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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.

A contribution to improved radiotherapy for muscle invading urinary bladder cancer

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Cystectomy has traditionally been regarded the treatment of choice for muscle invading urinary bladder cancer (UBC) in most countries, except for in the UK and in a few other centres in Europe and USA. Radiotherapy has been offered patients considered unfit for cystectomy. However, since the contraindications of surgery are frequent among UBC patients, a substantial amount of patients with muscle-invading UBC (typically 50%) are still managed primarily with radiation.

A tri-modality, organ-sparing treatment (trans-urethral resection and radio-chemotherapy) has been introduced for UBC, like in the management of a range of other common malignancies. This approach may provide as high control rates as cystectomy yet maintain a higher quality of life in selected patient groups.

Both in the radical radiotherapy and in the combined modality approach, high radiation doses are needed to improve local disease control. Radiation dose escalation requires improved conformation of dose distributions. This PhD programme aimed to develop improved conformal radiotherapy procedures in the management of patients with muscle-invading UBC.

Initially, computer-controlled movement of the linear accelerator collimator jaws during beam delivery was applied to shape so-called partially wedged beams (PWBs), that were designed specifically to tailor the dose distribution in bladder irradiation closer to the defined bladder target. The dosimetric verification and treatment planning implementation of this beam delivery concept were addressed. Particular attention was given to the BMS-96 diode array system, as it was adapted to dynamic beam dosimetry. Next, the potential clinical impact of these beams was analysed retrospectively in a set of urinary bladder treatment plans. The PWBs were documented to improve the dose homogeneity inside bladder targets as well as to reduce normal tissue (small intestine and rectum) doses. Using previously published clinical data as input to normal tissue complication probability (NTCP) models, the application of PWBs were found to permit radiation dose escalation with 2–6 Gy in up to 60% of the patients without increasing the overall NTCP above the risk resulting from the standard treatment. This analysis also revealed the uncertainty in the present NTCP models, an issue we addressed in a separate study. This study illustrated both the uncertainty in currently available small intestine and rectum radiation tolerance data as well as the differences between the probit and relative seriality models. Consequently, the results of the prospective studies on the late effects after pelvic irradiation that are underway both in our clinic and elsewhere are highly needed. Finally, as an important prerequisite for prescribing escalated radiation doses is to determine adequate target volume margins, we quantified the internal organ motion and patient set-up variation by weekly repeated CT scans and electronic portal images. New treatment margin proposals were derived, and were used to update the margins we use in bladder irradiation. We also generated rectum and intestine motion data that in the future can be applied to supplement the static treatment planning scan used in DVH/NTCP analysis to predict the risks for experiencing late effects. Currently, a clinical trial of radiation dose escalation in bladder irradiation using the PWB principle is underway. In this study we are testing the feasibility of escalating the dose to the whole bladder target from 64 Gy to 68 Gy while maintaining a low level of treatment-induced complications.

November 2002

An epidemiologic study of epithelial ovarian malignancies—With a focus on hormone-related factors

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The main purpose of this thesis was to examine estrogen and progestin effects of hormone replacement therapy, in relation to the risk for epithelial ovarian neoplasias. Other intentsions were to assess reproductive factors, oral contraceptive use, previous gynecologic surgeries, and family history of ovarian cancer according to the risk of ovarian neoplasias, and to connect these findings to different hypotheses regarding ovarian carcinogenesis. Finally, part of the work investigated whether preventative life-style modifications exist against these malignancies.

All results were based on a case-control study conducted in all of Sweden between October 1st, 1993 and December 31st, 1995. Subjects were 828 women with newly diagnosed, histologically confirmed epithelial ovarian neoplasias and 3899 population controls, all 50–74 years of age. For all epithelial ovarian cancer analyses (EOC, n=655) we used the histology classification reported by field pathologists, while borderline ovarian tumor data (BOT, n=193) were analyzed according to the reviewed histologies. Data were collected through mailed questionnaires. Odds ratios (OR) and 95% confidence intervals (CI) were estimated by the use of unconditional logistic regression.

Our key findings were elevated risks of EOC among ever users compared with never users of both unopposed estrogens (OR=1.43, 95% CI 1.02–2.00), and estrogens supplemented with sequential progestins (OR=1.54, 95% CI 1.15–2.05), while no excess risk appeared with the use of estrogens continuously combined with progestins (OR=1.02, 95% CI 0.73–1.43). Increased risks were seen for serous, mucinous, and endometrioid EOC, and for all EOC the greatest risk increase appeared with hormone use exceeding 10 years. Parity reduced EOC risk (OR=0.61 95% CI 0.46–0.81) for uniparous compared with nulliparous women, and multiparity decreased EOC risk futher. Ever compared with never use of oral contraceptives protected from EOC overall (OR=0.73 95% CI 0.59–0.90), and for serous, endometrioid, and clear-cell, but not mucinous EOC. The protection increased with duration of oral contraceptive use, and persisted up to 25 years after cessation of use. The risk of EOC appeared to decrease with incomplete

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pregnancies, early menopausal age, late age at first birth, unilateral oophorectomy, and coffee consumption; increase with ovarian cancer family history; and was unrelated to menarcheal age, lactation, irregular menses, menopausal symptoms, and alcohol use. Most epidemiologic findings on EOC risk seem consistent with the retrograde transporation hypothesis, which may operate through retrograde menstrual bleeding. A life-style including leisure time physical activity and a normal body mass index (BMI) may reduce EOC risk.

We observed no protection from serous (OR=1.40 95% CI 0.87–2.26) and mucinous BOT (OR=1.04 95% CI 0.61–1.79), among ever compared with never users of oral contraceptives, and duration of use was unrelated to BOT risk. An excess risk of serous BOT appeared among ever compared with never users of unopposed estrogens (OR=2.07 95% CI 1.08–3.95), but not for HRT including sequential or continuous progestins. No association between HRT and mucinous BOT was seen. The risk of BOT seemed to decrease with parity, lactation, incomplete pregnancies, tubal ligation, and leisure time physical activity; increase with irregular menstrual cycles, ovarian cancer family history and alcohol use; and was unrelated to age at first and last birth, and menopausal symptoms. The OR of serous BOT for the highest compared with the lowest BMI category was 6.47 (95% CI 3.09–13.5). We suggest that hormonal situations where estrogens are not counteracted by progestins may increase the risk of serous BOT.

