne, Germany), standardized for bioactive mistletoe lectin (ML), has antitumoral potencies *in vitro* and in animal tumor models.

In vitro studies on human tumor cell lines and xenografts showed Lektinol to be highly cytotoxic (ie toward breast, lung, prostate and renal cell cancers).

The *in vivo* antitumoral effects of Lektinol were examined in different subcutaneously growing murine neoplasms following repeated intraperitoneal treatment of 0.3–3–30–300 ng ML/kg. Marked tumor growth inhibition was observed with Renca renal carcinoma, C8 colon 38, and F9 testicular teratoma. The antimetastatic effects of Lektinol were investigated in the B16 melanoma model in mice. Following a single intravenous injection of the melanoma cells, the daily treatment with 3–30–150 ng ML/kg significantly reduced the formation of lung metastases. In parallel, Lektinol enhanced several immune parameters (ie the number of $MAC\text{-}1^+$ mononuclear cells and $CD4^+8^+$ thymocytes in the tumor-bearing animals). In a further study, the effects of locally administered Lektinol were evaluated in the MB49 urinary bladder carcinoma model in mice. After a single instillation of the tumor cells, Lektinol was given repeatedly by intravesical administration of 3–30 ng ML/0.1 ml/animal. Lektinol showed a distinct effect on survival ratio, growth of primary bladder tumors and the formation of multiple metastases.

A42

High-risk breast cancer patients: comparison of lymphocyte phenotypes and function *in vitro*

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The design of a phase III trial in high-risk breast cancer patients is characterized by a dose-dense sequential control (arm B), and a high-dose chemotherapy (HDC) arm with short induction phase and tandem HDC (arm A). It is known that following such a treatment there are profound changes in lymphocyte phenotypes and lymphocyte function. Here we show data on the changes *in vitro* during the different treatment cycles, beginning with the tests 1995.

The lymphocyte membranes were tested using commercially available antibodies (Ortho) and the CytoronAbsolute cytofluorograf. The lymphocyte functions were investigated in a H-3-thymidin incorporation test after stimulation with various stimuli (IL-2, IFN-γ, CD3, ConA, Pokeweed, PHA and *Candida* antigen). In some of the patients, in the supernatant of the lymphocyte proliferation test several cytokines were determined (IL-2, IL-5, IL-10, IL-12, IL-13, IL-16, IFN-γ, GM-CSF and TNF-α). From 80 examined patients, 16 died and eight recurred. In arm A there were 57 patients (11 died and six got a recurrence). Arm B contained 23 patients (five died and two recurred). $CD3^+$ cells were relatively equal in arm A and B patients, whereas there was an enormous difference in the $CD4^+$ and $CD8^+$ cells after end of therapy. In the HDC patients $CD4^+$ cells declined during the follow up and $CD8^+$ cells increased. In arm B there was a normal decline during the therapy and a recovery after 24 months. In a few patients of arm A we observed an elevation in B-cells (CD19), shortly after the end of therapy. The functions of the cells will be presented in tables. All patients had sustained changes of their lymphocyte situation at least for 24 months.

A43

The role of subareolar methylene blue in identifying the sentinel node in patients with invasive breast cancer

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Background: Recent studies have demonstrated that the sentinel node biopsy (SNB) is a reliable and minimally invasive method for determining the axillary node status in patients with breast cancer. However, the methods used for identifying the sentinel node (SN) are heterogenous with variable success rates. Some studies have reported low success rates with methylene blue (MB) dye for the identification of the SN. The present study aims to examine the accuracy of a simple method using subdermal injection of MB in the subareolar region.

Patients and method: A total of 35 women with operable invasive breast cancer undergoing axillary lymphadenectomy were recruited at our centre over a 4-month period (April–July 2000). The SN was identified in the axilla after injecting 1 ml of 1% MB in the subareolar region. The technical success rate, sensitivity and negative predictive value of this simple method were calculated. Furthermore, the cost-benefit of using MB rather than isosulfan blue as the labelling agent was determined.

Results: The SN was successfully identified in 34 (97%) out of 35 patients. Thirteen (37%) out of 35 patients had metastasis in the axillary nodes. The SN correctly predicted the presence of axillary disease in 12 (92.3%) out of 13 cases. The negative predictive value for SN was 96% (22/23). We have estimated that the use of MB rather than isosulfan blue as the labelling agent would save approximately £1.3 million/year in the UK, should the SNB become the standard of care.

Conclusion: Subareolar MB for identifying the SN in patients with operable invasive breast cancer provides a simple and reliable technique that can be used widely.

A44

High-dose chemotherapy with peripheral blood progenitor cell support in breast cancer: WSG AM01 and MM01

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The WSG as a German interdisciplinary group initiated 05/94 a large multicentre phase III trial to evaluate adjuvant high-dose chemotherapy in high-risk breast cancer. About 100 centres all over Germany participated. The second trial in metastatic breast cancer (MBC) was initiated in 4/97 in co-operation with the German Intergroup. The main characteristics of the two trials are listed in Table 1.

Table 1

	AM 01	MM01
Patients	Adjuvant N>9	M1, PR or CR after conventional induction, ER
Arm A	Tandem-E,C,TT	STAMP V
Arm B	EC ×4 → CMF ×3 q2w + G-CSF	Tandem STAMP V
Actually randomized	368	180/480
Status	Open	Open
Treatment related mortality (%)	0%	1.5%

The first trial tests high-dose Tandem E90C3000T400 + PBPC versus dose-dense conventional chemotherapy with G-CSF support. All patients received irradiation of the chest wall and the supraclavicular lymph nodes, and tamoxifen in case of ER+ tumors. The trial will probably be closed by the end of the year. The first interim analysis was done in 1/99, and the second one is planned in 1/02. Interim data will be presented. The trial in MBC randomizes chemosensitive, ER-negative patients to 1× versus 2× STAMPV. The first interim analysis was done in 12/00. Data will be presented.

A45

Human estrogen receptor-α (ER-α) transactivation by selective estrogen receptor modulators (SERMs) on VIT regulatory region in ER-α-negative breast cancer cell line Evsa-T transiently transfected by ER-α

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The action of 11 selective estrogen receptor modulators (SERMs) was investigated in two breast cancer cell lines, the estrogen receptor-α-positive ($ER\text{-}\alpha^+$) MCF-7 and the ERα– Evsa-T.

