nomenon in both groups. For the first group of patients the support may ease their concern, for the second group preventive measures may be discussed.

(Participating Center of the Multicenter Project: 'Familial breast cancer' supported by Deutsche Krebshilfe, Germany)

Thursday, February 26, 1998 9.00–18.00

Biology/Metabolism

P10 Multistep carcinogenesis of sporadic breast cancer

H.G. Schnürch, D. Niederacher, H.X. An, I. Ellenberger, C.R.C. van Roeyen, J.Y. Cho, M.W. Beckmann. *Dept. Obst. & Gyn., Heinrich-Heine-University Medical Center, Moorenstr. 5, D-40225 Düsseldorf, Germany*

Introduction: Breast cancer emerges as a multistep process with transformation of normal cells via steps of proliferation, atypia and in situ carcinoma. Cytogenetic and molecular genetic analysis of breast cancer samples indicate that tumor development involves the accumulation of various genetic alterations including amplification of oncogenes and mutation or loss of tumour suppressor genes. Microdissection of histological sections is needed to correlate the specific histological change and the genetic alteration. Up to date, no studies with prove of a direct sequential genetic motif are published, but the concomitant analyses of various genetic alterations in early lesions may correlate the histological finding with a biological function.

Methods: In a prospective study, after microdissection (1) DNA from 127 breast cancers with matched normal tissue was isolated and (2) in 20 cases comparative analyses with benign and precursor lesions (DCIS, CLIS) of the same breast were performed. Oncogene amplification (erbB2, int-2, c-myc, cdk4) was measured by quantitative differential PCR. Allele loss of tumor suppressor genes (p53, BRCA1, BRCA2, HIC1, MTS1/p16, NME1) was analysed by PCR-based microsatellite polymorphisms detecting differences in short tandem repeat sequences, which are informative for assessment of loss of heterozygosity (LOH). Fluorescent labelled PCR products were analysed and quantitated by polyacrylamid gel electrophoresis in an automated DNA sequencer (A.L.F.™Pharmacia, Freiburg, Germany). Results were analysed with Fragment Manager™ software.

Results: Prior to the study, DNA extraction from microdissected sections stained with different methods (hematoxylin, toluidin) as well as the linearity of the PCR reactions had to be validated. Oncogene amplification was found in 24% for erbB2, 19% for int2, 18% for c-myc, and 12% for cdk4. LOH could be detected in 57% for TP53, 48% for HIC1, in 38% for BRCA1, in 35% for BRCA2, in 8% for MTS1/p16. The sequential analyses shows LOH of TP53, LOH HIC1 followed by LOH of BRCA1 and c-myc amplification. In microdissected in situ lesions of the same breasts oncogene amplification and LOH could be demonstrated. These findings show identical alteration as seen in invasive samples.

Discussion: Quantitative differential PCR and microsatellite analyses combined with detection of fluorescent labelled PCR products by an automated laser DNA sequencer are powerful tools in determination of genetic alterations. Especially in combination with microdissection where small lesions are analysed, they proved to be useful as analytical methods. The accumulation and the combination of different genetic alterations may lead to a hint to the time frame of multistep carcinogenesis. The simultaneous analysis of histology and genetics of precursor lesions offers the opportunity for a biological description of the histological picture. The results of this pilot study support the concept of multistep carcinogenesis in breast cancer. LOH of TP53 in precursor and invasive lesions point to a key function of TP53 in breast cancer development.
(DFG Be 1215/6-2)

P11 Loss of retinoic acid receptor β expression in breast cancer and morphologically normal adjacent tissue but not in the normal breast tissue distant from the cancer

M. Widschwendter, J. Berger, G. Daxenbichler, A. Widschwendter, A.G. Zeimet, H. Schröcksnadel, O. Dapunt. *Department of Gynecology and Obstetrics, University of Innsbruck, Anichstr. 35, A-6020 Innsbruck, Austria*

Retinoids and their receptors (RARs, retinoic acid receptors; RXRs, retinoid X receptors) play an important role in maintaining the balance between proliferation and apoptosis. Recently, Deng and coworkers (*Science* 274: 2057–2059, 1996) reported a loss of heterozygosity on chromosome 3p24 in breast cancer specimens and the morphologically normal appearing adjacent tissue. The 3p24 locus includes, among other genes, the region coding for RAR-β. This study was designed to determine whether there are abnormalities in the expression of retinoid receptors in surgical specimens of patients with breast cancer.

In fourteen patients, transcripts of nuclear retinoid receptors were detected by *in situ* hybridization in formalin-fixed, paraffin-embedded specimens by means of digoxigenin-labeled riboprobes specific for RAR-α, -β and -γ.