January 2003

Dendritic cells in immune and gene therapy against cancer

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Dendritic cells (DC) are extensively used for vaccine therapy due to their superior T cell stimulatory capacity. However, the modest therapeutic effects reported by most clinical trials using DC, together with the rapid developing field of DC biology suggests important considerations for improvement of the clinical outcome. The general aim of this thesis is to develop DC-based cancer vaccines thereby facilitating the translation of such therapeutic approaches to the clinic. An ancillary aim of this study is to better understand the factors that affect the apoptosis of DC particularly during their differentiation from monocyte precursors. Mature DC are effectively resistant to Fas-mediated apoptosis. One of the underlying mechansisms that mediate this resistance is the observed upregulation of Bcl-X_L that accompanies DC maturation. These results provide additional support for the use of mature DC as cellular adjuvants since they not only represent a more potent T cell stimulatory population, but also may mediate a more protracted effect due to their resistance to Fas induced apoptosis.

The mode of antigen delivery is pivotal to the generation of specific immunity. In this study, a comparison of viral and non-viral gene delivery techniques to human DC derived from peripheral blood precursors or $CD34^+$ stem cells were performed. DC differentiated from both types progenitor cells were receptive to non-viral transfection using mRNA and electroporation. In addition, the number of transfected cells was comparable to what could be achieved by andeo- and retro-viral transduction. These findings encourage the use of non-viral methods for transfection of DC as problem of neutralizing antibodies and prohibitively expensive safety tests associated with viral delivery may limit their use in the clinic. Certain viruses can lead to inhibition or maturation of DC or even induce their apoptosis. Furthermore, the effect of viral transduction on cell physiology and APC activity has not been satisfactorily elucidated. Transduction of monocyte derived-DC using adenovirus (AdV) results in generation of DC that are phenotypically and functionally 'activated'. Taken together, the transfer of antigenic epitopes at high efficiency to human monocyte-derived DC as well as the DC-activating effects are strong arguments for their clinical application of AdV-transduced DC-based vaccines.

The final aspect of the thesis utilizes prostrate specific antigen (PSA) as a model tumor antigen for generation of specific T cell response, using DC transfected with different delivery methods. Stimulation with AdV transduced TNFα treated DC resulted in the generation of PSA-specific T cells that predominantly produced IL-10. However, stimulation with untreated, lipopolysaccharide, or anti-CD40 treated AdV transduced DC resulted in generation of IFNγ, but not IL-10, producing PSA-specific T cells indicating that the predomiant population was Th1 T cells. In conclusion, conventional antigen delivery and DC maturation methods may influence the functional characteristics of the generated DC and may produce T cell populations with aberrant functions, that in certain instances may be counter productive to the anti-tumor immune reponse desired with vaccination strategies.

January 2003

DNA-dependent protein kinase in normal and malignant cells—With special reference to anti-tumour agent sensitivity

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The DNA-dependent protein kinase (DNA-PK) is a DNA double strand break (DSB) repair enzyme, essential for the cellular response to DNA damaging agents. DNA-PK is also of importance for lymphoid development, since it includes two processes, V (D)J recombination and Ig switch recombination, during with DSBs are naturally occurring events. We observed that DNA-PK is differently expressed in normal as well as malignant haematological cells of different degrees of maturation. CD34 positive cells and cells from patients with acute lymphocytic leukaemia (ALL) display high levels of DNA-PK, whereas mature B-lymphocytes and chronic lymphocytic leukaemia (CLL) cells show low levels of DNA-PK. Following antigen presentation of normal lymphocytes, the expression of DNA-PK is increased. In parallel with this, multiple myeloma cells were found to display high frequency of DNA-PK positive cells.

In contrast to CLL cells from untreated patients, CLL cells from previously treated patients show high levels of DNA-PK protein expression, suggesting a role for DNA-PK in acquired drug resistance. In CLL cells we found a correlation between DNA-PK and etoposide sensitivity. This is to be expected, since etoposide induce DNA DSBs. Unexpectedly, DNA-PK activity was found to correlate with vincristine sensitivity in both CLL and acute myeloid leukaemia (AML) cells, indicating that DNA-PK may be involved in the reponse to anti-tumour drugs others than DNA DSB inducers.

The tumour cell line, M059J, deficient in DNA-PK activity, is known to be sensitive to DNA DSB inducing agents, e.g. X-ray (low linear energy transfer, LET). These cells are also more prone to

undergo X-ray induced apoptosis, compared with the DNA-PK proficient cells, M059K. Ionising radiation with high ionisation density (high LET) were found to induce apoptosis to similar extent in M059J and M059K cells, however, the course of events seems to be different between the two cell lines. Our interpretation is that the detected difference is related to giant cell formation and 'mitotic catastrophe'. While M059K cells show an expected G2 accumulation and release from the G2 block, M059J cells accumulate in both S and G2/M, followed by apoptosis.

In view of the increased sensitivity to DNA damaging agents in cells deficient in DNA-PK, it is tempting to suggest that DNA-PK is a suitable target for sensitisation of cells resistant to anti-cancer agents. DNA-PK is inhibited by trifluoperazine (TFP) in vitro, by direct inhibition, and in vitro, by induction of apoptosis. The findings presented in this thesis indicate that the role of DNA-PK in predicting therapy results in various tumours should be further explored, as well as the possibility of using DNA-PK as a target in tumour therapy.

January 2003

Mechanisms of immune escape—Implications for immunotherapy against cancer

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Tumor cells can be recognized and killed by cytotoxic T cells specific for certain tumor antigens. Immune mediated selection pressure along with genetic instability of tumor cells result in a growth advantage of tumor cells that acquire a less immunogenic phenotype. Increased understanding of immune escape mechanisms is crucial for the development of effective immunotherapy against cancer. This thesis deals with several aspects of immune escape and tumor induced immune suppression.