Our experiments were conducted by transient transfection of these cells by a reporter plasmid carrying the luciferase gene under the transcriptional control of the minimal promoter tk and the regulatory region of vitellogenin A1 gene (Vit-tk-Luc). This latter region is known to include a perfect estrogen responsive element (ERE). Evsa-T cells were cotransfected with an expression vector for the human ER-α.
Estradiol (E_2) always increased transcription of Vit-tk-Luc basal activity in both cell lines. Pure antiestrogens repressed it in MCF-7 cells, and had no effect in Evsa-T cells. Interestingly, in Evsa-T cells as compared with MCF-7 cells, SERMs for which the chemical structure contain clusters that mimic hydrophobic substituents linked to the 11β-position of estradiol conferred greater transcription. Of note, deletion of one half of the ERE site did not affect transcription in Evsa-T cells, but abrogated it in MCF-7 cells. Moreover, substitution of Vit by an AP-1 site failed to activate transcription in each case.
Our results show that some SERMs may act as strong agonists on transcription mediated by transfected ER-α in ER-α⁻ breast tumors with poor prognosis for antihormone therapy. We speculate that additional binding sites for transcription factors, as well as different coactivators, would be involved in this enhancement of activity.

A46

MMP-9 production by T cells from mammary tumor bearers is upregulated by tumor-derived VEGF

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Matrix metalloproteinase-9 (MMP-9) has been shown to be important in tumor invasion and metastasis, and may be implicated in lymphoreticular cell extravasation. T cells from D1-DMBA-3 mammary tumor-bearing mice exhibit an overproduction of MMP-9 compared with the levels expressed in T cells from normal mice, both at the transcriptional and translational levels. This upregulation is more pronounced in animals bearing large tumors. We have previously characterized several tumor derived factors in our system using the *in vitro* DA-3 tumor cells derived from the *in vivo* D1-DMBA-3 tumors (ie PGE_2, GM-CSF and phosphatidyl serine). Treatment of normal T lymphocytes with these factors yielded no increased production of MMP-9. TNF-α and IL-6, although not expressed by the tumor cells themselves, are greatly increased in the tumor bearers' lymphoreticular cells and in their sera. Exposure of normal T cells to these two cytokines also failed to upregulate MMP-9 production. Vascular endothelial growth factor (VEGF) has been shown to be produced by many tumors. Using a VEGF-specific ELISA, we determined that the DA-3 tumor cells, as well as the T lymphocytes from tumor bearers, express high levels of this growth factor. Treatment of normal T cells with VEGF resulted in an overproduction of MMP-9. These results indicate that VEGF may be responsible for the elevated levels of MMP-9 observed in T cells from tumor-bearing mice.

A47

A retrovirus similar to MMTV associated with human breast cancer

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We investigated whether a retrovirus similar to the mouse mammary tumor virus (MMTV) is implicated in human breast carcinogenesis.
Using PCR and specific primers, a 660-bp sequence homologous to the env gene of MMTV was detected in 38% of the human breast cancers. This sequence was absent in normal tissues and other tumors [1]. Samples from several geographical locations have higher or lower frequencies.
The MMTV-like sequence was expressed as RNA in most positive specimens [2]. The complete 9.9-kb proviral sequence of an MMTV-like agent has now been amplified and sequenced in two breast cancers. Structural features of this provirus suggest that it is replicative competent [3].
Primary cultures of env positive tumors show budding retroviral particles, and the supernatant particulate fractions show RT activity, presence of MMTV-like genes by RT-PCR and viral particles by electron microscopy [4]. Experiments to prove infectivity are in progress.
Whether this virus is MMTV or a related human mammary tumor virus is not certain, or is it known how humans are infected. However, if a retrovirus is indeed involved in human breast carcinogenesis, then preventive strategies can be planned.

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A48

Effect of interferon-γ (IFN-γ) on transforming growth factor-β (TGF-β) regulation of sialomucin complex/Muc4

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Sialomucin complex (SMC, rat Muc4) is a heterodimeric glycoprotein complex consisting of a mucin subunit ASGP-1 (Ascites sialoglycoprotein-1) and a transmembrane subunit ASGP-2, which is highly overexpressed on the surface of ascites 13762 rat mammary adenocarcinoma cells. The complex is produced from a single gene and polypeptide precursor. SMC is developmentally regulated in normal rat mammary gland by multiple and complex mechanisms, with levels in the lactating gland being 100-fold those in the virgin gland. SMC transcript levels are enhanced in normal rat mammary epithelial cells by fetal bovine serum, insulin, and IGF-1 by an ERK-1/2-dependent pathway. SMC is post-transcriptionally regulated by Matrigel (extracellular matrix) by inhibition of SMC precursor synthesis. SMC is also post-transcriptionally regulated by TGF-β by disruption of SMC precursor processing into mature ASGP-1 and ASGP-2. The inhibition of SMC levels by TGF-β occurs by an ERK1/2-independent pathway, suggesting that the SMAD or another pathway may be involved in this effect. Interestingly, the inhibition of SMC levels by TGF-β can be blocked by treatment with IFN-γ, which has been shown to block TGF-β effects via a Jak/Stat-dependent pathway. This effect is dose-responsive and is dependent on the order in which the cytokines are added, suggesting that the balance of signaling inputs is important in determining the expression level of SMC. Thus, SMC is regulated by multiple mechanisms, and the delicate interplay of the pathways involved serves to maintain normal levels of the complex and repress potential deleterious effects of overexpression.

A49

Apoptosis-related factors in nonpalpable breast tumors: an immunohistochemical study. Correlation with the mammographic image

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Objectives: Retrospective evaluation of the mammographic appearance of nonpalpable breast cancers and correlation with apoptosis-related factors.
Method: Patients with nonpalpable breast lesions ($n=211$) were evaluated between 1989 and 1999. All patients underwent preoperative mammographically guided needle-excision biopsy. Specimen radiography was always followed. Histological examination revealed 55 cancers (26%; 30 ductal invasive [54.5%], 18 ductal *in situ* [32.7%], five lobular invasive [9%] and two lobular *in situ* [3.8%]). In 41 out of 55 carcinomas, immunohistochemistry was conducted, using monoclonal antibodies against bcl-2, fas and DNA fragmentation, and polyclonal antibody for bax.
Results: Mammography revealed malignant microcalcifications in 42 out of 55 patients (76%) and opacity with undefined borders (greater diameter <1 cm) in 13 out of 55 patients (24%). In 16 out of 41 carcinomas (41%) there was immunostain positivity for bcl-2. In seven out of 16 patients (17.5%) mammography showed microcalcifications, whereas opacity was observed in nine out of 16 patients (22.5%). Twenty-three out of 41 carcinomas (56%) were positive for fas. In 14 out of 23 patients (60%) mammography showed microcalcifications and opacity in 11 out of 23 (47%). Thirty out of 41 carcinomas (73%) were positive for bax and DNA fragmentation. In 17 out of 30 (58%) carcinomas that were positive for DNA fragmentation, mammography showed microcalcifications, whereas opacity was revealed in 16 out of 30 (53%) patients. In 18 out of 30 (60%) carcinomas that were positive for bax, mammography showed microcalcifications, whereas opacity was detected in 14 out of 30 (46%) carcinomas.
Conclusion: Our results suggest the existence of significant correlation between mammographic appearance and expression of apoptosis factors in nonpalpable breast cancers, although the number of patients evaluated was relatively small.

