Acta Oncologica Vol. 40, No. 1, pp. 111–119

Abstracts of Theses from the Nordic Countries

Short abstracts of theses on oncologic subjects are published under this heading. The abstract should contain background, problems, results and conclusions and be an independent informative unit that can be read without access to the thesis. It should not contain references to literature, figures or tables in the thesis. A suitable size is about 500 words. The abstract can be sent to Acta Oncologica together with information about department, faculty and university and date of dissertation.

Vascular endothelial growth factor and prostate cancer

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The vasculature plays an important role in the regulation of growth and cell death in the normal and malignant prostate. Castration induces an involution of the prostate gland and its vasculature, and replacement of testosterone stimulates endothelial cell proliferation and normalises vascular volumes and blood flow several days before organ regrowth. Inhibition of new blood vessel formation. i.e. angiogenesis, reduces prostate tumour mass. Understanding of the mechanism behind the vascular responses may therefore provide important knowledge of the mechanisms responsible for the growth of prostatic tissues. Vascular endothelial growth factor (VEGF) is a potent angiogenic stimulator which is over-expressed in prostate cancers. The overall purpose of this thesis was to investigate VEGF and VEGF-receptors expression in prostatic tissues and their androgenic regulation.

Expression of VEGF and VEGF-receptors was analysed by combined reverse transcription (RT) and polymerase chain reaction (PCR), in combination with Western blot, immunohistochemistry, and enzyme linked immunosorbent assay (ELISA). VEGF and its receptors expression levels were studied in the rat ventral prostate and the Dunning R3327 PAP rat prostate tumour, as well as in non-malignant and tumour areas of prostate core biopsies from patients with advanced cancer, before and within two weeks after castration therapy. Dunning prostate tumour sublines, representing different tumour grades, were examined for their VEGF expression and vascular density. VEGF and vascular density were also analysed in a group of patients with benign prostatic hyper-plasia and assigned to treatment with either the 5α-reductase inhibitor finasteride or placebo.

$VEGF_{12}$, $VEGF_{165}$, and $VEGF_{189}$, together with VEGFR-1 and VEGFR-2 were detected in the rat and human prostate, and in prostate tumours. Castration decreased VEGF levels in the non-malignant rat and human prostate and generally also in the human prostate tumours where castration also induced tumour cell apoptosis. Castration did not alter the expression of VEGF in the androgen-sensitive R3327 PAP rat prostate tumour. VEGF and VEGF-receptor expression were increased in all Dunning tumour sublines compared with normal rat prostate. VEGF expression were higher in the metastatic than in the non-metastatic tumours or in tumours with low metastatic capacity. A decrease in $VEGF_{165}$ expression was observed in finasteride-treated patients when compared with the placebo-treated group. Serum VEGF levels were not significantly changed after hormonal treatment.

To conclude, castration-induced reduction of VEGF and corresponding vascular regression could be of importance for tumour cell death. Increased levels of VEGF appears to be associated with angiogenesis and metastatic capacity and may contribute to prostate cancer progression.

May 2000

Hypothermia and radiation—Effects on DNA chromatin and cellular survival

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The effect of ionising radiation on cells can be modulated by several physico-chemical factors. The aim of this study was to investigate if hypothermic (sub-physiological) temperatures (2°C-28°C) may influence the effect of x-rays on cellular damage in human normal fibroblasts and in a human breast adenocarcinoma cell line, MCF-7.

The induction of double-strand breaks (dsb) was measured by constant field electrophoresis. Dsb induction was found to decrease with decreasing temperature when DNA from the malignant cell line MCF-7 was irradiated under different temperature conditions. This temperature effect was most prominent in a non-scavenging environment (PBS) with a dose modifying factor 3.4 found between 37°C and 2°C. This difference was abolished by a high concentration of scavenger (2.0 M DMSO), which indicates that the temperature effect is mediated by the indirect effects of ionising radiation. No difference between the two temperatures was found when intact MCF-7 cells were irradiated. However, the rejoining of dsb during the fast phase was slower in MCF-7 cells irradiated with 40 Gy at 37°C compared to cells irradiated at 2°C. A possible explanation may be that the damage is more complex and requires longer time to restore.

The induction of chromatin damage was quantified in normal fibroblasts and MCF-7 cells by measuring the supercoiling ability of DNA in individual cells using the fluorescent nucleoid assay. Radiation-induced chromatin damage was decreased by low temperature in both normal fibroblasts and MCF-7 cells. The temperature during irradiation (2°C or 37°C) was more important than the temperature during a preincubation for 1 h. No difference was found in the protective effect of low temperature between normal and transformed cells.

MCF-7 cells irradiated at 2°C with 2, 3 and 4 Gy had a higher clonogenic survival than cells irradiated at 37°C using a colony forming assay in which the ability of cells to divide more than 6 times is quantified. The dose modifying factor was 1.23. No difference in cellular growth using the crystal violet assay was found in MCF-7 cells or normal fibroblasts during 19 days following irradiation at 2°C or 37°C.

In conclusion, hypothermic temperatures during irradiation seemed to influence induction and rejoining of dsd as well as the induction of chromatin damage. The higher clonogenic survival found in cells irradiated at low temperature may reflect the modulation of these types of damage by the temperature.

May 2000

Prognostic factors in breast cancer

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Breast cancer is increasing in the industrialised countries. Due to early detection and adjuvant treatment with radiotherapy, hormones and chemotherapy, mortality has decreased. The different adjuvant treatments have adverse effect. It is an important task to estimate the risk of recurrence for the individual patient in order to tailor her individual treatment. This thesis aims at identifying predictors of disease development in primary and disseminated breast cancer with emphasis on histopathological features.

Histologic grade was strongly correlated to breast cancer mortality in 630 patients with primary breast cancer. The combination of grade, tumour size and lymph node status in the Nottingham Prognostic Index provides a powerful instrument separating patients in groups with excellent, good, intermediate and poor prognosis.

Grade was more sensitive than S-phase fraction in identifying high-risk patients and patients with very good prognosis. Presence of cancer cells in blood- and lymph vessels close to the tumour in patients with grade 3 tumours increased the risk of locoregional recurrence 6-fold as compared to patients with grade 1 or 2 tumours without such vascular invasion.