We demonstrate the paradoxical finding that IFN-γ treatment of short-term ovarian cancer cell lines (OVAC) protected these from cytotoxic T lymphocyte (CTL) lysis. This was depenent on enhanced signaling to inhibitory NK receptors (iNKR), CD94/NKG2A, which were expressed by the CTLs. The ligand for CD94/NKG2A is HLA-E, which is a non-classical HLA class I molecule. HLA-E expression depends on its association with leader sequence peptides derived from classical and non-classical HLA class I molecules. Furthermore, the signaling capacity of the HLA-E/peptide complex is influenced by specific properties of the peptide that is bound to the HLA-E molecule. The leader sequence peptide of another non-classical HLA class I molecule, HLA-G, provides a particularly strong inhibitory signal to CD94/NKG2A receptors when bond in the peptide groove of the HLA-E molecule. Ovarian carcinomas were found to frequently express HLA-G at the protein level and HLA-E on a transcriptional level. The expression of both these molecules was induced by IFN-γ. We speculated that the increased intracellular accessibility to HLA-G leader sequence peptides (Gsp), followed by increased surface expression of HLA-E/Gsp complexes, was the underlying mechanism behind the IFN-γ mediated protection of tumor cells. In support of this possibility we were able to mimic the effect of IFN-γ by exogenously adding synthetic HLA-G leader sequence peptides to untreated tumor cells. It is concluded that IFN-γ may shift the balance towards inhibitory signaling to the CTLs, turning off the lysis of otherwise sensitive targets. The role of such ligand - iNKR interactions deserves further attention in future attempts of immunotherapy against ovarian carcinoma.

The importance of inhibitory NK receptors was further emphasized by the phenotypic aanalysis of ovarian tumor associated T and NK cells. There was a bias towards expression of inhibitory CD94/NKG2A receptors on the tumor infiltrating $CD56^+$ T cells as compared to $CD56^+$ T cells derived from PBL of patients and healthy donors. Further, there was an over-representation of regulatory, non-cytotoxic, $CD56^{bright}$ NK cells among the tumor associated lymphoctyes. This was associated with high expression of inhibitory CD94/NKG2A receptors on this subset of NK cells. Expression of inhibitory receptors by a large number of tumor associated lymphocytes is likely to decrease their cytotoxic activity against autologous tumors.

Hydrogen peroxide, released by tumor associated macrophages severly impairs the cytokine production and cytolytic function of T and NK cells. It was demonstrated that the Th1 cytokine production of T cells with an activated/memory ($CD45RO^+$) phenotype was more sensitive than naïve T cells to the influence of H_2O_2. Furthermore, the reduced Th1 cytokine production was associated with a block of NK-κB activation. The majority of tumor infiltrating lymphocytes is of a $CD45RO^+$ phenotype and our results may explain why T cells that reside in tumor lesions often display anergic properties. Based on these results we speculated that exogenous supply of antioxidants may protect immune cells from the attack of free radicals. This hypothesis was tested on 12 patients with advanced colorectal cancer who were given high doses of dietary vitamin E during a period of two weeks. In a majority of patients the supplementation of vitamin E leads to enhanced IL-2 and IFN-γ production by their T cells. Moreover, CD4/CD8 ratios were increased after treatment with vitamin E. It is concluded that the administration of dietary vitamin E may counteract tumor induced immune suppression and form an effective supplement to more specific immunotherapy.

February 2003

Targeted CD52 therapy in lymphoid malignancies—A clinical and immunological study

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There is a great need for developing new treatment alternatives in low grade non Hodgkin's lymphoma (NHL) including chronic lymphocytic leukemia (CLL). The CD52 antigen, which is expressed on almost all lymphoid malignancies, can be utilized as a target for CD52-directed monoclonal antibody therapy (alemtuzumab, Campath-1H). The aim of this thesis is to evaluate the clinical, safety and immunological effects of alemtuzumab in low grade NHL or CLL.

In the first trial, 50 patients with low grade NHL received alemtuzumab as i.v. infusions (30 mg tiw). The OR rate was 20%; marked anti-tumor effects were observed in blood (CR 94%) and bone marrow (CR 32% and PR 24%). At the time of initiating this study, antiviral and antibacterial prophylaxis was not routinely given and grade III–IV infections developed in one third of the patients. The trial served as the basis for alemtuzumab's subsequent development in lymphocytic leukemias and T-cell lymphomas.

In the second study, 41 patients were received subcutaneous (s.c.) alemtuzumab therapy for up to 18 weeks. The OR rate was 81% (intention to treat). Median time to treatment failure was 18+

months. CR or nodular PR in bone marrow was achieved in 66%, which often required 18 weeks of therapy. Grade 1–2 local injection site reactions was observed in most patients, but disappeared within 2 weeks upon continued therapy. Four cases of CMV reactivation were observed (fever without pneumonitis, rapidly responding to ganciclovir). This trial shows a considerably higher response rate on alemtuzumab than in advanced B-CLL. A prolonged duration of therapy appears to be important for maximal efficacy. S.c. administration seemed to reduce the incidence and severity of general 'first dose' side effects and may also reduce the costs of therapy.

In the third study, B cells from healthy volunteers and patients with previously untreated B-CLL were analyzed by flow cytometry (MESF units) to quantify receptor intensity of CD20, CD22 and CD52 (which all are potential therapeutic target antigens). Receptor intensity analysis revealed that the expression of CD52 was >30 times greater than that of CD20 and CD22. A substantial variability over the time was seen and most pronounced for CD20. The effects of in vivo cytokine (interleukin-4) therapy on receptor density were also studied but a clear impact on receptor intensity expression could not be observed. Thus, effects described in vitro in other studies were not easily translated into the in vivo situation.

In the fourth trial, 22 patients with advanced mycosis fungoides/Sézary's syndrome (MF/SS) were treated with alemtuzumab for 12 weeks. The OR rate was 55%, including 32% CRs. Less heavily pretreated patients had an OR rate of 80% vs. 33% in refractory patients. Itching, self-assessed by VAS was reduced from a median of 8 before treatment to 2 at end of the therapy. CMV reactivation occurred in 4 patients (18%) and 3 patients had fever of unknown origin, which resolved following i.v. antibiotics. Except for CMV, all serious infectious adverse events occurred in the refractory patients. This study shows that alemtuzumab has a major activity in MF/SS patients.

In a last study, immunological cell subpopulations were studied before and after therapy as well as during long-term unmaintained follow-up in CLL patients who had received alemtuzumab as first line therapy. CD4 and CD8 T cells were profoundly depleted from blood and remained at $\leq 25\%$ of the baseline levels for >6 months in most patients. Also normal B cells, NK-, and NK-T cell populations were markedly suppressed. Monocytes and granulocytes were less affected by the therapy. The degree of suppression and time to recovery appeared not to be dose-dependent. No late occurring infections were observed. Antigen (CD52) negative T-cell populations were detected in blood and persisted for a prolonged period of time.

Alemtuzumab is a new monoclonal antibody with significant effect in lymphoid malignancies, it induces long-lasting immune suppression and antibiotic prophylaxis is needed to prevent infectous complications.