We found RAR-α expressed in all specimens, whereas RAR-γ was expressed in 100% of normal breast tissue, but only in 11 out of 14 tumorous lesions. RAR-β was found in all cases of normal breast tissue localized distant from the tumor, but in 13 out of 14 cases it was completely absent in the tumor and the morphologically normal appearing tissue adjacent to the tumor. One possibility to explain the suppression of RAR-β is a mutation in the promoter region. Sequencing the DNA extracted from paraffin-embedded tumor tissue of the corresponding breast cancer specimens, we were not able to detect any mutation in the retinoic acid responsive element (RARE).

Our results clearly indicate a crucial role of RAR-β in the carcinogenesis of breast cancer.

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P12 In vitro cultivation of human mammary epithelial cancer cells. Study of their phenotypic characteristics and biologic behavior

S. Saxena, A.K. Jain, K.K. Pandey. *Institute of Pathology, ICMR and Safdarjung Hospital, New Delhi, India*

The successful long-term growth of tumor cells from primary breast tumor explants is a rare event. To define the characteristics of tumor cells which govern their ability to grow in vitro as primary culture as well as continuous or established cell lineage, human mammary epithelial cancer (HMEC) cells from 18 cases of unselected primary breast cancer were propagated in culture. Propagation of HMEC cells in vitro as monolayer in primary culture was successful in 10 out of 18 (55.5%) cases, which showed continuous proliferation of tumor cells only up to 6–8 passages before they reached senescence. An investigation of the effects of phenotypic expression of estrogen receptors (ER), the progesterone receptors (PgR), C-erb B_2 oncoprotein and epidermal growth factor receptors (EGFR) on the capacity of HMEC cells to grow in vitro as monolayers showed that expression of ER and EGFR is required for controlling tumor proliferative activity in vitro. Expression of ER made the growth of HMEC cells more difficult, while expression of EGFR protein made their growth in vitro easier. Phenotypic characteristics of floating HMEC cells were found to be different from those grown on coverslip as adherent cultures, suggesting a selective growth of HMEC cells of a specific phenotype in culture. This suggests that cell lines are not appropriate tool for chemosensitivity and radiosensitivity studies because neither the primary cells nor passaged cells represent the heterogeneous population of original tumor. Cultured HMEC cells in subsequent passages showed a decrease in their proliferative capacity, alterations in phenotypic characteristics and development of morphological features of terminal differentiation, resulting in senescence.

P13 Involvement of plasminogen activator inhibitor PAI-1 in *in vitro* growth of human breast cancer cell lines

S. Prifti[1], N. Fersis[2], M. Sillem[1], M. Szekelyi[2], B. Runnebaum[1], G. Bastert[2]. [2]*Dept. of Obstetrics and Gynaecology, Ruprecht-Karls-Universität Heidelberg, Germany;* [1]*Div. of Gynae. Endocrinology and Reproductive Medicine, Ruprecht-Karls-Universität Heidelberg, Germany*

Introduction: Effective proteolysis of extracellular matrix is a critical factor for tumor growth and metastasis. The integrity of extracellular matrix is affected by the activity of several different classes of proteinases, like serine proteases such as plasmin, generated by the urokinase pathway of plasminogen activation. The later is regulated by a specific cell-surface uPA receptor (uPAR) and by two inhibitors (PAI-1, PAI-2). It has been proposed that high levels of PAI-1 may protect the tumor against degrading itself and in this way promoting tumor growth. We have addressed the question whether human breast cancer cell lines express in a different manner components of the plasminogen activation system and whether this expression is correlated with their *in vitro* growth rate.

Material and Methods: 1×10^4/ml cells of 2 estrogen receptor (ER) as well as progesterone receptor (PR) negative (BT-20, MX-1) and 1 ER, PR positive (MCF-7) human breast cancer cell lines were cultivated in DMEM/F12 serum-free medium up to 6 days. suPAR, uPA and PAI-1 immunoreactivity were assayed by Elisa. MTT-assay as described elsewhere (Mossmann, 1986) was applied to estimate the *in vitro* growth capacity.

Results: In all media, uPA- and uPAR immunoreactivity was detected. MCF-7 cells did not express any PAI-1, whereas BT-20 tumor cells secreted a 7 fold higher PAI-1 amount (0.57 ng/ml) than the MX-1 cell line (0.08 ng/ml). Comparable levels of urokinase plasminogen activator were measured by all three cell lines. Again, BT-20 cells exhibited higher levels of uPAR concentration than MX-1 or MCF-7 cells. Interestingly BT-20 and MCF-7 tumor cells had similar in

vitro growth kinetics. On the other hand, MX-1 cells were growing much slower than the above cell lines.