The mortality of young women with breast cancer has decreased very little since 1960. Women under 37 years of age had increased tumour size, more metastatic lymph nodes and doubled rate of high grade tumours as compared to older women.

Disease-free interval and survival in patients with distant recurrence were strongly associated to histologic grade and hormone receptor content. Patients with grade 3 hormone receptor negative tumours had a median survival of 10 months after recurrence while only 15% of women with receptor positive, grade I tumours have so far died after a median follow-up time of 5 years after recurrence. The Nottingham Prognostic Index and assessments of presence of tumour cells in vessels provide important information about the risk of locoregional and distant recurrence in breast cancer. Treatment decisions, counselling and follow-up programmes should be based on such assessments. For patients with metastatic breast cancer, tumour grade, estrogen receptor status and serum-c-erbB-2 predict the course of the disease.

May 2000

Effects of IL-1β secretion on lymphoid tissue and on antitumor immune responses

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Interleukin (IL)-1beta is one of the most pleiotropic and potent cytokines, produced predominantly by activated monocytes and macrophages. IL-1 plays an important role in normal immunoregulatory processes but also in pathophysiological inflammatory responses.

In the present study, we developed a model for active IL-1beta secretion. A retroviral vector containing a cDNA construct encoding the mature form of IL-1b, fused to a signal sequence (ssIL-1beta), was used to transduce target cells. Analysis of cell lysates and supernatants of the transfected target cells showed that the addition of a signal sequence resulted in high extracellular release of the protein, whereas the protein retained intracellularly if the signal sequence was absent. We further evaluated how local and active release of IL-1beta from tumor cells influenced an antitumor immune response against the poorly immunogenic murine B16 melanoma. We showed that the in vivo tumor growth of IL-1beta-transduced tumor cells was significantly reduced when IL-1beta was locally secreted in the tumor area. The tumor growth inhibition correlated with infiltration of macrophages, CD4 + T cells and dendritic cells. We further demonstrated that vaccination with irradiated IL-1beta-secreting tumor cells, stimulated a T cell dependent, long lasting anti-tumor immunity against the wild type B16F10 tumor. In contrast, non-transduced irradiated tumor cells possessed little or no ability to stimulate systemic anti-tumor immunity.

To evaluate the biological effect of over-expression of IL-1beta in lymphoid tissue, we further generated transgenic mice that expressed the ssIL-1b gene construct under the control of a mouse immunoglobulin enhancer. We found that these mice developed a number of abnormal features such as lymphoid hyperplasia, aberrant architecture of lymphoid organs, hypergammaglobulinemia and appearance of activated CD4 + T cells.

In conclusion, IL-1beta may be therapeutically useful to augment antitumor immune responses, when locally secreted during the vaccination phase. However, demonstrated by our transgenic model, IL-1beta might in a more chronic setting lead to pathological conditions such as unregulated proliferation and activation of lymphocytes.

May 2000

Aggressive lymphoma

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Aggressive lymphoma is a rapidly growing tumour of lymphocyte origin, potentially curable with chemotherapy. In a trial by the Nordic Lymphoma Group, 405 patients with aggressive lymphoma were included, and randomised to receive either the standard chemotherapy regimen, CHOP, or a weekly multidrug regimen, MACOP-B. In this trial, MACOP-B was not superior in terms of response, failure-free or overall survival, but was associated with more non-haematological toxicity and with more pronounced negative effects on health-related quality of life (HRQOL). In this study, we were able to validate the prognostic impact of the International Prognostic Index (IPI). In 92 patients, assessment of HRQOL before, during, and after chemotherapy was performed. In this population, chemotherapy was associated with relatively little negative impact on HRQOL, compared to a reference population. In a multivariate analysis of prognostic factors, pre-treatment global quality of life was identified as an independent prognostic factor.

In 259 patients, immunohistochemical analysis of Ki-67, BCL2, p53 and P-glycoprotein was performed. In a multivariate model, high BCL2 expression, high p53 expression and both very high and low Ki-67 expression were associated with poor prognosis, and were shown to provide additional prognostic information to the IPI. Assessment of BCL2 is proposed to be included as a routine procedure in patients with aggressive lymphoma.

In 44 patients with diffuse large B-cell lymphoma (DLBL), frozen lymphoma tissue was available, enabling assessment of

BCL6 rearrangement with Southern blot. *BCL6* rearrangement was detected in six of 43 evaluable patients (14%) and among these, a trend towards superior overall and failure-free survival was noted.

In a consecutive series of 81 patients with cytogenetically analysed DLBL, the prognostic impact of cytogenetic aberrations of previously proposed prognostic importance, was analysed. In univariate analysis, der(lq)(21–23), was associated with inferior overall survival. Among patients receiving anthracyclin-based chemotherapy, der(lq)(21–23) remained an adverse prognostic factor in a multivariate analysis, stratified for IPI.

The implications of these results in relation to current findings in prognostication and treatment of patients with aggressive lymphoma is discussed.

June 2000

HPRT mutational spectra and microsatellite DNA instability in HNPCC and lung cancer patients

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The general aims of this work have been to explore the use of microsatellite DNA length variation and mutational spectra of the hypoxanthine guanine phosphoribosyltransferase *(HPRT)* locus in T-cells as tools for a better understanding of human somatic mutagenesis in vivo. In particular (a) how inherited mismatch repair (MMR) deficiency may affect the stability of microsatellite DNA and the mutational spectrum at the *HPRT* locus, and (b) how the mutational spectrum at the *HPRT* locus is influenced by smoking, and by predisposition to lung cancer among never smokers.

Microsatellite DNA length variation (MSDLV) was studied in DNA from T-cell clones and peripheral blood lymphocytes, using suitable markers for PCR analysis and polyacrylamide gel electrophoresis. T-cell cloning in medium containing 6-thioguanine was used to select for *HPRT* mutant clones, and the mutations were further classified and characterised by PCR-based methods and DNA sequencing.