February 2003

Allogeneic stem cell transplantation in children—Identification and prevention of complications—Adoptive transfer of EBV-immunity

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Allogeneic stem cell transplantation (SCT) can cure leukaemia, aplastic anaemia, and some inborn errors of metabolism and immunodeficiencies in children. As only 1/3 of patients who need SCT have an HLA-identical sibling, the use of alternative donors has increased, to over 50% of all paediatric SCTs at our centre. The immunological interactions and the clinical course are much more complex in such transplants. The aim of this study is to investigate and find means to manage some of the problems in paediatric SCT, particularly those inherent in matched unrelated donor (MUD) or partially mismatched donor transplants.

In a single centre study, we compared the outcome of all 59 children, who underwent MUD SCT, with case controls receiving sibling donor grafts. The 5-year probability of survival was 52% for MUD vs. 77% for sibling recipients ($p = 0.014$). In ALL, the survival of the MUD (77%) and sibling group was equal. In SAA, survival was 43% vs. 86% ($p = 0.09$) and in metabolic disorders 63% vs. 89% ($p = 0.025$). The incidence of acute graft-versus-host-disease (GVHD) and the transplant related mortality (TRM) were higher in the MUD group, while eight relapses occurred in each group. The relapse rate was lower in children with chronic GVHD. These results support the use of MUDs when a sibling donor is not available. They also prompted us to examine further whether GVHD is associated with a graft-versus-leukaemia (GVL) effect, also in childhood acute leukaemia. In all 169 children, who had SCT for ALL and AML at our centre, median time to relapse was 24 months in patients with chronic GVHD and 6 months in those without. The 5-year probabilities of relapse were 30 and 45% ($p = 0.01$). Patients with chronic GVHD had a better survival, 77 vs. 51% ($p = 0.01$). In a Cox regression model, chronic, but not acute GVHD decreased the risk of relapse (RR 0.44) and was predictive of an increased relapse-free survival (RFS) (RR 1.7), most apparent in late-stage disease and in ALL. This is in support of a GVL-effect in childhood leukaemia related to chronic GVHD.

To investigate further which factors impact on survival, relapse, TRM and GVHD we included all 181 children transplanted due to leukaemia at our unit. At the end of follow up 54% of the patients were alive, 27% had died in relapse, while 19% had died of other causes. The 5-year probabilities of acute GVHD grade II–IV, TRM, and RFS were 21%, 18% and 49%. Survival was similar in recipients of related (55%) and unrelated grafts (48%). In multivariate analysis, stage of disease ($\geq$CR 2) was an independent predictor for relapse and death. The risk of relapse increased significantly after 1992. A donor positive for 3–4 herpes viruses, increased the risk of acute GVHD, TRM and death, while a female to male transplant increased the risk of TRM, particularly in combination with a mismatch. HLA-matching independently improved survival, RFS and TRM. In children with leukaemia, an unrelated donor was not a risk factor for any of the five endpoints analysed. Instead, other donor characteristics such as HLA-matching, herpes virus serology, immunisation and sex were more important for outcome.

In mismatched donor SCT, the graft is T-cell depleted (TCD) to prevent life-threatening GVHD. TCD, however, increases the risk of Epstein-Barr virus (EBV)-associated lymphoma (PTLD), rejection and relapse. For six recipients at risk to develop PTLD, EBV-specific cytotoxic donor T lymphocyte (CTL) lines were generated by stimulation with EBV-transformed lymphoblasts, several weeks before SCT. Monitoring of the EBV-DNA load with semiquantitative PCR demonstrated that 4 of 5 recipients of TCD grafts and one Wiskott-Aldrich patient had a 4- to 5-log increase of EBV-DNA within 1–3 months after SCT, predicting a high risk of PTLD. In the other three recipients of unmanipulated grafts, the increases were moderate. Two to 4 infusions of 1×10^7 of the EBV-CTLs/m2 resulted in a 2- to 3-log decrease of EBV-genomes in four patients and in stabilisation of the virus load on a moderate level in one case. One child, who received a T-cell culture lacking in EBV-specificity, progressed to fatal PTLD. The results suggest that a rapid increase of the EBV-load occurs in the absence of EBV-

specific T-cell precursors, after TCD or in the presence of immunodeficiency. Infusion of EBV-CTLs early after SCT appears to prevent EBV-PTLD.

To investigate the increased EBV-DNA load and the state of viral latency in these patients, we analysed EBV-DNA in serum, applied limiting dilution analysis of EBV-DNA in mononuclear cells and RT-PCRs for viral gene expression. The increased virus load is suggested to be due to an expansion of a latently infected B-cell compartment that contains less than 10 EBV genome copies per cell and expresses EBERs, LMP-2A and occasionally LMP 1 and EBNA 1. This is compatible with latency forms I–II in non-proliferating B-cells, but not latency III. Most likely this is due to proliferation of EBV+ B cells somewhere in the lymphatic compartment, outside peripheral blood, as we did not find evidence of active replication of virus.

February 2003

Retinoids in experimental neuroblastoma therapy

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Retinoids are analogues of vitamin A, with documented activity against various malignant cell types. Neuroblastoma is a childhood tumour of the sympathetic nervous system that shows a complex clinical and biological heterogeneity, often with poor outcome despite intensive multimodal therapy.

The aim of the thesis was to investigate effects of retinoid treatment in vitro on human neuroblastoma cells, and in vivo on human neuroblastoma xenografts in nude rats. The ultimate goal was to find a new retinoid treatment for children with neuroblastoma.

Oral treatment with 9-*cis* RA in vivo resulted in a significant inhibition of neuroblastoma tumour growth, but with major toxic side effects. Further experiments showed that 9-*cis* RA might not be suitable for clinical use in children with neuroblastoma, because of its short half-life, low bioavailability and toxic profile in rats.

Ro 13-6307 was established to be a morphologically differentiating retinoid, able to reduce proliferation and induce G1 growth arrest in both MYCN amplified and non-amplified neuroblastoma cell lines in vitro. Further experiments showed that oral Ro 13-6307 could inhibit neuroblastoma tumour growth in vivo with limited toxicity. In vitro and in vivo results indicated that Ro 13-6307 was at least as effective as the clinically established retinoid 13-*cis* RA. These results demonstrate that Ro 13-6307 is a potential retinoid for clinical oral therapy of children with neuroblastoma.

Despite promising results demonstrating that fenretinide induces apoptosis in neuroblastoma cells in vitro, no significant reduction in neuroblastoma tumour growth was observed after oral treatment with fenretinide in vivo. Five different doses were evaluated, but no significant inhibiting effect on tumour growth or morphological changes were found in treated compared to untreated tumours. Other alternatives for fenretinide administration should be investigated in future experimental and clinical studies.