A50

C-erbB-2 overexpression in primary breast cancer: relationship to clinical and histopathological parameters of patients with breast cancer

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Objective: Amplification of c-erbB-2 oncoprotein has been described in 10–35% of primary breast cancers. Breast tumors with immunohistochemical overexpression of c-erbB-2 protein seem to be more aggressive. We evaluated the impact of c-erbB-2 overexpression on clinical and histopathological parameters of patients with breast cancer.
Method: Primary tumors from 417 patients, who were treated at our Department for breast cancer, were studied. Immunostaining of c-erbB-2 oncoprotein was carried out utilizing the monoclonal antibody CB11.
Results: C-erbB-2 overexpression was seen in 72 out of 417 (17%) of primary breast tumors. Patients with positive immunohistochemistry (IHC) for c-erbB-2 were significantly younger ($P=0.015$), on average. The number of involved lymph nodes was higher in patients with positive IHC ($P=0.014$). Nearly all IHC positive tumors (98.6%) were invasive ductal carcinomas, whereas all but one lobular carcinoma were negative. Tumors with negative IHC more often demonstrated positive estrogen ($P=0.001$) and progesterone ($P=0.001$) expression than did patients with positive IHC. There was a significant relation of c-erbB-2 IHC and nuclear grading, Ki67 and p53. No association was found with menopausal status, tumor size, T-staging and presence of metastases.
Conclusion: (1) Overexpression of c-erbB-2 oncoprotein in primary breast cancer tumors may be an indicator of the extent of lymph node metastases in patients. (2) Lobular carcinomas represent a defined subtype of breast carcinomas.

A51

Whole-body hyperthermia in the treatment of breast cancer

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Hyperthermia has two major effects on cancer. (1) Tumor cells can be killed, because they are more sensitive to heat than normal cells. Thereby, membrane components can also be released. Both events can induce antitumoral immunity. (2) It can revert chemoresistance of tumors. The patients in this study were postsurgery and treated with radiotherapy and/or chemotherapy. Additionally to chemotherapy, nonspecific immune stimulation was applied. All patients were studied more than 5 years after the primary diagnosis. A total of 105 patients received the above-mentioned therapy. The 35 patients of the 'hyperthermia group' received whole-body hyperthermia treatment. The distribution of tumor at various staging was practically identical in both groups, as was the median follow-up period: 70 months. In the control group (105 patients) 12 patients died and 61 developed metastasis within a mean period of 36 months. On the contrary, in the hyperthermia group (35 patients) no patient died and only three developed metastasis within 52 months.

A52

Identification and refinement of two regions on chromosomal arm 15q involved in breast cancer progression

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Loss of heterozygosity constitutes a major mechanism of genetic abberations in breast cancer, and strongly indicates the involvement of tumor-suppressor genes in the affected chromosomal regions. Ascertainment and refinement of such deleted regions by highly polymorphic microsatellite markers is a prerequisite for the identification of candidate genes and the isolation of novel genes. Prelimary results from our group indicate the existence of genes located on chromosomal arm 15q that may be involved in breast cancer progression to metastatic stage (Wick *et al*, Oncogene 1996). In this study a panel of 210 primary breast carcinomas, 28

metastases and 17 local recurrencies from primary breast carcinomas were analyzed for loss of heterozygosity by the use of 16 highly polymorphic markers spanning the chromosomal region 15q11-21. After PCR amplification, microsatellite markers were separated by PAGE. LOH15q was seen in 30 out of 45 (67%) metastases and recurrences, but only in 50 out of 210 (24%) primary tumors ($P<0.01$). We identified two subregions defined by microsatellite markers D15S514 (15q15) and CYP19 (15q21.1). LOH15q21.1 was most frequently detected in progressive tumor stages. Importantly, analysis of LOH in several other chromosomal regions (ie BRCA1 and BRCA2, TP53, RB1, ATM) did not demonstrate a general increase in LOH frequencies, indicating that LOH15q is a specific event associated with tumor progression. We are currently analyzing candidate genes located in the regions of interest.

A53

Role of stromelysin-3 in mammary tumor progression

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Numerous studies have provided evidence that, although the transformation of epithelial cells is the *sine qua non* condition for the development of carcinomas, the nature of the connective/stromal tissue environment is crucial for tumor progression. Matrix metalloproteinases (MMPs) that interact with stromal components have been shown to contribute to malignancy in both the early and late stages of tumor progression in human and mouse. Therefore, these studies are of interest to improve our understanding of malignant processes. In this context, the 11th member of the MMP family (MMP11), also named stromelysin-3 (ST3), fulfills this paradigm. It was discovered in 1990 because of its overexpression in a cDNA library established from a human breast cancer biopsy. Later, clinical trials showed that high levels of ST3 expression correlated with a lower survival rate among patients with breast, head and neck, or colon cancer. Therefore, the possibility that ST3 might play a role during tumor progression was promising for the diagnosis, prognosis and design of new treatment. During the past 10 years, numerous experiments have been performed to enhance the knowledge of the biological function of ST3, and to evaluate its clinical relevance. From the data, ST3 appears to be a unique member of the MMP family, exhibiting peculiar features and function.

A54

High-dose chemotherapy in breast cancer: Dutch randomized studies

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The role of high-dose chemotherapy in the adjuvant treatment of breast cancer will eventually be defined by a range of randomized trials that still require years for maturation. Two underpowered single-institution studies (from the MD Anderson Cancer Center and from the Netherlands Cancer Institute) failed to show an advantage for high-dose therapy. A randomized Scandinavian study compared prolonged and intensive chemotherapy without stem-cell support with brief chemotherapy followed by the STAMP-V regimen. The intensive conventional treatment arm was shown to be superior in terms of relapse-free survival. Two large studies comparing conventional dose adjuvant chemotherapy with high-dose chemotherapy have been reported in abstract form: the American Intergroup study (ASCO 1999) and the Dutch National Study (ASCO 2000). The American study shows fewer relapses in the high-dose arm. The Dutch study suggests a modest disease-free survival advantage for the high-dose arm, but further follow up is required to ascertain statistical significance ($P=0.057$, two-sided, at the early analysis). In 2002, a 24% reduction in hazard rate will be detectable with 80% power. Both the efficacy and toxicity of high-dose therapy may depend on the drugs, dosage and schedules selected. In the Dutch study, a regimen was employed that is similar to the frequently used CTCb (STAMP-V) regimen. The carboplatin dose is, however, twice as high, and the agents are administered as short-term infusions rather than as continuous 96-h infusions. This may have an impact on the activation of the prodrug cyclophosphamide; the activation route is strongly inhibited by the presence of even low concentrations of thiotepa.