The background frequency of MSDLV in peripheral lymphocytes was determined using three microsatellite markers (D2S123, D9S180, D10S197). 3 out of 1028 alleles studied in T-cell clones of normal healthy subjects, showed altered microsatellite size compared to other clones from the same individual. Thus the background MSDLV was estimated to 2.9×10^{-3} We then analysed the MSDLV and *HPRT* mutant frequency (MF) in a breast cancer patient belonging to a hereditary non-polyposis colorectal carcinoma (HNPCC) family, with two different mutations in her *hMLH1* genes. This compound heterozygote showed a frequency of microsatellite length variation of 18.9% per allele, which was 50 times higher than the background frequency. The *HPRT* MF of 34.5×10^{-6} was elevated 2–3 times compared to controls. The *HPRT* mutational spectrum of this patient was significantly different from normal, with a shift from base pair substitutions towards frameshifts, especially + 1 bp insertions, and deletions. Also two new base pair mutations not reported earlier were seen and two of the clones studied had two mutations each, which is very unusual. We concluded that the patient was likely to have a mild MMR deficiency in her somatic cells due to the mutations in both of her *hVLH1* genes, and that this was the cause of her microsatellite instability (MSI), increased *HPRT* MF and abnormal *HPRT* mutational spectrum.

The *HPRT* mutational spectrum was studied in 73 T-cell clones each from smoking and non-smoking lung cancer patients. The proportions of different types of mutations were not significantly different between smokers and non-smokers, although the smokers had less deletions. The distribution of base pair substitutions was non-random, with clustering at previously identified hotspots at positions 143, 197 and 617 of the *HPRT* coding sequence. One additional hotspot at position 606 was observed, in smokers only. The frequency of GC > TA transversions (13%) was higher in smokers than in non-smokers (6%). Conversely smokers had a lower frequency of GC > AT transitions (24%) than non-smokers (35%).

We concluded that there was a minor effect of smoking on the *HPRT* mutational spectra, with a trend for increase of GC > TA transversions and decrease of GC > AT transitions, in the smokers compared to the non-smokers. This is consistent with the in vitro mutagenicity of benzo(a)pyrene, one of the prominent carcinogens of tobacco smoke. In conclusion, these results show that analysis of *HPRT* mutations may contribute to the understanding of somatic mutagenesis in vivo, and that the mutational spectrum at the *HPRT* locus may reflect abnormalities of repair and extensive environmental exposure, such as tobacco smoking

June 2000

Molecular cytogenetic investigations of chromosomal abnormalities in prostate and urinary bladder cancers

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Tumorigenesis is suggested to be caused by base pair mutations and chromosomal abnormalities leading to inactivation of tumor suppressor genes and activation of oncogenes. However, still it is not clear for the vast majority of these changes how they contribute to the tumorigenesis of the prostate and bladder cancers. This study focused on the molecular cytogenetic investigations of chromosomal alterations in prostate and bladder cancers and the estimation of the significance of the chromosomal alterations in relation to the clinical practice.

Interphase cytogenetics with fluorescence in situ hybridization (FISH) is demonstrated as a powerful technique suitable for studies of the specimens from the prostate and bladder cancers. Deletion of the short arm of chromosome 8 has been reported to be a frequent phenomenon in prostate cancer. Distal deletion of 8p23 was found in 50% of the tumors. Interstitial deletion of 8p22 was found in 53% of the tumors. In total 71% of the specimens demonstrated deletions of any type in chromosome 8p. The degree of 8p deletion was well correlated with tumor grade and tumor metastases. These data support the hypothesis that tumor suppressor gene(s) may be located in the chromosomal region 8p. Deletion of 8p may be of crucial importance for the pathogenesis of prostate cancer.

Numerical aberrations of chromosome 16 and loss of 16q24 were studied by FISH in 31 primary and 22 metastatic tumors. Numerical aberrations of chromosome 16, 16q24 deletion and alteration of E-cadherin expression were respectively found in

29%, 35% and 29% of the primary tumors, and 73%, 73% and 73% of the metastases. High tumor grade and DNA aneuploidy were also found to have significant correlation with metastases. Deletion of chromosome 16q24 and/or decreased E-cadherin function appeared at a high frequency in metastases of prostate cancer. The results suggest that they may be important risk factors, contributing to a metastatic potential of the tumor.

When summarizing the clinical significance of chromosomal alterations, we found that multiple chromosomal changes were related to tumor metastasis and high malignant grade. Deletion of 8p22 was significantly increased in diploid metastases, while deletion of 16q24 was commonly associated with gross DNA changes. Alterations of chromosomes 8 and 16 together with other tumor properties such as high-grade, DNA aneuploidy and high S-phase fraction were not only significantly related to the poor prognosis and the short term cancer specific survival, but deletions of 8p and 16q may indicate specific steps in the pathway of prostate cancer progression.

Using interphase FISH technique, we found mutations of the p53 gene well correlated with the deletions and overexpression in transitional cell carcinoma of the urinary tract. Deletions of p53 were significantly associated with tumor grade, stage, S-phase fraction, and DNA ploidy, while p53 overexpression correlated only with grade. The close correlation between p53 deletion and the malignant potential of human urothelial tumors indicates the clinical importance. We also studied the alterations of chromosomes in both tumor and histologically normal appearing mucosa from the same urinary bladder. Chromosomal alterations in the tumor were also frequently found in the 'normal' mucosa. Normal mucosa with certain genetic abnormalities could indicate the risk of recurrence of bladder cancer, particularly in superficial tumors.

In one paper we reported on the chromosomal composition of 24 uroepithelial carcinomas and five cell lines from bladder cancer and eight established prostate cancer cell lines by spectral karyotyping (SKY). In general, more chromosomal alterations were detected in high-grade invasive bladder carcinomas than in low-grade papillary tumors indicating a progressive accumulation of acquired genetic alterations in bladder carcinogenesis. A clear correlation was observed between tumor staue and grade and specific net chromosomal imbalances involved. The SKY analysis showed complex karyotypes for all cell lines. Several complex chromosome translocations and pinpointed rearrangement breakpoints were also revealed. Identification of chromosomal alterations in these cancers by SKY may prove to be helpful in attempts to clone the genes involved in cancer tumorigenesis of prostate and bladder cancers. Most recurrent breakpoints were observed to participate in deletion and translocation events in these tumors suggesting the importance of tumor suppressor and oncogenes in these regions.