Proton magnetic resonance spectroscopy was found to be a suitable method for detecting metabolic alterations in neuroblastoma cells in vitro undergoing fenretinide-induced apoptosis. It was possible to monitor the kinetics in the treatment response and to distinguish between fenretinide-sensitive and -resistant cells. These findings suggest that proton magnetic resonance spectroscopy is a potential clinical non-invasive tool to monitor early tumour response to retinoid treatments.

In conclusion, retinoids were shown to inhibit growth of human neuroblastoma cells in vitro and in vivo, however the effect depends on the retinoid in use. Dosing, scheduling, and toxicity are important factors determining the therapeutic efficacy of retinoids in vivo. Ro 13-6307 may be a retinoid for future clinical therapy of children with neuroblastoma.

February 2003

Modeling of multi-step oral carcinogenesis in vitro—Assessment of growth, differentiation and apoptosis markers

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Human oral mucosa, especially the buccal epithelium, is worldwide a common site for cancer. Cancer development frequently results in inactivation of tumor suppressor p53, a central regulator of growth and programmed cell death, and deregulated expression of structural elements like cytokeratins. The overall aim of this study was to investigate if the multi-step process of carcinogenesis can be modeled and studied from a mechanistic stand point utilizing cultured normal (NOK), immortalized (SVpgC2a) and malignant (SqCC/Y1) human buccal keratinocytes.

Organotypic epithelia of the respective cell lines, as derived from serum-free culture on a collagen gel containing oral fibroblasts, showed morphological features ranging from normal tissue to carcinoma in situ. The respective epithelia showed sharp differences in immunochemical expression of keratins. NOK expressed many of the same keratins as buccal mucosa, whereas loss of keratins in SVpgC2a and their retention in SqCC/Y1 showed similarities to oral dysplasia and well-differentiated squamous cell carcinoma. Assessment of tissue homeostatic functions demonstrated that NOK exhibited a terminal squamous differentiation (TSD) and apoptosis-capable phenotype, that responded to fibroblast-mediated proliferation with increased apoptosis and to elevation of Ca^{2+} by induction of TSD. In contrast, SVpgC2a and SqCC/Y1 exhibited hyper-proliferative, TSD-deficient and hyper-apoptotic phenotypes that failed to respond to the above stimuli. Immunochemical expression of tumor suppressor p53 was scattered in NOK, heterogeneous in SVpgC2a and negative in SqCC/Y1. Exclusively for NOK, p53 expression increased with proliferation and decreased with TSD, moreover, expression of Bax, a gene associated to apoptosis in many cell types, correlated with TSD. Further evaluation of NOK and SVpgC2a in various conditions for up to 17 days consistently showed several-fold higher proliferation and apoptosis rates in SVpgC2a. Micro-array analysis of NOK and SVpgC2a in monolayer culture confirmed the respective keratin protein profiles to the mRNA level, and indicated expression of keratins not previously reported for buccal epithelium. Under sparse or confluent culture, SVpgC2a exhibited relatively higher cloning ability and growth rate as well as lower responsiveness to contact inhibition than NOK. Apoptosis and TSD were regulated in NOK in response to increasing cell density whereas SVpgC2a showed resistance. Cultures of NOK showed obligatory dependence for the growth supplement, pituitary extract, whereas SVpgC2a

showed independence, and thus, SVpgC2a could be cultured at chemically defined conditions. Immunochemical assessments in NOK showed increased Bax expression under conditions that increase TSD and decrease apoptosis, providing further evidence for the dissociation of Bax expression from apoptosis in keratinocytes.

A composite in vitro model for malignant transformation of oral epithelium is described. Characterization of NOK, SVpgC2a and SqCC/Y1 demonstrated that the multi-step process of malignant transformation of buccal keratinocytes clearly associates with alterations in basic cellular functions and mechanisms that regulate tissue homeostasis and build-up of the cytoskeleton. Overall, standardized, highly defined culture conditions, different cell densities and co-culture models provide useful means of investigating mechanisms underlying oral cancer development.

February 2003

The attenuation of the p53 response to DNA damage in rodent liver preneoplastic enzyme-altered foci

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In connection with life-time bioassays in rodents, the liver is one of the organs most frequently affected. During the carcinogenic process, single preneoplastic hepatocytes develop into hepatocellular adenoma or carcinoma (HCC). Preneoplastic hepatocytes are identified on the basis of their overexpression of the inactivating phase II enzyme glutathione-S-transferase Pi (GST-P). In the presence of continuos exposure to carcinogen, initiated hepatocytes expand clonally to form hepatic enzyme-altered foci (EAF). Such development of EAF might be considered to be an adaptive response and their pattern of gene expression may provide mechanistic information concerning the action of a putative carcinogen.

Our studies have focused on the response of the tumor suppressor p53 to DNA damage in EAF. Activated by several types of cellular stress, the p53 protein is involved in regulating cell cycle arrest and apoptosis. The overall aim of our project was to characterize the attenuated p53 response to DNA damage in preneoplastic EAF lesions and the possible role of this attenuation in the development of EAF by diethylnitrosamine (DEN).

To this end, after receiving an initiating neonatal dose of DEN female Sprague-Dawley rats were exposed to DEN or the non-genotoxic agent phenobarbital (PB), which induced the development of EAF in both cases. A challenging dose of DEN was also administered 24 hours prior to sacrifice to elicit a p53 response in these EAF.

Only EAF arising from treatment with DEN exhibited an attenuated p53 response in comparison to that of surrounding, non-EAF tissue and PB-induced EAF. This attenuation was enhanced by prolonging the period of treatment, as well as in larger EAF. The attenuated p53 response to DEN-induced DNA damage was also present in primary co-cultures, of hepatocytes isolated from EAF (GST-P-positive hepatocytes) and from non-EAF tissue (GST-P-negative hepatocytes).

Treatment of such co-cultures with $CoCl_2$, which mimics hypoxia, resulted in nuclear accumulation of p53 in the GST-P-positive cells. This finding demonstrates that a p53 response may be evoked by hypoxic stress, but not by genotoxic chemicals. Additional studies with the P13 kinase inhibitors caffeine and wortmannin, as well as with ATM antisense oligonucleotides indicated that ATM is involved in signalling to p53 following DEN-induced damage of DNA. Immunohistochemical analysis of the livers of DEN-treated rats and Western blotting of macroscopic EAF tissue revealed lowered expression of ATM in these tissues. Thus, down-regulation of ATM may to some extent explain the attenuated p53 response to DEN exhibited by EAF.