Discussion: Our study shows that human breast cancer cell lines exhibit a different expression pattern of the urokinase plasminogen activator system components but a link between this expression and their *in vitro* growth capacity could not be demonstrated. Our findings of higher PAI-1 amount in combination with a higher growth rate by BT-20 cells are in line with the proposed protective mechanism against the proteolytic degradation of tumor cells themselves mediated by PAI-1. Based on our observation that non-PAI-1 producers, MCF-7 cells, also exhibited a high growth capacity we suggest, that disturbance of balance between activator and inhibitor may also result in upregulated *in vitro* tumor growth. Therefore, a direct role of PAI-1 in growth rate of breast carcinoma is, at least *in vitro,* rather unprobable.

P14 Adjuvant tamoxifen treatment in breast cancer induces no activation of blood coagulation

C. Oberhoff, U.H. Winkler, A.E. Schindler. *University Hospital Essen, Dept. of Gynecology, D-45122 Essen, Germany*

Based on the incidence of thromboembolic complications (1–14%) during the clinical adjuvant breast cancer trials, tamoxifen is considered as a potentially thrombogenic drug. Studies evaluating changes of hemostasis during tamoxifen treatment report very conflicting results and the cause-and-effect relationship has never been established.

To asses potential effects of antiestrogen treatment on the hemostatic system, we studied blood coagulation and fibrinolysis in 20 postmenopausal women with breast cancer receiving 20 mg tamoxifen daily as an adjuvant therapy. Blood sampling was done before and after the 1st, 3rd and 6th month of treatment. Blood collection was done according to standard protocols.

Pretreatment values of procoagulation [fibrinogen (Fbg), factor VII (FVII)], thrombin-antithrombin-complex (TAT), anticoagulation [antithrombin III (ATIII), protein C (PC), protein S (PS)] or plasminogen and plasminogen activator inhibitor were found within the normal range, whereas tissue-plasminogen activator (t-PA), D-dimer fibrin degradation products (DDIMER) and prothrombin-fragment 1 + 2 (Frag 1 + 2) were elevated. On therapy an initial decrease of all measured parameters was observed during the first month of treatment, followed by consistent plasma levels up to the end of the observation period. This effect was significant for Fbg, FVII, AT III, PC, PS and t-PA. Fibrin degradation products decrease continuously. The analysis of blood coagulation inhibitors revealed decreased AT III (13%), PS (27%) and PC (29%) during the first month of treatment. However, all values remained within the normal range (>70%). No cumulative effects on anticoagulation were seen on therapy.

Our pretreatment data are consistent with an activated hemostatic system (acute-phase-reaction) after major surgery. We can not exclude, that the decrease of hemostatic parameters during the initial phase of tamoxifen treatment refers to the timing of blood collection (<14 days after surgery). The decrease of blood coagulation inhibitors was not associated with a concomittant increase of in vivo coagulation markers (Frag 1 + 2, TAT, DDIMER). Therefore our results are likely to reflect only the resolution of postoperative activation and does not translate into a drug related thrombogenic effect. The epidemiological findings suggesting an increased risk for thromboembolic complications may easily be explained by tumor-induced hypercoagulability, additional anti-tumor therapy or individual predisposing risk factors for thrombosis (inherited or defects of blood coagulation).

P15 Blood coagulation and fibrinolysis after oral or intravenous cyclophosphamide containing adjuvant CMF-chemotherapy

C. Oberhoff, U.H. Winkler, A.E. Schindler. *University Hospital Essen, Dept. of Gynecology, D-45122 Essen, Germany*

Epidemiological data suggest an increased incidence of thromboembolic complications (2–17%) during CMF-chemotherapy in breast cancer patients. Several studies report a decrease of blood coagulation inhibitors (protein C and S) induced by adjuvant CMF-chemotherapy containing oral application of cyclophosphamide. Because of an increased alkylating activity after oral administration, the aim of our study was to asses potential different effects of oral (p.o.) and intravenous (i.v.) application of cyclophosphamide during adjuvant CMF-Chemotherapy for breast cancer.

We studied parameters of blood coagulation and fibrinolysis in 20 patients receiving 6 courses of chemotherapy containing of cyclophosphamide (100 mg/m^2 p.o. days 1–14 or 600 mg/m^2 i.v. days 1, 8), methotrexate (40 mg/m^2 days 1, 8) and 5-fluorouracil (600 mg/m^2, days 1, 8). Blood collection was done before the application of the chemotherapy at days 1 and 8 according to standard protocols.