Molecular cytogenetic techniques that we apply in this thesis are useful for the observation of genetic alteration in urinary bladder and prostate tumors. Our results show that some specific chromosomal alterations are closely related to tumor progression and the biological activity of the tumor. They may play an important role in tumorigenesis and tumor development. In the future the assessment of these genetic changes in clinical samples may become important diagnostic and predictive tools.

September 2000

Assessment of population screening—The case of mammography

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Out of seven randomised controlled trials on mammography screening performed world wide, four have been performed in Sweden. Between October 1976 and December 1982 women aged 40–74 years were randomised in Malmö, Kopparberg and Östergötland, Göteborg and South East Stockholm, 158 737 to the invited group and 127 527 to the control group. At the initiative of the Swedish Cancer Society, the Swedish trialists agreed to merge the trials to get a larger study base and to perform an independent determination of cause of death.

Initially the trialists sent a magnetic tape to the Department of Epidemiology and Public Health, Umeå university, containing information on personal identification number, date of randomisation. and randomisation group for each woman. The files were merged and sent to the six Regional Oncologic Centres and to Statistics Sweden to get information on date of breast cancer diagnosis and date and cause of death. For all deceased breast cancer cases all available information, i.e. medical records, autopsy protocols, histo-pathology reports and cause of death certificates were collected. Cause of death was then determined by an independent End Point Committee (EPC) consisting of four physicians independently using two end-point measures, *breast cancer as the underlying cause of death* and *breast cancer present at death*. Out of 1313 deceased breast cancer cases there was disagreement regarding 132 cases (10%), and these were discussed at consensus meetings. Data were analysed using two models: the *follow-up model*, where all women were followed from date of randomisation through date of follow-up, and the *evaluation model*, where breast cancer cases diagnosed after first screening round of the control group were ignored to minimise dilution.

Based on follow-up through 1989 there was a significant reduction in breast cancer as the underlying cause of death according to the EPC in the invited group, compared with the control group, in women 40–74 years at randomisation (RR = 0.76; 95% Cl: 0.66–0.87). The effect was most pronounced in women 50–69 years at randomisation (29%), and lowest (13%) and non-significant in women 40–49 years at randomisation. The effect was independent of end-point measure and source for cause of death determination. The age-adjusted relative risk for the total mortality was 1.00 (95% Cl 0.98–1.02).

It was also shown that the excess mortality method gave valid estimates of the effect of invitation to screening, similar to the resource-demanding procedures described above, especially in the age group 50–69 years at entry. This is of importance as it is easy to calculate and relies on objective official sources, the Cancer and Cause of Death Registries, and is independent of cause of death determination.

The first follow-up showed that Statistics Sweden as a source for cause of death determination produced reliable estimates that were, if anything, conservative. Thus, it became the only source for the second follow-up through 1993. Moreover, only the evaluation model was used, as dilution using the follow-up model was expected to increase with increasing follow-up time. The follow-up focussed on the age group 40–49 years at entry. With a median trial time of 7.0 years and a median follow-up of 12.8 years the relative risk was 0.77 (95% Cl: 0.59–1.01) and close to significance. Numbers of prevented breast cancer deaths per 10 000 invited to screening were 6.3, 11 and 19, respectively, in the age groups 40–49, 50–59 and 60–69 years. The corresponding figures for number of years of life saved were 188, 222, and 238. Thus, if number of life-years saved is prioritised, the lower age limit for invitation to screening should be 40 years.

Whether or not the results can be generalised and the impact maintained when moving from efficacy to effectiveness, i.e. when

implementing the RCT results in the service-screening programme, remains to be studied. It is suggested that a mass-screening register be established and that models and methods for its use and analysis be developed to better monitor the programme and its quality assurance.

September 2000

Leukaemic relapse after allogeneic haematopoietic stem cell transplantation and the use of the graft-versus-leukaemia effect

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Survival after allogeneic haematopoietic stem cell transplantation (HSCT) has improved over the last decades due to improvements in immunosuppressive regimens and supportive care. Today the major risk of treatment failure is recurrent leukaemia, a risk which has stayed relatively constant over the years. This study was undertaken to assess factors that may affect the risk of relapse after HSCT and to explore new potential treatment strategies.

Among 306 transplanted patients, significant risk factors for relapse in multivariate analysis comprised acute leukaemia, as compared to chronic myeloid leukaemia (CML) ($P = 0.003$), total body irradiation (TBI) compared to busulfan treatment ($P = 0.011$), gram-negative prophylaxis with ciprofloxacin during conditioning with cyclophosphamide (CY) ($P = 0.024$), graft-versus-host disease (GVHD) prophylaxis using a combination of methotrexate (MTX) and cyclosporine (CSA), compared to monotherapy ($P = 0.037$) and absence of chronic GVHD ($P = 0.050$). Following this, ciprofloxacin is no longer given during CY conditioning.

Chronic GVHD has a major impact on the leukaemia-free survival (LFS). Four hundred and fifty-one bone marrow transplant recipients were analysed for factors predictive of chronic GVHD. Significant factors in multivariate analysis were increasing recipient age ($P < 0.001$), acute GVHD grades (I-IV) ($P < 0.001$), immune female donor to male recipient ($P = 0.006$) and CML, compared with all other diagnoses ($P = 0.014$). In patients with HLA-identical sibling donors and GVHD prophylaxis with MTX and CSA, increasing recipient age ($P < 0.001$) and CML ($P = 0.007$) were significant factors. Furthermore, multivariate analysis of patients with unrelated donors showed recipient age ($P = 0.006$) to be the only significant risk factor for chronic GVHD.