Upon examining the combined p53–MDM2 response in rat liver at different time-points following a single injection of DEN, significant temporal and spatial variations were observed. Midzonal areas demonstrated a transient combined p53–MDM2 response 6–24 hours after the DEN challenge, whereas in centrilobular areas this response culminated 24–72 hours after injection. MDM2 was constitutively expressed in midzonal areas. Furthermore, following repeated treatment with low doses of DEN, GST-P-positive EAF were found to be particularly prevalent in this same zone.

Finally, the influence of the p53 gene dosage on the development of p53-negative preneoplastic lesions was investigated. Treatment of p53 (+/+) and (+/−) mice for 15–20 weeks with DEN revealed a genotype-dependent difference in the numbers of p53-negative preneoplastic hepatic lesions obtained, with p53 (+/−) mice developing significantly fewer p53-negative lesions than p53 wild-type (+/+) mice. However, the total number and average size of all preneoplastic lesions were similar in these two types of mice.

In conclusion, these findings indicate that an attenuated p53 response to DNA damage confers a growth advantage on preneoplastic focal lesions in the liver. The selective pressure for focal lesions exhibiting such p53 attenuation can be modulated by altering the p53 gene dosage or by exposure to xenobiotics. These observations indicate that the attenuated p53 response in preneoplastic lesions is an adaptive response to genotoxic stress.

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Langerhans cell histiocytosis—A clinical and immunological study

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Langerhans cell histiocytosis (LCH), previously known as histiocytosis X, eosinophilic granuloma, Hand-Schüller-Christian or Letterer-Siwe disease, is a rare disease with a reported incidence in childhood of 5.4 cases per million children per year. The disease can present at any age but young children are most often affected. It is characterized by an accumulation of abnormal and clonal Langerhans cells in various organs such as the skin, bone, lymph nodes, lungs, liver, spleen and bone marrow. The course of the disease is unpredictable, varying from a spontaneously healing isolated bone or skin lesion, to a chronic relapsing course resulting in permanent consequences, or to a multi-organ disease that can be fatal.

The overall aim with this thesis has been to clarify the long-term course and outcome of patients with LCH, to investigate methods to detect and monitor disease activity and, finally, to elucidate if the HLA system may play a role in the pathogenesis of LCH. Initially a follow-up study of 49 patients, with reviewed and verified pathology findings, seen at Department of Pediatrics at the Karolinska Hospital 1962–1989 was performed, 40 of whom underwent physical examination and laboratory tests, as well as imagining

(n = 38). The 5-yr survival of 77% (17/22) in multi-system disease was significantly lower as compared to the 100% (27/27) survival of children who had single-system disease at diagnosis ($p = 0.014$). Late sequelae of the disease or its treatment had developed in 42% of the follow-up patients, leaving only 51% (23/45) of all patients alive and healthy, at a median follow-up of 16 years, with the corresponding figure being 67% (16/24) for single-system and 33% (7/21) for multi-system disease ($p = 0.026$). The late sequlae included diabetes insipidus (15%), severe CNS-complications (10%, affecting 25% of multi-system LCH patients), and late-stage pulmonary disease (11%). The serious permanent consequences in the lungs and the CNS were further studied.

High resolution computed tomography (HRCT) revealed late-stage radiographic abnormalities of the lungs (cysts and/or emphysema) in 24% of the patients at follow-up. These patients more often had multi-system rather than single-system LCH ($p = 0.01$), were significantly older at diagnosis ($p < 0.001$), and had been more heavily treated with chemotherapy and/or radiotherapy. They were also more frequently smokers ($p < 0.0001$) and most (70%) had had lung involvement at diagnosis. In two adults, diagnosed with LCH in childhood, smoking preceded pulmonary involvement by three years. We conclude that LCH patients should avoid smoking tobacco and that patients with LCH should be informed about smoking-related pulmonary morbidity.

In pulmonary LCH the physical examination is often unremarkable. Chest-X-ray is a valuable screening method for detection and monitoring. HRCT is more informative in monitoring the extent of the disease. Pulmonary function tests (PFT) give a better picture of the current lung function, and it is particularly valuable for monitoring alterations in pulmonary function. Prolonged monitoring of the lungs is suggested for smokers and patients with known pulmonary involvement.

One of the most feared permanent consequences of LCH is severe neurological impairment. Patients with LCH–CNS were examined with positron emission tomography (PET) scan. The ability to detect metabolic changes in the CNS with PET provides additional information about the activity of the disease, and PET may also be a valuable method to assess the efficacy of therapeutic measures in LCH–CNS.

Although recent studies have revealed clonality in LCH lesions and that Langerhans cells are immature with regard to phenotype as well as function, the underlying cause of this enigmatic disease remains unknown. The histopathological features of single-system and multi-system LCH are highly similar, but it is not yet known whether these manifestations have a common cause or whether each has a separate and distinct cause. In an attempt to elucidate if the HLA system may play a role in the pathogenesis of LCH, the largest reported pediatric cohort of ethnically homogeneous LCH patients (n = 84) was analyzed for HLA associations. We found that patients with single-system LCH more often had the phenotype *HLA-DRB1*03* as compared to patients with multi-system disease, suggesting an immunogenetic heterogeneity in the two clinical entities of LCH and indicating that the *HLA-DRB1*03* phenotype may play a protective role against developing multi-system LCH.

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Cancer risks and immunological effects in agriculture

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In general, farmers and agricultural workers are characterized by relatively low mortality rates when considering all causes of death and deaths due to all types of cancer. However, several studies have shown that this occupational category tend to be at increased risks of developing the following forms of malignancies: leukaemia, lymphomas, multiple myeloma, and cancers of the skin, lip, prostate, stomach, brain, and connective tissue. Although the reported excesses have not been consistent in different studies, they nevertheless suggest that agricultural exposures, especially pesticides, contribute to cancer aetiology through different mechanisms of action. It has been hypothesised that immunotoxic effects may play a role, since most of the malignancies found to be increased in workers employed in agriculture are also elevated in patients suffering from immunodeficiencies. The present studies were conducted to ascertain whether agricultural activities and exposures influence the risk of developing cancer, and to evaluate the immunological effects of phenoxy herbicides as possibly important for the aetiology of lymphomas.