In both treatment groups the pretreatment values of procoagulation [fibrinogen, factor VII (FVII)], anticoagulation (antithrombin III, protein C (PC), protein S (PS), fibrinolysis (plasminogen, tissue-plasminogen activator) and antifibrinolysis (plasminogen-activator-inhibitor) were found within the normal range. Thrombin-antithrombin-complex and D-dimer fibrin split products were elevated. On therapy a decrease of FVII (20–35%), PC activity (20–40%) and antigen (25–38%) was observed from day 1 to 8 in both treatment groups. This effect was only significant ($p < 0.005$) for protein C. Whereas the plasma levels of FVII returned to pretreatment values within the treatment free period, a distinct cumulative effect was demonstrated for protein C with the occurrence of pathological values below 60% of normal range. There was no significant difference within the two treatment groups, but the effect was pronounced with oral cyclophosphamide.

Our data confirm the results of other authors reporting an acquired deficiency of protein C associated with adjuvant CMF-chemotherapy. We observed no significant difference whether cyclophosphamide was given p.o. or i.v.. In absence of any significant cumulative decrease of other vitamin-K-dependent coagulation factors (FVII, PS), the simultaneous decrease of PC activity and antigen, indicates a specific defect of the vitamin-K-dependent synthesis of protein C in the liver. Further analysis is mandatory to evaluate if cyclophosphamide, methotreaxate or 5-fluorouracil cause this effect.

P16 Adjuvant Goserelin depot in premenopausal women with early breast cancer: Ovarian function, bone mineral density and survival. Preliminary data

A. De Matteis, G. D'Aiuto, G. Landi, F. Nuzzo, V. Labonia, D. Montedoro, E. Rossi, I. Capasso, M. Pizzorusso. *Istituto Tumori, Naples, Italy*

Ovary suppression with Goserelin depot is alternative to ovarian ablation: in metastatic breast cancer Goserelin depot yelded objective response in 36% of patients. Ovarian ablation in women aged under 50 was associated with 6% fewer recurrences or deaths after 15 years. Studies are ongoing in order to evaluate the effectiveness of Goserelin depot as adjuvant treatment in the prevention of relapse and reduction in mortality.

We report our experience about 75 premenopausal patients with early breast cancer treated after surgery with Goserelin depot 3.6 mg subcutaneously every 28 days for two years. Median age was 43 years (range 31–50), all patients had regular menses, 36 patients were N+ and 39 were N−. ER status was positive in all patients but one in which was unknown.

One patient had bilateral breast cancer.

Owing to administration of Goserelin depot amenorrhea occurred after the first depot in 11 patients and after the 2nd depot in 64 women.

Spotting was observed in 8 patients and stopped after 10 depots.

At the end of 26 depots regular menses resumed in most patients (73%), on average after 5.3 months.

Weight gain was observed in 61% of patients, in 28.1% of patients weight was unchanged, weight loss occurred in the remaining women. All patients complained of hot flushes, sweating and impairment of libido. Metrorrhagia occurred in 3 patients at the end of therapy: 2 patients underwent hysterectomy. A decline in Bone Mineral Density was observed in patients studied with Dual Energy X-ray Absorptiometry (DEXA). A second primary tumor occurred in four patients: myeloid chronic leukemia, kidney cancer, oat cell carcinoma, second primary breast cancer. At a median follow-up of 51 months overall survival was 90.5% and disease free survival 70.2%.

P17 Weight gain associated with breast cancer adjuvant chemotherapy

G. Fried, K. Drumea, N. Yereslav, B. Vizel, N. Haim. *Department of Oncology, Rambam Medical Center, Haifa, Israel*

Purpose: Weight gain (WG) is one of the most common, distressful and less appreciated toxicity of adjuvant chemotherapy (CT) of breast cancer (BC). We retrospectively evaluated WG in the adjuvant CT of BC associated with cyclophosphamide, methotrexate, 5-fluorouracil (CMF) or cyclophosphamide, doxorubicin, 5-fluorouracil (CAF).

Methods: The pretreatment and post-treatment weight was determined in all patients and was recorded in the file.

Results: Between 1/94 and 12/95 131 BC pts were treated at our center with adjuvant CMF or CAF. Data were available for 65 CMF and 24 CAF treated pts. WG (range: 1–20, median, 8 kg) was recorded in 62 pts (70%). Weight loss (range: 2–9, median, 4 kg) was recorded in 10 pts (11%) and the remaining 17 pts (19%) maintained their weight during CT. WG was more pronounced in CMF than in CAF (51 pts, 78% vs 11 pts, 46%, $p < 0.004$). WG of >10 kg was noticed in 22 CMF treated pts (34%) vs 2 CAF treated pts (8%). Other factors that significantly affected WG included menopausal status {80% in pre vs 43% in postmenopausal ($p < 0.004$)} and obesity before therapy {100% for pts with pretreatment weight >130% of ideal body weight (IBW) vs 36% for pts with weight <130% IBW ($p < 0.0002$)}. There was no significant influence on WG for the type of surgery (lumpectomy vs mastectomy) or for CT induced amenorrhea.

Conclusion: WG is a common side effect of adjuvant CT for BC and its