To try to stimulate the graft-versus-leukaemia (GVL) effect we evaluated the use of a low-dose CSA regimen (starting at 1 mg/kg/day i.v.) of short duration, in combination with 4 doses of MTX. CSA was tapered off from 2 months after HSCT and discontinued at a median of 6.6 months. In comparison with retrospective controls the low-dose regimen resulted in a increased risk of mild acute GVHD (78% vs 57%, $P < 0.01$) and chronic GVHD (60% vs 24%, $P < 0.001$). A high CSA dose was the strongest predictive factor for relapse in multivariate analysis ($P = 0.03$). The regimen appears safe as the risk of moderate to severe acute GVHD, TRM and extensive chronic GVHD did not increase. This treatment may enhance the GVL effect, thus reducing the risk of relapse after HSCT.

Donor lymphocyte infusions (DLI) have so far been the most effective way to treat recurrent leukaemia after HSCT. In an evaluation of 44 leukaemic patients receiving DLI at our centre, an initial response was seen in 15 out of 22 patients (68%) with CML. However, 4 patients among the responders (27%) relapsed within two years after DLI, resulting in a three-year current-LFS (cLFS) of 46%. None of the patients with haematological relapse achieved a remission lasting over a year after DLI, compared to a three-year cLFS of 85% for patients in cytogenetic ($n = 10$) or molecular ($n = 3$) relapse. For patients with non-CML diseases the results are less promising, especially for patients relapsing within one year after HSCT. The cLFS at 18 months was merely 13%, suggesting that early and aggressive treatment may be important for these patients.

Transduction of T-cells with a suicide gene prior to DLI can potentially allow control of subsequent GVHD. In the optimisation of T-cell expansion for retroviral transduction, we found X-VIVO 15, among other serum-free media, to give the highest rate of serum-free expansion after 21 days in culture (a median of 79-fold expansion, range 20–117). Equal percentages of $CD4^+$ and $CD8^+$ cells were obtained and the cytokines released into the media showed a type1 cytokine pattern. Transduction with the LN vector, carrying the neomycin resistance gene, and G418 selection resulted in a 14-fold increase in cell numbers. Human serum increased expansion rates for all media.

$CD3^-CD56^+$ natural killer (NK) cells have been shown to mediate potent anti tumour effects with limited non-specific alloreactivity. Peripheral blood mononuclear cells cultured for 21 days in Cellgro, stem cell growth medium with interleukin-2 (500 U/ml) and anti CD3 (OKT3) expanded 193-fold (median, range 21–277) and contained 55% (median, range 7–92) $CD3^-CD56^+$ cells. The expanded cell population lysed 26 to 45% of the K562 target cells in a 1:1 effector to target ratio, signifying substantial cytotoxic efficacy. These high-yield $CD3^-CD56^+$ cells have been termed cytokine-induced natural killer (CINK) cells and may be prepared and used in a DLI setting.

October 2000

Cholesterol gallstone disease in the gallbladder-Mechanisms of gallstone formation and cancer development

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We studied bile lipid analyses from 145 cholesterol gallstone patients, 23 patients with pigment stones, and 87 gallstone free patients. During cholecystectomy the gallbladder was punctured and bile aspirated for analyses. Cholesterol saturation and molar percentage of cholesterol was higher, total lipid concentration lower and nucleation time shorter in the gallstone patients. NT was also shorter in patients with multiple stones. There was no difference in deoxycholic acid between gallstone and gallstone free patients.

A bile sample and liver biopsy was obtained from 19 patients undergoing (VBG) for morbid obesity. ACAT activity and hepatic content of free cholesterol did not differ between obese and non-obese patients. Obese patients did not have more saturated gallbladder bile than the non-obese controls. They had a normal nucleation time and their gallbladder bile did not contain any cholesterol crystals. We conclude that obese patients without

gallstones usually have a normal esterification rate of cholesterol in the liver. In obese patients, the gallbladder bile contained less total lipids than in non-obese patients. ACAT activity in the liver does not play a role in cholesterol gallstone disease.

Thirty-two patients with cholesterol gallstone disease were treated with 2 g vitamin C daily for the two weeks preceding laparoscopic cholecystectomy. High doses of vitamin C did not alter plasma lipids, cholesterol synthesis or bile acid synthesis in the livers of cholesterol gallstone patients. No major effect on the composition of the major biliary lipids could be detected. The increase in the secondary bile acids in bile during vitamin C treatment could either be due to increased dehydroxylation activity on bile acids in the intestine or be a result of a direct effect on the intestinal microflora. The prolongation of the nucleation time could be due to a vitamin C-mediated effect on pro- or anti-nucleating substances in the bile of cholesterol gallstone patients.

DNA ploidy and cell cycle composition were studied by flow cytometry in formalin fixed archival specimens from 52 gall bladder carcinomas. Aneuploidy was detected in 69% of the gallbladder carcinomas. S-phase increased from $5.0\% \pm 3.0$ in diploid tumours, to $8.6\% \pm 5.0$ in aneuploid tumours ($p = 0.004$). Patients with diploid tumours had a better cancer-related survival than those with aneuploid tumours ($p = 0.02$). The higher frequency of aneuploidy in single stone patients may be explained by the less intense and frequent gallstone symptoms and complications resulting in operation at a more advanced stage of tumour development.

Ninety-four cholecystectomized patients were included in the study. Eighty-three had cholesterol stones, 36 being younger than 35 and 47 older than 60 years. Eleven were gallstone free. There was a low frequency of histopathological lesions in the different groups. Dysplasia existed in 5%, and metaplasia in 5% of all gallbladders with gallstones. One patient in the older gallstone group showed an aneuploid gallbladder cancer. One of the patients in the older gallstone group showed aneuploidy without any other signs of histopathological malignancy in the mucosa. The increased cell proliferation in the gallbladder mucosa of cholesterol gallstone patients, strongly related to inflammation, may explain the role of gallstones in the development of gallbladder cancer.