Different types of cancer were considered in relation to farming in two case-control studies, focused one on men and the other on women. A decreased risk of postmenopausal breast cancer (odds ratio [OR] 0.4) and an excess of skin melanoma (OR 2.7) and bladder cancer (OR 2.7) were observed in the women. The main finding in the men was an excess of prostate cancer (OR 1.5). Further analyses showed that this malignancy was associated with exposure to chlorinated compounds (OR 2.5), and, more specifically, to DDT (OR 2.1) and dicofol and tetradifon (OR 2.8).

Mortality in selected groups of farmers and their relatives was investigated in two cohort studies. Farmers and their wives showed reduced mortality from all causes and all cancers although there was a tendency towards increased risk of leukaemia, particularly among wives of pesticide users (standardised mortality ratio [SMR] 2.4). Adult sons and daughters of green-house owners exhibited increased mortality from all types of cancer (SMR 1.9).

A study was also performed to compare pre- and post-exposure values of selected immunological parameters in a group of ten farmers exposed to phenoxy herbicides. The most notable findings were reduced levels of the following: circulating natural killer (NK) cells (-38%), NK cytotoxic activity (-53%), and lymphocyte response to mitogenic stimulation (-50%).

April 2003

Optimization of compliance in epidemiologic research and disease prevention—with special emphasis on Pap-smear screening

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The aim of this thesis was to investigate factors affecting attendance in screening for cervical cancer, and to evaluate various measures aimed at increasing compliance to self-administered postal questionnaires and screening for cervical cancer.

A population-based randomized controlled trial including 2 000 men and women aged 20–79 years and living in Sweden was conducted to investigate compliance to postal questionnaires. In a randomized 2^3 factorial design three factors were tested: 1)

preliminary notification or not, 2) questionnaire length, and 3) mention of a possible telephone contact or not.

Preliminary notification increased the response rate by 7%, a short questionnaire with 5%, whereas mention of a possible telephone contact did not influence attendance. Combinations of preliminary notification and short questionnaires increased the response rate by 16%, whereas young age, male gender and urban residence lowered the response rate.

The relation between non-attendance to screening for cervical cancer (Pap smear screening) and sociodemographic factors, gynecological examinations, risk behavior, general health behavior, knowledge, attitudes and beliefs was investigated in a population-based case-control study with 430 non-attenders and 514 attenders at Pap smear screening in Uppsala County.

Non-attendance was more likely among women who had *not* used oral contraceptives, who had *not* taken their own initiative to a Pap smear, who had visited different gynecologists, and who had visited a physician very often or not at all. Regular condom use, living in rural/semirural areas, and *not* knowing the recommended screening interval were all associated with non-attendance, whereas socioeconomic status was not, when tested in a multivariate model.

Multivariate analysis also showed that non-attendance was more likely among women who did *not* perceive cervical cancer to be as severe as other malignancies, who did *not* perceive the benefits of a Pap smear, who had time-consuming and economical barriers, and who did *not* feel anxious about the test results or cervical cancer. The results were strengthened with increasing time since the last smear or if self-reported attendance status was used instead of true attendance.

Non-attenders also kept holding on harder to their preferences than did attenders, stating that they would not participate if their preferences were not met and were less likely to intend to participate in future screening. Among the non-attenders, 57% underestimated the time lapse since the last smear.

Modifications of the invitation and call-recall system for Pap smear screening was investigated in a randomized controlled trial including all 12 240 women invited to organized screening during 17 weeks in 2001 in Uppsala County. Three successive interventions were tested: 1) modified invitation vs. the standard invitation letter, 2) reminder letter vs. no reminder letter, and 3) phone reminder vs. no phone reminder.

Whereas the modified invitation did not increase attendance, a reminder letter increased the proportion attending by 9%, and a phone reminder by 31%. Combinations of modified invitation, written reminder and phone reminder almost doubled attendance within 12 months, and the number of detected cytologic abnormalities was more than tripled.

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Localization and characterization of genes involved in parathyroid tumor development

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The overall goal of this thesis has been to identify new genes and characterize them regarding the involvement in parathyroid tumor development. The parathyroid glands are responsible for the control of calcium homeostasis. Parathyroid tumors usually have a benign growth pattern, with less than 1% of the tumors being malignant. The definition of hyperparathyroidism (HPT) is that a patient has inappropriately high levels of parathyroid hormone (PTH) in relation to extracellular calcium. HPT is a common endocrinopathy and is generally caused by a monoclonal parathyroid adenoma. However, the etiologies behind the tumor development needs to be further elucidated.

Secondary HPT (SHPT) arises in patients with renal failure, resulting in asymmetric hyperplasia of the parathyroid glands, but the mechanisms behind the tumorigenesis is unknown. A comparative genomic hybridization (CGH) analysis was performed in order to find numerical aberrations in SHPT tumors. Among 16 parathyroid glands from eight patients investigated, chromosomal abnormalities was detected in one gland only. The detected loss of chromosome 11 was coupled with a mutation in the *MEN1* tumor suppressor gene in 11q13, shown to be responsible for some familial and sporadic forms of HPT. Hence, the majority of secondary HPT glands are chromosomally stable and can be expected to harbor more discrete genetic alterations, below the detection level of CGH.

We characterized the expression profiles in parathyroid adenomas and normal tissues, using the oligonucleotide microarray technology. Among the upregulated genes in adenomas vs. normal parathyroid, we identified several putative oncogenes implicated in cell growth and transcription regulation, such as the *Cyclin D1* and the *C-jun* proto-oncogenes. Loss of chromosome 11q is the best studied chromosomal alteration in parathyroid adenomas. Hence, we examined expression distinction between adenomas with or without 11q loss. Unsupervised hierarchical clustering separated the tumor subsets well, and also the normal parathyroid samples.

Based on a positional candidate approach, the disease gene for the hyperparathyroidism-jaw tumor (HPT-JT) syndrome was isolated. Thirteen different heterozygous, germline, inactivating mutations in a single gene were identified in fourteen families with HPT-JT. The proposed role of *HRPT2* (1q25) as a tumor suppressor was further supported from mutation screening in 48 parathyroid adenomas with cystic features, which revealed three somatic inactivating mutations. *HRPT2* is a ubiquitously expressed, evolutionarily conserved gene, with a predicted protein of 531 amino acids, referred to as parafibromin.