A55

Novel liposomal vectors for an enhanced gene transfer *in vitro*

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Highly efficient gene transfer methods are basic requirements for a successful gene therapy. Liposomal vectors based on cationic lipids have been proven to be an attractive alternative to viral vector systems concerning production and safety. The major disadvantage of liposomes is their distinct lower transduction rate. To improve the transduction efficiency in comparison with commercially available liposomal vector systems, we have synthesized two novel cationic lipids. In *in vitro* experiments with these novel liposomal vectors, we examined gene transfer efficiency and cytotoxicity. As controls we used the commercially available DC-Chol and FuGene™. We analyzed the cytotoxicity of our new lipids with a dual-reporter-gene-assay and gene transfer efficiency via FACS-analysis in seven gynaecological cancer cell lines. With the new lipids, in different cell lines we achieved equivalent or better transduction rates compared with the results obtained with DC-Chol or FuGene™. Apart from improved transduction rates, cytotoxicity was very low in all cases. These promising *in vitro* results led to further analysis of possible usability of our new lipids in *in vivo* experiments.

A56

Pathway pathology: the wnt and erbB mammary tumors

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Human mammary cancer is frequently associated with the erbB pathway, whereas 'spontaneous' mouse mammary tumor virus (MMTV)-induced mammary tumors are associated with the wnt-1 pathway. Many members of both pathways have been studied in genetically engineered mice. Using examples from the UCD Mutant Mouse Pathology Laboratory, we studied the characteristics of both pathways and found that they have unique, identifiable phenotypes. These observations are the foundation for pathway pathology. Members of the wnt1 pathway tend to form variations of the classical, MMTV-induced, type A, B and P tumors described by

Dunn. Wnt1 tumors are expansile, surrounded by dense stroma, develop around central ducts, retain myoepithelial differentiation, and frequently have squamous metaplasia. Examples include the following: wnt1, wnt10b, APC1, GSK, CKII, B-Catenin, and FGF mice. In contrast, members of the erbB pathway are more likely to resemble human tumors, to be invasive, lose myoepithelial differentiation, form solid nodular asymetrical masses budding from individual ducts, have less stroma, and be less metaplastic. Examples include the following: erbB2, PyV-mT, mutants and bigenics of erbB and PyV-mT, src, and ras. Interestingly, GEM tumors initiated by nuclear factors do not tend to have the characteristics of either of these pathways. Examples: myc and lef1. These observations suggest that the principles of pathway pathology can be applied to human tumors of the breast and other organs. This work was supported by the DAAD (AR, individual grant), the State of California, BCRP JB-0014, and RO1CA89140 from NCI.

A57

CD31 expression by cells of extensive ductal *in situ* and invasive carcinomas of the breast

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CD31 is a surface molecule mediating homo- and heterotypic interactions that control leukocyte trafficking through the endothelial layer. Monoclonal antibodies against CD31 are used as markers of neovascularization. Assessment of angiogenesis in 270 breast carcinomas revealed expression of CD31 in a single case of large (5.2 cm in diameter) high nuclear grade ductal carcinoma, in both *in situ* and invasive components. Expression was limited to the cell membrane, suggesting an adhesion function of CD31 in epithelial cells. At variance with invasive breast carcinomas, angiogenesis is not considered as a prognostic parameter in DCIS, and consequently anti-CD31 MoAb are not included in standard testing. Thus, a reasonable explanation for our finding was that CD31 expression might be underscored in DCIS cells. Therefore, we focused on 32 ductal carcinomas *in situ* (DCIS) larger than 2 cm, pure or associated with invasive ductal carcinoma (IDC). Cancer cells of seven extensive, high nuclear grade DCIS associated with IDC were $CD31^+$. CD31 was expressed by cells of DCIS the were able to colonize lobules and large ducts extending to the nipple (Paget's disease). It was also expressed by IDC, but only in association with CD44. Normal epithelium and hyperplastic epithelial lesions were consistently $CD31^-$. We conclude that CD31 expression is a feature acquired by breast cancer cells in DCIS model. Secondly, CD31 expression mainly correlates with tumor cell spreading within the ductal system; and, finally, the invasive phenotype requires the coexpression of CD31 and CD44.

A58

Neuroendocrine breast carcinomas of aged women may express apocrine differentiation markers

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Neuroendocrine (NE) carcinomas of the breast are a rare entity that diffusely expresses NE markers. We here demonstrate that NE breast carcinomas in elderly women can also express apocrine immuno-phenotype, and analyze the histological and clinical aspects of such differentiation. A selected series of 50 NE tumors (positive for NE markers in >50% of the cells) was tested for the immunocytochemical expression of gross cystic disease fluid protein-15 (GCDFP-15). About 50% of moderately (G2) and well-differentiated (G1) NE breast carcinomas coexpressed the apocrine marker. In these cases specific mRNA for GCDFP-15 (PIP) and for chromogranin A was demonstrated using *in situ* hybridization (ISH). Carcinomas of the alveolar subtype (G2) and poorly differentiated carcinomas (G3) were pure NE carcinomas, devoid of apocrine differentiation. The steroid receptor status of these lesions was evaluated to test a possible involvement of androgen receptors (AR) in apocrine differentiation. The level of AR and the mean age of patients at diagnosis were significantly higher in apocrine than in nonapocrine differentiated tumors. The histological grade and the expression of estrogen receptor significantly influenced the prognosis of these NE carcinomas, either pure or NE-apocrine differentiated. In conclusion, NE breast carcinomas may exhibit divergent apocrine differentiation that might be regulated by the activation of AR in elderly patients. In addition, the possibility to use Chs or GCDFP-15 serum values in the follow up of these patients, as demonstrated in two cases of the present series, can justify the immunophenotyping of the tumors.

A59

Constitutional genomic instability of 9p23-24 in *BRCA2* mutation carriers

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Germ-line mutations in the *BRCA2* gene account for a large proportion of familial breast cancer cases in females, and for the majority of familial breast cancers in males. Recent studies provide evidence for a role of the BRCA2 protein in the maintenance of genomic integrity by involvement in DNA repair and recombination. In order to identify genetic damage resulting from mutated *BRCA2* in humans, we analyzed constitutional karyotypes of *BRCA2* mutation carriers. FISH anlysis from lymphocytes of patients of breast cancer families with germ-line *BRCA2* mutation revealed additional constitutional chromosomal alterations on 9p23-24. The rearrangements observed include inversions, duplications and amplifications. Additionally, a high level of random somatic chromosomal abnormalities on 9p23-24 has been shown. The 9p rearrangements are complex in all families analysed, showing that this chromosomal region has suffered a number of intrachromosomal recombinations. The topography of the 9p rearrangements can differ among family members, even within an individual that can have cell populations with different 9p rearrangements. Collectively, these results point to an association of mutant *BRCA2* with genomic instability and gene alteration in 9p23-24, in at least a subset of *BRCA2* mutation carriers.