October 2000

Genetic analysis of neurofibromatosis type 2 (NF2) patients and NF2-associated tumors with emphasis on chromosome 22 deletions

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Neurofibromatosis type 2 (NF2) is an autosomal dominant disease with the hallmark of bilateral vestibular schwannomas. NF2 patients may also develop schwannomas at other locations as well as meningiomas, neurofibromas and ependymomas. NF2 shows a distinct clinical variability ranging from very mild to severe forms. Since identification of the *NF2* gene in 1993, mutation-screening analyses have been performed. Mutations were, however, not found in numerous cases and the mechanism behind tumor development in NF2 is not fully understood. The correlation between type of *NF2* gene mutation and clinical phenotype of patients does not provide a clear-cut explanation of the clinical variability. Also, absence of *NF2* gene mutations in constitutional tissue from a related disorder, such as schwannomatosis, points towards a possible existence of an additional NF2-related locus from chromosome 22, which may modify the disease phenotype. This implies the necessity of further clarification of the genetic factors involved in NF2.

Deletions on chromosome 22 and the inactivation of *NF2* tumor suppressor gene are critical steps for meningioma formation. However, 40% of the tumors do not show aberrations of chromosome 22 or mutations in the *NF2* gene. This suggest that alternative mechanisms are responsible for the development of a large fraction of meningiomas. We analyzed 25 meningiomas, which do not display chromosome 22 deletions, for genetic abnormalities by CGH (comparative genomic hybridization) to metaphase chromosomes. Two tumors showed loss of chromosome 1p and 3p which suggest that deletions of both 1p and 3p may contribute to meningioma turnorigenesis. In schwannomas, detailed mutation analyses of the *NF2* gene showed that 60% of the tumors carry inactivating mutations. Thus, the mechanism behind the development of 40% of schwannomas is unknown. We studied 50 sporadic and NF2-associated schwannomas by high resolution LOH (loss-of-heterozygosity) on chromosome 22 and other chromosomes. Chromosome 22 deletions were detected in over 80% of the cases. Four tumors showed LOH not involving the *NF2* locus. All exons of the *NF2* gene were sequenced in these tumors and mutations were detected only in one case. Thus, additional regions chromosome 22 may harbor mutations possibly involved in schwannoma tumorigenesis. We also identified an early-onset NF2 patient with a large constitutional deletion on chromosome 22. Constitutional deletions of the entire *NF2* gene were previously described in NF2 patients; two large deletions encompassing 700–800 kb have been reported in mildly affected subjects. Our severely affected case showed a much larger deletion stretching approximately 5 Mb towards the telomere. It may therefore be hypothesized that the severe phenotype in this patient is a result of a combined mutation in *NF2* gene and in a putative modifier gene. Further mapping of deletions in this candidate region was performed by analysis of 116 NF2 patients for deletions on 22q. Analysis was carried out using high-resolution microarray-CGH on a genomic array covering at least 90% of a 7.4 Mb interval of 22q, around and distal to the *NF2* locus. This is a novel approach for high-resolution detection of chromosomal abnormalities, both in constitutional and tumor-derived DNA. Deletions were detected in nine severe, nine moderate and six mild patients. Deletions in severely and moderately affected patients varied in size, whereas cases with mild NF2 displayed deletions affecting the *NF2* locus only. This result indicates that the geneotype/phenotype correlation is unlikely to exist and supports the notion that the *NF2* modifier gene may exist in the vicinity of the *NF2* locus.

Finally. the tumor suppressor gene *SMAECB1* was tested for mutations in meningioma and schwannoma. *SMARCB1* is mutated in malignant rhabdoid tumors and is located on 22q11.2, a region frequently deleted in meningiomas. Fourty-three meningiomas and twenty-one schwannomas were tested for mutations in exons 2 through 8. However, no mutations were detected, suggesting that the *SMARCB1* gene is not frequently involved in the pathogenesis of these tumors. We also identified the mouse ortholog and characterized different splice forms of this gene, both in human and in mouse.

October 2000

Molecular progression and clonality of urinary bladder cancer

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The general aim of this work was to investigate the clonality of multifocal bladder tumours, chromosomal deletions and model the initiation-progression of bladder tumours at a molecular level using microsatellite analysis. In a population-based study, we found correlation between stage and grade and the prevalence of loss of heterozygosity (LOH) at all observed chromosome 13 loci and stage and grade, respectively. Statistically significant correlation was found for 13q14.3, the Rb locus, and between the number of loci with LOH at 13q and tumour stage and grade, respectively. Clonality was studied using superficial multifocal bladder tumours. The given patient had always lost the same microsatellite allele when LOH was encompassed, suggesting monoclonality of the tumours. In the majority of the cases the polyclonality was excluded with at least $1–2 \times 10^{-16}$ probability, calculated using binomial distribution. At chromosome 9, at least three consensus regions were found to be deleted at superficial multifocal bladder tumours, one at 9p (9p21-22) and two at 9q (9q21-22 and 9q32-34). All the regions appeared to be equally important and during the development of tumours all these regions were finally affected. At chromosome 3 at three loci on chromosome 3p25-26, 3p14.2 and 3q27 had frequent LOH in multifocal superficial bladder tumours. The phylogenetic-type analysis suggested that the FHIT region at 3p14.2 contain often the very first alterations at chromosome 3. The tumour-adjacent surrounding tissues were found to contain similar alterations as the tumour tissues. On chromosome 9 all the patients analysed showed LOH in at least one surrounding sample and over 60% of them showed LOH also on chromosome 3. On chromosome 9 three distinct clusters of LOH were found, 9p21-22, 9q13-22 and 9q31-34.2 and at chromosome 3p (D3S3050) and at 3q27 (D3S2418).