The cellular function of Menin, the product of the *MEN1* tumor suppressor gene, is still unknown. We have in mouse characterized the Men1 ortholog and identified a menin interacting partner. The *Men1* gene was shown to be expressed in all organs investigated, at both RNA and protein levels. Four splice variants upstream of the translation initiation site were found and confirmed. The mRNA–ISH and Western blot analysis revealed *Men1* RNA expression during the entire spermatogenesis. This suggests that menin may have a crucial function during the development of spermatids in agreement with the infertile phenotype of homozygous *MEN1* humans. By a Yeast-two hybrid assay, the putative transcription factor gene *pem*, was identified and confirmed to be a menin interacting protein in mouse.

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Prognosis in acute myeloid leukaemia and influence of monocytic markers—Epidemiological, clinical and experimental studies

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The aim of the first part of this thesis was to describe an unselected series of AML patients in adults (≥ 15 years) from the three

counties Örebro, Värmland and Södermanland 1987–1992, including cases un-referred from district hospitals. In the study of incidence and registration, also ALL and unspecified AL were included due to delimitation difficulties. Four registries were used for case finding and diagnoses were verified from medical records. 260 cases of acute leukemias were found giving a mean annual incidence of $6.5/10^5$ in adults. Under-notification, diversity and lack of specificity of codes, and selection of good prognosis patients were problems noted for the Cancer Registry. Supplementation with cases from the Cause of Death Registry substantially improved the coverage. The incidence of AML in adults was $5.4/10^5$/year and the median age 69.5 years. 56% had received standard induction treatment, 28% low-dose treatment and 16% no cytostatic treatment. Median survival for all patients was 5.8 months and the probability of survival at 5 years was 9.3%. In a multivariate Cox proportional hazard analysis, age, LDH and kidney function were independent prognostic variables for survival. Cytogenetics was missing in many patients but had a strong influence on outcome in univariate analysis.

The aim of the second part was to investigate whether the 'monocytic markers' ANAE, CD14 and lysozyme correlate to prognosis in AML. In a study of 65 AML patients, there was a marked difference in CD14 and ANAE positivity in bone marrow cells for some cases. Patients expressing 10% or more CD14 had a poorer prognosis, in the total material as well as when non-monocytic AML was analyzed separately. In a retrospective study of 232 AML patients treated in Örebro and Umeå, a linear relation was rejected and a bimodal relation was found between serum lysozyme and prognosis. Lysozyme values below 20 or above 80 mg/l were indicative of better outcome than values in the range 20–80 mg/l. The bimodal relation to prognosis was verified in multivariate logistic regression and Cox proportional hazard analyses with inclusion of the factors age, de novo/secondary AML, cytogenetic risk group, lysozyme, leukocyte count, lactic dehydrogenase (LDH), and kidney function. Lysozyme was a selected prognostic factor for CR frequency ($p = 0.0003$), overall survival ($p < 0.0001$) and CR duration ($p = 0.0005$). The hazard ratios (HR) for lysozyme < 20, 20–80 and > 80 mg/l regarding overall survival were 1.0, 3.3 (95% CI 2.1–5.1) and 0.7 (95% CI 0.4–1.2). A bimodal relation to prognosis was found both in non-monocytic and monocytic AML. Our finding should be regarded as a new hypothesis and controlled in other studies.

The aim of the third part was to investigate effects of lysozyme on the leukemia cell line K562. Hen egg white lysozyme (HEL) concentrations of 800–12 800 mg/l gave increased proliferation with 1.4–2-fold higher cell counts and a tendency to higher viability compared to control incubations. HEL increased the proportion of cells in S-phase and decreased the proportion in G2/M-phase with an associated broadening of the peaks and higher fluorescence intensity signals in the DNA histograms. The threshold for effects of lysozyme was high, but corresponding levels of endogenous lysozyme are probably present in near cell environments of a not negligible proportion of patients with AML.

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Telomere analysis of normal and neoplastic hematopoietic cells—Studies focusing on fluorescence in situ hybridization and flow cytometry

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The telomeres are specialized structures at the end of the chromosomes composed of the repeated DNA sequence $(TTAGGG)_n$ and specific proteins bound to the DNA. The telomeres protect the chromosomes from degradation and end to end fusions. Due to the end-replication problem, the telomeric DNA shortens every cell division, forcing the cells into senescence at a critical telomere length. This process can be counteracted by activating a specialized enzyme, telomerase, which adds telomeric repeats to the chromosome ends leading to an extended or infinite cellular life span. Telomerase activity is absent in most somatic tissues but is found in germ cells, stem cells, activated lymphocytes and the vast majority of tumor cells and permanent cell lines. Hence, telomerase has been suggested as a target for cancer treatment as malignant cells almost exclusively express the enzyme and in that context telomere length measurements will be of great importance.

Telomere length is traditionally measured with a Southern blot based technique. A new method for telomere analysis of cells in suspension, called flow-FISH, was developed based on fluorescence in situ hybridization using a telomeric peptide nucleic acid (PNA) probe, DNA staining with propidium iodide and quantification by flow cytometry. Flow-FISH had high reproducibility and the telomere length measurements showed good correlation with Southern blotting results. The flow-FISH technique also allows studies of cells in specific phases of the cell cycle and the replication timing of telomeric, centromeric and other repetitive sequences were analyzed in a number of cells. Like previous studies, centromeres were shown to replicate late in S phase while the telomere repeats were found to replicate early in S phase or concomitant with the bulk DNA, which is opposite to the patterns described in yeast.

In benign immunopurified lymphocytes from tonsils, high telomerase activity was found in germinal center (GC) B cells. This population also had high hTERT mRNA levels and displayed a telomere elongation as shown by flow-FISH and Southern blotting. Combined immunophenotyping and flow-FISH on unpurified tonsil cells confirmed the results.

Chronic lymphocytic leukemia (CLL), the most common leukemia in adults, can be divided into pre-GC CLL, characterized by unmutated immunoglobulin V_H genes and worse prognosis, and post-GC CLL, with mutated V_H genes and better prognosis. In 61 cases of CLL, telomere length was measured with Southern blotting and V_H gene mutation status was analyzed. A new association was found between V_H mutation status and telomere length, where cases with longer telomeres and mutated V_H genes (post-GC CLL) had better prognosis than CLL with short telomeres and unmutated V_H genes (pre-GC CLL). A larger study of 112 CLL cases was performed using flow-FISH. The same correlation between telomere length and V_H mutation status was found but gender seemed to be of importance as telomere length was a significant prognostic factor for the male CLL patients but not in the female group. Age of the patients and spread of disease seemed to affect the prognostic value of V_H gene mutation status.

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