A60

Heterocyclic amines (HCAS) and risk of breast cancer

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HCAs are mutagenic and carcinogenic compounds formed in meat and fish prepared by high-temperature cooking methods, such as frying, grilling and barbecuing. The precursors are amino acids,

reducing sugars and creatine, found specifically in muscle meat. One of the HCAs, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), the most abundant HCA in the Western diet, has been found to be a mammary gland carcinogen in rats. Studies in rodents have also shown that PhIP is distributed to the mammary gland and excreted into breast milk. Several epidemiologic studies have found a moderately increased risk of breast cancer with higher intake of red meat. Zheng *et al* (*JNCI* 1998) conducted a case–control study within the cohort of the Iowa Women's Health Study to investigate the potential role of meat and HCAs and the risk of breast cancer. A questionnaire was mailed to women in the cohort who had breast cancer diagnosed during the period from 1992 to 1994, and to a random sample of cancer-free cohort members to obtain information on usual intake of meats and cooking practices. Color photographs showing various levels of doneness for hamburger, beefsteak, and bacon were included. Using a HCA database (Sinha *et al*: *Food Chem Toxicol* 1998), dietary intake of 2-amino-3, 8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3, 4, 8-trimethylimidazo[4,5-*f*]quinoxaline (DiMeIQx) and PhIP were estimated. Multivariate analysis was performed on data from 273 cases and 657 control subjects who completed the survey. Well-done red meat intake was associated with increased risk of breast cancer (Zheng *et al*: *JNCI* 1998). The odds ratios (95% confidence interval) for categorical analysis of PhIP, with first quintile as the referent group, were as follows: second quintile 1.1 (0.6–1.8); third quintile 1.2 (0.7–1.9); fourth quintile 1.4 (0.8–2.3); and fifth quintile 1.9 (1.1–3.4) – *P* value for trend 0.001 (Sinha *et al*: *JNCI* 2000). There was no statistically significant increase in risk with either MeIQx or DiMeIQx. Both animal carcinogenicity studies and epidemiologic evidence suggests that consumption of PhIP may increase the risk of breast cancer, but this hypothesis needs to be investigated further. Simple changes in cooking methods could eliminate the presence of PhIP in foods, if it is conclusively found to increase the risk of breast cancer.

A61

Control of apoptosis in breast by growth factors and extracellular matrix: targets for therapeutic intervention

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Cell survival is adhesion-dependent in normal breast epithelium. Survival requires the integrin class of extracellular matrix (ECM) receptors. We have demonstrated that specific ECM such as basement membrane promote cell survival, whereas others, including collagen I, do not. Basement membrane proteins are largely absent around invasive breast cancer cells. Thus, cancer cells have lost their specific ECM-dependency, presumably due to inappropriate activation of adhesion-regulated survival enzymes. Such enzymes represent potential targets for cancer intervention, particularly where there is sufficient redundancy of signalling on basement membrane to provide reduced or no dependency in normal cells.

We have shown that pp125FAK mediates integrin survival signals in breast epithelia, and phosphatidylinositol 3-kinase overcomes apoptosis induced by dominant negative pp125FAK. Signals downstream of pp125FAK regulate apoptosis through a control on the activity of the proapoptotic protein Bax.

Signal transduction through growth factor receptors can be regulated by adhesive interactions via integrins. We have discovered that pharmacological inhibition of epidermal growth factor receptor signalling strongly induces apoptosis in breast epithelia. The mechanism of apoptosis induction appears not to be through Bax activation, but rather through dephosphorylation of the proapoptotic protein Bad.

Thus, different classes of potent survival regulators (ie adhesion and soluble factors) determine apoptotic cell fate within the same cells through independent control of different mitochondrial acting proapoptotic proteins. Our results broaden the scope for future strategies of cancer intervention.

A62

Normo- and hyperbaric oxygenation of tumors: from bench to bedside

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Tumor hypoxia is an important factor limiting the efficacy of sparsely ionizing radiation and O_2-dependent chemotherapy. Because the tumor pO_2 is the result of a dynamic steady state between oxygen supply and O_2 consumption of the tumor cells, hypoxia could be reduced by improving the O_2 supply for instance by breathing hyperoxic gas mixtures to increase the arterial oxygen partial pressure. This technique seems to be the most effective method to improve tumor oxygenation, and thus to enhance the efficacy of standard radiotherapy and chemotherapy in experimental malignancies, as well as in human tumors. However, the role of varying inspiratory pCO_2 on tumor oxygenation has been discussed controversially. Although carbogen (95% O_2 + 5% CO_2) is used in the clinical setting, it remains unclear whether the beneficial therapeutic effects are more pronounced than with pure oxygen. Because in some tumor entities oxygenation is inadequate and anisotropic, normobaric hyperoxia is often not sufficient to completely eradicate tumor hypoxia. In these cases, breathing of hyperoxic gases under hyperbaric conditions (2–3 atm) may be sufficient to lead to therapeutic results. However, studies on experimental tumors in animals as well as clinical trials in patients showed nonuniform results concerning the therapeutic benefit of hyperbaric hyperoxia, depending on the tumor entity, site of growth, or tumor vascularization. Especially, squamous cell carcinomas of the head and neck region seem to benefit from additional HBO therapy during radiotherapy, although several technical problems of irradiation during hyperbaric conditions are presently not satisfactorily resolved.

A63

Serological Her2/neu-determination in patients receiving Herceptin®

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Objective: Treatment with Herceptin® is one of the most promising therapies for patients with metastatic breast cancer whose tumors overexpress the HER2/neu protein. Recent studies provide evidence that patients receiving herceptin have a significant benefit. Taking into account this clinical success and, additionally, the favorable side-effect pattern, addition of Herceptin in first-line treatment of metastatic breast cancer is considered the most encouraging therapeutic option. However, prognostic and/or predictive markers justifying the therapy have not been available until now. Therefore, we designed a retrospective study in order to evaluate the serological Her2/neu determination accompanying a Herceptin therapy.

Method: The sera samples of 10 Herceptin-patients were collected immediately after standard hematological investigation. Serological Her2/neu was quantified using the Her2/neu Kit (Bayer Diagnostics, Munich, Germany). Automatical determination was performed on the immunoanalyzer Bayer Immuno 1™. The values were analyzed in terms of the clinical course of each patient. Each assay was performed in duplicate.
Results: The 10 patients were observed 15 months (median), range 6–21. Two patients had visceral metastases, two patients bone metastases and six patients developed multiple occult metastases. Seven patients had suffered a relapse. In all these seven patients the serological Her2/neu concentration increased strikingly at time of progress. The Her2/neu levels of the three patients with stable disease did not change during the observation period.
Conclusion: Serological Her2/neu concentrations paralleled the clinical course of a patient with metastatic breast cancer receiving Herceptin therapy. Prospective studies should be designed in order to demonstrate the prognostic/predictive value of serological Her2/neu determination.