October 2000

Late side-effects following radiotherapy after mastectomy in breast cancer patients—A long-term follow-up

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The clinical records of 150 breast cancer patients treated with radiotherapy post-mastectomy in the mid-late sixties have been retrospectively analysed. The cohort has now been followed up to 34 years. The incidence and the prevalence of various forms of late injuries have been studied to determine the latency and the dose response relationships. None of the patients received chemotherapy as part of their primary treatment. Almost all of the patients continued to be checked at regular intervals at our Oncology Department. Detailed records are available for the entire 34 years of follow-up period. Radiotherapy was delivered to the parasternal, axillary and supraclavicular lymph node regions. The prescribed dose was either 11×4 Gy (treated with ^{60}Co photons) or $14–15 \times 3$ Gy (treated with both ^{60}Co photons and electrons). The recalculation of dose at the brachial plexus in each patient was performed in the early seventies and expressed in CRE units. The received dose has now been converted into BED_3 units and from that into the equivalent dose in 2 Gy fractions in order to evaluate dose response relationships. This group of patients has many long-term survivors, which is the reason that they were able to express some of the very slowly evolving injuries. There was progression of many of the late side effects in the period between 5 and 34 years. The more serious morbidities such as brachial plexus neuropathy and paralysis have increased progressively over the whole 34-year follow-up period. Other unexpected findings included unilateral vocal cord paralysis among 5% of the patients, after a median latency of 19 years. A comparison is presented of the latency and the frequency of fibrosis, oedema, brachial plexus neuropathy, paralysis and vocal cord paralysis in the different series and in the total group. There was a significant reduction of the incidence of the various morbidities and the latency was longer in patients treated with smaller doses per fraction and with smaller fields. Dose response relationships are shown at 5, 10 and 30 years after irradiation for fibrosis, brachial plexus neuropathy and paralysis.

The neuropathy seems to be closely linked to the dose delivered to the nerves. For a small range of prescribed dose to the regional lymph nodes (42–45 Gy), there is almost a 2-fold range of dose at the brachial plexus. The use of large daily fractions, combined with hotspots from overlapping fields, contributed to the severity of the complications. Clear dose response curves are seen for all these late radiation injuries. The incidence seen at 5 years does not represent the full spectrum of injuries. Doses that seem safe at 5 years can lead to serious complications at later times.

October 2000

Angiogenesis in prostate cancer—Studies on its prognostic role and importance for prostate growth

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Despite recent advances in early detection and improvements in therapy, the ability to prognosticate and cure many men with prostate cancer remains elusive. Consequently, there is an urgent need to identify new prognostic markers and therapies for this large group of patients. However, to fmd these, it is necessary to gain further insight into the molecular and cellular mechanisms involved in the control of normal and malignant prostate growth. The purpose of this study was therefore to investigate the role of the vasculature in prostate growth and its prognostic role in prostate tumors.

In the adult male rat, castration induced an involution of the prostate vasculature and the prostate gland. Testosterone supplementation given to castrated rats caused a rapid onset of endothelial cell proliferation, and normalized vascular volumes several days before epithelial and ventral prostate organ volumes were normalized. Thus, prostate endothelial cells are likely to be important targets for androgen action, and proliferation and growth of the glandular epithelial cells after testosterone supplementation may therefore be dependent upon this early regeneration of the prostate vasculature. When castrated testosterone supplemented rats were treated with the potent angiogenesis inhibitor TNP-470, ventral prostate growth was restrained but not completely inhib-

ited, further supporting the assumption that the vasculature plays a central role in the control of prostate growth.

Endothelial cell proliferation in the adult male rat was considerably higher in the reproductive organs than in other organs, probably because testicular factors, presumably testosterone, stimulate endothelial cell proliferation in male reproductive tissues. These results imply that the endothelial cells in the male reproductive organs are not as quiescent as previously believed.

In human prostate tumors, an increased vascular density was related to poor clinical outcome and was an independent prognostic marker of cancer specific survival in patients with intermediate grade tumors. However, the density of tumor associated macrophages was not an independent prognostic factor for cancer specific survival and was only weakly correlated to angiogenesis.

In conclusion, the vasculature appears to play a central role in prostate growth control and tumor vascular density is a promising prognostic factor in prostate cancer.

October 2000

Prognosis in carcinoma in situ of the breast

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The incidence of breast cancer is rising steadily in Sweden and the proportion of carcinoma in situ (CIS) has increased appreciably, most likely due to mammography screening. The aim of this study was twofold: to examine risk factors for subsequent invasive breast carcinoma and breast cancer death after primary ductal carcinoma in situ (DCIS) and to study the biology in the progress between in situ and invasive carcinoma.

In cohort-study based on 3398 women with a primary CIS reported to the Swedish Cancer Registry (SCR) 1980–1992, women diagnosed in 1989–1992 ran a relative risk of 0.1 (CI 95%, 0.0–0.9) from dying of breast cancer as compared with women diagnosed in 1980–1982. Women in counties with mammography screening ran a relative risk of 0.2 (CI 95%, 0.0–2.1) for breast cancer death in comparison with women in non-screening counties.

In a case-control study derived from all 4661 women with primary CIS reported to the SCR 1960-1992, we investigated risk factors for subsequent invasive breast carcinoma (n = 118) and breast cancer death (n = 39). Large size and multifocality were found to increase the risk for breast cancer death. Postoperative radiotherapy and mastectomy lowered the risk for ipsilateral invasive cancer.

The standardised incidence rates (SIR) for invasive breast cancer were estimated in the cohort from 1980–1992. The SIR after primary DCIS and primary lobular carcinoma in situ (LCIS) was 4.5 (CI 95%, 3.7–5.5) and 4.0 (CI 95%, 2.1–7.5), respectively.

New histopathological classification systems for DCIS were evaluated in 195 women consecutively diagnosed with primary DCIS between 1986–1994. One group with highly differentiated lesions was defined with the EORTC classification system and had an excellent prognosis.

Histopathological grade and expression of p53, c-erbB-2, Ki 67, hormone receptors, Bcl-2 and angiogenesis were compared in 626 women with either a pure DCIS, a small invasive carcinoma or a lesion with both an invasive and in situ component. When grade was taken into account, no change in tumour markers could be detected that signalled the progression from an in situ stage to invasiveness. All tumour markers correlated to grade and their distribution was very similar in the two components of mixed lesions.

October 2000

DNA fragmentation in cultured cells exposed to high linear energy transfer radiation

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The DNA double-strand break (DSB) is a critical lesion which, if not completely restored, can have serious biological consequences. The relative biological effectiveness (RBE) of many severe endpoints are closely related to radiation quality, with increased effectiveness at elevated ionization density. Data presented provide information about the influence of radiation quality on the initial processes causing DNA damage, and the mechanisms leading to its restoration. Such information will increase the understanding of radiation action mechanisms in mammalian cells.