A64

Downregulation of macrophage IL-12 production by tumor-derived IL-11

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We have previously shown that macrophages from mammary tumor-bearing mice have a profound downregulation of IL-12, which has been implicated in the low levels of IFN-γ and generally depressed lymphoreticular cells functions in this tumor model. The tumor used in our studies constitutively produces several factors that have immunosuppressive activity (ie granulocyte macrophage-colony stimulating factor [GM-CSF], prostaglandin E_2 [PGE_2], and phosphatidyl serine [PS]). Of these factors, PGE_2 and PS have been shown to exert effects on macrophage functions. Recently, we found that these tumor cells express IL-11 at both the transcriptional and translational levels, as evidenced by RT-PCR and by Western blots. Treatment of normal macrophages with IL-11 resulted in a downregulation of IL-12. In further studies using a murine IL-11 ELISA, we observed low constitutive levels of this cytokine in the supernatants of macrophage cultures from normal mice, which is upregulated upon stimulation with LPS. Importantly, macrophages from tumor bearers have higher production of IL-11 than their normal counterparts. Our results suggest that tumor cell derived IL-11, in conjunction with the elevated levels of this cytokine in tumor-bearing animal macrophages, play a role in the depressed IL-12 production, leading to the impaired immune functions observed during mammary tumorigenesis.

A65

Chromosomal instability and cancer predisposition: insights from studies on the breast cancer susceptibility gene *BRCA2*

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Inherited mutations in the breast cancer susceptibility gene *BRCA2* predispose to breast, ovarian and other cancers. *BRCA2* encodes a 3418-amino-acid protein that localizes to the nucleus of dividing cells. The biological functions of the protein and its role in tumour suppression remain uncertain.
We have identified an essential function for BRCA2 in DNA repair by homologous recombination. In BRCA2-deficient cells, DNA breaks introduced into chromosomal substrates are inefficiently repaired by homology-directed mechanisms, although repair by nonhomologous end-joining is unaffected. BRCA2 interacts with the RAD51 recombination protein, a functional homolog of bacterial RecA. The correct intracellular localization and function of RAD51 are dependent upon BRCA2, suggesting a mechanistic basis for its role in repair.
Loss of BRCA2 induces chromosomal instability characterized by spontaneous breakage, in appropriate mitotic exchanges and chromosomal fusions. Evidence will be presented that the repair of DNA breaks that arise spontaneously during DNA replication require BRCA2 for their error-free resolution. Loss of this function may foster carcinogenesis by increasing the rate of spontaneous mutation.
Paradoxically, BRCA2-deficient cells undergo cell cycle arrest rather than the unrestrained proliferation that is typical of neoplastic transformation. We find that mutations inactivating cell cycle checkpoints that regulate assembly of the mitotic spindle reverse proliferative arrest, and foster transformation, in BRCA2-deficient cells. These findings have implications not only for the evolution of tumours following BRCA2 loss, but also for the mechanisms by which cells perceive and respond to chromosome breakage.

A66

Herceptin clinical trials: past, present and future

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Herceptin is the humanized monoclonal antibody targeting the Her-2-Neu oncogene, which, when amplified, connotes a poor prognosis for the 25% of breast cancer patients with gene amplification. Pivotal clinical trials in metastatic breast cancer have established clinical benefit in approximately one-third of patients treated with one or two prior chemotherapy regimens for metastatic disease; almost half of patients when used as a first-line single agent; and in even higher percentages when used in combination with cytotoxic chemotherapy. Antitumor response rates can be maximized by selecting patients for treatment based on gene amplification tests (eg FISH) rather than on immunohistochemistry. Although Herceptin is very well tolerated subjectively, it was, unexpectedly, found to be cardiotoxic, especially when used in conjunction with anthracyclines. This finding has made the development of adjuvant programs a challenge, but several such adjuvant protocols in earlier stage disease are in progress or planned. In addition to adjuvant protocols, newer Herceptin combinations with other cytotoxics and hormonal therapy will also be discussed. Herceptin is the first (hopefully of many) targeted therapies that could revolutionize breast cancer therapy in the decade to come.

A67

Radioprotective and tumor antiangiogenic effect of the novel synthetic superoxide dismutase (SOD) mimetic compounds

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We have developed and tested several synthetic superoxide dismutase (SOD) mimetic metalloporphyrin compounds to determine their ability to protect/ameliorate radiation-induced (RT) normal

tissue injury, and, at the same time, to produce significant antitumor activity. In rats with R3230 AC mammary adenocarcinoma tumors, a significant inhibition of tumor growth was observed after intraperitoneal administration of 6 mg/kg of manganese(III) tetrakis (N-ethylpyridinium-2-yl) porphyrin (MnTE-2PyP). Furthermore, rats that received MnTE-2PyP had a significant inhibition of the postradiation tumor regrowth. Animals pretreated with MnTE-2PyP (6 mg/kg intraperitoneal) 24 h before implantation of R3230 mammary adenocarcinoma show a significant inhibition of tumor angiogenesis. MnTE-2-PyP significantly delayed development of RT-induced lung injury in rats after 28 Gy of right hemithoracic irradiation. The magnitude of the change in breathing rate is, on average, reduced by 30%, indicating the ability of MnTE-2 PyP to significantly reduce the severity of RT-induced lung injury. Six months after the treatment, a significant increase in hydroxyproline content per gram of dry or wet lung was observed in animals receiving radiation only. Administration of 6 mg/kg of MnTE-2-PyP before RT resulted in a significant reduction in hydroxyproline content. Furthermore, we have found a significant association between the radioprotective effect of MnTE-2-PyP and changes in plasma levels of transforming growth factor-β. This association suggests a possible role of SOD mimetics in activation/regulation of cytokines that are involved in development of radiation-induced lung injury. This new strategy of utilizing a single compound with antitumor activity to simultaneously protect normal tissues could allow a higher dose of radiation to be delivered to the tumor without increasing the risk of complications, and could further improve breast-conserving cancer therapy.

A68

Ultrasound-guided pO_2 measurement in breast cancer patients before and after hyperthermia treatment

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The significance of tumor hypoxia extends beyond conventional radiation resistance. It has been found that tumor hypoxia affects drug resistance, angiognesis, cytokine production, cell cycle control, apoptosis and development of distant metastases. Recently, it has been reported that hyperthermia improves tumor oxygenation in both canine as well as human soft tissue sarcoma. This study describes a new optimized technique for pO_2 measurement in breast cancer patients using ultrasound-guided placement of Eppendorf polarographic oxygen probes. Locally advanced breast cancer patients, participating in a phase I/II study of neoadjuvant liposomal doxorubicin/paclitaxel/hyperthermia treatment, were the subjects of this study. Tumor oxygenation was measured before and 24 h after hyperthermia treatment. Advantages of the ultrasound-guided pO_2 probe placement are the following: accuracy with visualization and verification of the Eppendorf electrode placement in tumor tissue; monitoring of the electrode movement through the tumor tissue during the measurement; ability to avoid electrode placement near or in large blood vessels by using color Doppler imaging; and spatial reproducibility of the second measurement. Despite progress in the technology that can be used to measure tumor hypoxia, accurate and verifiable placement of the oxygen probes in tumor tissue is of tremendous importance. Ultrasound-guided pO_2 probe placement should become standard technique to improve accuracy and reliability in the assessment of tumor oxygenation for disease sites in which it is appropriate.

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A69

Haplotype analysis in German families with recurrent *BRCA1* and *BRCA2* mutations

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Association analysis was performed for 19 different *BRCA* mutations (*BRCA1*: 14; *BRCA2*: 5), which were detected at least three times in the German population. The aim of this study is the identification of founder mutations and hot-spot mutations that are specific for the German population. Patients were genotyped for three intragenic markers (D17S855, D17S1322 and D17S1323) in the *BRCA1* gene and for closely flanking markers (D13S1698, D13S171 and D13S267) in the *BRCA2* gene. Statistical analysis was performed with an exact test of goodness-of-fit (Müller *et al*: 1991). The genotype data for the three markers analyzed each in the *BRCA1* and *BRCA2* genes are in concordance with the presence of probable common haplotypes. Therefore, most of the frequent mutations detected are likely to be founder mutations. Suprisingly, four C→T transitions in the *BRCA1* gene, which had been expected to result from independent mutational events, are probably also founder mutations. In contrast, the 4-bp deletion in the *BRCA1* gene (4184del4bp) and the most frequent mutation 3034delA in *BRCA2* are recurrent mutations, for which no significant association with specific founder alleles could be shown. Testing further informative family members to define the specific haplotype is the aim of our current investigations.

A70

On-site audits of the two clinical trials reported from South Africa involving high-dose chemotherapy (HDC) therapy for breast cancer

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An investigator at the University of the Witwatersrand in Johannesburg has reported (*J Clin Oncol* 1995; **13**:2483–2489, and *Proc Am Soc Clin Oncol* 1999, **18**:2a) two clinical trials purporting to be randomized prospective evaluations of HDC in comparison with treatment administered in conventional doses. One involved metastatic breast cancer, and the other high-risk primary disease. In both trials two cycles of a regimen devised at this institution combining cyclophosphamide, mitoxantrone, and etopside were used for the HDC. A total of 90 patients were reported in the metastatic disease study, and 154 (or 151) were said to have been treated in the high-risk study. Two separate on-site audits of available patient records have been performed.

Results: Of the 154 (or 151) patients allegedly entered into the high-risk study, medical records for only 58 patients (all appearing to have received HDC) were made available for review. Of the 90 patients allegedly entered on the metastatic study (based on information provided by the investigator), records for only 61 could be found. Only 25 of these appeared to have received protocol treatment (22 receiving HDC). The remainder could not be verified to have received the purported study therapy. Many of the patients reviewed for both studies did not meet the stated eligibility criteria, and there was no evidence of any acceptable randomization process. The reported results of these two studies cannot be used

as a basis for evaluating HDC for either metastatic or high-risk primary breast cancer.

A71

Diminished milk fat secretion and premature mammary gland involution in episialin/MUC1 transgenic mice

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Background: Episialin (MUC1, EMA, CA15.3) is a membrane-associated mucin and is frequently overexpressed in adenocarcinomas. If overexpressed, it inhibits cell adhesion, promotes invasiveness, and protects against cytotoxic T-cells *in vitro*. To study the effects of episialin *in vivo*, we developed an episialin transgenic mouse model.
Method: Transgenic FVB mice were developed expressing the human episialin gene under the control of the glucocorticoid inducible MMTV promoter. Two transgenic founder lines were selected: one expressing relatively low levels (F64) and one expressing high levels of episialin (F8), both in a variety of glandular epithelia.
Results: Juvenile mice, either transgenic or not, showed significant growth retardation at day 13 of age if fostered by a F8 transgenic mother. In the F8 mammary gland, large intracellular fat droplets were present just beneath the apical membrane of the luminal epithelial cells. In addition, the fat content in milk of fostering F8 transgenic mice was significantly reduced. This suggests that the accumulation of large intracellular fat droplets is the result of hampered fat secretion machinery in the mammary glands of these transgenic mice. Moreover, the mammary glands of the F8 transgenic mice already showed histological signs of premature involution after 13 days of lactation. Moreover, lactoferrin levels in milk of mice lactating for 13 days were higher in F8 mice than in nontransgenic mice, confirming that episialin overexpression induces premature involution.
Conclusion: Overexpression of episialin strongly inhibits fat secretion, and critically affects timing of involution of the lactating mammary gland.

A72

Patterns of cytokines and lymphocyte subsets in patients with breast and prostate cancer treated with a standardized mistletoe extract preparation

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In an open, multicentre clinical trial (GCP), 136 patients with cancer were included to develop a quality of life instrument. After completion of primary cancer therapy, patients received subcutaneous injections of a standardized mistletoe extract preparation corresponding to a dose of approximately 5 ng mistletoe lectin twice weekly for 3 months. In 19 patients with breast or prostate cancer, immunological parameters were determined at baseline, and after 6 and 12 weeks of treatment. FACS analysis revealed a slight increase in T lymphocytes ($CD3^+$, $P = 0{,}13$) and B lymphocytes ($CD19^+$, $P = 0.01$) during treatment. The ability of patient blood cells to release cytokines was tested by measuring cytokine concentrations in the supernatants of cell cultures stimulated with two different mitogens (PHA or PWM). A mean increase was seen for IL-2, IL-10 and TNF-α ($P < 0.05$, PHA method after 12 weeks of treatment). For IL-1-α, IL-6, IFN-γ and IL-12 no clear effects were observed. However, the PHA and PWM methods produced different results for some cytokines.
Conclusion: Mild immunomodulatory effects were observed in patients with breast and prostate cancer during treatment with a mistletoe extract preparation standardized to mistletoe lectin (Lektinol®). Changes in the different cytokines and lymphocyte subsets remained within the physiological range.