Human cells were irradiated with accelerated ions having linear energy transfer (LET) values in the range 40–225 keV/μm, and ^{60}Co-photons. Detailed analyses of the DNA fragment distributions were performed in the size-range 5 kilobasepairs to 6 megabasepairs by pulsed-field gel electrophoresis.

A non-random fragmentation of DNA was evident, with an elevated number of small and medium-sized fragments for ion irradiation, and the total number of breaks increased by 80–110% when these fragments were included in the analyses. The RBE for DSB induction was 1.2–1.5. A two-fold increase of the number of breaks induced per nitrogen ion passing the cell nucleus was found when LET was increased from 80 to 225 keV/μm, indicating a possible role of particle track structure in DSB induction. Furthermore, the ability to repair DNA was closely related to radiation quality, with an increased proportion of unrejoined breaks for densely ionizing radiation. Surprisingly, the majority of breaks were rapidly rejoined even following exposure to high-LET radiation. The proportion of breaks restored by the slow phase showed a five-fold increase for the highest LET tested, compared with photons. The results presented nominates the complexity of breaks as one determining factor for reduced reparability reported following high-LET exposure.

October 2000

Deoxyribonucleoside kinases in nuclear and mitochondrial DNA precursor synthesis—Phosphorylation of anti-cancer nucleoside analogs in different subcellular compartments

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Nucleoside analogs have been developed as anticancer and antiviral agents. The main mechanism of action of nucleoside analogs is the incorporation of the corresponding analog triphosphate into

DNA where it interferes with DNA replication. The phosphorylation to the triphosphate form by cellular or viral enzymes is therefore required for the activation of nucleoside analogs. The first step of phosphorylation, catalyzed by nucleoside kinases, is regarded as being rate-limiting for most nucleoside analogs. DNA replication occurs both in the nucleus and in the mitochondria. Thus, both nuclear and mitochondrial DNA can be targeted by nucleoside analogs.

My research mainly addresses questions regarding nucleoside analog phosphorylation in the mitochondria. First, one of the mitochondrial deoxyribonucleoside kinases, deoxyguanosine kinase (dGK), was characterized with regard to its kinetic properties for natural substrates as well as for clinically important nucleoside analogs. We showed that dGK could efficiently phosphorylate the nucleoside analogs araG, CdA and dFdG. Overexpression of dGK in the mitochondria of pancreatic cancer cell lines enhanced the sensitivity to dGK phosphorylated nucleoside analogs. These data suggested that this mitochondrial deoxyribonucleoside kinase plays a role for nucleoside analog phosphorylation. Furthermore, we created a cell model by targeting genetically engineered dCK to the different subcellular compartments of a dCK deficient cell line. The expression of dCK in the nucleus, the cytosol or the mitochondria restored the sensitivity to dCK phosphorylated nucleoside analogs. By using autoradiography, we showed that nucleoside analogs phosphorylated by dCK in the mitochondria were predominantly incorporated into mitochondrial DNA, while nucleoside analogs phosphorylated in the nucleus or cytosol were incorporated into nuclear DNA. We concluded that incorporation of nucleoside analogs into nuclear or mitochondrial DNA was determined by the intracellular phosphorylation site. We also showed that nucleoside analogs phosphorylated in the mitochondria could initiate apoptosis similar to nucleoside analogs phosphorylated in the nucleus or the cytosol. The same cell model was used to study the cytotoxicity of nucleoside analogs in combination with the ribonucleotide reductase inhibitor hydroxyurea. The results showed that the combination of nucleoside analogs and hydroxyurea resulted in synergistic effects when the nucleoside analogs were phosphorylated in the nucleus or the cytosol but not when phosphorylated in the mitochondria. We have also shown by autoradiography that araG was predominantly incorporated into mitochondrial DNA while araC was incorporated into nuclear DNA. This finding may contribute to explain the selective cytotoxicity of araG in T-lymphocytes.

In summary, our results showed that mitochondrial kinases play a role for nucleoside analog activation. We believe that our findings are important in the development of improved therapeutic strategies involving nucleoside analogs.

October 2000

Human glandular kallikrein 2 (hK2) in prostate cancer—Clinical and methodological studies

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Human glandular kallikrein 2 (hK2) is a predominantly prostate produced protein with many similarities to prostate-specific antigen (PSA). Just like for PSA, elevated levels of hK2 in serum can be found in men with prostate cancer.

We created an assay where hK2 in serum could, reliably and with negligible cross-reactivity with PSA, be measured down to 0.030 ng/mL. The assay measured free hK2 and hK2 complexed to ACT with a -6% bias to free hK2. Gel-filtration of serum with elevated hK2 concentrations showed that the predominant form of hK2 in serum was free hK2.

We then studied the utility of hK2 measurements in the differentiation of men with benign prostatic hyperplasia (BPH) from men with prostate cancer. The addition of hK2 to measurements to free and total PSA improved the differentiation of men with BPH from men with clinically localized prostate cancer.

In order to study weather hK2 was useful in the clinical staging of prostate cancer, we measured hK2 in preoperative sera derived from prostatectomized men. The levels of hK2 was found to be significantly different in men with organ confined prostate cancer compared to men with nonorgan confined prostate cancer. High concentrations of hK2 makes a nonorgan confined prostate cancer likely.

The utility of hK2 measurements were also studied in a population of men participating in the Göteborg screening study for prostate cancer. The men studied were all biopsied due to PSA concentrations of 3.0 ng/mL or higher. We could demonstrate that hK2 used in combination with free and total PSA (hK2 $\times$ total PSA/free PSA) improved the differentiation of men with and without prostate cancer. By this combination we saw a significant improvement in sensitivity at high levels of specificity compared to by use of percent free PSA or total PSA. Two years after the first screening, the same cohort of men were called for a new serum PSA test. Again, we studied the men biospied due to elevated PSA and found that hK2 in combination with free and total PSA improved the separation of men with and without cancer, however less significant than in the first round of screening. We found that hK2 increased almost two-fold, and significantly higher, in men with cancer over a two year period compared to in men without cancer.

October 2000