A73

Design and profile of low-molecular-weight receptor tyrosine kinase inhibitors for antiangiogenic therapy

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Angiogenesis occurs physiologically during embryogenesis, ovulation and wound healing, and pathologically in inflammation, psoriasis and tumor growth. Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), appears to be a key factor in pathological situations that involve neovascularization as well as enhanced vascular permeability. Our aim was to design synthetic low-molecular-weight molecules that, by blocking the VEGF/VEGF receptor system after oral administration, can be used therapeutically. One compound we developed is PTK787/ZK 222584, a potent inhibitor of VEGF receptor tyrosine kinases that is active in the submicromolar range. It also inhibits other class III kinases, like the PDGFR-β tyrosine kinase, c-Kit and c-Fms, but at higher concentrations. It is not active against kinases from other receptor families such as EGFR, FGFR-1, c-Met and Tie-2, or intracellular kinases such as c-Src, c-Abl, PKC-α. PTK787/ZK 222584 inhibits VEGF-induced autophosphorylation of KDR, and endothelial cell proliferation, migration and survival in the nanomolar range in cell-based assays. In concentrations up to 1 μmol/l, PTK787/ZK 222584 does not have any cytotoxic or antiproliferative effect on cells that do not express VEGF receptors. After oral dosing (50 mg/kg) to mice, plasma concentrations of PTK787/ZK 222584 remain above 1 μmol/l for more than 8 h. PTK787/ZK 222584 induces dose-dependent inhibition of VEGF- and PDGF-induced angiogenesis in a growth factor implant model, as well as a tumor cell-driven angiogenesis model after once daily oral dose (25–100 mg/kg). In the same dose range, it also inhibits the growth of xenografted human carcinomas either solid or in ascites formation, as well as a murine renal carcinoma and its metastasis in syngeneic, orthotopic models. Histological examination of tumors reveals inhibition of microvessel formation in the interior of the tumor. PTK787/ZK 222584 is very well tolerated and does not impair wound healing or hematopoietic recovery after concomitant cytotoxic anticancer agent challenge.
Compounds that inhibit VEGF, such as PTK787/ZK 222584, have the potential to provide a novel, effective and well-tolerated therapy for the treatment of solid tumors, and may provide a new therapeutic approach for the treatment of other diseases where angiogenesis plays an important role.

A74

The mechanism of tamoxifen in breast cancer prevention

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Tamoxifen (TAM) is known to have a dual mechanism of action: (1) to compete with 17β-estradiol (E_2) at the receptor site and to block the promotional role of E_2 in breast cancer; and (2) to bind DNA after metabolic activation and to initiate carcinogenesis. Recent large clinical trials indicate that TAM is also an effective chemopreventive agent against breast cancer. The mechanism is unknown. Because E_2 requires activation by epoxidation to bind DNA forming DNA adducts [1], and the same is true for TAM [2], the question is whether this preventive effect of TAM against breast cancer is contributory to the possibility that TAM, as an effective competitor for epoxidation, prevents the formation of E_2 epoxide and consequently breast cancer. Evidence will be presented to show that, indeed, when incubated together with E_2 for epoxidation, TAM was able to dramatically reduce the formation of E_2 epoxide as measured by both the loss of the ability of E_2 to inhibit nuclear RNA synthesis, and the reduced binding of [^{3}H]labeled E_2 to nuclear DNA. Identical results were obtained when TAM and estrone (E_1) were used. These results suggest that the breast cancer preventive effect of TAM is through a competitive epoxidation mechanism with E_1/E_2.

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Late Submissions

A75

CD95 ligand expression in dedifferentiated breast cancer

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CD95 ligand expression has been observed in various malignancies. Studying the CD95 ligand (CD95L) and receptor (C95) system in benign and malignant breast tumours from 48 patients mRNA and protein expression were determined by quantitative RT-PCR and immunofluorescence. mRNA levels of CD95 correlated inversely ($r = 0.90$; $P < 0.01$) and CD95L positively ($r = 0.89$; $P < 0.01$) with histopathological grading of the breast tumours. CD95 mRNA levels were low in high-grade carcinomas, but high in benign mammary tissues. In contrast, CD95 mRNA levels were low in adenomas, but increased 20-fold in grade I, 120-fold in grade II and 310-fold in grad III breast cancer.

Since CD95L acts as an efficient inducer of apoptosis in CD95+ cells, apoptotic cells were identified on the tissue sections. Tumour infiltrating lymphocytes and stroma cells in close proximity to CD95L expressing breast cancer underwent apoptosis. As a functional test, CD95+ target cells were cultured on breast cancer sections. The target cells underwent apoptosis when cultured on breast cancer sections, but could be rescued when CD95L was specifically blocked by a CD95-Fc fusion molecule.

In conclusion, the findings suggest an inverse regulation of CD95 and CD95L expression during dedifferentation of breast cancer and that killing of bystander cells by the CD95L expressing tumour could be involved in tissue invasion.

A76

CD95 ligand expression mediates immune escape in breast cancer

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Interaction of CD95 and its ligand (CD95L) plays an important role in the regulation of immune response, since CD95+ lymphocytes may be killed after engagement of the CD95 receptor. Studying the CD95/CD95L system in 40 cases of breast cancer, the malignant cells expressed CD95L, but lost CD95 expression, when compared to non-malignant mammary epithelia. In addition, four breast cancer lines expressed CD95L, which was further enhanced, when the cells were treated with IFN-γ. This was functionally relevant, because Jurkat T cells incubated on breast cancer cells underwent CD95L specific apoptosis and the rate of apoptosis was demonstrated by inhibition of matrix metalloproteinases, CD95L expressed on breast cancer cells could also be shed from the cell membrane into the culture supernatant and supernatants derived from breast cancer cell cultures induced CD95L specific apoptosis in Jurkat T cells. Interestingly, in breast cancer patients depletion of CD4+ and CD8+ peripheral blood lymphocytes was tightly correlated with CD95 ligand expression in the tumours, which is suggestive for a relationship between CD95 ligand expression by tumour and systemic immunosuppression.

A77

Resistance to CD95-mediated apoptosis in breast cancer is not due to somatic mutation of the CD95 gene

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Resistance to CD95 (Apo-1/Fas)-mediated apoptosis is a typical feature of breast cancer cells. Recent studies identified deleterious mutations of the CD95 gene not only in a variety of B cell lymphomas but also in a number of solid tumour entities.

Therefore, we amplified and sequenced selected regions (including regulatory promoter regions and the last exon coding for the death domain) of the CD95 gene from 48 breast cancer cases and 10 breast cancer cell lines but no mutation was found. In the presence of both polymorphic allele, loss of heterozygosity was excluded in 27 informative cases. We conclude that relevant somatic mutations of the CD95 gene occur, if at all, at a very low frequency and are not the primary cause for resistance to CD95-mediated apoptosis in breast cancer.